Ascorbate and its interaction with plasma membrane redox systems

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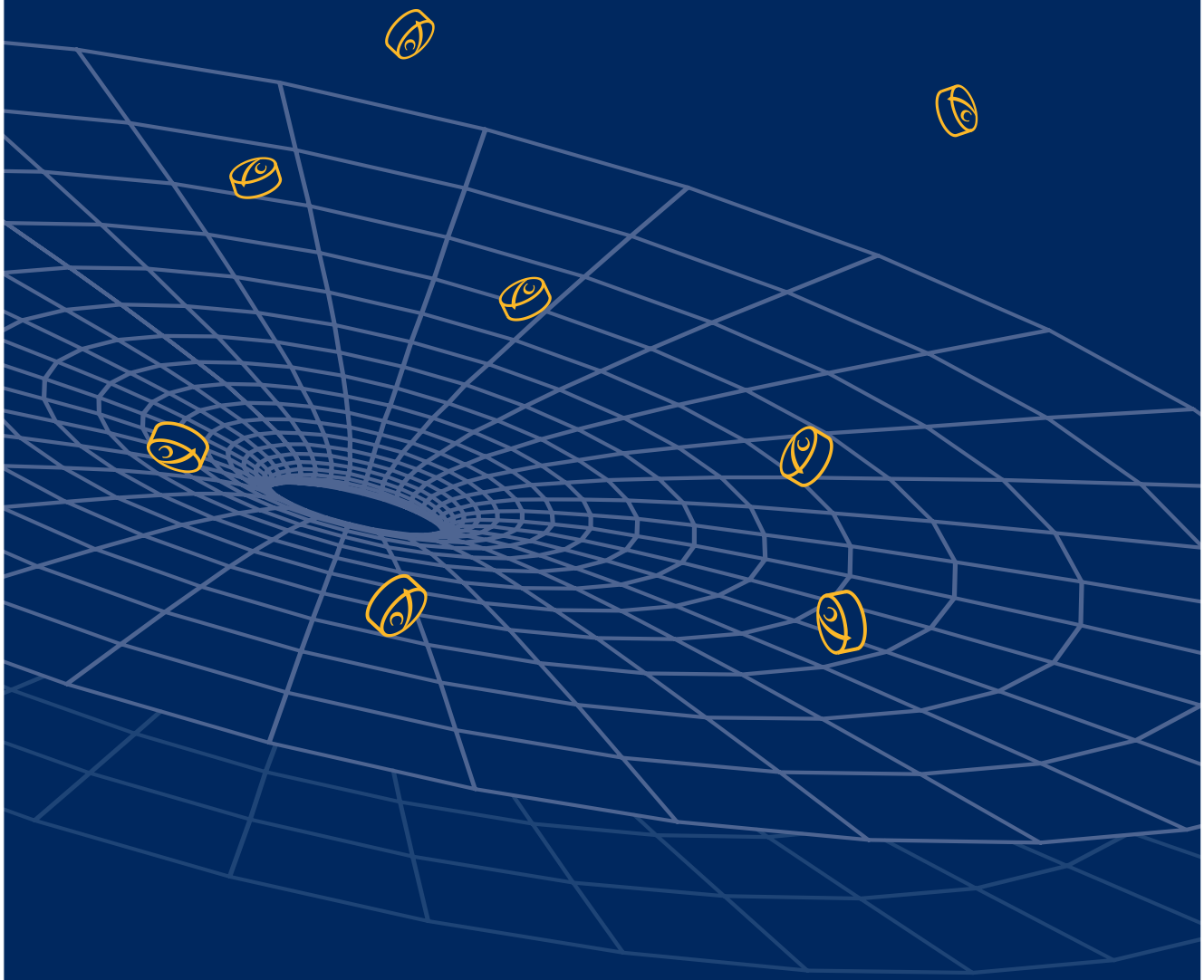

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Note to the cover:

The redox systems described in this thesis form a gate in the plasma membrane of cells, that allows electrons from ascorbate (vitamin C) to cross to the exterior face of the cell. The cover symbolizes such a passageway in the membrane bilayer.

"The great tragedy of science --
the slaying of a beautiful hypothesis by an ugly fact."
Thomas Huxley

Voor Judith

Abbreviations

AFR	ascorbate free radical
Asc	L-ascorbic acid
DHA	dehydroascorbic acid
$DiSC_3(5)$	3,3′-dipropylthiadecarbocyanine iodide
DTPA	diethylenetriaminepentaacetic acid
EDTA	ethylenediaminetetraacetic acid
ESR	electron spin resonance
FIC	ferricyanide
FOC	ferrocyanide
GSH	glutathione
HPLC	high performance liquid chromatography
$Ni(en)_3^{2+}$	tris(ethylenediamine)nickel(II) chloride
PBS	phosphate buffered saline
*p*CMBS	*para*-chloromerucribenzenesulfonic acid
RDA	recommended dietary allowance
ROS	reactive oxygen species
TEMPO	2,2,6,6-tetramethylpiperidine-N-oxyl
TEMPOL	4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl

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Chapter 1

Introduction

Redox reactions in biology

Amidst the wide array of chemical reactions that make up life, redox reactions play a central and indispensable role. The transfer of one or more electrons from an electron donor -a reductant- to an electron acceptor -an oxidant- is the hallmark of a redox (**Red**uction **Ox**idation) reaction. They occur in all main catabolic and anabolic pathways, and can be closely guided by specialized enzyme systems, or proceed spontaneously after an incidental encounter of the reactants.

The cell contains many enzymes that mediate redox reactions. Though it would be most appropriate to designate them as oxido-reductases, many other names have been used historically, and remain today. Well known examples are oxidases, reductases, dehydrogenases or oxygenases. In spite of the different names, all mediate the transfer of electrons from a donor to an acceptor. Redox enzymes are present in most cellular compartments, either dissolved in cell-water, or associated or integrated with a membrane. Mitochondria are the richest source of membrane-associated redox proteins, but they can be found in virtually all cell membranes, including the plasma membrane.

The studies presented in this thesis investigate redox reactions at the plasma membrane. Many cells can reduce *extracellular* molecules with electrons originating from an *intracellular* electron donor. Thus, the reactants of this redox reaction are separated by the plasma membrane of the cell. One or more systems must be present in the plasma membrane that assist in the electron transfer from the intracellular donor to the extracellular substrate. It is conceivable that a protein exists that traverses the membrane to form a channel for the electrons. However, for many plasma membrane redox activities the involvement of a protein has not been clearly established, nor has such a protein been isolated.

Ascorbate, or vitamin C, is an important reductant. One of its functions is to react with, and thus neutralize, oxidants and radicals to prevent them from damaging the cell. Thus, it plays an important role in the protection of the cell against oxidative stress. Some studies indicate that it can also interact with redox systems in the plasma membrane, and enhance the reduction of extracellular substrates by the cell. It was suggested that ascorbate is the intracellular electron donor for a plasma membrane redox system, which contributes to the efficacy of ascorbate as an anti-oxidant.

This thesis presents a study on the interaction of ascorbate with plasma membrane redox systems. The interaction will be characterized, the components of the redox system in the plasma membrane will be explored, and the physiological relevance

of these systems will be studied. This chapter presents some concepts and mechanisms governing biological redox reactions in general, and plasma membrane redox systems in particular. Thus, many of the subjects that are discussed in this thesis will be introduced. First, the basic forces that drive redox reactions will be explained in a section on thermodynamics. Next, an overview on oxidative stress provides information on radical reactions, anti-oxidant defense systems, and the chemistry and biology of ascorbate. Subsequently, the structural components of many redox mediators are discussed. Finally, various aspects of plasma membrane redox systems will be reviewed. This includes an overview of intra- and extracellular substrates, different systems that have been identified and isolated, and possible relations with cell proliferation. Also, the relevance of ascorbate to plasma membrane redox systems will be explained. The introduction will be concluded by an overview of this thesis.

Thermodynamics of redox reactions

Redox potential

As all chemical reactions, redox reactions are governed by thermodynamic laws. These laws dictate that a (redox) reaction will proceed spontaneously only if the

Redox Couple	E'_0 (mV)
$HO^{\bullet}$, H^+ / H_2O	2310
$O_2^{\bullet -}$, 2 H^+ / H_2O_2	940
α-tocopheroxyl$^{\bullet}$ / α-tocopherol	500
Ferricyanide / Ferrocyanide	360
H_2O_2, H^+ / H_2O, $HO^{\bullet}$	320
AFR, H^+ / ascorbate	282
CoenzymeQH$^{\bullet}$ / CoenzymeQH_2	200
CoenzymeQ / CoenzymeQH$^{\bullet}$	-36
DHA / AFR	-174
NAD^+ / NADH	-320
$NADP^+$ / NADPH	-324
O_2 / $O_2^{\bullet -}$	-330

Table 1. Standard redox potentials of some biologically interesting redox couples. Under standard conditions, an oxidant can react with a reductant below it. However, the rate of a reaction could still be very low.

free energy G in a system is decreased. In a redox reaction, the change in free energy is determined by the difference in redox potential E between the reactants. This redox potential is always defined for a redox-couple, the oxidized and the reduced form of the reacting molecule. It can be measured as the electrical potential generated in an electric cell under standard conditions, relative to a hydrogen electrode. The redox potential of this reference electrode is, by definition, 0 V. The redox potential of a redox couple under standard conditions is denoted as E_0. In biochemistry, it is more common to use the redox potential under standard conditions at a pH of 7 instead of 0. This is indicated by the prime in E'_0. Values for E'_0 are known for many biologically relevant redox couples. Table 1 shows a selection of well-known reactions (1, 2). During a reaction electrons will generally tend to flow from the redox-couple with a low, more negative, E'_0 to one with a higher, more positive E'_0. However, the actual redox potential E of a redox couple will almost always be different from E'_0, as temperature, concentrations and pH will be different from the standard conditions. Especially the ratio of the concentrations in a redox couple will have a strong effect on the redox potential. This is expressed in the Nernst equation : $E= E'_0 + RT/zF \ln [Ox]/[Red]$, where R is the gas constant, T the absolute temperature, F Faraday's number and z the number of charges that are transferred.

When the standard redox potentials of the reactants are known, it can still be difficult to predict whether a reaction is possible. A reactant can be inaccessible for a reaction when it is sequestered in a protein or a membrane. A lipophilic reactant can be localized in a biological membrane, and can thus be concentrated more than a hydrophilic molecule. Even when a reaction is thermodynamically favorable, its actual rate depends on the kinetic properties of the reaction, and could still be very low. Also, the rate could be increased dramatically by the presence of an enzyme. Though standard redox potentials can be valuable tools to assess a reaction, a well-designed experiment may be the only way to show that it occurs biologically.

Membrane potential

When the reactants are separated by the (plasma) membrane, electrons must be transported across the membrane for a reaction to proceed. Under those conditions, the reaction is also affected by the membrane potential at that membrane. The membrane potential is the result of the uneven distribution of ions at both sides, and by differences in permeability for those ions. Due to these differences, a charge separation results across the membrane, generating a potential across the

membrane as described by the Goldman-Hodgkin-Katz equation. The inside of most cells is negatively charged compared to the extracellular medium. However, large potential differences are found between cell-types, ranging from about -100 to 0 mV. In a redox reaction across the plasma membrane, charge that is transported will 'feel' the membrane potential, and the rate of the reaction will be affected. When the inside of the cell is negative, the potential will promote electrons to move to the extracellular face. Conversely, electrons are drawn into the cell by a potential that is positive inside. In addition to being affected by the existing membrane potential, a plasma membrane redox reaction can also contribute to the potential, as charge is transported across the membrane. If left uncompensated, this build-up of charge will eventually inhibit the redox reaction.

Oxidants and Anti-oxidants: the role of vitamin C

Oxidative stress

Through evolution, an important part of life has evolved to the point where it depends on oxygen for its survival. Though the use of oxygen brought many advantages, it has proved to be a mixed blessing. While it is essential for our biological systems, we are constantly bathing in a chemical that also poses a threat to us. This apparent contradiction is sometime referred to as the 'oxygen paradox' (3). The dangers of oxygen stem from the inherent reactivity of oxygen itself and, even more, the reactivity of the many (radical) forms of oxygen that may result from metabolic processes in the organism. Those reactive oxygen species, or ROS, can quickly react with cellular components, such as proteins, nucleic acids and membrane lipids. Oxidative reactions damage these components, resulting in e.g. the dysfunction of an enzyme, or even a mutation in DNA. Oxygen is not the only molecule capable of inflicting oxidative damage. Other oxidants may be endogenous, or originate from our environment. Thus, we are surrounded by a variety of chemicals capable of causing oxidative damage to the biological system. This puts a strain on the organism that is commonly referred to as oxidative stress.

When mitochondria and oxidative phosphorylation emerged during the evolution of cells, a rich source of ROS was introduced inside them. Without appropriate defense mechanisms, these ROS have the potential to induce intolerable damage. It is therefore no surprise that a wide range of mechanisms have been put in place to eliminate ROS and other oxidants before they can do any serious damage (4). As a first line of defense, a number of anti-oxidant enzymes and molecules are

present, such as superoxide dismutase, catalase, ascorbate, glutathione and the tocopherols. These systems work by interacting with various oxidant molecules, and converting them to less harmful compounds that can easily be disposed of. Though these anti-oxidants are efficient scavengers of oxidants, their effectiveness is much greater when working together. A well described example is the cooperation of ascorbate and α-tocopherol, in which α-tocopherol scavenges a radical inside the lipid bilayer, resulting in a tocopheryl radical. Ascorbate can reduce the tocopheryl radical, but then forms an ascorbate radical in the cytoplasm. The ascorbate free radical can spontaneously disproportionate, or be reduced by cellular enzymes (5, 6). Thus, the radical originating from the membrane is removed from the system using two different anti-oxidants.

As a rule, an anti-oxidant is a reducing chemical that can react with damaging oxidants or radicals that are encountered. As reviewed by Rose *et al.*, the ideal free radical scavenger must be present in adequate amounts, capable of reacting with a variety of radicals, suitable for compartmentalization, well available through synthesis or diet, suitable for regeneration, retained in the kidney and preferably non-toxic before and after a scavenging reaction (7). Many biological molecules have at least some of these properties, but ascorbate stands out as a particularly potent anti-oxidant. However, other anti-oxidant systems, though perhaps not as versatile as ascorbate, play equally important physiological roles. For example, α-tocopherol specializes in the protection of lipid membranes, which are not accessible for ascorbate. The anti-oxidant enzyme superoxide dismutase has a specificity limited to only one oxidant, but is essential in the disposal of superoxide anions. The various anti-oxidant mechanisms supplement each other, overlap in certain areas, and all contribute in a more or less specialized way to the prevention of oxidative damage.

Radical reactions

Oxygen Chemistry

The primary target of anti-oxidants are reactive oxygen species (3, 8-10). These include several redox forms of oxygen itself, but also reactive forms of oxygen in a composite molecule with other elements, e.g. nitric oxide, HOCl, or lipid hydroperoxides. Oxygen is preferably reduced in a one electron step. This is due to the peculiar spin-state of the outer electrons of O_2. In most molecules, electrons with opposite spins are paired in an orbital, as parallel spins in one orbital are not allowed. O_2 contains two unpaired electrons with parallel spins in separate orbitals,

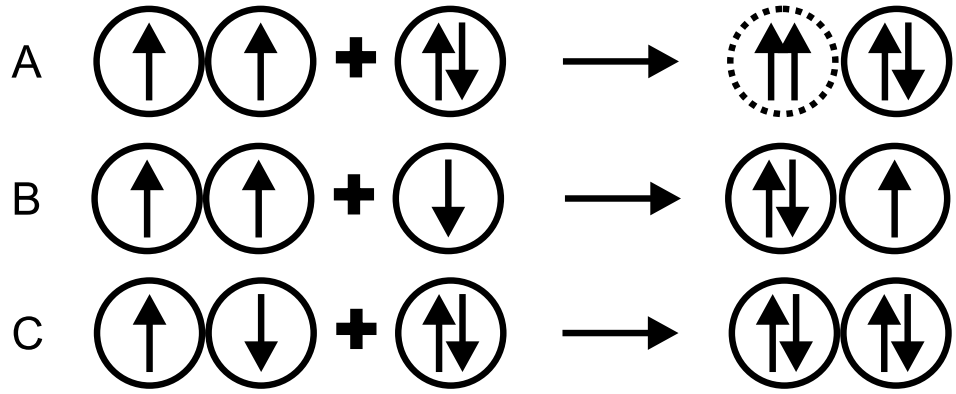


Figure 1. Spin-state and reactivity of oxygen. Parallel spins are not allowed in a single orbital, represented by the circles in the figure. Normal (triplet state) oxygen has two single parallel electrons in separate orbitals. A two-electron interaction (A) would result in parallel spins, and is therefore not possible. A single electron interaction (B) is thus preferred. Singlet oxygen is capable of two-electron interactions (C), and is therefore much more reactive. (Figure modified from Fridovich (7))

and essentially is a bi-radical molecule (•O-O•). In a two-electron reduction, an anti-parallel electron pair would be added to the oxygen molecule, resulting in the forbidden state of two parallel electrons in a single orbital of the oxygen molecule (Figure 1). Inversion of a spin is possible, but relatively slow. Oxygen is therefore preferably reduced in a one-electron step. A total of four one-electron steps results in the complete reduction of oxygen, the first step leading to the superoxide anion (Figure 2). The superoxide anion is not extremely reactive, and can interact both as a reductant (e.g. with Fe^{3+}) and as an oxidant (e.g. with catecholamines). Two molecules of superoxide can also react with themselves, a process called dismutation. This reaction results in ground state O_2 and hydrogen peroxide (H_2O_2). H_2O_2 is not a radical, but it is an oxidant. Also, it is easily converted into other reactive oxygen species, most notably the hydroxyl radical. This redox state of oxygen is particularly reactive, and will rapidly oxidize the first molecule that is

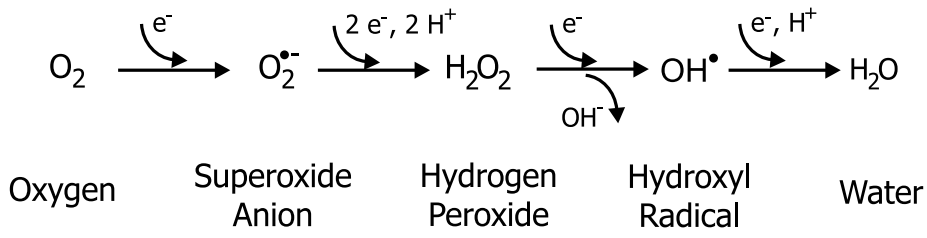


Figure 2. Redox states of oxygen. Four electrons (and four protons) are needed to reduce O_2 to two molecules of water.

available. The reduction of the hydroxyl radical finally yields water, the completely reduced form of oxygen.

Ground state O_2 can also gain reactivity by a change in the spin of its outer electrons. Electromagnetic energy can excite an electron, causing its spin to flip. This yields singlet oxygen ($^1O_2$), in which the outer electrons have anti-parallel spins, allowing two-electron interactions (Figure 2C). These are more likely to occur than one-electron reactions, making singlet oxygen much more reactive than normal, triplet state, oxygen.

Radical chain reactions

Reactive oxygen species can interact with other molecules to produce a cascade of other reactive compounds. A notorious reaction is the metal-catalyzed generation of the hydroxyl radical, which can involve iron or copper ions. The superoxide anion can reduce Fe^{3+} yielding Fe^{2+} and O_2. Hydrogen peroxide, which is always present as a dismutation product of superoxide, reacts with Fe^{2+} to form the hydroxyl radical and Fe^{3+} in the so-called Fenton reaction (11). Iron is subsequently recycled by superoxide (or another reductant), and the cycle can repeat itself. The net reaction is a metal catalyzed formation of O_2 and hydroxyl radical from H_2O_2 and $O_2^{\bullet-}$, also called the Haber-Weiss reaction (Figure 3). Because of this reaction, the presence of free iron or copper is very dangerous physiologically. Iron should therefore always be sequestered in proteins like transferrin and ferritin, while copper is sequestered similarly by ceruloplasmin. Another dangerous chain of reactions can occur in lipid membranes. The interaction of lipids with e.g. ionizing radiation or a radical can result in the formation of lipid radicals ($L^{\bullet}$), which quickly

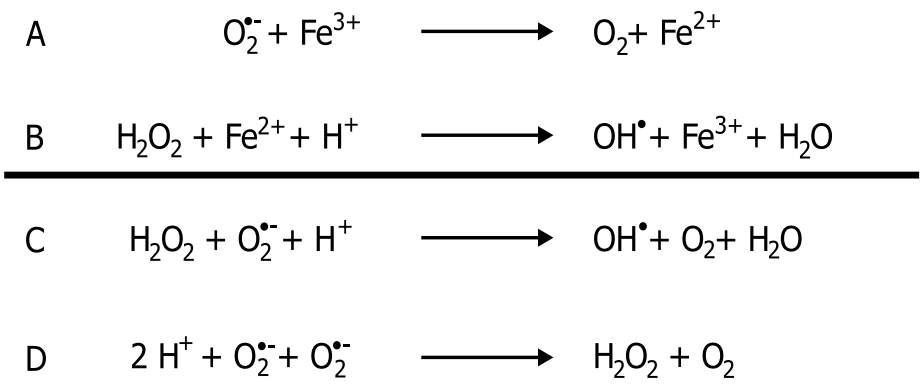


Figure 3: The Haber-Weiss reaction. The Haber-Weiss reaction (C) is catalyzed by metal ions. This is due to reactions A and B, the latter being the classical Fenton reaction (11). The superoxide anion is often released, e.g. by mitochondria. Hydrogen peroxide can be formed from the superoxide anion by dismutation (D).

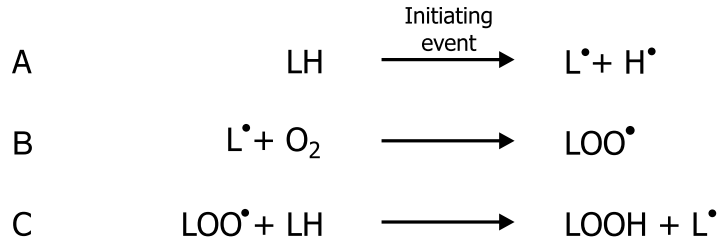


Figure 4. Lipid Peroxidation chain reaction. The reactions above illustrate how a single initiation reaction (A) in a polyunsaturated lipid LH can result in a cascade of propagation reactions (B and C) that produce lipid hydroperoxides. α-Tocopherol can break the propagation chain by reacting with a lipid peroxyl radical LOO•.

transform to lipid hydroperoxides (LOO•). However, these hydroperoxides react with undamaged lipids to form new lipid radicals. Thus, a single initiation can result in many lipid hydroperoxides, compromising the function of the membrane (Figure 4) (8). α-Tocopherol is known as a 'chain-breaking' anti-oxidant, because it can react with a lipid hydroperoxide without propagating the reaction, thereby stopping the chain of events.

Anti-oxidant defenses

Prevention

The best way to counter oxidative stress is to prevent the formation of reactive species. Presumably, evolution has selected for metabolic pathways that minimize their release. All dangerous intermediates in the oxidative phosphorylation have been carefully sequestered in protein complexes, thus preventing unwanted interactions with oxygen that could yield ROS. In spite of this design, some (radical) intermediates from mitochondrial metabolism can interact with oxygen. This reaction releases significant amounts of ROS, and probably form the primary source of oxidative stress in our body. Also, the constant auto-oxidation of hemoglobin in the blood results in ROS (12, 13). It has been estimated that an average person can produce as much as 1.75 kg of superoxide per year (14). Apparently, the formation of ROS cannot be prevented. It is therefore essential to have defenses against this constant onslaught of reactive compounds.

Interception

A large variety of oxidants can be encountered, making it almost impossible to use specific defenses. Only the two ROS that are most common, superoxide and hydrogen peroxide, can be removed by specific enzymes. Superoxide is removed

by superoxide dismutases, which exist in several varieties with Cu, Zn or Mn in the active center. The dismutation yields hydrogen peroxide, which can be removed by two different enzymes. Both catalase and glutathione peroxidase reduce it to O_2 and water, albeit through different mechanisms. No enzymes exist for the conversion of the hydroxyl radical, presumably because of its short lifetime; it will have reacted with another molecule before a chance encounter with a specific enzyme.

The other anti-oxidant defenses do not have a specific substrate, but will react with most oxidants and radicals they encounter. This reaction is 'suicidal', i.e. the anti-oxidant is consumed in the process. However, for many anti-oxidants regenerative systems exist to prevent the need for a massive intake or synthesis of these compounds. Well-known biological anti-oxidants are ascorbate, α- and other tocopherol isoforms, carotenoids, glutathione, bilirubin and uric acid. Many other molecules exist with anti-oxidant properties, and several are used in the food industry. Examples of such xenobiotic molecules are butylated hydroxytoluene, butylated hydroxyanisole and ascorbic acid derivatives. These compounds are now part of most modern diets. However, not much is known about their possible contribution to our anti-oxidant defense. A lot of interest exists in other, natural, anti-oxidant components in the diet. Polyphenolic flavinoids from fruits, vegetables, teas or wines could contribute to our defenses, both by scavenging oxidants and by the chelation of metal ions which could catalyze oxidation reactions (15). Also, food products are now often supplemented with 'classic' anti-oxidants like the vitamins C and E to increase their nutritional value, but also to benefit from the public interest in health foods.

Repair

A final countermeasure against oxidative damage consists of the repair of inflicted damage. When the first lines of defense have failed, important (macro) molecules can be damaged by oxidants. Damaged components must either be repaired or removed, as they can otherwise threaten the cell, or possibly even the entire organism. In proteins, oxidative damage mostly occurs at cysteine residues, producing unwanted disulphide bonds or protein cross-linking. However, other residues can also be modified. Disulphide bonds can be repaired by reductases, but many other types of damage cannot be repaired. In those cases, proteins have to be degraded by the proteasome, a large complex of proteases that recognizes oxidative damage on proteins (3). It is believed that recognition occurs through patches of hydrophobic amino acids exposed by the oxidative damage.

Proteolysis releases the composing amino acids, which can be used for *de novo* synthesis of new proteins.

Separate mechanisms exist for the repair of biological membranes. Phospholipase A_2 has a higher affinity for damaged membrane lipids than for normal lipids. The enzyme releases the damaged fatty acid, leaving a lysophospholipid for re-acylation. It has also been suggested that lipid hydroperoxides can be reduced without prior removal of the fatty acid moiety. Thus, it is possible to repair the membrane structure after oxidative damage is inflicted.

Finally, nucleic acids are also prone to oxidative attack. A large variety of damage in DNA has been described, including base modifications, changes in the backbone, and strand breaks. This damage can block DNA polymerases and transcription, but can also be mutagenic. Several repair mechanisms have been identified that repair this damage, though the repair of mitochondrial DNA seems to be less efficient than the repair of nuclear DNA (16). When DNA damage is beyond repair, the cell has to resort to apoptosis. This drastic measure protects the rest of the organism against loss of control over proliferation that may result from the damaged genome.

Chemistry and metabolism of ascorbate

Ascorbate is an important molecule in biology, both as a cofactor in several biosynthetic pathways, and as an anti-oxidant. In most species, ascorbate is synthesized in the liver or kidney. However, humans, primates, guinea pigs, passerine birds and flying mammals are not capable of *de novo* synthesis of ascorbate. An essential enzyme in the biosynthetic pathway, L-gulono-γ-lactone oxidase (EC 1.1.3.8), is not present in these species. This enzyme mediates the last step in the ascorbate biosynthetic pathway originating from glucose. Perhaps as a result of the availability of ascorbate in the diet, this enzyme has been lost in evolution. As a result, these species now completely depend on dietary intake to supply the required amounts of ascorbate. In a normal diet, the daily requirement to prevent acute disorders is usually met without problems. However, the aberrant diet that was common in the long sea voyages at the end of the 15th century was deficient in ascorbate, and resulted in high mortality among sailors due to scurvy. Not until 1753, scurvy was recognized as related to diet. The concept of deficiency diseases was established for the first time, when the Scottish naval surgeon James Lind showed that scurvy could be cured and prevented by ingestion of the juice of oranges, lemons, or limes (17). Only later it was found that scurvy was accompanied by problems in collagen metabolism. Indeed, ascorbate has been found to be a

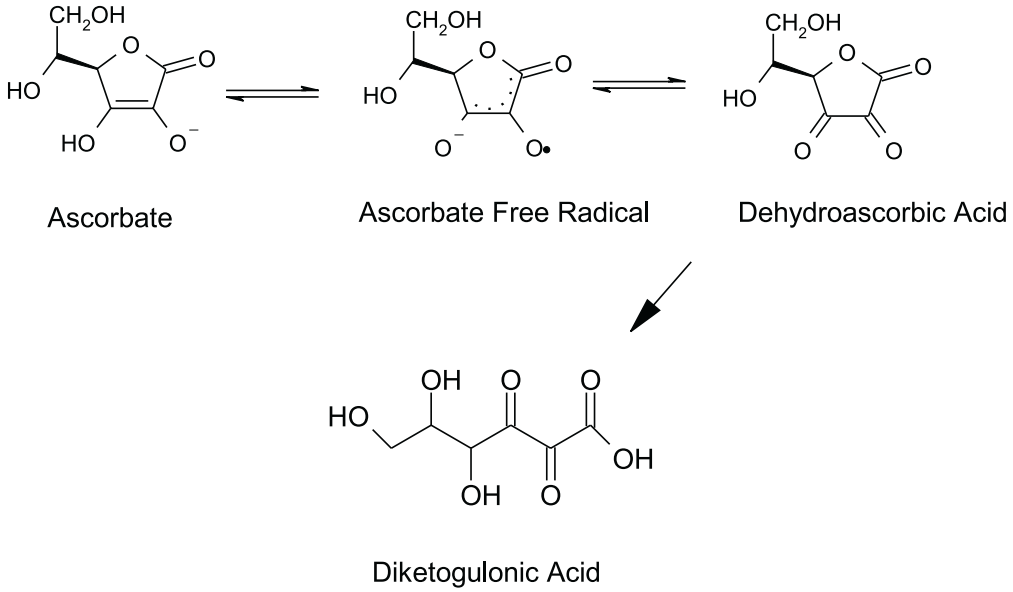


Figure 5. Structure of ascorbate and its derivatives. Ascorbate can be oxidized in two successive one-electron steps to ascorbate free radical and dehydroascorbic acid. The unpaired electron in ascorbate free radical is distributed over the ring structure, stabilizing the molecule. The ring-structure of dehydroascorbic acid can be lost in a hydrolysis reaction that is biologically irreversible.

cofactor for the enzyme prolyl hydroxylase, which modifies the polypeptide collagen precursor in order to facilitate the formation of collagen fibers. Though an impaired collagen synthesis has long been considered the only result of ascorbate deficiency, later research has revealed an important role of ascorbate in many other biosynthetic pathways (18). These include carnitine synthesis, catabolism of tyrosine, synthesis of norepinephrine by dopamine β-oxygenase, and the amidation of peptides with C-terminal glycine to activate hormone precursors. In fungi, additional pathways exist requiring ascorbate as a cofactor. Finally, ascorbate is an excellent anti-oxidant, removing oxidants and radicals before they can inflict damage on essential cellular components (7). Though ascorbate deficiency primarily results in insufficient collagen synthesis and scurvy, it may cause problems in many other systems as well.

Chemical and physical properties

The structure of ascorbate is shown in figure 5. It resembles a pentose sugar, but has two double bonds that allow the redox-chemistry characteristic for the molecule. Ascorbate can ionize at the C_2 and C_3 positions, which have pK values of 4.17 and 11.57, respectively. Ascorbate is therefore mainly present as a monovalent anion at physiological pH. When ascorbate participates in a redox reaction, the hydroxyl

groups at C_2 and C_3 are oxidized to ketones. The abstraction of a first electron from the molecule yields the Ascorbate Free Radical (AFR). The conjugated bonds in AFR allow the unpaired electron to be distributed over the molecule. The radical is stabilized by these resonance structures, and is therefore less likely to react with other molecules. It also causes the relatively long half-life of AFR, of up to a second. AFR has pK values of 1.10 and 4.25, and is a monovalent anion at physiological pH. The oxidation of AFR yields dehydroascorbic acid (DHA). Most commonly, this is the result of a disproportionation (or dismutation) reaction of AFR with another molecule of AFR, yielding one ascorbate and one DHA molecule. In DHA, all hydroxyl groups in the ring have been replaced by keto groups, and the double bond in the ring has also been lost. Around a keto group, carbon atoms prefer to be bonded at an angle of 120°. However, the bonds in DHA are forced into a sharper angle. Thus, the molecule is highly strained, and therefore not very stable. This strain is partially relieved by a reversible hydration of the molecule, yielding a bicyclic structure (19). Nevertheless, the ring structure of DHA is easily hydrolyzed to form a linear molecule, 2,3-diketo-L-gulonic acid. While the oxidation of ascorbate to AFR and DHA can easily be reversed, this ring opening reaction of DHA is biologically irreversible, and results in loss of the vitamin.

The different ionized forms of ascorbate have different redox properties. Therefore, the redox-chemistry of ascorbate is highly pH dependent. For instance, the rate of auto-oxidation with oxygen is much higher at a more alkaline pH, due to the relatively higher concentration of the ascorbate dianion (20, 21). Though most biological systems have a fixed physiological pH, some pH differences occur that can shift equilibria. For instance, the adrenal chromaffin granule is acidified by a H^+-ATPase. The pH gradient across the vesicle membrane drives the equilibrium towards the reduction of intravesicular AFR (22).

Ascorbate transport

At neutral pH, ascorbate mainly exists as a monovalent anion. Due to this charge and its size, ascorbate hardly diffuses across bio-membranes. Instead, carrier mechanisms are required for transport of the molecule. Ascorbate from the diet is absorbed through the epithelium of the gut. An ascorbate transporter, which co-transports sodium to power movement against the concentration gradient, mediates the transport into the cytoplasm of the epithelial cells. Recently, the sodium-dependent ascorbate transporters SVCT1 and SVCT2 were cloned, and found to be differentially expressed in many tissues, including gut and kidney (23-25). The presence of the transporter in the kidney explains the resorption of ascorbate

from urine after glomerular filtration, which prevents loss of the vitamin by excretion. However, the SVCT transporters are not expressed ubiquitously in all cells. Cells lacking the SVCT transporter must therefore use alternative transport systems to acquire ascorbate. This is done by the transport of DHA, the oxidized form of ascorbate. DHA is efficiently shuttled through the GLUT-1 glucose carrier, but also through the GLUT-3 and 4 isoforms (26-28). Transport can therefore be inhibited by excess glucose, and also by inhibitors of glucose transport. In contrast to the active transport of reduced ascorbate, DHA is translocated by facilitated diffusion. In the cytoplasm, DHA can be quickly reduced to ascorbate. This prevents loss of DHA by hydrolysis, and maintains a gradient of DHA into the cell. Also, ascorbate is metabolically trapped, as ascorbate can only slowly leave the cell. Thus, cells can accumulate ascorbate at concentrations exceeding the serum concentration.

It is not completely clear how ascorbate leaves the cell after e.g. uptake into epithelial gut cells. Transport is not energy-dependent, as it will be down a concentration gradient. It remains to be shown whether proteins are involved in this step, or whether the slow rates of diffusion across the plasma membrane are sufficient for the transport requirements of ascorbate (29). However, recent experiments on hepatocytes and intact liver revealed saturation kinetics and temperature dependency for ascorbate efflux, providing some evidence for a facilitated process in those tissues (30). Thus, protein-mediated efflux of ascorbate from hepatic or other tissues seems to be a compelling model for this often ignored step in ascorbate trafficking.

Intracellular cycling of ascorbate redox forms

It is important for the cell to maintain ascorbate in its reduced form in order to maintain proper anti-oxidant levels, and to prevent loss of the vitamin from degradation. Significant amounts of oxidized ascorbate have to be dealt with intracellularly, either from intracellular oxidative events, or from the accumulation of DHA through the GLUT-1 transporter. Therefore, mechanisms are available to reduce both oxidized forms of ascorbate, AFR and DHA. In part, reduction can occur by a simple chemical reduction by glutathione (31, 32). However, enzymatic reactions appear to play an important role. Some enzymes, such as glutaredoxin (33) and protein disulfide isomerase (34) also depend on glutathione to reduce DHA, while thioredoxin reductase and 3α-hydroxysteroid dehydrogenase rely on NADPH (35, 36). Himmelreich *et al.* reported that DHA can also be reduced during transport into the cell (37). The radical form of ascorbate, AFR, can also be reduced, for instance by a reductase that is present in the mitochondrial membrane. There

are strong indications that this activity is caused by the NADH-dependent cytochrome b_5 reductase and cytochrome b_5 on the outer mitochondrial membrane (38, 39). In the cytoplasm, thioredoxin reductase not only reduces DHA, but also AFR (35, 40). In plants, other cytoplasmic AFR reductases were found (41-43). However, AFR can also non-enzymatically disproportionate to DHA, and then be reduced by the other systems in the cell. Thus, a variety of possibilities exist to keep ascorbate in the reduced state. However, the capacity for regeneration can differ considerably between cell-types.

Pro-oxidant aspects of ascorbate

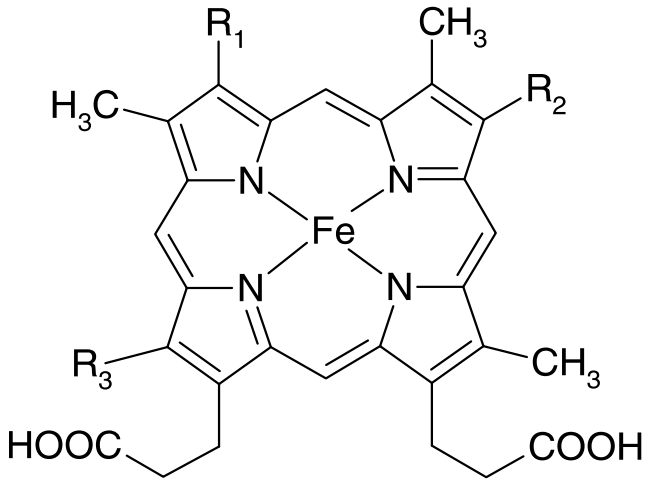
Ascorbate is generally presented as a powerful anti-oxidant molecule, implicated in the protection against numerous diseases (44). Health authorities therefore recommend a minimum dietary intake of ascorbate, known as the Recommended Dietary Allowance (RDA, approximately 60 mg/day). However, many others believe that the maximal benefit of ascorbate is reached at much higher doses. Such behavior is possibly not without danger, as pro-oxidant effects have also been reported after administration of ascorbate. Pro-oxidant reactions of ascorbate can result from its interaction with Fe^{3+} or Cu^{2+} ions, generating hydroxyl radicals by Fenton-type reactions. Free metal ions should be very rare under physiological conditions due to sequestering by one of several macromolecules, but nevertheless oxidative damage due to ascorbate has been shown *in vivo*. A lot of discussion has resulted from a publication showing an increase of the oxidation product 8-oxoadenine in lymphocyte DNA after ascorbate supplementation (45). Other clinical trials showed inconsistent results, but nevertheless a majority of them indicated mainly beneficial effects of ascorbate on oxidative damage to the cell (46). Therefore, the present consensus is that the anti-oxidative properties of ascorbate outweigh any pro-oxidant properties it may have, and that the consumption of the RDA of ascorbate is essential for human health. This view can be altered by certain pathological conditions. Several disorders, such as β-thalassemia and haemochromatosis, result in iron overload. The presence of ascorbate could increase the oxidative load inflicted by the presence of free iron, aggravating the disease. In these cases, a more limited intake of ascorbate could prove beneficial (46, 47). The consumption of mega-doses of ascorbate by healthy persons is still disputed. Tissue saturation with ascorbate appears to occur at a few hundred milligrams per day, indicating that there is no point in administering doses in excess of that amount. Nevertheless, many consumers still persist in taking up to grams of ascorbate per day.

Mediators in cellular redox processes

A variety of mediators, consisting of macromolecules and smaller compounds, is available to the cell to manage redox processes. Though a large number of different mediators have been identified, they use only a small number of mechanisms to accept and donate electrons. Proteins have a limited capacity to participate in redox reactions. Their thiol and tyrosine residues are capable of redox chemistry, e.g. as described for the enzyme ribonucleotide reductase (48). To be capable of more versatile reactions, many redox proteins therefore use a prosthetic group to assist in the transfer of one or more electrons. Most systems have been identified during the study of oxidative phosphorylation and photosynthesis. The redox chains in those processes have been thoroughly characterized, and all components have been isolated and identified. Though many plasma membrane redox systems remain to be characterized, they are likely to consist of the same building blocks as e.g. mitochondrial redox enzymes.

Cytochromes

Cytochromes are participants in cellular redox reactions that have been studied extensively (49). They consist of a polypeptide backbone, which can either be soluble, membrane associated or membrane integrated. One or more prosthetic heme groups are linked to the polypeptide to accept electrons, or participate in other reactions. In different cytochromes, the nature of the heme group may vary, as well as the way in which they are linked to the peptide backbone. These differences have a strong impact on the properties of the cytochrome, and have therefore also been used to classify the cytochromes. A heme group consists of a porphyrin ring system, with usually an iron atom in its center. As shown in figure 6, four different heme types are known, corresponding to *a*, *b*, *c* and *d*-type cytochromes. The heme groups are linked to the peptide backbone by non-covalent interactions. In addition, *c*-type cytochromes have a covalent thioether link between the porphyrin ring and the protein backbone. The non-covalent links can include hydrophobic interactions of the protein with the heme ring, and interaction of the heme iron with a histidine residue perpendicular to the plane of the heme. The sixth coordination position of the heme, on the other side of the plane, can be either free or occupied by another residue. This may be histidine, but also cysteine, methionine, tryptophan, lysine or tyrosine. When the sixth position remains free, it can usually also interact with ligands from outside the polypeptide. The ligand could be e.g. oxygen in the case of hemoglobin, but also cyanide or carbon monoxide. The properties of a given cytochrome can be reviewed using



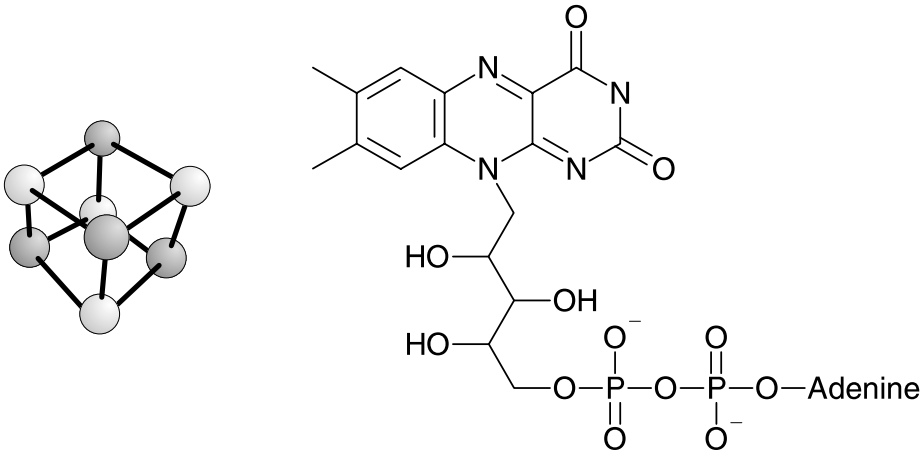
Heme Type	R_1	R_2	R_3
Heme *a*	$-C_{17}H_{29}O$	$-C=CH_2$	-CH=O
Heme *b*	-C=CH2	--	$-CH_3$
Heme *c*	$-\underset{\text{S-Protein}}{\overset{}{\text{CH}}}-CH_3$	$-\underset{\text{S-Protein}}{\overset{}{\text{CH}}}-CH_3$	--
Heme *d*	$-\underset{\text{OH}}{\overset{}{\text{CH}}}-CH_3$	$-C=CH_2$	--

Figure 6. Hemegroups of the cytochromes. Cytochromes can be classified according to their heme group. All have the same basic structure, but can have different side-groups. These are presented in the table on the bottom. In addition, a heme *d* also has a slightly different heme ring (siroheme). The nomenclature of the cytochromes follows that of the ring, i.e. a cytochrome *b* contains a heme *b*.

spectroscopy. Both oxidized and reduced forms of cytochromes have specific spectral properties, depending on the type of heme, their ligandation, and their surroundings. Thus, the spectral properties of a cytochrome can identify it as a member of a certain subset, or exclude it from others. The highly characteristic absorption maxima of cytochromes are also often used in their nomenclature.

Iron-sulfur clusters

Iron is the reactive core of a cytochrome. Other proteins also use iron as a prosthetic group, but in a completely different configuration. One or more complex clusters of iron and sulfur can be found in iron-sulfur proteins, also called non-heme iron proteins. The iron-sulfur clusters can be found in a wide array of compositions, the simplest consisting of two iron and two sulfur atoms. Shown in figure 7A is a more complex cluster of four iron and four sulfur atoms. Though multiple iron atoms are



A: Fe_4S_4 Iron-Sulfur Cluster

B: Flavin Adenine Dinucleotide (FAD)

Figure 7. Iron-sulfur clusters and flavins. Iron-sulfur clusters can be found in many configurations, the smallest being two sulfur and two iron atoms. Shown is a cluster of four iron and sulfur atoms, represented by dark and light spheres (A). Flavins can be found as Flavin Adenine Dinucleotide (FAD) (B), or as Flavin MonoNucleotide (FMN), which lacks the adenosine monophosphate group that is present in FAD.

present, the clusters can only undergo one-electron oxidation and reduction reactions, distributing charge over the whole cluster. The clusters play a versatile role in biology, not only mediating in redox reactions, but also sensing iron and oxygen, and storing iron in ferritin (50). Iron-sulfur proteins can be found both in membranes and in solution, but remain to be shown in the eukaryotic plasma membrane.

Flavins

An important group of redox proteins use a flavin moiety as a prosthetic group (Figure 7B). Flavins are molecules with a conjugated bond system, which allows them to form relatively stable radicals (51). Thus, they can exist in three redox states; reduced (FH_2), the radical form ($FH^{\bullet}$), and an oxidized form (F). This allows flavins to engage in both one- and two-electron redox reactions. The flavin moiety is found in two slightly different forms, known as flavin mono nucleotide (FMN) and flavin adenine dinucleotide (FAD). Both forms differ by an adenine group in the side chain, but share the same reactive site.

Metal ions

Finally, many proteins contain metal ions bound directly to amino acid residues. The ions in these metallo-enzymes can often exist in multiple redox states, and are therefore well suited to participate in redox reactions. Common metals in active sites are zinc, copper, iron, molybdenum, manganese and cobalt. The involvement of these metals is not limited to redox reactions, but also extends to other types of reactions. In those cases, the charge of the metals usually plays a role in the reaction mechanism.

Multiple prosthetic groups

The mechanisms mentioned above represent the main ways employed by proteins to mediate redox reactions. Proteins can contain one or more of these components, and can also mix different types. For instance, an important protein involved in the respiratory burst of neutrophils is a flavocytochrome, a protein containing both a heme and a flavin moiety. Thus, proteins can contain a combination of prosthetic groups best suited for their purpose. In all cases, the prosthetic groups are tightly bound to the protein backbone. After their biosynthesis, prosthetic groups and apo-enzymes are therefore rarely encountered separately.

Diffusable mediators

Cells contain many other redox mediators that are not immobilized in a protein, but can freely diffuse in water or lipid compartments. These compounds transiently bind to proteins as cofactors, or do not require protein mediation at all. The best known redox cofactors are the water soluble pyridine nucleotides NADH and NADPH. They can be regarded as the universal redox currency of the cell, supplying reducing equivalents for many reactions, including oxidative phosphorylation. After an oxidation, they are reduced by cellular metabolism in e.g. the glycolytic pathway and citric acid cycle. In lipid membranes, coenzyme Q is an important mediator in redox reactions. Due to its freedom of movement in the membrane, it can act as an electron shuttle between different systems. Like flavins, it can exist in three redox states, among which a stable radical form. It therefore participates in both one- and two electron reactions.

Many redox-active compounds in the cell also have an important anti-oxidant function. Good examples of such molecules are glutathione and ascorbate, which both act as a cofactor in enzymatic processes, but also are anti-oxidants in the absence of a protein. In fact, α-tocopherol is the only anti-oxidant which does not

seem to act as a co-factor, though it has been shown to affect e.g. protein kinase C signaling processes in the cell by an unknown mechanism (52, 53).

Plasma membrane redox systems

History

Most of the research on plasma membrane redox processes started in the 1970's. Experiments revealed that membrane preparations, but also intact red cells, nucleated cells and tissues, could reduce certain redox dyes (54-60). Biological membranes are impermeable to the dyes that were used. As the dyes could not enter the cell, and no reducing compound was found to leave the tissue, reduction by intact cells had to be the result of electron transport across the plasma membrane. Ferricyanide emerged from these investigations as the best substrate to study plasma membrane redox reactions, due to its low toxicity and low permeation, and the convenient assays to determine its conversion.

Substrates for plasma membrane redox systems

Intracellular substrates

Plasma membrane redox systems transport electrons across the membrane. This transport is usually directed out of the cell, requiring an intracellular electron donor. Early studies on isolated membranes revealed that NADH could reduce ferricyanide only in presence of these membranes. It therefore seemed likely that NADH was the natural intracellular substrate of the plasma membrane redox system, though the presence of other donors could not be excluded. In isolated membranes it is difficult to discriminate enzymes which are only present on e.g. the inner face of the membrane from those having a transmembrane structure. Nevertheless, these and further experiments have firmly established the important role of NADH as electron donor for the reduction of extracellular substrates. However, it was found that NADH is not the only source of reducing equivalents that can be used. In some systems, electrons are exclusively supplied by NADPH. A well known example is the reduction of extracellular oxygen by neutrophils in the respiratory burst response (61). The study of the reduction of ferricyanide revealed that electron donors may not be limited to pyridine nucleotides. Intracellular ascorbate stimulated ferricyanide reduction, and was later suggested to be an intracellular electron donor for this reduction (62-64). Many other reductants are available in the cell,

but none of them have been identified as an alternative electron donor for transmembrane reducing activities. Thus, depending on the redox system, the source of reducing equivalents seems limited to NADH, NADPH or ascorbate.

Extracellular substrates

Though plasma membrane redox systems have most frequently been characterized with the artificial electron acceptor ferricyanide, several other compounds have been used or proposed as substrates. Some of those substrates could have a physiological relevance. It is not clear to what extent different redox systems are involved in the reduction of these substrates.

Ferricyanide - Ferricyanide consists of trivalent iron (Fe^{3+}) with six CN^- ligands. Thus, the ferricyanide complex carries three negative charges: $Fe(CN)_6^{3-}$. Due to this high negative charge it cannot pass the plasma membrane. However, it is readily reduced by redox systems in the plasma membrane, yielding ferrocyanide, in which the iron ion is reduced to Fe^{2+}. Ferrocyanide ($Fe(CN)_6^{4-}$) is charged even stronger, and will not permeate the membrane either. Due to these non-permeant properties, and the convenient assays that are available to monitor the reaction, ferricyanide is ideally suited to study plasma membrane redox processes. However, its value can be debated due to its promiscuous character. Ferricyanide is frequently used as a general oxidant for e.g. many kinds of cytochromes. If multiple redox systems exist in the membrane, with different physiological substrates, ferricyanide is likely to be reduced by most of them.

A number of other mildly oxidizing dyes, such as indophenol, nitroblue tetrazolium and indigo tetrasulfonate are also readily reduced by plasma membrane redox systems (55, 64, 65). It is conceivable that many other oxidants, in addition to being reduced by anti-oxidants like ascorbate, can also be reduced directly by plasma membrane redox systems. Thus, the systems would be a part of the anti-oxidant defenses of the cell.

Oxygen - The reduction of oxygen with a single electron yields the superoxide anion, a reactive form of oxygen. Though the release of such reactive molecules is usually avoided in biological systems, neutrophils and macrophages actively generate them to attack foreign bodies, such as bacteria. The superoxide anion and other ROS react with and damage bacteria, resulting in their death. This generation of superoxide is usually called the respiratory burst, referring to the increased consumption of oxygen that can be observed. The protein complex responsible for the respiratory burst has been cloned and characterized extensively

(61). It was found to be a tightly controlled system, that uses intracellular NADPH as its electron source.

Non-transferrin bound iron – Many organisms can reduce Fe^{3+}, either as a free ion or chelated, to Fe^{2+}. This reduction is important for the subsequent transport of the iron to the cytoplasm, where it can be inserted into intracellular stores. Such transport mechanisms have been identified in yeast, plant roots and in the gut. However, similar systems were also found in cells that usually accumulate iron using transferrin. This transport protein sequesters iron in the bloodstream to prevent the generation of reactive oxygen species by Fenton type reactions, and is also essential for the receptor-mediated accumulation of iron. When any free iron is inadvertently present in the bloodstream, the ferric reductases apparently provide an alternative pathway for its removal. Thus, oxidative reactions can be prevented.

Ascorbate free radical and dehydroascorbate - Ascorbate is continuously subject to oxidation, both intra- and extracellularly. Therefore, many systems exist to ensure a swift regeneration of the vitamin. It has been shown that the oxidation of ascorbate in a solution can be slowed down by the addition of cells. This could be the result of the reduction of extracellular AFR by a plasma membrane redox system (66-70). Though the enzymatic nature of this stabilization of ascorbate has been questioned, most data indicate that extracellular AFR can be reduced enzymatically by a number of different cell-types (71). Other groups have shown that the fully oxidized form of ascorbate, DHA, can also be reduced extracellularly (37, 72). Thus, both AFR and DHA can be reduced by plasma membrane redox systems, preserving the level of ascorbate in the extracellular fluid.

Ascorbate and plasma membrane redox systems

The capability of cells to reduce extracellular oxidants has been studied for many years now. In 1979, Orringer and Roer discovered a significant increase in reduction of ferricyanide by erythrocytes after the accumulation of intracellular ascorbate by the cells (62). They concluded that ascorbate had left the erythrocyte to reduce ferricyanide, was reabsorbed as DHA, and reduced for a subsequent cycle. However, later studies revealed that ascorbate did not need to leave the cell to enhance ferricyanide reduction. Instead, its reducing equivalents were apparently transported across the membrane to extracellular ferricyanide (63, 73).

Functions

The physiological function of the ascorbate-dependent reductases has not yet been identified. The possibilities are similar to those mentioned earlier for NADH-dependent reductases; protection against oxidants, regeneration of extracellular ascorbate, or modulation of cell proliferation. The only experimental data were obtained using ferricyanide as an electron acceptor.

Molecular mechanism

Several mechanisms have been proposed to account for the effects of ascorbate. The process seems similar to NADH-dependent ferricyanide reduction. Proteins were isolated that reduce ferricyanide using NADH as an electron donor. Analogously, a protein could be present in the plasma membrane capable of using ascorbate as a substrate. Several findings support the involvement of a protein. The reduction of ferricyanide has saturable dose-response characteristics for both ferricyanide and ascorbate (74, 75).

Identification of plasma membrane redox systems

NADPH oxidase and related proteins

Not many plasma membrane redox systems have been isolated, and even less have been characterized at the molecular level. Only the respiratory burst NADPH oxidase of the neutrophil has been isolated, cloned and studied in detail. The oxidase, named cytochrome b_{558} or gp91phox (*g*lyco-*p*rotein, estimated weight *91* kDa, from *ph*agocyte *ox*idase) was found to contain a FAD group and two *b*-hemes to enable the electron transfer (61, 76, 77). Upon activation of the system, electrons are transported from intracellular NADPH to the FAD moiety. The heme groups are placed above each other between the transmembrane helices of the protein, and carry the electron from FAD, across the membrane, to extracellular oxygen (Figure 8). This reduces oxygen to superoxide ($O_2^{\bullet-}$), which can dismutate to H_2O_2, or form other reactive species. These will be cytotoxic to e.g. ingested microorganisms. The reactive oxygen species are usually generated in phagocytic vacuoles, but can also be produced at the plasma membrane.

The expression of the proteins from the respiratory burst complex is limited to neutrophils and macrophages. It is therefore not likely that it is also involved in other plasma membrane redox activities, such as the ferricyanide reduction found in many cells. Homologues of the phagocyte oxidase have been described in other

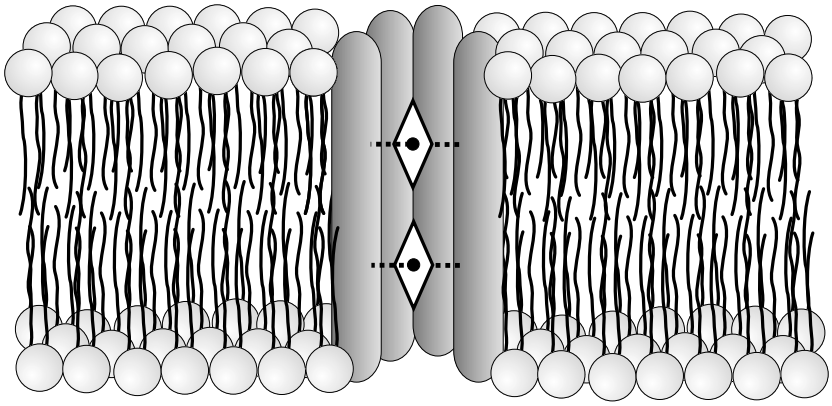


Figure 8. Bis-heme motif. Both cytochrome b_{561} and gp91phox share a structural similarity. They have two *b*-type hemes bound to histidine residues on their transmembrane helices. These heme groups are important in the transfer of electrons over the membrane.

tissues, and have different functions. In the thyroid gland, H_2O_2 is required for the synthesis of thyroid hormone by thyroperoxidase. A membrane protein has been isolated and cloned that uses NADPH to generate H_2O_2, but not $O_2^{\bullet-}$ (78, 79). The protein, which has only been found in the thyroid, strongly resembles gp91^{phox}. Another human homologue of gp91^{phox} is the Mox1 gene, an $O_2^{\bullet-}$ producing protein that is linked to cell proliferation (80). Yeast homologues of gp91^{phox} have yet another function. The Fre1 and Fre2 proteins do not reduce oxygen, but extracellular iron (81). In spite of the structural similarities, gp91^{phox} does not reduce iron, whereas Fre1 does not reduce oxygen to $O_2^{\bullet-}$ (82, 83).

Ascorbate converting systems

A number of membrane redox proteins are involved in the conversion of ascorbate or its oxidized forms. In humans, a cytochrome b_{561} is known from chromaffin granules of the adrenal gland (84-87). This cytochrome reduces ascorbate radicals in the lumen of the granule using reduced ascorbate on the cytoplasmic side of the membrane as an electron source. Cytochrome b_{561} has two heme groups in its transmembrane domain, which is similar to cytochrome *b* of mitochondrial complex III and to proteins related to gp91^{phox} (Figure 8). It is not clear whether cytochrome b_{561} is also expressed in the plasma membrane, but in plants evidence has been found for a cytochrome in the plasma membrane with properties very similar to the mammalian cytochrome b_{561} (88, 89). Also, *Arabidopsis* sequencing programs revealed the existence of (hypothetical) proteins with sequence homology to human cytochrome b_{561} (Acc.# CAA18169, gi:2980793). Thus, it is conceivable

that cytochrome b_{561} or similar proteins are also present in mammalian plasma membranes.

Plants also contain the enzyme ascorbate oxidase (AO), which mediates the oxidation of ascorbate by oxygen, generating AFR (90). Though AO is not a membrane redox protein, it is worth mentioning as a valuable tool in the study of ascorbate-dependent redox systems. It has frequently been used to either eliminate reduced ascorbate from reaction mixtures, or as a source of AFR to allow study of the interactions of the radical with plasma membrane redox systems. Also, it was the first ascorbate converting enzyme that was characterized at high resolution by X-ray diffraction.

NADH-dependent plasma membrane redox systems can also reduce extracellular AFR back to ascorbate, thus maintaining the level of anti-oxidants (70, 91-93). Finally, the reduction of fully oxidized ascorbate, DHA, has also been reported at the plasma membrane. Extracellular DHA was reduced to ascorbate, but reduction was also found during the transport of DHA to the intracellular space (37, 72). It is still unclear what proteins are involved in these reductions.

Ferric reductases

In mammals, transferrin is the primary mediator for transport and for the receptor mediated accumulation of iron. However, an alternative mechanism must be available for iron that is not transferrin bound. Also, a mechanism must exist to absorb iron from the lumen of the gut, which lacks transferrin. Several studies indeed revealed a capacity of cells to transport non-transferrin iron into the cytoplasm (94-97). Moreover, it was found that a reduction step was essential for transport of the iron, which is mainly present as Fe^{3+}. Thus, reductases in the plasma membrane allow iron to be transported independent of transferrin. In fact, reductases may also play a role in transferrin dependent transport. After binding to its receptor, transferrin is internalized by endocytosis. The resulting endosome is acidified, upon which Fe^{3+} is released from transferrin. Still, a free iron ion must be transported across the endosomal membrane to the cytoplasm. Recently, Nramp2 and DCT1 were identified as transporters of Fe^{2+} and other divalent cations, and were also found to be expressed in the endosomes (98-100). It is therefore likely that a ferric iron reductase is also part of the transport system in the endosome. However, human ferric reductases, both from the plasma membrane and from endosomes, remain to be isolated and characterized at the molecular level.

In contrast, components of an iron transport system have been cloned in yeast, and also in *Arabidopsis* (101). *Saccharomyces cerevisiae* contains a *b*-cytochrome called Fre1, capable of reducing iron (81, 83, 102). Fe^{2+} released by Fre1 then interacts with the Fet3/FTR1 protein complex capable of transporting Fe^{2+} across the plasma membrane. Strikingly, however, the Fet3 component of this complex oxidizes iron back to Fe^{3+} before transport by FTR1. Thus, in yeast reduction of iron is involved in transport, but transport protein nevertheless uses oxidized iron as its substrate. New data suggest that the transport of oxidized iron, analogous to the yeast model, is also possible in humans. Ceruloplasmin is a soluble oxidase homologous to the yeast Fet3, and is capable of oxidizing Fe^{2+} to Fe^{3+}. Also, the activity of ceruloplasmin was shown to stimulate non-transferrin iron transport. It was hypothesized that a trivalent metal-ion transporter is present that operates in concert with ceruloplasmin similar to the Fet3/FTR1 complex in yeast (103). However, this view remains controversial, as other studies indicated that ceruloplasmin is involved in the efflux of iron from the cells, rather than the influx (104). Though plasma membrane reductases are apparently involved in the transport of non-transferrin bound iron, the pathways and molecular components remain to be identified, especially in humans.

Cytochrome b_5 reductase

Cytochrome b_5 reductase is a common NADH-dependent enzyme for the reduction of cytochrome b_5, but has also been associated with plasma membrane redox activities. It exists as a membrane associated protein in most tissues, and as a soluble protein in erythrocytes (105). A deficiency in cytochrome b_5 reductase results in methemoglobinemia, a disease in which the reduction of oxidized hemoglobin, methemoglobin, is impaired (106). However, the cytochrome and its reductase are also involved in many other metabolic reactions (107). Membrane bound cytochrome b_5 reductase can be found on the endoplasmic reticulum, mitochondria, and also on the plasma membrane. The protein does not cross the membrane, but is anchored by a hydrophobic, myristoylated N-terminal tail. Nevertheless, it has been associated with a trans-plasma membrane reductase activity. Cytochrome b_5 reductase was shown to be a coenzyme Q reductase (108). It was suggested that the reductase reduced extracellular AFR through coenzyme Q, using the coenzyme as an electron shuttle across the membrane (69, 109). Thus, in spite of the fact that the reductase itself does not cross the membrane, extensive data show that it does play a role in the reduction of extracellular substrates.

Purified proteins

Various other groups have isolated active protein fractions, which remain to be characterized at the molecular level. The majority of these studies were on NADH-ferricyanide reductase activities, often yielding proteins of about 30 kD (110-113). It is difficult to verify the physiological membrane orientation of such proteins, as their activity is tested without an intact membrane environment. Thus, a protein may therefore be identified as an NADH:ferricyanide reductase while it is only active on the inner side of membrane. It is conceivable that some of the 30 kD proteins that were isolated are in fact cytochrome b_5 reductase, which has a mass of 34 kD and the capacity to reduce ferricyanide. As described, it was suggested that it could drive the reduction of extracellular ferricyanide through coenzyme Q. However, other protein fractions had properties, like glycosylation, that distinguished them from the b_5 reductase. Further study could reveal whether the proteins have a novel sequence, and whether they are involved in the reduction of extracellular substrates.

Non-protein electron transporters

It has been suggested that electrons can be transported across the plasma membrane without involvement of a specialized protein. Small lipid-soluble compounds existing in different redox states diffuse across a membrane, potentially moving an electron. Candidate molecules for such a phenomenon are α-tocopherol, coenzyme Q and menadione (69, 114-119). They could cycle through the plasma membrane, be oxidized at the cell surface by e.g. ferricyanide, and reduced at the intracellular face by an enzyme system, ascorbate, GSH or other reductants. Indeed, it was found that liposomes containing α-tocopherol could transfer electrons from ascorbate to extravesicular ferricyanide (120). Also, extensive evidence has been produced for the essential role of coenzyme Q in the reduction of extracellular AFR (69, 109). A yeast mutant, defective in the synthesis of coenzyme Q, was also deficient in the reduction of AFR, while the mutation could be rescued by the addition of exogenous coenzyme Q (121). On the other hand, the involvement of such electron carriers has been disputed in other publications, e.g. questioning the mobility of longchained quinones like coenzyme Q_{10} (122). As yet, it is not clear what the relative contributions are of low-molecular weight carriers, protein systems or combinations of both in the reduction of extracellular substrates. Also, these contributions may differ depending on the intra- and extra-vesicular substrates of the redox reaction.

Effects on cell proliferation

All of the functions discussed so far concern the rather straightforward reduction of an extracellular substrate by the cell. However, plasma membrane redox systems have also been implicated in the area of cellular proliferation and differentiation. A network of signaling molecules integrates information on the viability of the cell, metabolic condition, DNA integrity and extracellular signals from circulating hormones, neighboring cells or autocrine loops. Based on this information, a decision can be made to start cell division. Cell division is under such tight control to prevent unbridled growth of tissue in an organism. Loss of control can lead to e.g. cancer, and should be avoided at all cost in multi-cellular organisms. Due to the highly branched structure of signal transduction pathways, many levels exist at which proliferation could be influenced. One factor that has been found to promote cell division is a moderately oxidizing environment. Oxidizing conditions are usually regarded as harmful, and indeed, at higher levels, will damage cells. However, several groups have reported increased proliferation when proper levels of oxidants are added (65, 123-126). Even endogenous proteins that produce oxidants have been linked to proliferation. Mox1, a homologue of the respiratory burst oxidase $gp91^{phox}$, is a human membrane protein that produces superoxide anions. Transfection of cells with the Mox1 gene increases proliferation, whereas it is inhibited by antisense oligonucleotides against Mox1.

Some of the oxidants that were reported to promote cell proliferation are ferricyanide and AFR. It was suggested that their mitogenic effects could be mediated by plasma membrane redox activity. However, different views exist on the mechanism leading from electron transport to increased cell division. It seems likely that a cascade of events should eventually trigger a mitogenic signal transduction pathway. Indeed, it has been reported that oxidants can have effects on signaling pathways controlling the cell (127, 128).

Role of changes in pH

The flow of electrons across the membrane can result in the concomitant movement of protons, and thus in changes in the intracellular pH. For instance, the reduction of extracellular ferricyanide has been found to be accompanied by the extrusion of protons (59, 129), and cytoplasmic alkalinization. In turn, cellular alkalinization has been linked to a mitogenic response (130). Thus, changes in the intracellular pH may link the reduction of ferricyanide to effects on cell proliferation. However, conflicting reports show intracellular acidification after ferricyanide treatment, and

a link of acidification to a mitogenic response, albeit in a different cell-line (131, 132). Thus, controversy exists on the role of intracellular pH on cell proliferation, and also on the effect of ferricyanide reduction on intracellular pH.

NADH / NAD^+ ratio

The reduction of extracellular ferricyanide or AFR causes the consumption of intracellular NADH. It has been suggested that the altered ratio of NAD^+ versus NADH resulting from this consumption could be responsible for the stimulation of cell proliferation by such compounds (133, 134). This was supported by other data that correlated changes in NADH levels to the proliferative state of cells (135).

A special case of growth stimulation by ferricyanide is in so-called ρ^0 cells (136-138). ρ^0 cells are cells that have been cultured for a prolonged period in the presence of low concentrations of ethidium bromide. The cells lose their mitochondria due to this treatment, and therefore depend on glycolysis for their energy needs. This results in excess levels of NADH, which is normally consumed by oxidative phosphorylation to produce ATP. Consumption of NADH by a plasma membrane redox system releases NAD^+, allowing glycolysis to proceed. Thus, here the redox system might act as a safety valve for NADH levels.

It is not clear how the reduction of NADH levels can promote cell division in normal cells, capable of oxidative phosphorylation. In contrast with ρ^0 cells, normal cells can presumably maintain an optimal NADH/NAD^+ ratio, while unnecessary consumption of NADH withdraws their reducing potential from ATP synthesis. It is therefore likely that this specific way to promote growth only applies to cells with defective oxidative phosphorylation.

Hydrogen peroxide

Extensive literature exists on the interaction of hydrogen peroxide (H_2O_2) with signal transduction pathways. It was found that H_2O_2, which freely crosses the membrane, causes inactivation of protein tyrosine phosphatases (PTPs) (123, 139). Thus, an increased tyrosine phosphorylation level can result from H_2O_2, and the activation of several mitogenic pathways. The stress-related transcription factor NFκB is also strongly associated with the effects of H_2O_2 (140). These signaling events could be responsible for the increase in cell proliferation that has been found after treatment of cells with H_2O_2 (123, 124). It has even been shown that the activation of receptor tyrosine kinases by endogenous growth factors induces

the production of H_2O_2, and thus that it may be an essential component of the signaling machinery in the cell (141).

Though the effects of the oxidant H_2O_2 on signaling and proliferation seem to be well established, it does not seem likely that plasma membrane redox systems are involved in the mechanism. In fact, H_2O_2 could be an alternative for plasma membrane redox systems as the explanation for the effects of oxidants on cell proliferation. It has been suggested that the formation of semiquinones from the reactions of extracellular oxidants with quinones in the membrane, could lead to the formation of H_2O_2 (142). This H_2O_2 could subsequently induce proliferation in the cell.

Ascorbate and cell proliferation

The addition of ascorbate to serum-starved cells has mitogenic effects very similar to that of ferricyanide (143, 144), though growth inhibition has also been reported (145, 146). In spite of the fact that ascorbic acid is an anti-oxidant, a reducing agent, its effect has also been linked to plasma membrane redox systems. Not ascorbate itself, but its oxidation product might be a substrate for plasma membrane reductases. Ascorbate will always auto-oxidize at some rate, and a transmembrane process can subsequently reduce the resulting ascorbate radicals. Thus, AFR can induce transmembrane electron transport, similar to ferricyanide. Data have been presented indicating that indeed AFR, and not ascorbic acid or DHA, induces increased proliferation in cells (134, 143). However, AFR was produced in these studies by mixing ascorbate and DHA, an unreliable method which cannot produce a stable concentration of the radical. In attaching cells, the effects of ascorbate may be partly due to its role in the formation of the extracellular matrix, as ascorbate is a well-known co-factor in the biosynthesis of collagen (147). However, ascorbate also affects proliferation in cells in suspension, and an effect on collagen synthesis can thus not be the only explanation. A possible alternative would be a non-specific anti-oxidant effect of ascorbate in the absence of serum. At normal serum levels, albumin can act as a scavenger for ROS in the medium. In absence of serum, an excessive amount of oxidative stress may be inflicted on the cells, impairing cell division. Such oxidative injury could be prevented by ascorbate. It was shown that ascorbate could prevent apoptosis resulting from the removal of serum (148, 149). Thus, the anti-oxidant function of ascorbate could contribute to higher cell numbers not by promoting cell proliferation, but by inhibiting cell death.

Plasma membrane redox systems and proliferation

Though it may be tempting to speak of 'the mitogenic effect of oxidants', the type of oxidant is probably an important factor in the pathway leading to proliferation. Some oxidants easily permeate the membrane to induce intracellular effects, whereas others must use different means to pass a signal across the membrane. The involvement of plasma membrane redox systems is possible, but alternative explanations have also been suggested. In the case of ascorbate, it is even uncertain whether its effect is mediated by the oxidant effects of the ascorbate radical, or through e.g. an anti-oxidant or collagen synthesis effect. Even if plasma membrane redox systems are indeed involved in the effectuation of a proliferative response, the molecular mechanisms remain to be solved.

Outline of this thesis

In this thesis, a study is described on the interaction of ascorbate with plasma membrane redox systems. Though it was known that ascorbate could promote the reduction of extracellular ferricyanide, more detailed information was hardly available. For instance, it was unclear how the ascorbate-dependent reduction of substrates relates to the reduction in the absence of ascorbate, or what the physiological substrates of these systems could be. Experiments described in this thesis provide answers to these questions, and are aimed to further investigate the mechanism that allows reducing equivalents to be transported across the membrane.

Chapter 2 explores the mechanisms leading to AFR formation in cell-free systems. Autoxidation of ascorbate, the disproportionation reaction and metal-ion-catalyzed oxidation of ascorbate are discussed. It is concluded that the equilibrium constant of the disproportionation reaction should not be used to predict AFR concentrations in a solution, as the actual concentration can deviate significantly from calculated values under some conditions.

Chapter 3 of this thesis deals with the reduction of ferricyanide by HL60 cells. The reduction rate can be significantly increased by adding ascorbate or DHA to the cells. The mechanism of this stimulation was studied, and it is concluded that ascorbate can be an intracellular substrate for a ferricyanide reductase. Inhibitor studies indicated that the ascorbate-dependent system is distinct from the NADH-dependent system that reduces ferricyanide in the absence of ascorbate.

Chapter 4 describes an ascorbate-dependent redox process in erythrocytes. Evidence was found that these cells can reduce extracellular AFR using intracellular ascorbate as an electron source. Thus, it is concluded that AFR can be a physiological substrate for the ascorbate-dependent redox system in the erythrocyte.

Chapter 5 shows that the ascorbate-dependent reduction of AFR by the erythrocyte has an electrogenic character. This confirms the transmembrane nature of the reaction. Also, it supports the hypothesis that a protein in the plasma membrane is responsible for the reductase activity.

Chapter 6 reports a study on the possible involvement of cytochrome b_{561} in the ascorbate-dependent reduction of extracellular AFR. Antibodies could not detect the cytochrome in extracts of erythrocyte membranes, nor could its mRNA be detected in erythrocyte precursor cells. It is therefore highly unlikely that cytochrome b_{561} is involved in this redox activity of the erythrocyte.

Chapter 7 describes technical aspects of the study of ascorbate and its interaction with cells.

The results presented in this thesis are reviewed in a general discussion in chapter 8.

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CHAPTER 2

Electron spin resonance study on the formation of ascorbate free radical from ascorbate: the effect of dehydroascorbic acid and ferricyanide

This chapter was adapted from MM Van Duijn, J Van der Zee, and PJA Van den Broek, Electron spin resonance study on the formation of ascorbate free radical from ascorbate: the effect of dehydroascorbic acid and ferricyanide. Protoplasma, 205(1-4): 122-128, 1998.

Summary

Ascorbate free radical is considered to be a substrate for a plasma membrane redox system in eukaryotic cells. Moreover, it might be involved in stimulation of cell proliferation. Ascorbate free radical can be generated by autoxidation of the ascorbate dianion, by transition metal-dependent oxidation of ascorbate or by an equilibrium reaction of ascorbate with dehydroascorbic acid. In this study, we investigated the formation of ascorbate free radical, at physiological pH, in mixtures of ascorbate and dehydroascorbic acid by electron spin resonance spectroscopy. It was found that at ascorbate concentrations lower than 2.5 mM, ascorbate free radical formation was not dependent on the presence of dehydroascorbic acid. Removal of metal ions by treatment with Chelex 100 showed that the rate of autoxidation under these conditions was less than 20% of the total oxidation. Therefore, it is concluded that at low ascorbate concentrations generation of ascorbate free radical mainly proceeds through metal-ion-dependent reactions. When ascorbate was present at concentrations higher than 2.5 mM, the presence of dehydroascorbic acid increased the ascorbate free radical signal intensity. This indicates that, under these conditions, ascorbate free radical is formed by a disproportionation reaction between ascorbate and dehydroascorbic acid, having a K_{equil} of $6 \cdot 10^{-17}$ M. Finally, it was found that the presence of excess ferricyanide completely abolished ascorbate free radical signals, and that the reaction between ascorbate and ferricyanide yields dehydroascorbic acid. We conclude that, for studies under physiological conditions, ascorbate free radical concentrations cannot be calculated from the disproportionation reaction, but should be determined experimentally.

Introduction

Ascorbate is considered to be one of the main anti-oxidants in biological systems (1-4). It can be oxidized by radicals and oxidants in two successive one-electron steps (5). The first one-electron oxidation gives ascorbate free radical, which can subsequently be oxidized to dehydroascorbic acid. This latter compound is unstable and is irreversibly degraded to potentially toxic compounds (6). In order to prevent accumulation of toxic ascorbate metabolites, cells are equipped with efficient ascorbate regenerating systems. One way to achieve this is by transporting extracellular DHA to the cell interior after which it can be reduced to ascorbate (7). Alternatively, it has been reported that a plasma membrane localized redox system may be involved in ascorbate regeneration (8, 9). This system would reduce extracellular AFR to ascorbate at the expense of intracellular reducing equivalents.

Interestingly, it has been claimed that this redox system is involved in regulation of growth of leukemic cells (10). Moreover, links to intracellular signal transduction pathways have been reported (11-13).

In order to study AFR-driven processes in cells, the generation of known amounts of AFR is a prerequisite. Experimentally, this has been achieved in two ways. One method makes use of the enzyme ascorbate oxidase which catalyzes the oxidation of ascorbate to AFR (14). The other method to generate AFR is by using mixtures of ascorbate and DHA, where AFR is formed in an equilibrium reaction (15, 16). Though the equilibrium results from both symproportionation and disproportionation reactions, it will be referred to as the disproportionation reaction. In mixtures of ascorbate and DHA, AFR concentrations can be calculated from ascorbate and DHA concentrations and the equilibrium constant. A number of studies on the effect of AFR on cells, and especially on plasma membrane redox systems, use the latter method to estimate AFR concentrations (10, 17-19). Recently, however, it was reported that addition of DHA to an ascorbate solution did not increase the AFR concentration, suggesting that AFR was not formed by the equilibrium reaction (20). The aim of this study was to determine whether the disproportionation reaction can indeed be used to estimate AFR concentrations. Since it has been assumed that potent oxidants, like ferricyanide, do not significantly influence the reaction between ascorbate and DHA (19), the influence of ferricyanide on AFR levels was also investigated. AFR formation was determined by electron spin resonance (ESR) spectroscopy and it is concluded that for studies under physiological conditions, the disproportionation reaction does not adequately describe AFR formation.

Materials and methods

Ascorbate and DHA were obtained from Aldrich, Chelex 100 from Bio-Rad and TEMPO (2,2,6,6-tetramethylpiperidine-N-oxyl) came from ICN Biochemicals. All solutions were prepared in ultrapure water that was prepared using a Millipore Milli-Q ultrapurification system. Experiments were performed at room temperature with air-saturated solutions in 0.2 M sodium phosphate buffer, pH 7.4. Stock solutions of ascorbate and DHA were always made up freshly in buffer in plastic tubes and kept on ice. When necessary, the pH was adjusted to 7.4. It was checked, by measuring the absorbance at 265 nm, that the ascorbate concentration of the stock solution remained constant during the course of the experiments. A solution of DHA could be kept for 60 min on ice without significant degradation. For chelex treatment, the 0.2 M sodium phosphate buffer, pH 7.4, was treated overnight with Chelex 100 resin by the batch method (21).

DHA concentrations were determined as described by Vera et al (22). Briefly, DHA samples were incubated with 5 mM dithiothreitol. This converts DHA into ascorbate, which was determined from its absorption at 265 nm, with an extinction coefficient of 14,500 M^{-1} cm^{-1} (21).

ESR spectra were obtained using a JEOL-RE2X spectrometer operating at 9.36 GHz with a 100-kHz modulation frequency, equipped with a TM_{110} cavity. The samples were transferred to the quartz flat cell by means of a rapid sampling device (23). In all experiments the complete spectrum of AFR was recorded within 3 min after preparing DHA solutions. The total area under the ESR absorption curve is proportional to the amount of paramagnetic species in the sample and this can be used to quantify AFR. The concentrations of AFR were determined by double integration of the ESR spectra with TEMPO as a standard. The TEMPO spectra were obtained with the same instrument settings as used for the AFR spectra, except for receiver gain. Saturation effects were accounted for and a microwave power of 40 mW was used (24). ESR spectrometer settings were: 1 Gauss modulation amplitude, 0.1 s time constant, 6 G/min scan rate.

Oxygen consumption was measured with a Clark-type electrode connected to a YSI Model 5300 Biological Monitor.

Ferricyanide concentrations were measured spectrophotometrically at 420 nm.

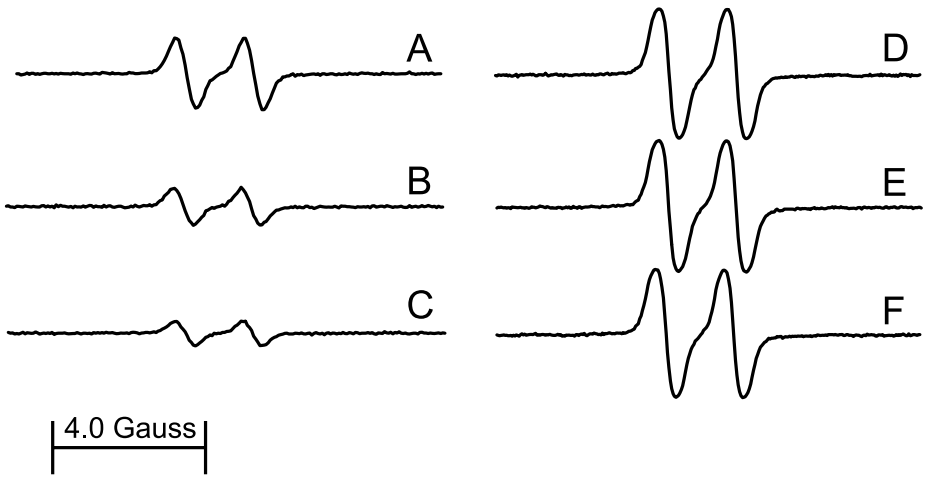

Figure 1: AFR generation from 6 mM ascorbate and 6 mM ascorbate plus 6 mM DHA. A) ESR signal obtained from a solution of 6 mM ascorbate in 0.2 M sodium phosphate, pH 7.4; B) as for A but with 50 µM EDTA; C) as for A but with 50 µM DTPA; D) 6 mM ascorbate plus 6 mM DHA in 0.2 M sodium phosphate, pH 7.4; E) as for D but with 50 µM EDTA; F) as for D but with 50 µM DTPA. Spectrometer settings were as described in Materials and Methods.

Results

Generation of ascorbate free radical

Ascorbate free radical can be detected by ESR spectroscopy as a doublet with hyperfine splitting a^{H4} = 1.8 G (25). This doublet can readily be observed in a solution of 6 mM ascorbate in phosphate buffer, showing that ascorbate free radical is formed in solution (Fig. 1A). Addition of the chelators EDTA or DTPA, to remove trace amounts of Cu^{2+} or Fe^{3+} ions respectively, decreased AFR signal intensity considerably (Figs. 1B-C). This indicates that a large part of the AFR formation from ascorbate was mediated by catalytic amounts of these metal ions, according to the reaction (21, 26, 27):

$$\text{Ascorbate} + Me^{n+} \longrightarrow \text{AFR} + Me^{(n-1)+} \qquad \text{(Reaction I)}$$

Addition of 6 mM DHA to a 6 mM ascorbate solution caused an increase in AFR signal intensity as compared to the signal obtained from ascorbate alone (compare Fig. 1D to A). In the presence of DHA the chelators DTPA and EDTA did not have any effect on AFR signal intensity (Fig. 1E, F). These data suggest that, at these concentrations, AFR formation is determined by a disproportionation reaction between DHA and ascorbate (15, 16):

$$\text{Ascorbate}^- + \text{DHA} \longleftrightarrow 2\ \text{AFR}^- + H^+ \qquad \text{(Reaction II)}$$

However, when these experiments were performed with 1 mM ascorbate, the results were different (Fig. 2). The AFR doublet was again readily observed in a solution of ascorbate (Fig. 2A) and addition of EDTA and DTPA gave a similar reduction in AFR signal intensity as was found with 6 mM ascorbate (not shown). Chelex 100 treatment of the phosphate buffer, which removes both iron and copper ions, also considerably decreased signal intensity, confirming that AFR formation was mainly mediated by reaction I. The background signal that is observed after Chelex 100 treatment (Fig. 2B) is thought to arise from autoxidation of the ascorbate dianion (21):

$$\text{Ascorbate}^{2-} + O_2 \longrightarrow \text{AFR}^- + O_2^{\bullet -} \qquad \text{(Reaction III)}$$

Figure 2C shows that addition of 1 mM DHA to 1 mM ascorbate did not increase signal intensity, compared to the situation where only ascorbate was present (Fig. 2A). This is in clear contrast with the results obtained with 6 mM DHA and ascorbate (Fig. 1A,D). In Chelex-100-treated buffer, however, addition of 1 mM DHA to 1 mM ascorbate did cause an increase in AFR signal intensity as compared

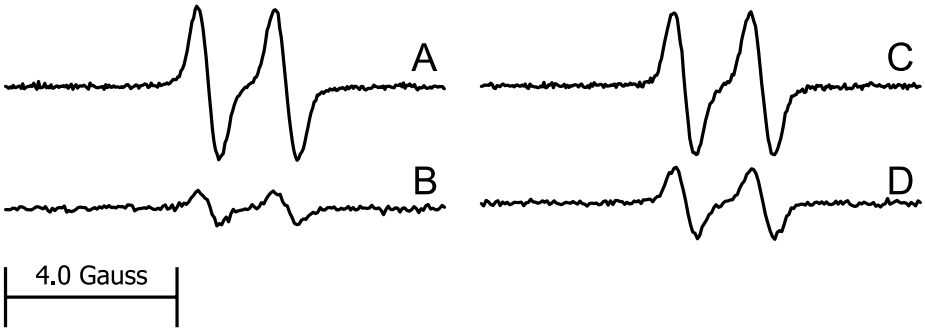

Figure 2: AFR generation from 1 mM ascorbate and 1 mM ascorbate plus 1 mM DHA. A) ESR signal obtained from a solution of 1 mM ascorbate in 0.2 M sodium phosphate, pH 7.4; B) as for A, but in buffer treated with Chelex 100; C) 1 mM ascorbate plus 1 mM DHA in 0.2 M sodium phosphate, pH 7.4; D) as for C, but in buffer treated with Chelex 100. Spectrometer settings were as described in Materials and Methods.

to ascorbate alone (compare Fig. 2D to B). In the presence of EDTA or DTPA the same signal was obtained as shown in Fig. 2D (data not shown).

These results suggest that, when metal ions are present, reaction I determines AFR formation at low ascorbate and DHA concentrations, whereas at higher ascorbate and DHA concentrations reaction II prevails (Figs. 1 and 2). Therefore, we decided to determine AFR generation at various concentrations of ascorbate and DHA, both in 'regular' buffer and in buffer treated with Chelex 100 (Fig. 3).This was compared to the amount of AFR formed in ascorbate solutions in regular buffer. It was found that, in regular buffer, at ascorbate concentrations lower than 2.5 mM the formation of AFR was independent of the presence of DHA, and was mainly driven by metal ions. Only at higher concentrations, DHA could augment AFR formation, showing that reaction II determined the AFR concentration.

In reaction I, reduced metal ions are reoxidized in an O_2-dependent way (28). Thus, it can be expected that addition of ascorbate to a solution would induce O_2 consumption. Figure 4 shows that this is indeed the case. It should be noted that after 5 min of incubation only about 20 % of the oxygen was consumed. This shows that AFR measurements by ESR spectroscopy were not oxygen-limited, since these measurements never took more than 3 min. In the presence of DHA similar results were obtained (data not shown).

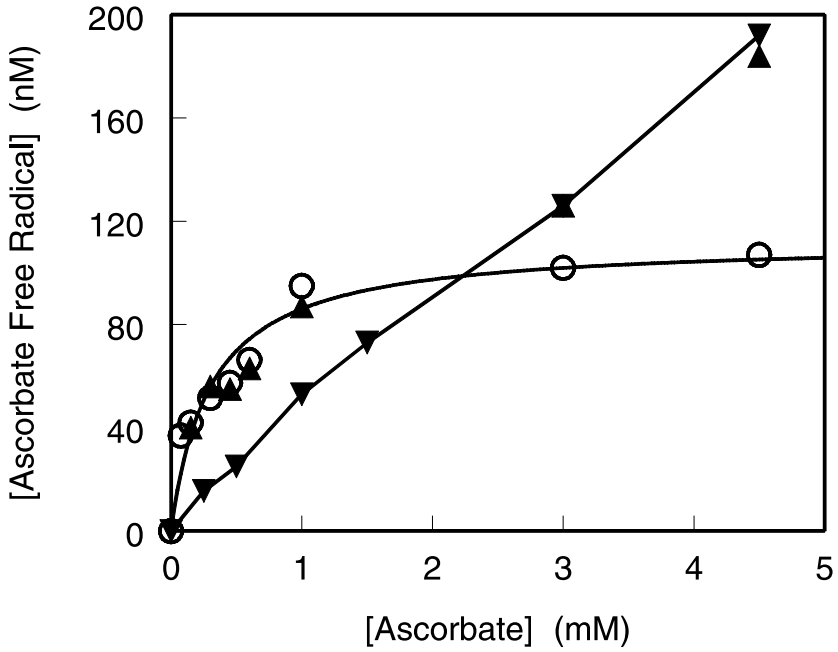

Figure 3: Dependence of the AFR generation on the ascorbate concentration. AFR generation was measured in 0.2 M sodium phosphate, pH 7.4, containing ascorbate (○) or equimolar concentrations of ascorbate and DHA (▲), or in buffer treated with Chelex 100, containing equimolar concentrations of ascorbate and DHA (▼). Spectrometer settings were as described in Materials and Methods.

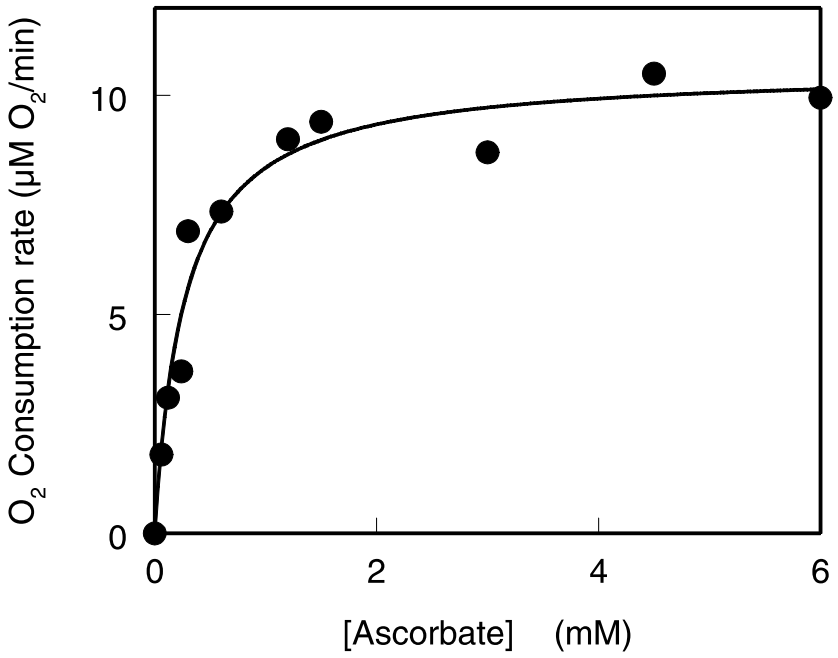

Figure 4: Dependence of the oxygen consumption rate on the ascorbate concentration. The initial rate of oxygen consumption was measured in 0.2 M sodium phosphate pH 7.4, containing various amounts of ascorbate.

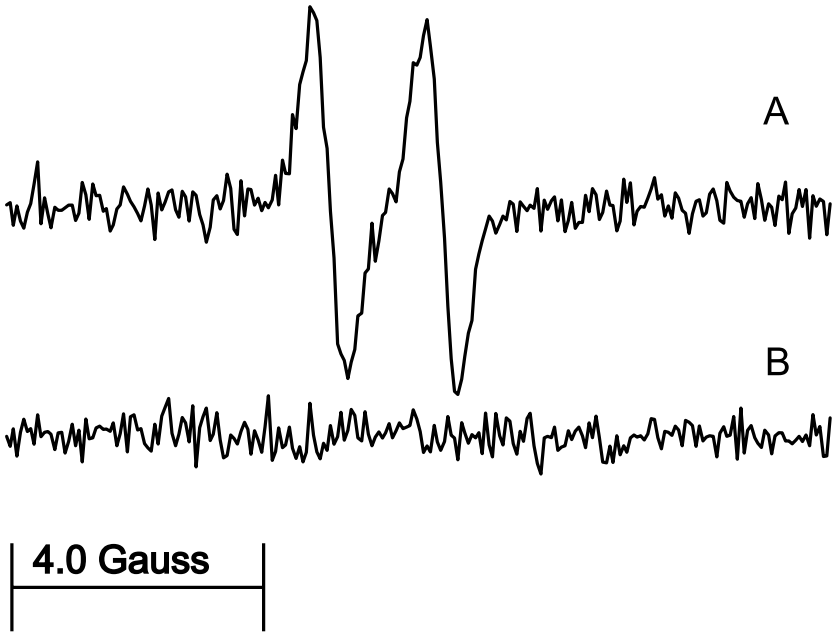

Figure 5: The influence of ferricyanide on the formation of AFR. A) 25 µM ascorbate in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4; B) as for A but with 1 mM ferricyanide. ESR settings were as in figure 1.

Equilibrium constant

The disproportionation reaction is an equilibrium reaction for which the equilibrium constant can be described as (29):

$$K_{equil} = \frac{[AFR^-]^2 [H^+](K_a + [H^+])}{[Ascorbate]_{total} [DHA] K_a} \qquad \text{(Equation 1)}$$

with $[ascorbate]_{total} = [ascorbate^-] + [H\text{-}ascorbate]$

and $K_a = [ascorbate^-][H^+]/[H\text{-}ascorbate]$.

At a particular pH, K_{equil} can be determined by measurement of the AFR concentration by ESR spectroscopy in a solution of known concentrations of ascorbate and DHA. DHA is unstable at pH 7.4 and at room temperature, and decays with a half-life of about 12 min (data not shown). Therefore, AFR measurements were performed within 3 min after mixing ascorbate and DHA. After correction for decay of DHA, it was found that, at pH 7.4, in a mixture with an initial concentration of 30 mM ascorbate and 30 mM DHA, K_{equil} amounted to $6 \cdot 10^{-17}$ M.

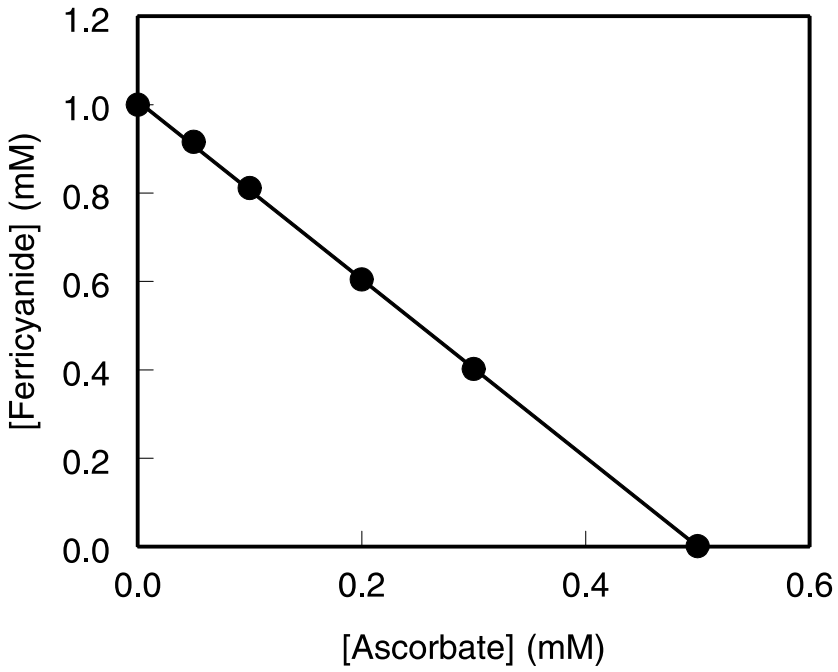

Figure 6: The influence of ascorbate on the ferricyanide concentration. Ferricyanide (1 mM) was mixed with various amounts of ascorbate in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4. Ferricyanide concentration was determined from its absorbance at 420 nm.

Effect of ferricyanide

In a number of studies on plasma membrane redox systems, ascorbate or ascorbate plus DHA were incubated simultaneously with ferricyanide (19, 30). Though ferricyanide is a potent oxidant, it was assumed that it did not significantly affect the AFR concentration (19). To determine the AFR concentration under these experimental settings, ESR studies were performed. Figure 5 shows that incubation of 25 mM ascorbate with 1 mM ferricyanide completely abolished the AFR signal, indicating that ascorbate was completely converted to dehydroascorbic acid. This was corroborated by the experiments presented in figure 6, which show that upon addition of ascorbate to 1 mM ferricyanide, the concentration of ferricyanide decreased with a stoichiometry of two mol ferricyanide per mol of ascorbate added.

Discussion

Ascorbate free radical can be formed from ascorbate in solution by various mechanisms (reactions I-III). Autoxidation of the ascorbate dianion (reaction III) results in the formation of AFR, and is dependent on pH and ascorbate concentration (24). The experiments performed in this study were all done at physiological pH 7.4, where autoxidation is low (Fig. 2B). A second mechanism (reaction I) involves the

transition-metal-ion-catalyzed oxidation of ascorbate (21) Under normal laboratory conditions buffers are always contaminated by trace amounts of iron and copper (21). A third mechanism (reaction II) involves an equilibrium reaction between ascorbate and DHA yielding two AFR molecules (16). Finally, AFR can also be generated enzymatically with ascorbate oxidase.

Our results show that at ascorbate concentrations lower than 2.5 mM the metal-ion-dependent reaction prevails (Fig. 3). First, removal of metal ions by Chelex 100 treatment decreased the AFR signal by at least 80%, showing that the rate of autoxidation is less than 20% of the total rate under these conditions (Fig. 2). Second, addition of DHA did not affect AFR signal intensities in 'regular' buffer (Figs. 2 and 3). Only when metal ions were removed, by addition of EDTA or DTPA (data not shown) or by treatment with Chelex 100 (Fig. 2D), the addition of DHA increased AFR signal intensity. This shows that, when the concentration of metal ions was low, the disproportionation reaction determined the AFR concentration. In regular buffer the disproportionation reaction will also take place, but at ascorbate concentrations lower than 2.5 mM the AFR concentration is determined by metal-ion-dependent oxidation of ascorbate. Thus, it is concluded that, in ascorbate-DHA mixtures, the actual AFR level strongly depends on the reaction conditions, and especially on the presence of metal ions and on the concentrations of ascorbate and DHA.

It has been reported that the metal ions involved in this reaction are Fe^{3+} and Cu^{2+} ions (21). During ascorbate oxidation these ions are reduced to Fe^{2+} and Cu^{1+} and must be re-oxidized in order to remain catalytically active. This oxidation involves molecular oxygen and probably generates hydroxyl radicals (28). The results presented in figure 4 reveal that ascorbate stimulates O_2 consumption under conditions where AFR is formed. Moreover, it shows that O_2 consumption does not have a strong dependence on the ascorbate concentration. This is consistent with the view that the catalyst of the reaction is present in trace concentrations, and therefore that regeneration of the metal ions is rate limiting. It should be noted that oxygen consumption is not affected by the addition of DHA (data not shown). At concentrations above 2.5 mM, the final AFR concentration is determined by the equilibrium reaction, but autoxidation and metal ion dependent reactions still take place at the same level as without DHA. Thus, oxygen is consumed.

At ascorbate concentrations higher than 2.5 mM the disproportionation reaction determines the AFR concentration, as equimolar mixtures of DHA plus ascorbate gave higher AFR signal intensities than ascorbate alone (Figs. 1 and 3). The fact that under these conditions neither EDTA, DTPA nor Chelex 100 treatment influenced

the AFR concentration shows that the disproportionation reaction is not mediated by metal ions and strengthens the view that this mechanism is different from the metal-ion-dependent reaction.

Equilibrium constants for the disproportionation reaction have been determined by Von Foerster in the pH range 4-6.4, according to equation 2 (16):

$$K_{equil}^{Foerster} = \frac{[AFR]^2}{[Ascorbate][DHA]} \qquad \text{(Equation 2)}$$

Extrapolation of the data to pH 7.4 yielded a constant of 10^{-8} (15). A more general way of describing the equilibrium constant is the one presented in equation 1, which relates to equation 2 as:

$$K_{equil} = K_{equil}^{Foerster} \frac{[H^+](K_a + [H^+])}{K_a} \qquad \text{(Equation 3)}$$

Thus, the equilibrium constant of 10^{-8}, determined by Lumper et al, would, in equation 3, give a K_{equil} of $4 \cdot 10^{-16}$ M at pH 7.4, which is much higher than the value we determined in this study, i.e., $6 \cdot 10^{-17}$ M. The reason for this difference may be due to the settings of the ESR spectrometer. We utilized settings as determined by Buettner and Jurkiewicz, which give optimal signal-to-noise ratios (24). Secondly, we used a different calibration procedure, using the water soluble standard TEMPO, whereas Foerster et al used the solid standard diphenylpicryl-hydrazyl (16). Since AFR signals were generated in solution, a water-soluble standard should be used, rather than a solid one. Finally, our measurements were performed at pH 7.4, whereas the previously used constant of 10^{-8} was obtained through extrapolation from data at more acidic pH values.

Ferricyanide can react with ascorbate and AFR, according to the reactions:

$$Ascorbate^- + \text{ferricyanide} \longrightarrow AFR^- + \text{ferrocyanide} + H^+ \qquad \text{(Reaction IV)}$$

$$AFR^- + \text{ferricyanide} \longrightarrow DHA + \text{ferrocyanide} \qquad \text{(Reaction V)}$$

It has been suggested that reaction V proceeds more slowly than reaction IV, and that in mixtures of ascorbate and ferricyanide AFR would still be present (19). On the other hand, it has been claimed that AFR is more reactive towards ferricyanide than ascorbate (5), suggesting that once AFR is formed it would rapidly react to DHA. Our results confirm the latter view, as no AFR could be detected in mixtures of ascorbate/AFR plus ferricyanide (Fig. 5). Moreover, ascorbate caused a decrease of the ferricyanide concentration within seconds, with a stoichiometry of two mol

ferricyanide reduced per mol ascorbate added (Fig. 6). This shows that in mixtures where ferricyanide is present in excess, as used for some plasma membrane reductase measurements, no AFR is present.

AFR is referred to as a substrate for a plasma membrane redox system, and could play a role in cell proliferation. In several studies on these topics, AFR concentrations have been calculated using the disproportionation reaction (10, 17-19). Thus, it has been assumed that AFR concentrations would be higher in solutions containing ascorbate plus dehydroascorbic acid than in solutions of only ascorbate. The results presented in this chapter show that one has to be cautious with these conclusions. Especially at low ascorbate concentrations, the AFR concentration is not determined by the disproportionation reaction, but rather by the metal-ion-catalyzed oxidation of ascorbate. With ascorbate concentrations in the micromolar range huge differences occur between calculated AFR concentrations, based on reaction II, and the experimentally measured concentrations. This conclusion is not only valid for sodium-phosphate buffers but also for more physiological media, like RPMI supplemented with fetal bovine serum. In the latter medium we observed that, at low ascorbate concentrations, the AFR concentration was much higher than calculated on basis of the disproportionation reaction (data not shown), which indicates that the presence of proteins does not prevent metal-driven AFR formation. This once again shows that AFR concentrations cannot simply be calculated, but should be determined using ESR spectroscopy, as it is strongly dependent on medium conditions.

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CHAPTER 3

Ascorbate stimulates ferricyanide reduction in HL60 cells through a mechanism distinct from the NADH-dependent plasma membrane reductase

This chapter was adapted from MM Van Duijn, J Van der Zee, J VanSteveninck, and PJA Van den Broek, Ascorbate stimulates ferricyanide reduction in HL-60 cells through a mechanism distinct from the NADH-dependent plasma membrane reductase. J Biol Chem, 273(22): 13415-13420, 1998

Summary

The non-permeating oxidant ferricyanide is reduced by the plasma membrane redox system of HL60 cells. The rate of reduction is strongly enhanced by ascorbate or dehydroascorbate. The aim of this study was to determine the mechanism by which ascorbate and dehydroascorbate accelerate ferricyanide reduction in HL60 cells. Addition of ascorbate or dehydroascorbate to cells in the presence of ferricyanide led to the intracellular accumulation of ascorbate. Control experiments showed that extracellular ascorbate was rapidly converted to dehydroascorbate in the presence of ferricyanide. These data suggest that intracellular ascorbate originates from extracellular dehydroascorbate. Accumulation of ascorbate was prevented by inhibitors of dehydroascorbate transport into the cell. These compounds also strongly inhibited ascorbate-stimulated ferricyanide reduction in HL60 cells. Thus it is concluded that the stimulation of ferricyanide reduction is dependent on intracellular accumulation of ascorbate. Changing the α-tocopherol content of the cells had no effect on the ascorbate-stimulated ferricyanide reduction, showing that a non-enzymatic redox system utilizing α-tocopherol was not involved. *para*-(Chloromercuri)benzenesulfonic acid strongly affected ferricyanide reduction in the absence of ascorbate, whereas the stimulated reaction was much less responsive to this compound. Thus, it appeared that at least two different membrane redox systems are operative in HL60 cells, both capable of reducing ferricyanide, but through different mechanisms. The first system is the ferricyanide-reductase, which uses NADH as its source for electrons, while the novel system proposed in this chapter relies on ascorbate.

Introduction

Many eukaryotic cells contain a redox system in their plasma membrane, capable of reducing extracellular substrates using electrons from intracellular NADH (1). The system efficiently reduces the impermeable substrate ferricyanide. This is not the natural substrate for the redox system, but as yet there has been no conclusive evidence for the substrates of this system or for its primary function. It has been suggested that the system is involved in the maintenance of the redox state of SH-residues in membrane proteins (1) , the neutralization of oxidative stressors outside the cell (2) or in the uptake of iron through a non-transferrin pathway (3, 4).

This redox system deserves special attention because of its possible involvement in regulation of growth and differentiation. Activation of the redox system results

in stimulation of growth in serum-limited HeLa cells (5) and HL60 cells (6). Induction of differentiation in HL60 cells has been associated with transient changes in reductase activity (7). In other cells, activation of the redox system has been shown to modulate protein kinase C activity (8). These findings raise an interest in the role of the plasma membrane reductase in these cells, and the mechanisms through which it operates.

The reduction of ferricyanide by the plasma membrane reductase can be greatly stimulated by the addition of ascorbate and the oxidized form of ascorbate, dehydroascorbate (DHA). This has been found both in K562 cells (9), a leukemic cell line, and in human erythrocytes (10). Although several mechanisms have been proposed, the exact mechanism of this enhancement by ascorbate and DHA remains to be elucidated. For K562 cells, it was suggested that the stimulation of ferricyanide reduction by ascorbate was due to a plasma membrane-localized ascorbate free radical (AFR) reductase (9). This enzyme is supposed to catalyze the reduction of external ascorbate free radical using intracellular NADH. It was proposed that ferricyanide reacts with ascorbate to ferrocyanide and the ascorbate free radical. The latter would then be regenerated by the AFR-reductase to ascorbate, which can subsequently again react with ferricyanide.

Another mechanism is based on studies on erythrocytes and involves the accumulation of ascorbate in cells, where it may serve as an intracellular electron donor for a plasma membrane reductase (10). In many cells, accumulation of ascorbate is achieved through a facilitative glucose transporter, GLUT-1 (11, 12). This transporter efficiently transports DHA into the cell, but not ascorbate itself. Inside the cell, DHA is reduced to ascorbate. This metabolic trapping mechanism enables the cell to accumulate ascorbate at concentrations far exceeding that of its environment. However, recently, it was found that in erythrocytes, the enhancement of ferricyanide reduction by DHA was not affected by an inhibitor of the GLUT-1 transporter (13). This led to the conclusion that ascorbate was closely involved in the redox reaction, but that it acted both intra- and extracellularly. It was concluded that DHA could be regenerated to ascorbate independent of its cellular location. Thus, controversy exists on the mechanism of enhancement of ferricyanide reduction by ascorbate and DHA.

We have studied the effect of DHA and ascorbate on ferricyanide reduction by HL60 cells. It has been reported that HL60 cells contain a ferricyanide reductase and an AFR-reductase (14, 15). In this study, the possible role of both enzymes in the enhanced ferricyanide reduction was investigated. It is concluded that ascorbate-stimulated ferricyanide reduction does not involve an AFR-reductase.

Rather, it involves a mechanism where ascorbate is internalized and where intracellular ascorbate is involved in the reduction of extracellular ferricyanide by a novel redox system, different from the NADH-driven ferricyanide reductase.

Experimental Procedures

L-Ascorbic acid and L-dehydroascorbic acid were purchased from Aldrich, and potassium ferricyanide and *para*-(chloromercuri)benzenesulfonic acid (*p*CMBS) from Fluka. Cytochalasin B and α-tocopherol were from Sigma, L-buthionine-(RS)-sulfoximine (BSO) from Acros Chimica and phloretin from ICN.

HL60 myeloid leukemic cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 100 U/ml penicillin G, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 3 mM L-glutamine. Culture flasks were kept in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were harvested when the culture had reached a density of ~$9 \cdot 10^5$ cells/ml, and were washed twice in 20 mM Tris/150 mM NaCl at pH 7.4 (Tris/NaCl). Subsequently, the cells were suspended in this buffer for further use.

Ferricyanide reduction was determined as follows: $4 \cdot 10^6$ cells/ml in Tris/NaCl were incubated at 37 °C in a closed shaking waterbath. The reaction was started by the addition of 1 mM ferricyanide to suspensions containing various concentrations of ascorbate or DHA, and was followed for at least 60 min. Aliquots were centrifuged, and supernatants were assayed for ferrocyanide using the bathophenantroline disulphonic acid assay (16). The rate of ferrocyanide generation was used as a measure for the activity of the reductase.

Accumulation of ascorbate in cells was determined by incubating $4 \cdot 10^6$ cells/ml in Tris/NaCl in the presence of ascorbate or DHA, with or without 1 mM ferricyanide. The suspension was incubated at 37 °C in a closed shaking waterbath, and 1 ml aliquots were taken at set time points. After centrifugation, cells were washed twice in ice-cold Tris/NaCl, containing 100 µM of the GLUT-1 inhibitor phloretin (11, 13). The cell pellet was extracted with 600 µl methanol, which was diluted to 1 ml with water and 1 mM EDTA (end-concentration). After centrifugation, the supernatants were analyzed for ascorbate on an HPLC system with a Partisil SAX column (10 µm, 250 x 4.6 mm), eluting with a gradient starting at 7 mM potassium phosphate, pH 4.0, and changing to 0.25 M potassium phosphate, 0.5 M KCl, pH 5.0. Ascorbate was detected at 265 nm using a Jasco 870-UV detector. Extraction and HPLC analysis were validated using [^{14}C]-ascorbate (Amersham). The extraction

yielded over 95 % of the cell-associated radioactivity, and HPLC analysis revealed that 90% of intracellular radioactivity corresponded with ascorbate.

The stability of ascorbate and DHA was tested by incubating 25 µM of either compound with $4 \cdot 10^6$ cells/ml in Tris/NaCl, with or without 1 mM ferricyanide at 37 °C in a closed shaking waterbath. At set times 100 µl aliquots were mixed with 50 µl 0.1 M dithiothreitol (DTT), 600 µl methanol and 250 µl water. The extracts were spun down, and the ascorbate concentration in the supernatant was determined by HPLC.

Glutathione (GSH/GSSG) depletion was achieved by incubating cells with BSO (17). Cells were incubated at a density of $3.5 \cdot 10^5$ /ml in growth medium supplemented with 0 or 500 µM BSO. After 2 days at 37 °C, cells were centrifuged and washed twice with Tris/NaCl. Glutathione levels in cells, i.e. the total amount of GSH/GSSG, were measured using an enzyme cycling assay (18).

α-Tocopherol depletion was achieved by growing cells for at least six generations in medium, where fetal bovine serum was replaced by 5 µg/ml transferrin, 5 µg/ml insulin and 0.5% bovine serum albumin (19). α-Tocopherol supplementation of cells was achieved by growing cells for 2 days at 37 °C in culture medium, supplemented with 0, 30 or 100 µM α-tocopherol, at an initial cell density of $3.5 \cdot 10^5$ cells/ml.

α-Tocopherol was extracted and analyzed essentially as described by Thurnham *et al.* (20). Briefly, $5 \cdot 10^6$ cells were spun down and lysed with 10 mM SDS and ethanol containing α-tocopherol-acetate as an internal standard. The lysate was extracted using heptane, which was subsequently evaporated under N_2. The samples were dissolved in the mobile phase of the HPLC system (methanol/acetonitrile/ chloroform 47/47/6 (v/v)), injected on a Spherisorb ODS-2 column (250 x 4.6 mm), and eluted isocratically. Both α-tocopherol and α-tocopherol-acetate were detected at 292 nm.

ESR spectra were recorded on a JEOL-RE2X spectrometer operating at 9.36 GHz with a 100 kHz modulation frequency, equipped with a TM_{110} cavity. Samples were transferred to a quartz flat cell using a sampling device, which allowed sampling within seconds after mixing. ESR spectrometer settings were as follows: microwave power, 40 mW; modulation amplitude, 1 G; time constant, 0.1 s; scan rate, 6 G/min.

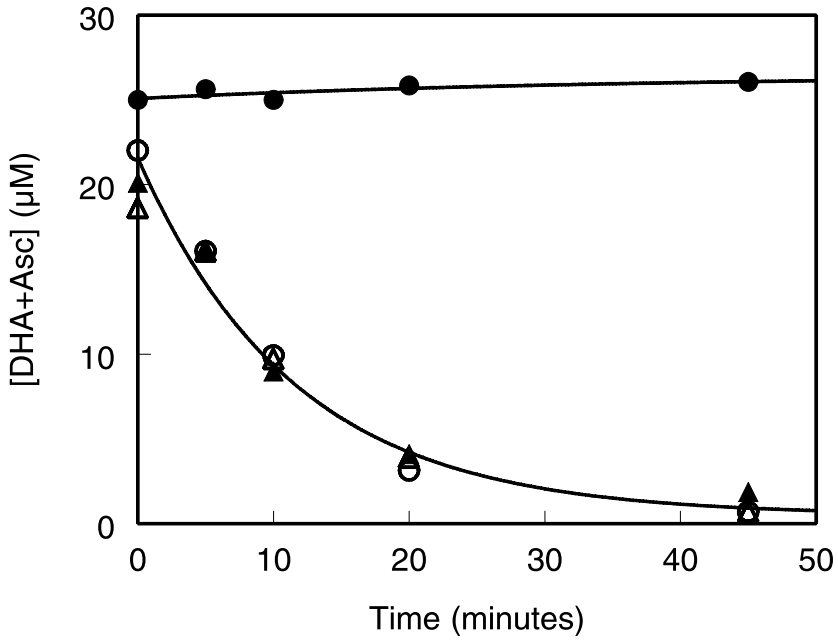

Figure 1: Stability of ascorbate and DHA. 25 µM DHA (▲), 25 µM DHA + 1 mM ferricyanide (△), 25 µM ascorbate (●) or 25 µM ascorbate + 1 mM ferricyanide (○) were incubated in Tris/NaCl in the presence of $4 \cdot 10^6$ cells/ml. Samples were taken and processed as described in the Experimental Procedures. Results represent total ascorbate (Asc) plus DHA concentration.

Results

Ascorbate and DHA metabolism

Several papers mention the rapid irreversible degradation of DHA in solution (11, 21), yielding 2,3-diketo-L-gulonic acid (22). Therefore, the stability of ascorbate and DHA in cell suspensions was first determined. DHA can be measured by addition of dithiothreitol, which converts DHA to ascorbate, after which the ascorbate concentration can be determined by its absorbance at 256 nm. Samples were mixed with DTT and methanol in order to determine the total, i.e. intra- and extracellular, amount of ascorbate and DHA. As expected it was found that DHA was rapidly degraded in a suspension at pH 7.4 (Fig. 1), with a half-life of 8-9 min. Ascorbate remained stable for at least 90 min. Ferricyanide had no effect on the rate of DHA degradation (Fig. 1). Addition of ferricyanide to ascorbate, on the other hand, resulted in a rapid decrease in ascorbate, with similar kinetics as found for DHA.

The data in figure 1 show that a residual amount of ascorbate or DHA can be observed after 45 min and remains relatively stable. HPLC analysis revealed that

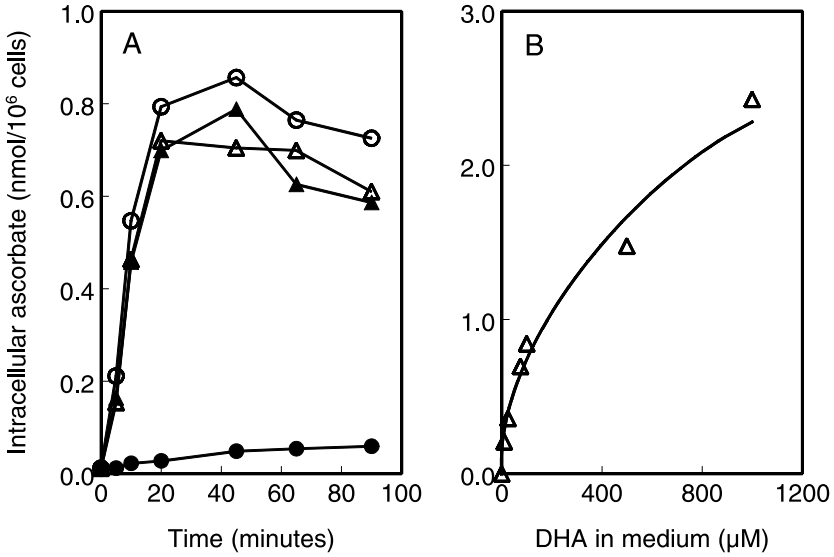

Figure 2: Accumulation of ascorbate in HL60 cells. (A) uptake after incubation with 25 μM DHA (▲), 25 μM DHA + 1 mM ferricyanide (△), 25 μM ascorbate (●) and 25 μM ascorbate + 1 mM ferricyanide (○). (B) ascorbate accumulated after 20 min incubation with various concentrations of DHA.

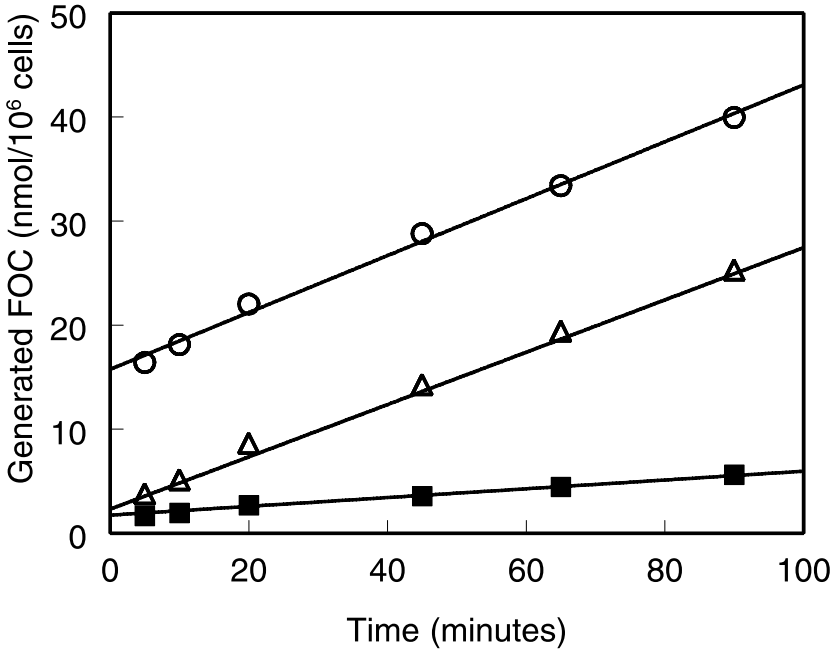

Figure 3: Ferricyanide reduction in HL60 cells. Cells were incubated with 1 mM ferricyanide (■), 25 μM DHA and 1 mM ferricyanide (△) or 25μM ascorbate and 1 mM ferricyanide (○). Ferricyanide reduction was determined as described in the Experimental Procedures. FOC is ferrocyanide.

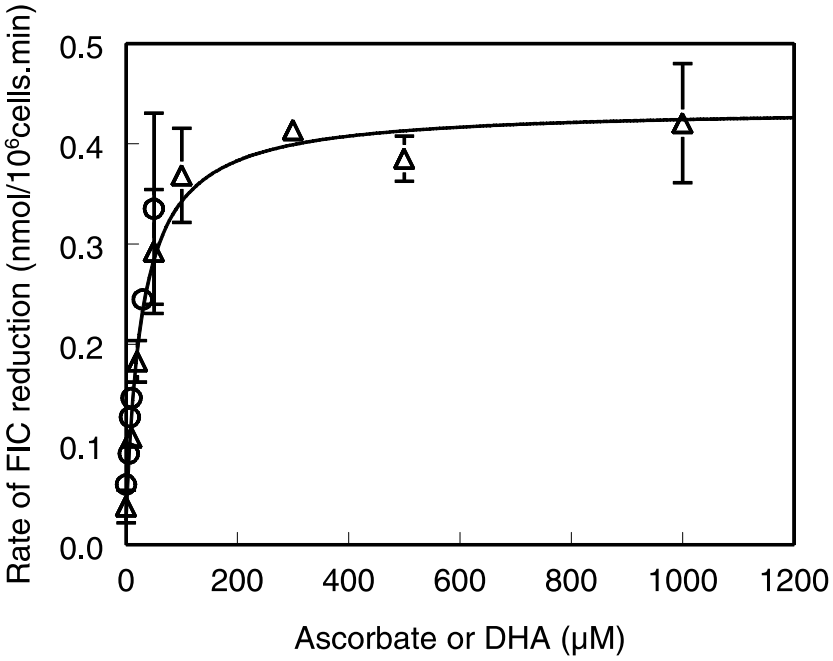

Figure 4: Dependence of the ferricyanide reduction rate on the concentration of ascorbate or DHA. Ferricyanide (FIC) reduction was measured in the presence of various concentrations of ascorbate (○) or DHA (△). Error bars represent the standard deviation.

this residue was ascorbate (data not shown) and corresponded with ascorbate accumulated in the cells (Fig. 2A). Cells incubated with ascorbate alone accumulated only a small amount of ascorbate. However, cells incubated with DHA, DHA and ferricyanide, or ascorbate and ferricyanide rapidly accumulated ascorbate, reaching maximum levels after 20 min of incubation (Fig. 2A). The maximum of approximately 0.8 nmol/10^6 cells corresponded with an intracellular concentration of 1.5 mM (assuming a cell diameter of 10 μm). After 45 min, the levels of ascorbate showed a slight decrease, though most of it remained in the cells. The accumulation of ascorbate proved to be dependent on the concentration of DHA in the incubation mixture (Fig. 2B).

Enhancement of ferricyanide reduction

Subsequently, the effect of ascorbate and DHA on ferricyanide reduction by HL60 cells was investigated. Without ascorbate and DHA, the reduction of ferricyanide to ferrocyanide was linear for at least 90 min (Fig. 3). In the presence of ascorbate and DHA, the rate of reduction was higher and the same linear kinetics were found for both compounds. It should be noted that, with ascorbate, a sudden increase in ferrocyanide levels could be observed within seconds after mixing of ferricyanide and ascorbate (Fig. 3). This increase, which also occurred in the absence

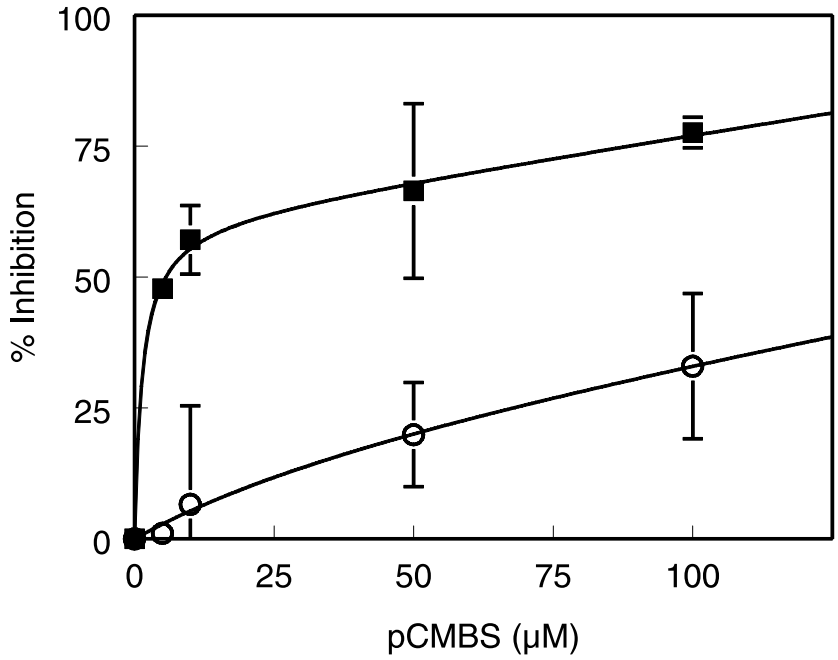

Figure 5: The effect of *p*CMBS on ferricyanide reduction. *p*CMBS was added at the indicated concentration 15 min before adding 1 mM ferricyanide (■) or 1 mM ferricyanide and 25 µM ascorbate (○). The effect of *p*CMBS is expressed as % inhibition of the control without *p*CMBS. Control ferricyanide reduction rates without and with ascorbate were 29.7 and 199.3 pmol/10^6 cells·min respectively. Error bars represent the standard deviation.

of cells, had a stoichiometry of two ferrocyanide molecules formed per molecule of ascorbate added and resulted from the direct reaction between ascorbate and ferricyanide, generating DHA and ferrocyanide.

In figure 4 the dependence of the ferricyanide reduction on the concentrations of ascorbate and DHA is shown. In the case of ascorbate, the dose-response curve could not be extended to concentrations above 50 µM: the immediate reaction of ascorbate with ferricyanide caused excessive depletion of ferricyanide from the system at higher concentrations. The concentration range for DHA was limited to 1 mM, as higher concentrations of DHA interfered with the bathophenantroline assay. The data in figure 4 show that the rate of ferricyanide reduction is dependent on the extracellular ascorbate or DHA concentration, and that it behaved in a saturable manner with a maximal rate of 0.41 nmol/10^6 cells·min and an apparent K_m of 30 µM.

*p*CMBS, a sulfhydryl reagent, can be an effective inhibitor of the plasma membrane ferricyanide reductase (23, 24). *p*CMBS inhibited the reduction of ferricyanide in the absence of ascorbate, causing a 50% inhibition at 5.6 µM (Fig. 5). It also inhibited the ascorbate-stimulated reaction, although to a lesser extent, with a

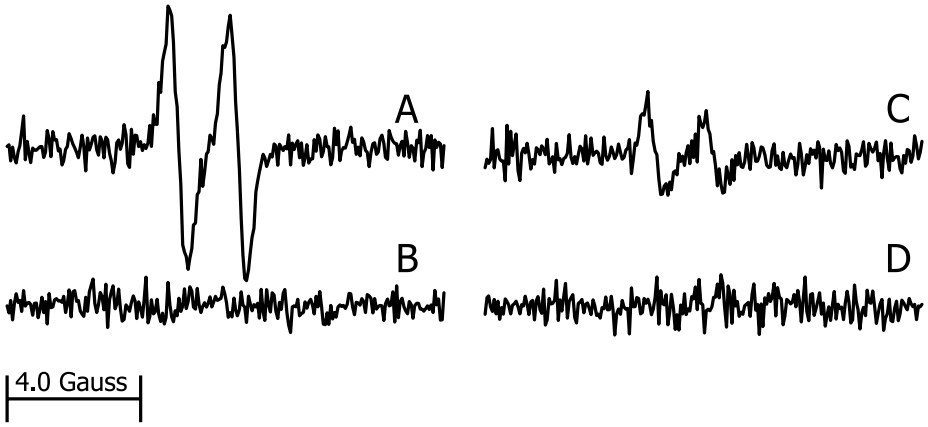

Figure 6: ESR spectra of the formation of the ascorbate free radical. (A) 25 µM ascorbate in Tris/NaCl; (B), as (A), but with 1 mM ferricyanide present; (C), as (A), but in the presence of $4 \cdot 10^6$ cells/ml; (D), as (B) but in the presence of $4 \cdot 10^6$ cells/ml.

50% inhibition estimated at 180 µM. This number was obtained through extrapolation of the data from figure 5. Concentrations above 100 µM *p*CMBS were not used, as they affected the intracellular accumulation of ascorbate.

Formation of the ascorbate free radical

The ascorbate free radical has been proposed as an intermediate in the accelerated reduction of ferricyanide by K562 cells (9). It is relatively stable and results from the one-electron oxidation of ascorbate. To test its involvement in the accelerated reduction of ferricyanide, the formation of the radical was measured under various experimental conditions (Fig. 6). The ascorbate free radical can readily be observed in a 25 µM ascorbate solution in buffer, with an ESR spectrum consisting of a doublet with hyperfine splitting $a^{H4} = 1.8$ G (Fig. 6A). Transition metal ions, which are always present in buffer solutions, mediate this formation of the ascorbate free radical (25). In the presence of 1 mM ferricyanide, no radical signal could be detected (Fig. 6B). The ascorbate free radical was also detected in the presence of HL60 cells, although the signal intensity was lower than in the absence of cells (Fig. 6C). This may be caused by a decreased ascorbate degradation, due to chelation of transition metal ions, or by an increased regeneration by an AFR-reductase. However, this was not further investigated, as it was considered to be outside the scope of this chapter. Again, in the presence of ferricyanide no radical signal could be detected (Fig. 6D).

Inhibitor	Ferricyanide Reduction		Ascorbate accumulation
	0 µM ascorbate	25 µM ascorbate	
None	38	206	667
20 mM D-glucose	28	78	149
20 mM 2-DOG	31	70	29
20 mM 6-DOG	23	94	96
Cytochalasin B			
Added before	39	50	<10
Added after	38	221	674

Table 1 : The role of the GLUT-1 transporter in ferricyanide reduction and ascorbate accumulation. Cells ($4 \cdot 10^6$/ml) were incubated with the compounds listed for 15 min at 37 °C prior to the addition of 1 mM ferricyanide and 25 µM ascorbate. In some experiments cytochalasin B was added 20 min after the addition of ferricyanide and ascorbate (i.e. added after). For cells incubated in the presence of ascorbate, its accumulation in HL60 was measured after 20 min of incubation and was expressed as pmol ascorbate/10^6 cells. Cells incubated in the absence of ascorbate did not contain any measurable ascorbate. Ferricyanide reduction (pmol ferrocyanide/10^6 cells·min) was determined as described in the Experimental Procedures.

Involvement of GLUT-1 transporter

Extracellular DHA is taken up by HL60 cells through a facilitative process, catalyzed by the GLUT-1 glucose transporter (11, 12). To test whether the activity of the GLUT-1 transporter was also involved in ascorbate or DHA-mediated ferricyanide reduction, the effect of several GLUT-1 substrates and inhibitors was studied. Table 1 shows that glucose and its analogs 2-deoxyglucose and 6-deoxyglucose strongly inhibited the ascorbate stimulated ferricyanide reduction, as well as the accumulation of ascorbate. Cytochalasin B, which inhibits GLUT-1 (11), was also an effective inhibitor. Ferricyanide reduction in the absence of ascorbate was hardly affected by these compounds, showing that they did not have an effect on the plasma membrane reductase.

Mechanism of enhanced ferricyanide reduction

Previous studies on erythrocytes suggest that ascorbate may serve as an intracellular electron donor for the plasma membrane reductase (26). The accumulation of ascorbate in HL60 leads to a similar concept, in which the reduction of ferricyanide and the oxidation of ascorbate are coupled. Comparison of the data in figures 2A and 3 shows that the amount of ferrocyanide formed exceeded the amount of

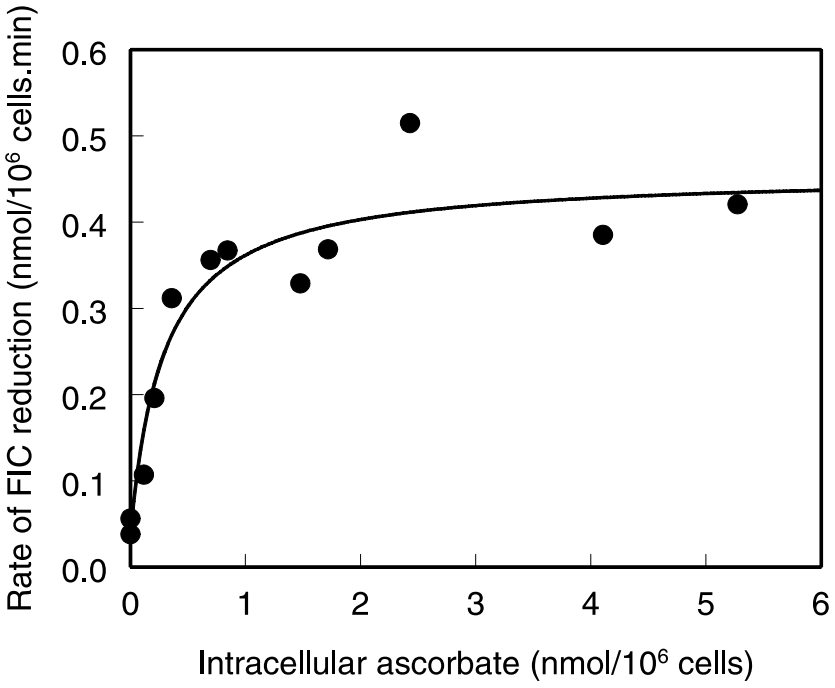

Figure 7: Correlation between the intracellular concentration of ascorbate and the rate of ferricyanide (FIC) reduction in HL60 cells. Data were obtained from uptake measurements and reductase assays in the same cell batch.

ascorbate in the cells: after 60 min, 12 nmol of ferricyanide was converted by 10^6 cells, while they contained 0.8 nmol ascorbate. As one molecule of ascorbate can reduce two molecules of ferricyanide, this amount of ascorbate accounted only for the reduction of 1.6 nmol ferricyanide. Thus, the presence of a recycling mechanism for ascorbate is required to explain the amount of ferricyanide reduced.

There are several ways by which intracellular ascorbate can reduce ferricyanide. It can either remain inside the cell and donate electrons to a redox system for ferricyanide reduction, or, alternatively, leave the cell to react with ferricyanide directly. In the latter case, DHA will be formed outside the cells, and has to re-enter the cell to be recycled to ascorbate. Thus, with a re-uptake step being essential, the stimulation of ferricyanide reduction should remain sensitive to GLUT-1 inhibitors throughout the entire incubation period. As shown in Table 1, this was not the case. Cytochalasin B inhibited only when added before the addition of ascorbate. When 5 µM cytochalasin B was added 20 min after ascorbate, no inhibition was observed. This indicates that ascorbate remains in the cell and that intracellular ascorbate mediates the accelerated reduction of ferricyanide. Replotting the data in figures 2B and 4 results in figure 7, which shows the relation between the intracellular concentration of ascorbate and the rate of ferricyanide reduction.

α-Tocopherol added to medium	α-Tocopherol in cells	Ferricyanide reduction	
		0 µM ascorbate	25 µM ascorbate
µM			
Grown with serum			
0	10	33	229
30	203	39	232
100	393	47	214
Grown serum-free			
0	0	29	242
30	202	45	292

Table 2: Effect of α-tocopherol on ferricyanide reduction. Ferricyanide reduction (pmol ferrocyanide/10^6 cells·min) and intracellular α-tocopherol levels (pmol/10^6cells) were determined as described in the Experimental Procedures. Cells grown with serum were cultured under standard conditions, whereas cells without serum were cultured in a medium with serum replaced by transferrin, insulin and bovine serum albumin.

This rate correlated to the intracellular concentration in a saturable manner, resembling Michaelis-Menten kinetics. A fit of the data resulted in an apparent K_m of 0.29 nmol/10^6 cells, which corresponds to 0.55 mM (assuming a cell diameter of 10 µm).

Glutathione is believed to be involved in the conversion of DHA to ascorbate inside the cell (27). Therefore, it was investigated whether glutathione depletion had an effect on the stimulation of ferricyanide reduction by ascorbate or DHA. Treatment of cells with BSO, an inhibitor of glutathione synthesis (17), caused a dramatic reduction in the cellular GSH/GSSG level, from 2.3 nmol/10^6 cells in the control to 0.07 nmol/10^6 cells. However, ferricyanide reduction rates were hardly affected by the depletion of glutathione. Also, ascorbate uptake measurements showed that intracellular ascorbate accumulation was not significantly affected by glutathione depletion (results not shown).

There have been reports suggesting a role for α-tocopherol in assisting the transport of electrons over the membrane (2, 28). Under standard culture conditions, cells have a very low α-tocopherol content, due to the low level of this compound in serum (29). In order to achieve α-tocopherol levels even lower than those in control cells, HL60 cells were cultured for several generations in absence of serum. Without this exogenous source of α-tocopherol, the α-tocopherol levels dropped below the detection limit of approximately 1 pmol/10^6 cells (Table 2).

Supplementation, on the other hand, caused at least a 20-fold increase in cellular α-tocopherol levels. Ascorbate-stimulated ferricyanide reduction was not significantly affected by supplementation or depletion of α-tocopherol.

Discussion

Ascorbate and DHA strongly increased the rate of ferricyanide reduction in HL60 cells, as has previously been found for K562 cells and erythrocytes (9, 10). For K562 cells, it was suggested that the stimulation of ferricyanide reduction by ascorbate was due to a plasma-membrane-localized AFR-reductase (9). However, it is highly unlikely that this mechanism explains our results. The reaction between ferricyanide and ascorbate is very fast, and the stoichiometry of the reaction amounted to 2 mol ferrocyanide formed per mol of ascorbate (Fig. 3). This indicates that the oxidation of ascorbate does not stop at the level of the ascorbate free radical, but that it continues to DHA. This was confirmed by ESR spectroscopy (Fig. 6). Addition of excess ferricyanide to a solution of ascorbate, as used in our experiments, resulted in the formation of DHA, not the ascorbate free radical. In the experiments with K562 cells a similar excess of ferricyanide was used, and it is therefore also unlikely that, in K562 cells, the enhanced reduction of ferricyanide by ascorbate is mediated by an AFR-reductase (9).

Another mechanism that has been proposed was based on studies on erythrocytes and involves the accumulation of ascorbate in cells, where it may serve as an intracellular electron donor for the plasma reductase (10). The data presented in this chapter also strongly suggest that intracellular accumulation of ascorbate in the cell is an essential part of the mechanism by which ascorbate or DHA has its effect on ferricyanide reduction. We found that only conditions that allowed the accumulation of ascorbate resulted in an accelerated reduction of ferricyanide (Fig. 2). In many cells, DHA is transported by a facilitative process, which is mediated by the GLUT-1 glucose transporter (11). Ascorbate, on the other hand, is not transported over the plasma membrane of HL60 cells. Our experiments corroborate this view, as ascorbate was accumulated in the cells only when DHA was present in the extracellular medium (Fig. 2). Several substrates and inhibitors of the GLUT-1 transporter blocked the accumulation of ascorbate, showing the involvement of this transport system (Table 1).

Intracellularly, DHA is reduced to ascorbate, as followed from control experiments using ^{14}C-labelled ascorbate. This conversion was also found in erythrocytes incubated with DHA (10). However, on incubation with ferricyanide erythrocytes

showed an efflux of DHA. As DHA is transported by the facilitative GLUT-1 transporter, it can be transported in both directions following a concentration gradient. Apparently, in erythrocytes, ferricyanide induced the formation of intracellular DHA from ascorbate, which could subsequently leave the cell. However, no significant efflux of DHA from HL60 cells could be observed, indicating the presence of an efficient regenerating system (Fig. 2). The major part of the DHA generated inside the cells was thus recycled to ascorbate, before it had a chance to be transported or hydrolyzed. Recently, Guaiquil et al. (12) reported that HL60 cells have an efficient ascorbate regenerating system, that does not require intracellular glutathione. The lack of effect of glutathione depletion on ascorbate accumulation found in our study corroborates this view. It was concluded that uptake of DHA into the cell, and its accumulation in the cell as ascorbate, are essential steps in the enhancement of ferricyanide reduction.

The stimulation of ferricyanide reduction by ascorbate was inhibited by GLUT-1 inhibitors or competing substrates (Table 1). This is in clear contrast with data recently obtained for erythrocytes, where it was found that inhibition of DHA transport by cytochalasin B did not impair the stimulation of ferricyanide reduction by ascorbate (13). This led to the conclusion that ascorbate was closely involved in the redox reaction, but that it acted both intra- and extracellularly. It was supposed that DHA could be regenerated to ascorbate independent of its cellular location. In HL60 cells this is clearly not the case. In these cells the GLUT-1 transporter is involved in stimulation of ferricyanide reduction by ascorbate and DHA.

The redox equivalents represented by intracellular ascorbate were exceeded many times (at least by a factor 8) by the total amount consumed by ferricyanide. This shows that ferricyanide reduction only lasts because ascorbate is continuously regenerated. Once ascorbate was accumulated in the cells, the addition of GLUT-1 inhibitors no longer had any effect on the rate of ferricyanide reduction (Table 1). Thus, the effect of ascorbate or DHA on ferricyanide reduction cannot be explained by an excretion and re-absorption of ascorbate from the medium. Instead, the data show that ascorbate has its effect inside the cell, serving either as a direct substrate for a redox system, or as an activator of such a system.

What redox system is responsible for the effect of ascorbate? It is possible that ascorbate-stimulated ferricyanide reduction uses the same system as the basal reduction of ferricyanide. However, when the effect of the inhibitor *p*CMBS on both the basal and the ascorbate-stimulated reduction is compared, this seems to be unlikely. The basal reduction is much more sensitive to inhibition by *p*CMBS than the accelerated reduction (Table 1). This indicates that the basal and the

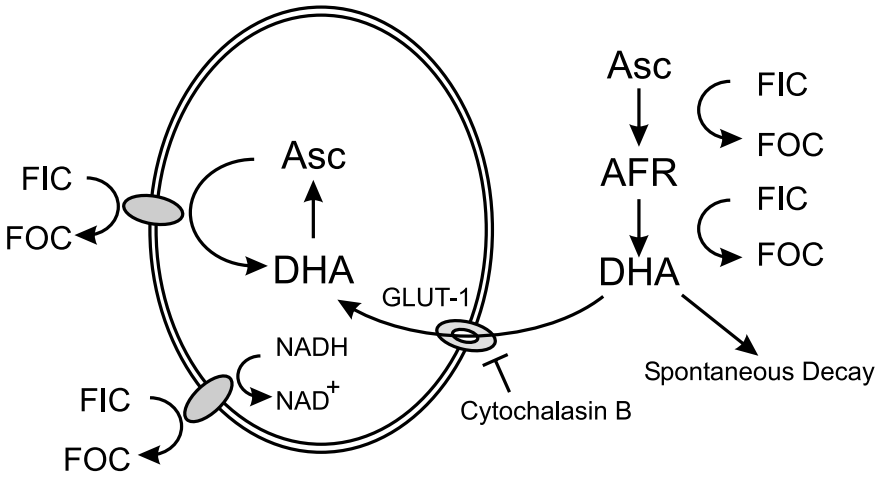

Figure 8: Model for the mechanism of the stimulation of ferricyanide reduction by ascorbate. FIC, ferricyanide; FOC, ferrocyanide; Asc, ascorbate.

accelerated reduction of ferricyanide are actually two separate processes, resulting in an additive reduction of extracellular ferricyanide. Summarizing our data, a model for the reduction of ferricyanide by HL60 cells is proposed in figure 8. At least two membrane redox systems are present in HL60 cells, both capable of reducing ferricyanide. The first system is the ferricyanide-reductase, which is thought to use NADH as its source for electrons. The second system is the one proposed in this chapter, and it relies on ascorbate for its reducing equivalents. It is interesting to note that under normal cell culture conditions there is only a limited supply of ascorbate to feed this system, since serum contains very low levels of ascorbate (30). Under physiological conditions, however, ascorbate is present at much higher concentrations. Therefore, this redox system may be a formidable addition to the cellular capability to counter oxidative processes at its surface, far exceeding the capacity of the NADH-dependent reductase.

The proteinaceous character of the basal ferricyanide-reductase seems well-established (24, 31-33), but the nature of the ascorbate-dependent system is not clear. It could involve either direct chemical reactions or a membrane-based enzyme (2, 28). There have been reports in the literature suggesting a non-enzymatic route of electron transport. In liposomes it was found that α-tocopherol, a natural anti-oxidant present in membrane lipids, mediated ferricyanide reduction by ascorbate without intervention of an enzyme system (2). Also, for erythrocytes, it was observed that α-tocopherol could augment ascorbate-induced ferricyanide reduction, but the involvement of a membrane-localized enzyme system could not be excluded (2). Conversely, the present results show that in HL60 cells the supplementation or depletion of α-tocopherol had no significant effect on the

efficacy of ascorbate in the stimulation of ferricyanide reduction (Table 2). Even in the total absence of α-tocopherol HL60 cells remain fully responsive to the addition of ascorbate. This unequivocally shows that α-tocopherol does not play a significant role in the stimulation of ferricyanide reduction by ascorbate and DHA in HL60 cells.

The intracellular level of ascorbate appeared to have a saturable dose-response relation with the observed rate of ferricyanide reduction, indicating that the reaction has Michaelis-Menten kinetics towards ascorbate. *p*CMBS inhibitor studies suggest the involvement of a protein, since this compound is well known to interfere with protein functions through its reactivity towards SH-groups. Thus, these experiments support the view that ascorbate-driven ferricyanide reduction proceeds through a redox system containing a proteinaceous component. However, further experiments will be needed to establish the exact nature of the system by which intracellular ascorbate can donate electrons to extracellular ferricyanide.

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CHAPTER 4

Erythrocytes reduce extracellular ascorbate free radicals using intracellular ascorbate as an electron donor

This chapter was adapted from MM VanDuijn, K Tijssen, J VanSteveninck, PJA Van den Broek, and J Van der Zee, Erythrocytes reduce extracellular ascorbate free radicals using intracellular ascorbate as an electron donor. J Biol Chem, 275(36): 27720-27725, 2000

Summary

Ascorbate is readily oxidized in aqueous solution by ascorbate oxidase. Ascorbate radicals are formed, which disproportionate to ascorbate and dehydroascorbic acid. Addition of erythrocytes with increasing intracellular ascorbate concentrations decreased the oxidation of ascorbate in a concentration-dependent manner. Concurrently, it was found, utilizing ESR spectroscopy, that extracellular ascorbate radical levels were decreased. Control experiments showed that these results could not be explained by leakage of ascorbate from the cells, inactivation of ascorbate oxidase, or oxygen depletion. Thus, this means that intracellular ascorbate is directly responsible for the decreased oxidation of extracellular ascorbate. Exposure of ascorbate-loaded erythrocytes to higher levels of extracellular ascorbate radicals, resulted in the detection of intracellular ascorbate radicals. Moreover, efflux of dehydroascorbic acid was observed under these conditions. These data confirm the view that intracellular ascorbate donates electrons to extracellular AFR via a plasma membrane redox system. Such a redox system enables the cells to effectively counteract oxidative processes and thereby prevent depletion of extracellular ascorbate.

Introduction

Ascorbate, a well-known anti-oxidant in biological systems, is capable of reducing a variety of oxidative compounds, especially free radicals (1, 2). The importance of sufficient levels of ascorbate has been well established in the past (3, 4). Primates and guinea pigs lack the ability to synthesize ascorbate from glucose and are dependent on dietary intake. Ascorbate deficiency may lead to scurvy and to oxidative injury resulting in necrosis or apoptosis. It may also cause malignant proliferation of cells as a consequence of oxidative DNA damage (5).

The oxidation of ascorbate is a two-step reaction in which single electrons are transferred. The first step yields a relatively stable radical, the ascorbate free radical (AFR). In the second step, AFR donates a second electron, yielding dehydroascorbic acid (DHA). These steps are reversible, but DHA can irreversibly be hydrolyzed to diketo-gulonic acid, which degrades further to potentially toxic compounds (6-8). The degradation of DHA is very fast, with a half-life of approximately 8 min at 37 °C (9, 10). Under oxidative stress, the consumption of ascorbate can be high, and without regeneration, ascorbate would soon be depleted. However, in order to restore ascorbate levels, systems exist that reduce AFR and/or DHA. Most of these systems are located in the cell. Reactions proceed spontaneously, such as glutathione-mediated ascorbate regeneration (11), or are

mediated by enzymes, such as thioredoxin reductase (12-14), glutaredoxin (15), and proteindisulfide isomerase (16). Moreover, an NADH:AFR reductase has been described that reduces intracellular AFR (17).

These pathways only convert oxidized ascorbate species that are present in the cell. Regeneration of extracellular ascorbate is more complicated. Reduction of extracellular DHA can involve transport of DHA into the cell, e.g., by the GLUT-1 glucose transporter (9, 18-20). This regeneration pathway, however, cannot prevent depletion of extracellular ascorbate. Ascorbate is trapped inside the cell after reduction and will only slowly leak back to the extracellular space. It is, therefore, essential that other regenerating pathways exist. Recently, Himmelreich et al. reported that, in erythrocytes, extracellular DHA can be reduced without entering the cell (21). Also, transmembrane AFR-reductases have been described in the plasma membrane of liver cells (22) and red blood cells (23, 24). It was suggested that the latter reduce extracellular ascorbate radicals using intracellular NADH, thereby generating ascorbate and NAD^+.

Previous observations indicated that alternative systems might exist in the plasma membranes of cells. It was found that intracellular ascorbate acted as electron donor for transmembrane electron transport, with ferricyanide as extracellular electron acceptor (9, 25-27). However, the actual substrate is still unknown, as ferricyanide is not a physiological compound. Because ascorbate is a natural compound in, for example, blood plasma, we hypothesized that under physiological conditions, AFR and/or DHA might act as the extracellular electron acceptor. Therefore, we investigated this hypothesis using erythrocytes as a model system. The data in this chapter provide the first experimental evidence for the presence of a system in the erythrocyte membrane that reduces ascorbate free radicals in the extracellular space using intracellular ascorbate as an electron donor.

Experimental Procedures

Chemicals were obtained from Sigma or Baker, unless indicated otherwise. L-Ascorbate oxidase (EC 1.10.3.3) was purchased as sticks containing 17 U of enzyme (Roche Diagnostics, Almere, The Netherlands) and was dissolved in phosphate-buffered saline (PBS). Tris(ethylenediamine)-nickel(II) chloride 2-hydrate ($Ni(en)_3^{2+}$) was synthesized according to State (28).

Erythrocytes were obtained from 1-day old citrate-anticoagulated blood, collected from healthy human volunteers by the Bloodbank Leiden/Haaglanden. The cells

were washed three times with PBS. The buffy coat of white cells was removed carefully with each wash.

Erythrocytes were loaded with ascorbate by resuspension of the cells to a hematocrit of 20% in PBS containing 2.5 mM adenosine as energy source (29) and DHA at concentrations as indicated under 'Results'. Control erythrocytes were treated similarly, but without DHA present. After 30 min of incubation at room temperature, erythrocytes were washed three times with PBS, and used within 1 h for subsequent experiments. All experiments were performed in PBS at room temperature.

Ascorbate concentrations in erythrocytes were determined by HPLC. Loaded, packed erythrocytes were mixed with three volumes of 7 mM potassium phosphate, 1 mM EDTA, pH 4.0, and frozen in liquid nitrogen. After thawing, hemoglobin was removed using an Amicon Micropartition System with 30 kD cut-off ultrafiltration membranes (Millipore, Etten-Leur, The Netherlands). 100 µl 0.5 mM EDTA, 450 µl methanol and 5 µl concentrated HCl were added to 200 µl of ultrafiltrate, and 100 µl of this mixture was injected on an Adsorbosphere SAX column (250 x 4.6 mm, Alltech, Breda, The Netherlands). Ascorbate was eluted with 7 mM potassium phosphate, 7 mM KCl, pH 4.0 at a rate of 1.5 ml/min. The LKB 2140 detector (LKB Bromma, Sweden) was set at 265 nm, and chromatograms were integrated on a personal computer, using Nelson 2600 revision 4.1 software (Nelson Analytical, Cupertina, CA, USA). After the elution of ascorbate, the column was regenerated with 0.25 M potassium phosphate and 0.5 M KCl, pH 5.0. Ascorbate concentrations are expressed relative to the water content of packed erythrocytes, which is 70% of the packed cell volume (26).

Ascorbate concentrations were determined by measuring the absorption at 265 nm in a Beckman DU-65 spectrophotometer (ε=14,500 M^{-1} cm^{-1}) or by HPLC analysis. For HPLC analysis, 200 µl of the solution was diluted with 100 µl of 0.5 mM EDTA, 450 µl of methanol and 5 µl of concentrated HCl, and subsequently injected and analyzed as described above. When present, erythrocytes were removed by centrifugation prior to the determination of ascorbate.

Ascorbate depletion from erythrocytes was carried out by treating cells with 1 mM 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL), as described by May *et al.* and Mehlhorn (30, 31). Briefly, 20% erythrocytes were incubated in PBS with 1 mM TEMPOL for 5 minutes at 37 °C. After subsequent centrifugation, this treatment was repeated twice, followed by three washes with PBS alone.

NADH levels in erythrocytes were modulated by incubation of 10% erythrocytes with 10 mM pyruvate, xylitol or glucose at 37 °C for 45 min. After centrifugation,

samples were taken for the NAD/NADH assay. The remaining erythrocytes were resuspended in their respective buffers including pyruvate, glucose or xylitol, and immediately used for an ascorbate oxidation assay. NAD^+ and NADH levels were determined using an alcohol dehydrogenase cycling assay modified from Wagner and Scott (32). Briefly, 200 µl packed erythrocytes were diluted in 1.3 ml extraction buffer, frozen in liquid nitrogen, thawed, and filtered using an Amicon Micropartition System with 30 kD cut-off ultrafiltration membranes. The filtrate was further analyzed as described by Wagner and Scott (32).

ESR spectra were recorded on a JEOL-RE2X spectrometer operating at 9.36 GHz with a 100 kHz modulation frequency and equipped with a TM_{110} cavity. Samples were transferred to a quartz flat cell using a rapid sampling device, which allowed the recording of spectra to be started within seconds after mixing. ESR spectrometer settings were as follows: microwave power, 40 mW; modulation amplitude, 1 G; time constant, 0.3 s; scan time, 5 min; scan width, 15 G.

Loading of erythrocytes with L-[carboxyl-^{14}C]ascorbate (Amersham International) was carried out by incubating a 20% suspension with 500 µM ^{14}C-labeled ascorbate and 1.7 U/ml ascorbate oxidase. After 30 min, the erythrocytes were washed three times with PBS, and resuspended at 10% hematocrit. After the experiment, the suspensions were centrifuged, and the supernatants were transferred to vials for liquid scintillation counting and for immediate HPLC analysis, as described above. Fractions were collected and analyzed by liquid scintillation counting. The scintillation cocktails used were Emulsifier Scintillator 299 and Flo-Scint IV (Packard).

^{14}C-Labeled DHA was prepared by incubation of [^{14}C]ascorbate with bromine, according to Washko et al., with every step on ice (33). Immediately after preparation, 1 mM ^{14}C-labeled DHA was added to either PBS or to a 10% suspension of ascorbate-loaded or control erythrocytes. After incubation, supernatants were collected, separated by HPLC and analyzed by liquid scintillation counting.

Oxygen consumption was measured using a YSI 5300 Biological Oxygen Monitor (YSI Inc., Yellow Springs, OH).

All experiments were performed at least three times and were evaluated using Student's t-test where applicable.

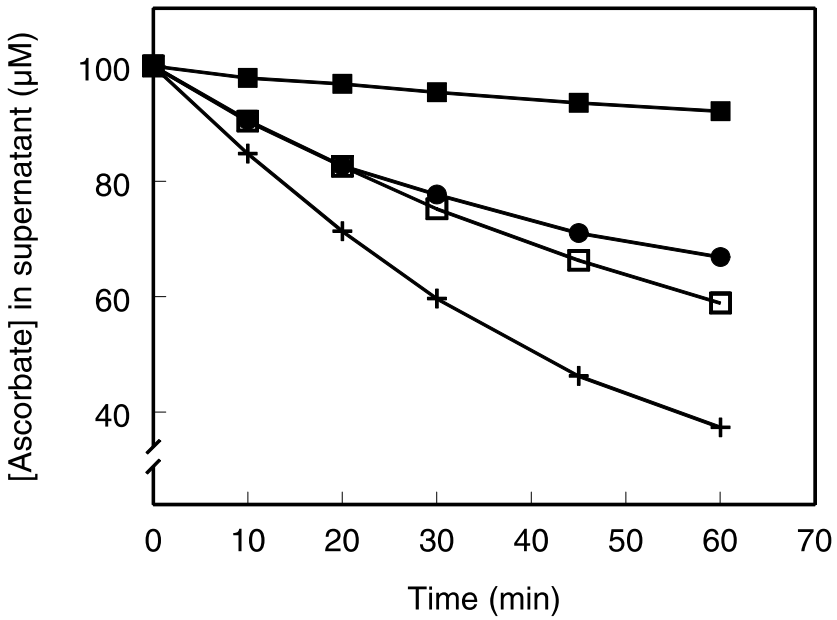

Figure 1. Oxidation of ascorbate by ascorbate oxidase. Ascorbate (100 µM) and 4 mU/ml ascorbate oxidase were incubated at room temperature in PBS (+), supplemented with 10% erythrocytes (●), 10% ascorbate-loaded erythrocytes (■) or 10% ascorbate-loaded erythrocytes treated with TEMPOL (□). At the indicated times, samples were taken, centrifuged and the ascorbate concentration in the supernatant was determined by measuring A_{265}. Ascorbate-loading was performed by incubating cells with 500 µM DHA as described under 'Experimental Procedures'. Results are expressed as the average of three experiments. S.E. values fall within the markers.

Additions	Ascorbate oxidation rate	NADH
	µM/min	% reduced nucleotide
None	1.30 ± 0.05	26 ± 7
Adenosine	1.06 ± 0.09	78 ± 5
Glucose	1.08 ± 0.10	52 ± 9
Xylitol	1.14 ± 0.04	58 ± 9
Pyruvate	1.39 ± 0.06	19 ± 5
TEMPOL	1.39 ± 0.06	14 ± 5

Table 1. Effect of energy sources and TEMPOL on NADH levels and the ascorbate oxidation rate. Erythrocytes that had not been loaded with ascorbate were preincubated with the additions listed. Subsequently, NADH levels and oxidation rates of extracellular ascorbate were determined. All treatments and assays were performed as described under 'Experimental Procedures'. NADH levels are expressed as a percentage of total nucleotides, i.e. NAD^+ + NADH (48 nmol/ml packed cells). All results are shown as an average ± S.E.

Results

Oxidation of ascorbate by ascorbate oxidase

Ascorbate can easily be oxidized in aqueous solution by spontaneous, metal-ion or enzyme-catalyzed reactions. In this study, the enzyme ascorbate oxidase was used to oxidize ascorbate. Incubation of ascorbate with ascorbate oxidase resulted in a decrease of the ascorbate concentration that was linear for 15 min (Fig. 1). After 60 min, approximately 60% of the ascorbate was oxidized. Ascorbate oxidation decreased upon addition of erythrocytes, and decreased even further by addition of erythrocytes, loaded with ascorbate. The addition of cells with high levels of intracellular ascorbate resulted in complete protection (Fig. 1). This suggested that intracellular ascorbate played an important role in the effect of erythrocytes on the oxidation of ascorbate. To test this, ascorbate-loaded cells were treated with TEMPOL, which depleted intracellular ascorbate for more than 90% (data not shown). As expected, the effect of ascorbate-loaded cells on ascorbate oxidation could be reversed by treatment with TEMPOL (Fig. 1). In subsequent experiments, erythrocytes were pre-incubated with various concentrations of DHA to determine the correlation between the intracellular ascorbate concentration and extracellular ascorbate oxidation. Figure 2 shows that the rate of ascorbate oxidation in the external medium was strongly dependent on the intracellular ascorbate concentration: with increasing intracellular ascorbate concentrations the oxidation of extracellular ascorbate decreased.

Figure 1 shows that addition of control erythrocytes (i.e. not loaded with ascorbate) decreased ascorbate oxidation with 30-40%. To test whether NADH contributed to this effect, erythrocytes were incubated with various compounds that are known to perturb the NADH/NAD^+ ratio (34, 35). A decrease in the intracellular NADH concentration resulted in an increase in the ascorbate oxidation rate, whereas an increase in NADH resulted in a concomitant decrease in the ascorbate oxidation rate (Table 1). This shows that NADH, as well as ascorbate, plays a role in the regeneration of extracellular ascorbate. To measure the relative contribution of endogenous ascorbate to the effect of control erythrocytes on ascorbate oxidation, cells were treated with TEMPOL. However, it was found that TEMPOL also reduced intracellular NADH levels (Table 1). It was, therefore, not possible to separate the effects of endogenous ascorbate and NADH. It should be noted that TEMPOL depleted NADH only in the case of control erythrocytes, and not in erythrocytes loaded with ascorbate.

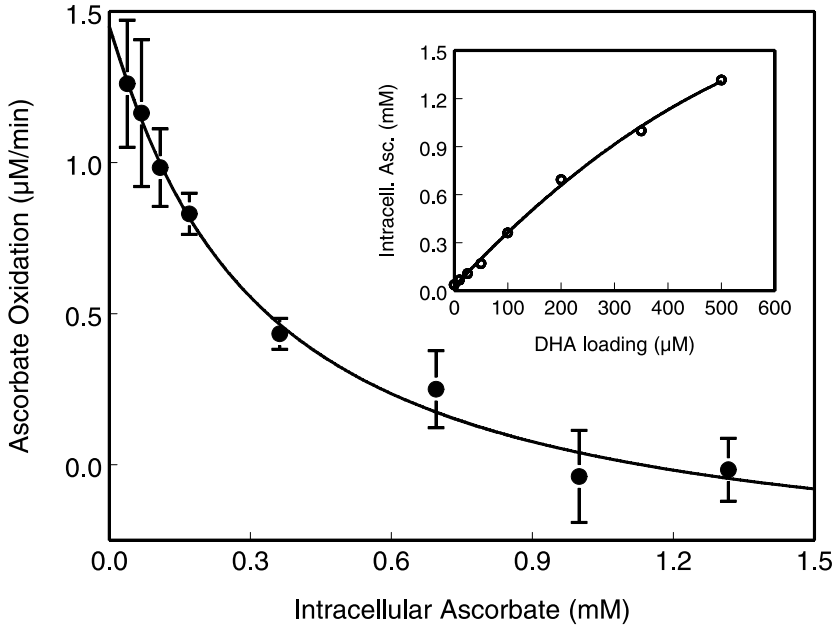

Figure 2. Correlation between the intracellular ascorbate concentration and the oxidation rate of extracellular ascorbate. Erythrocytes were loaded with ascorbate by treating cells with various concentrations of DHA, as described under 'Experimental Procedures'. Subsequently, a 10% suspension was incubated with 100 µM ascorbate and 4 mU/ml ascorbate oxidase. After 0 and 15 min, the amount of ascorbate in the supernatant was determined by measuring A_{265} and by HPLC, and the rate of ascorbate oxidation was calculated. Results of A_{265} measurements are presented as the average of three experiments ± S.E. Measurements using the HPLC method are not shown, but produced similar data. The intracellular ascorbate concentration was determined as described under 'Experimental Procedures'. Inset: Intracellular ascorbate concentration resulting from preincubation with various concentrations of DHA.

The effect of erythrocytes on ascorbate oxidation could be explained by inhibition of ascorbate oxidase by the erythrocytes. To study this hypothesis, control or ascorbate-loaded erythrocytes were incubated with ascorbate oxidase for 0 or 15 min. After removal of the erythrocytes, 100 µM ascorbate was added to the supernatants, and the rate of ascorbate oxidation was determined. It was found that incubation of ascorbate oxidase with control or ascorbate-loaded erythrocytes did not affect the activity of the enzyme. Oxygen is also required for ascorbate oxidase activity. Under our experimental conditions, oxygen levels did not decrease more than 20%, which showed that oxygen levels were not limiting during the experiments (data not shown).

A possible explanation for the protection by ascorbate-loaded erythrocytes could be leakage of ascorbate from the erythrocytes. More than 1 mM of ascorbate was accumulated intracellularly after incubation with DHA (Fig. 2, inset). Leakage of

Extracellular additions	Leakage
	% of total content
None	1.1 ± 0.3
100 µM ascorbate	1.3 ± 0.1
100 µM DHA	1.5 ± 0.1
4 mU/ml ascorbate oxidase	1.2 ± 0.2
14 mU/ml ascorbate oxidase	0.8 ± 0.1
100 µM ascorbate, 4 mU/ml ascorbate oxidase	3.0 ± 0.5
100 µM ascorbate, 14 mU/ml ascorbate oxidase	12.4 ± 1.2
100 µM ascorbate, 4 mU/ml ascorbate oxidase, 20 µM cytochalasin B	1.8 ± 0.3
100 µM ascorbate, 14 mU/ml ascorbate oxidase, 20 µM cytochalasin B	4.2 ± 0.4

Table 2. Leakage of radioactivity from erythrocytes containing [^{14}C]ascorbate. Erythrocytes were loaded with ^{14}C-labeled ascorbate, as under 'Experimental Procedures', washed, and incubated as a 10% suspension with the additions indicated in the table. Leakage after 15 min was determined by centrifugation of the suspensions. Radioactivity in the supernatant was determined by liquid scintillation counting. Values are shown as mean ± S.E. (n=8), relative to the amount of ^{14}C that would be released by lysis of all cells.

the accumulated ascorbate could result in an extracellular ascorbate concentration of up to 100 µM. To investigate leakage of intracellular ascorbate, erythrocytes were loaded with ^{14}C-labeled ascorbate. Table 2 shows that incubation of these erythrocytes caused some leakage of ^{14}C-labeled material, both in control cells and after addition of ascorbate, DHA or ascorbate oxidase. The addition of 100 µM ascorbate and 4 mU/ml ascorbate oxidase resulted in a small increase in leakage of radioactivity. This amounted to an extracellular concentration of 3 µM ^{14}C-labeled material. From the data in figure 2, it can be derived that complete protection against oxidation of ascorbate would need leakage of 20 µM of ascorbate. This means that efflux of ascorbate could maximally account for 15% of the observed effect. Only in the presence of a higher ascorbate oxidase concentration did leakage become substantial. Furthermore, HPLC analysis revealed that most of the radioactivity was released from the cells as [^{14}C]DHA, and not as [^{14}C]ascorbate (Fig. 3). Leakage of DHA could be inhibited by the addition of cytochalasin B, an inhibitor of the GLUT-1 transporter (Table 2). Thus, it can be concluded that leakage of ascorbate is not responsible for the decrease in ascorbate oxidation.

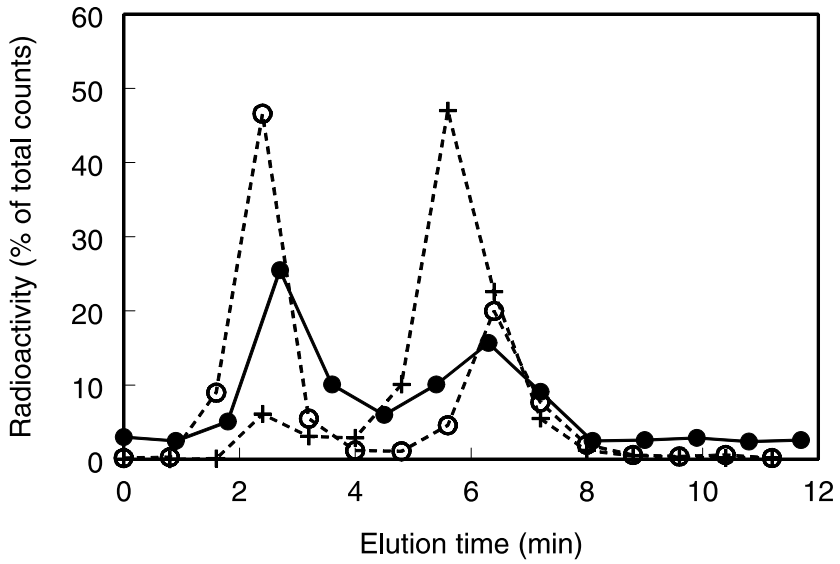

Figure 3. HPLC analysis of radioactive material released from erythrocytes loaded with [^{14}C]ascorbate and exposed to ascorbate and ascorbate oxidase. Erythrocytes were loaded with [^{14}C]ascorbate, as described under "Experimental Procedures". Subsequently, a 10% suspension was incubated with 100 µM ascorbate and 4 mU/ml ascorbate oxidase. After 15 min, the suspension was centrifuged, and the supernatant prepared for HPLC analysis, as described under 'Experimental Procedures'. Counts from the supernatant (●) are shown and compared with the peaks of [^{14}C]ascorbate (+) and [^{14}C]DHA (○). Data are expressed as percentage of the cumulated counts of all fractions. [^{14}C]DHA standard was prepared by a short incubation of [^{14}C]ascorbate with ascorbate oxidase.

Formation of AFR

The oxidation of ascorbate by ascorbate oxidase results in the formation of ascorbate free radical, which can easily be detected by ESR spectroscopy. The ESR spectrum consists of a doublet with hyperfine splitting a^{H4} = 1.8 G (Fig. 4A). Control experiments showed that AFR peak intensities were constant for at least 15 min. The data in figure 4 were obtained with a scan time of 5 min and therefore represent steady state levels of AFR. No signal was observed when ascorbate was omitted from the incubation mixture (data not shown). Removal of ascorbate oxidase resulted in a small AFR signal, which amounted to 15% of the signal in figure 4A (data not shown). This signal was due to autoxidation and transition metal-mediated oxidation of ascorbate (36). Addition of erythrocytes decreased AFR signal intensity by 10%, whereas the addition of ascorbate-loaded erythrocytes decreased the AFR concentration considerably (compare Figs. 4, A, C and E).

In the presence of ascorbate-loaded erythrocytes, AFR could be generated both inside and outside the cell. To distinguish between intra- and extracellular AFR,

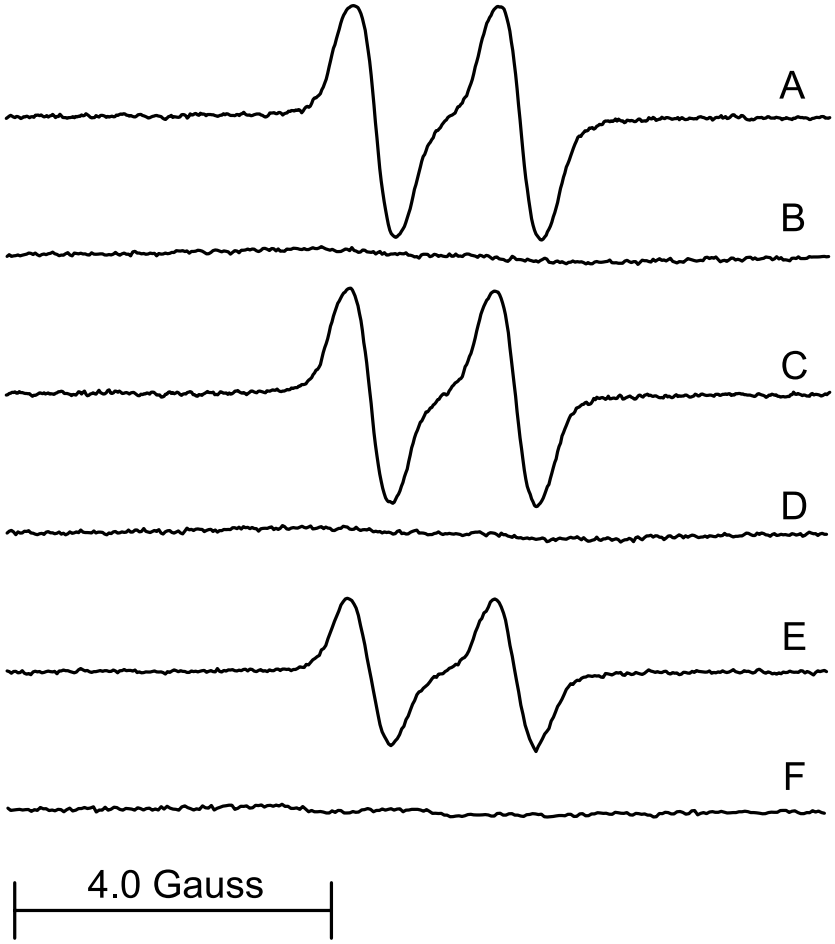

Figure 4. ESR spectra of ascorbate free radicals. A, 100 µM ascorbate and 4 mU/ml ascorbate oxidase; B, same as A, but with 5 mM $Ni(en)_3^{2+}$; C, same as A, but with 10% erythrocytes; D, same as B, but with 10% erythrocytes; E, same as A, but with 10 % ascorbate-loaded erythrocytes; F, same as B, but with 10 % ascorbate-loaded erythrocytes. Erythrocytes were loaded with ascorbate, using 500 µM DHA, as described under "Experimental Procedures".

the line broadening compound $Ni(en)_3^{2+}$ was used. This compound induces line broadening of the AFR signal, as is illustrated in figure 4B, without influencing its redox properties (37). Since $Ni(en)_3^{2+}$ cannot cross the plasma membrane, only the intensity of the extracellular AFR signal will be affected. Addition of 5 mM $Ni(en)_3^{2+}$ to an incubation of control or ascorbate-loaded erythrocytes in the presence of ascorbate and ascorbate oxidase, resulted in the line-broadening of the AFR signal (Figs. 5D and F). This shows that the AFR signal detected under these experimental conditions came from ascorbate radicals located outside the cell.

To determine the correlation between the intracellular ascorbate concentration and extracellular AFR, cells were preincubated with various DHA concentrations.

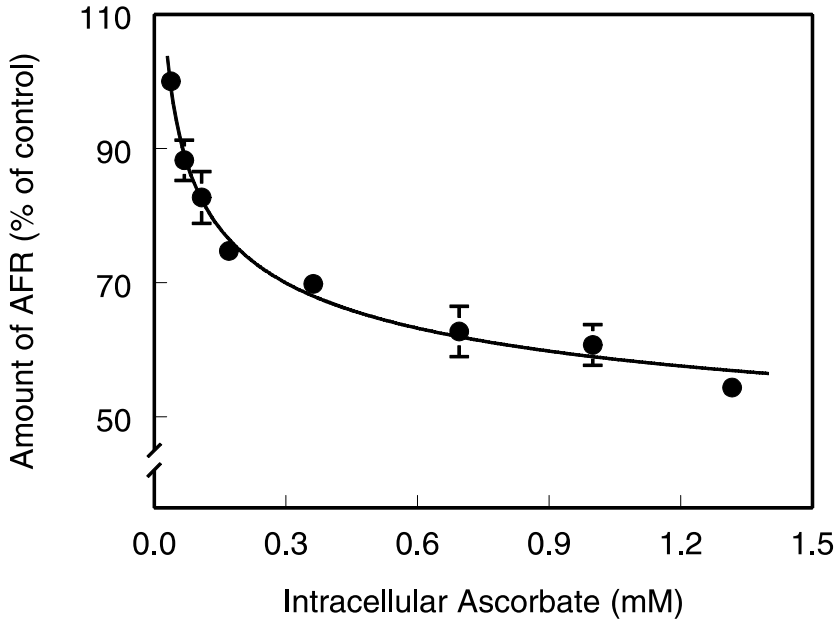

Figure 5. Correlation between the intracellular ascorbate concentration and the extracellular ascorbate free radical concentration. Experimental conditions were as described in figure 2. Immediately after addition of ascorbate and ascorbate oxidase, the samples were transferred to the flat cell, and ESR spectra were recorded and integrated. The levels are expressed, as an average ± S.E., relative to the amount of AFR found in the presence of unloaded erythrocytes.

Figure 5 shows the effect of increasing intracellular ascorbate concentrations on external AFR signal intensities. Ascorbate-loaded erythrocytes reduced the AFR signal in the extracellular medium in a concentration-dependent manner. The effect was similar to the effect observed in figure 2.

Formation of intracellular AFR

So far, our data seem to indicate that extracellular AFR is reduced by intracellular ascorbate. If the hypothesis is correct that intracellular ascorbate donates an electron to extracellular AFR, it can be expected that intracellular AFR is generated as a consequence of this reaction. The data in figure 4 suggest that under the present conditions, only extracellular AFR can be observed. The absence of an intracellular AFR signal is most likely due to the efficient regeneration of ascorbate in the erythrocyte. To overwhelm the reductive capacity of the cell, extracellular AFR levels were increased using high concentrations of ascorbate and ascorbate oxidase. Incubation of 1 mM ascorbate with 20 mU/ml ascorbate oxidase in the presence of 5 mM $Ni(en)_3^{2+}$ resulted in a broadened AFR signal (Fig. 6A). Subsequently, erythrocytes were added (20% suspension) and the spectrum in

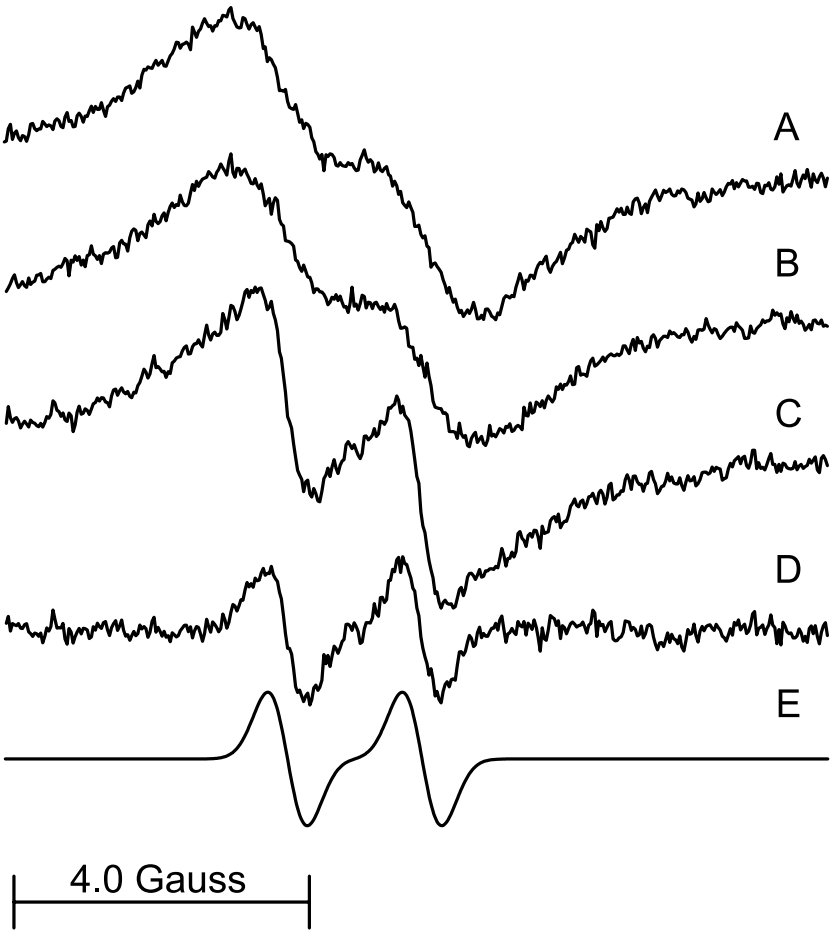

Figure 6. Detection of an intracellular AFR signal. A, ESR spectrum of 1 mM ascorbate, incubated with 20 mU/ml ascorbate oxidase in the presence of 5 mM $Ni(en)_3^{2+}$; B, same as A, but with 20% erythrocytes; C, same as A, but with 20% ascorbate-loaded erythrocytes; D, spectrum A was subtracted from spectrum C; E, simulation of D (hyperfine splitting constant a^{H4}=1.82 G; linewidth 0.27 G). Erythrocytes were loaded with ascorbate, using 500 µM DHA, as described in the Experimental section.

figure 6B was obtained, which is identical to the spectrum obtained without erythrocytes. When ascorbate-loaded erythrocytes were used, on the other hand, the ESR spectrum showed additional, slightly sharper peaks (Fig. 6C). This indicates that an additional AFR signal was formed that could not be broadened by $Ni(en)_3^{2+}$. Subtraction of curve A (Fig. 6) from C generated the spectrum given in figure 6D, which is typical for AFR in the absence of $Ni(en)_3^{2+}$ (simulated in Fig. 6E). This shows that at high levels of extracellular AFR, intracellular AFR can indeed be detected.

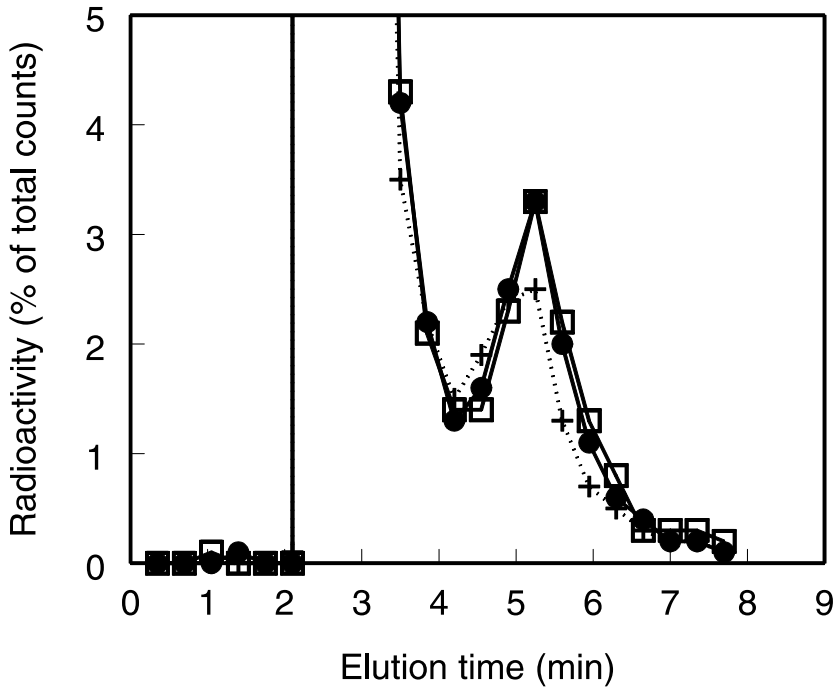

Figure 7. HPLC analysis of supernatants after incubation of [^{14}C]DHA in the presence or absence of erythrocytes. 1 mM [^{14}C]DHA, prepared with bromine as described in the Experimental section, was added to PBS (+) or to a 10% suspension of ascorbate-loaded (●) or control erythrocytes (□). After 10 min incubation at room temperature the samples were centrifuged, and the supernatant prepared for HPLC analysis as described in the Experimental section. Fractions were collected, and analyzed using liquid scintillation counting. In a control sample of [^{14}C]DHA at t=0, the peak eluting at 5.5 min was almost absent (not shown). Ascorbate elutes at 5.5 min, and DHA at 2.5 min. Data are expressed as percentage of the cumulated counts of all fractions.

Involvement of extracellular DHA

Although the ESR experiments indicated that extracellular AFR was reduced by the erythrocytes, we also investigated whether reduction of extracellular DHA played a role. [^{14}C]DHA was added either to PBS or to a 10% suspension of ascorbate-loaded or control erythrocytes. After 10 min, the supernatants were collected and injected on a HPLC system. A small peak at the retention time of ascorbate was found to develop during the incubation of DHA alone (Fig. 7). The presence of erythrocytes resulted in a slightly larger peak, but no differences were found between ascorbate-loaded and control erythrocytes.

Discussion

Addition of erythrocytes with increasing intracellular ascorbate concentrations decreased the oxidation rate of extracellular ascorbate by ascorbate oxidase (Fig. 1). At intracellular concentrations of 1 mM, ascorbate degradation was even completely prevented (Fig. 2). Removal of intracellular ascorbate by TEMPOL reversed the process (Fig. 1). In control experiments, inactivation of ascorbate oxidase and leakage of intracellular ascorbate could be eliminated as explanations for these findings. Thus, intracellular ascorbate directly influences the oxidation rate of extracellular ascorbate, even in the physiological range of 20-60 µM ascorbate (Fig. 2) (38). Addition of control erythrocytes, i.e. erythrocytes with only endogenous ascorbate, decreased the ascorbate oxidation with 30-40%. NADH also contributed to this effect, as the ascorbate oxidation rate was readily affected by changes in the concentration of NADH in the erythrocytes (Table 1). Unfortunately, the relative contribution of endogenous ascorbate and NADH could not be established experimentally, as depletion of endogenous ascorbate by TEMPOL also caused depletion of NADH (Table 1). Nevertheless, it can be concluded that the degradation of extracellular ascorbate is prevented by reactions that are driven by intracellular NADH and ascorbate. In erythrocytes loaded with ascorbate, the ascorbate-driven reaction prevails.

Ascorbate oxidase produces AFR from ascorbate in a direct reaction, followed by disproportionation of two AFR molecules to one molecule of ascorbate and one molecule of DHA (39). In order to restore ascorbate levels, either AFR or DHA has to be reduced to ascorbate. There have been reports on the reduction of extracellular DHA by K562 cells (40) and by erythrocytes (21), but our data did not indicate an important role for this process. A small amount of ascorbate or an ascorbate-like compound was formed on incubation of DHA in PBS (Fig. 7). Jung and Wells described a degradation product of DHA that can reduce DHA to ascorbate (41). In addition, degradation of DHA results in erythroascorbic acid, an analogue of ascorbate that could elute very similar in our HPLC system. These processes could contribute to the formation of the ascorbate-like peak at 5.5 min. The peak increased slightly in the presence of erythrocytes, irrespective of the presence of intracellular ascorbate. This small increase may be caused by a NADH-dependent redox system. Thus, although our data indicate that erythrocytes reduce some extracellular DHA, it is evident that this does not depend on the intracellular ascorbate concentration.

Reduction of ascorbate radical levels could be established using ESR spectroscopy (Figs. 4 and 5). A correlation was found between increasing intracellular ascorbate concentrations and the decrease in extracellular ascorbate oxidation, reflected

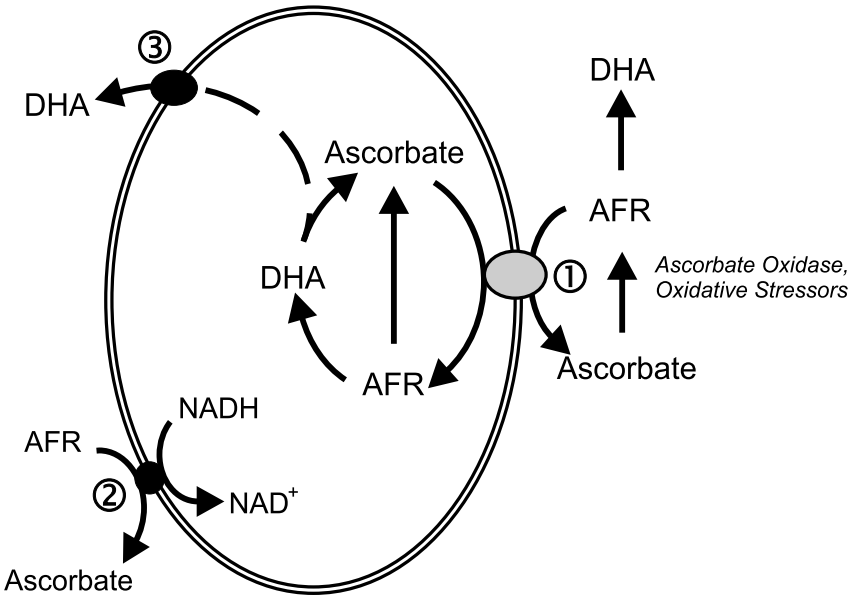

Figure 8. Model for the mechanism of the reduction of extracellular AFR by intracellular ascorbate. Extracellular AFR is reduced by intracellular ascorbate via an ascorbate:AFR reductase [1], as discussed in this chapter. AFR can also be reduced by an NADH-dependent AFR reductase [2]. The efflux of DHA from erythrocytes is shown as a dashed line through the GLUT-1 transporter [3].

either by the absorbance of ascorbate in the extracellular medium or by steady state levels of AFR (Figs. 2 and 5). This suggests that there is a close relationship between the intracellular ascorbate concentration and the reduction of extracellular oxidation products. A plausible explanation is that intracellular ascorbate supplies an electron that reduces extracellular AFR to extracellular ascorbate. In this process, intracellular ascorbate will be oxidized to an ascorbate radical. Either this radical can be reduced to ascorbate via intracellular NADH-AFR reductases or two radicals can disproportionate to ascorbate and DHA. At first, we were unable to detect intracellular AFR. This can be explained by the fact that with low levels of extracellular AFR the cell is able to regenerate intracellular ascorbate efficiently, and intracellular levels of AFR will be too low to be detected. Higher levels of AFR will cause a more severe oxidative stress over the plasma membrane and intracellular regeneration of ascorbate might be overwhelmed. This was indeed the case. Using $Ni(en)_3^{2+}$ to line broaden extracellular AFR signals, a small intracellular AFR signal could be detected (Fig. 6). This strongly suggests that intracellular ascorbate donates an electron to extracellular AFR, forming extracellular ascorbate and intracellular AFR.

The efflux of ^{14}C-labeled material from the erythrocytes provided additional evidence (Fig. 3 and Table 2). HPLC analysis of the extracellular medium revealed that ^{14}C-labeled material emerged as [^{14}C]DHA, rather than [^{14}C]ascorbate (Fig. 3). The efflux of ^{14}C-labeled material was increased in the presence of ascorbate and ascorbate oxidase. This confirmed that external AFR induced an oxidative stress over the membrane, which resulted in the oxidation of intracellular ascorbate. At high levels of AFR, the production of DHA exceeds the capacity of the cell for regeneration to ascorbate, resulting in efflux of DHA via the GLUT-1 transporter (Table 2).

Although there is good evidence for ascorbate-dependent electron transport across the membrane, it is still not clear what the functional components of this system are. Possible mechanisms are transport chains of a physico-chemical nature, e.g. an α-tocopherol electron shuttle, and protein-mediated systems such as an integral plasma membrane protein, complexes of multiple proteins, or proteins using a cofactor to traverse the membrane. However, the standard reduction potentials (E'_0) of α-tocopherol and ascorbate with their respective radicals differ more than 200 mV, which makes the transfer of an electron from α-tocopherol to AFR unlikely (1). Another putative co-factor is coenzyme Q, but its capability to move freely across the lipid bilayer has been questioned (27, 42). Moreover, capsaicin and dicumarol, inhibitors of coenzyme Q mediated electron transport, did not inhibit the decrease in the level of AFR in the presence of ascorbate-loaded erythrocytes (data not shown). The most likely form appears to be a single protein or a protein complex, accepting electrons from intracellular ascorbate and transporting them to extracellular AFR. Interestingly, a cytochrome with a similar function has been described in chromaffin cells from the adrenal medulla. In chromaffin vesicles inside these cells norepinephrine is synthesized at the expense of large amounts of ascorbate. A cytochrome b_{561} is present in the vesicle membrane, transporting electrons from cytoplasmic ascorbate to intravesicular AFR (43, 44). A similar or identical cytochrome could explain the ascorbate:AFR oxidoreductase activity in the erythrocyte. Indeed, evidence was found for the presence of a *b*-cytochrome in the erythrocyte membrane with spectral characteristics similar to those of cytochrome b_{561}. However, it has been reported that cytochrome b_{561} is not present in the erythrocyte (45). This was confirmed in recent work by our group. It is conceivable that a cytochrome similar to cytochrome b_{561} is responsible for the reduction of AFR by the erythrocyte, but so far, no proteins with structural homology to cytochrome b_{561} have been described.

The various processes in the erythrocyte are summarized in figure 8. Extracellular AFR is reduced by intracellular ascorbate via an ascorbate:AFR reductase [1]. Concurrently, intracellular ascorbate is oxidized to AFR, which can be reduced to ascorbate via intracellular NADH-AFR reductases or disproportionate to ascorbate and DHA. Efflux of DHA proceeds through the GLUT-1 transporter [3], which facilitates bi-directional transport of DHA over the plasma membrane. In addition, extracellular AFR can be reduced by a NADH-dependent AFR reductase [2]. Presently, it is not known whether the ascorbate-dependent AFR reductase discussed in this chapter is related in any way to other AFR reductases known from the literature. Generation of intracellular AFR, as well as efflux of DHA, was found by May et al. upon incubation of erythrocytes with ferricyanide (11, 29). However, recently May and Qu observed that pCMBS partly inhibited ascorbate-dependent ferricyanide reduction in erythrocytes, whereas it did not influence ferric iron reduction (46). We were unable to find any effect of *p*CMBS on the AFR reductive capacity (data not shown). This suggests that AFR and ferricyanide are not reduced by the same reductase, but could imply that the erythrocyte AFR reductase also catalyzes ferric iron reduction. Further studies are, however, required to identify the transmembrane reductase or reductases that are responsible for the ascorbate-dependent reduction of extracellular oxidants.

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CHAPTER 5

The ascorbate-driven reduction of extracellular ascorbate free radical by the erythrocyte is an electrogenic process

This chapter was adapted from MM VanDuijn, J Van der Zee, and PJA Van den Broek, The ascorbate-drive reduction of ascorbate free radicals by the erythrocyte is an electrogenic process. FEBS Lett, 491:67-70, 2001.

Summary

Erythrocytes can reduce extracellular ascorbate free radicals by a plasma membrane redox system using intracellular ascorbate as an electron donor. In order to test whether the redox system has electrogenic properties, we studied the effect of ascorbate free radical reduction on the membrane potential of the cells using the fluorescent dye 3,3'-dipropylthiadicarbocyanine iodide. It was found that the erythrocyte membrane depolarized when ascorbate free radicals were reduced. Also, the activity of the redox system proved to be susceptible to changes in the membrane potential. Hyperpolarized cells could reduce ascorbate free radical at a higher rate than depolarized cells. These results show that the ascorbate-driven reduction of extracellular ascorbate free radicals is an electrogenic process, indicating that vectorial electron transport is involved in the reduction of extracellular ascorbate free radical.

Introduction

Ascorbate, or vitamin C, is involved in the protection of an organism against a variety of oxidative agents (1, 2). However, the reaction with an oxidant consumes ascorbate, thus leading to loss of the vitamin. Oxidation of ascorbate usually takes place in two one-electron steps, the first of which results in the Ascorbate Free Radical (AFR) (3). AFR can be oxidized further, producing dehydroascorbic acid (DHA). Also, two molecules of AFR can disproportionate, forming one DHA and one ascorbate molecule. The ring structure of DHA is easily opened by an irreversible hydrolysis reaction (4). Hence, failure to quickly reduce oxidation products of ascorbate will lead to loss of the vitamin. To prevent ascorbate depletion, DHA and AFR can be reduced by a number of systems, most of which are located in the cytoplasm of the cell. The conversions are mainly enzymatic, by e.g. glutaredoxin, thioredoxin reductase, or AFR reductases, but a chemical reduction by glutathione alone has also been described (5-9). When an oxidation occurs extracellularly, the product DHA can be transported into the cell for reduction. For erythrocytes, it has also been described that AFR and DHA can be reduced extracellularly by redox enzymes in the plasma membrane, which are thought to use intracellular NADH as a source of reducing equivalents (10, 11). Recently, we found evidence for an alternative pathway for the reduction of extracellular ascorbate free radicals in the erythrocyte (Figure 1) (12). Not NADH, but intracellular ascorbate provided the reducing equivalents for this reaction, which may involve a transmembrane redox enzyme. The reaction has high similarity to a redox process in the adrenal chromaffin granules. In these granules, a cytochrome b_{561} is involved

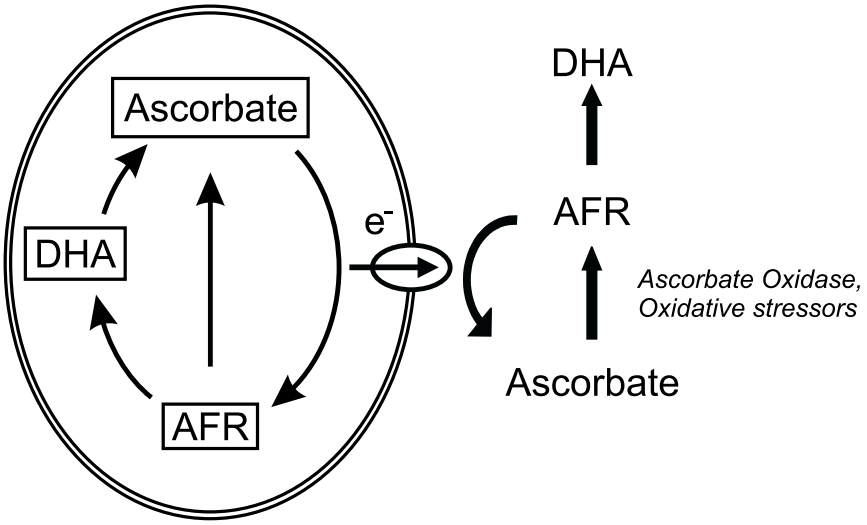

Figure 1: Model for the ascorbate-dependent reduction of AFR. Intracellular ascorbate can donate an electron to a system in the plasmamembrane, which subsequently reduces extracellular AFR. Thus, the net oxidation of extracellular ascorbate is decreased. Intracellular AFR is quickly regenerated to ascorbate. NADH can also serve as an intracellular electron donor for the reduction of extracellular AFR, but was omitted from this scheme.

in the transmembrane reduction of AFR by ascorbate (13-15). Earlier, we investigated whether this cytochrome was also expressed in the erythrocyte, but it was found that this was not the case (16). It is possible that another, similar, erythrocyte redox system is responsible for the reduction of AFR. On the other hand, it has also been suggested that electrons can be transported over the membrane by small lipid soluble molecules like α-tocopherol and coenzyme Q (17-19). Thus, the mechanism of the redox system remains unresolved.

This chapter describes a further investigation of the mechanism of the reduction of extracellular AFR by intracellular ascorbate. Previous data indicated that an electron is abstracted from intracellular ascorbate, and transported over the membrane. In principle, such a movement of charge is electrogenic, and could change the potential of the plasma membrane. Other transmembrane redox processes were indeed shown to be electrogenic. In cells and vesicles from different cell types and species, the reduction of extracellular substrates like ferricyanide was found to affect the membrane potential, resulting in depolarization (20-25).

To test whether the reduction of AFR by the plasma membrane redox system of erythrocytes has electrogenic properties, changes in the membrane potential were studied using a potential-sensitive fluorescent carbocyanine probe. Moreover, the effect of different membrane potentials on the reductase activity was determined. It is concluded that the ascorbate-driven plasma membrane redox system indeed

catalyzes electrogenic AFR reduction, and that it is not likely that small electron carriers are involved in the transfer of electrons over the plasma membrane.

Materials and Methods

Chemicals were from Sigma (Zwijndrecht, The Netherlands) unless specified otherwise. Ascorbate oxidase (EC 1.10.3.3) was purchased as sticks containing 17 U of enzyme (Roche Diagnostics, Almere, The Netherlands), and was freshly dissolved before use by gentle mixing for 30 min. Erythrocytes were obtained from one-day old citrate-anticoagulated blood, collected from healthy human volunteers by the Bloodbank Leiden/Haaglanden (Leiden, The Netherlands). The cells were washed three times in 20 mM Tris, 150 mM NaCl pH 7.4 (Tris/NaCl). The buffy coat of white cells was removed carefully with each wash.

Erythrocytes were loaded with ascorbate by resuspending cells to a hematocrit of 20% in Tris/NaCl with 500 µM DHA, and 2.5 mM adenosine as an energy-source (26). Control erythrocytes were treated similarly, but without DHA. After 30 min of incubation at room temperature, the erythrocytes were washed three times with Tris/NaCl, and used within one hour for subsequent experiments. As described before, ascorbate-loaded erythrocytes contained 1 mM ascorbate, while control erythrocytes contained up to 50 µM endogenous ascorbate (12). All experiments were performed in Tris/NaCl, or, where indicated, in a similar buffer where a part of the NaCl was replaced by KCl.

Membrane potentials were determined using the potential sensitive dye 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$, Molecular Probes, Leiden, The Netherlands) (27). 1 µM of dye was added to a 1% suspension of erythrocytes in a stirred plastic cuvette, and fluorescence was monitored using a Perkin-Elmer LS-50B fluorimeter and FL Winlab 3.0 software (Perkin-Elmer Benelux, Oosterhout, The Netherlands). The exitation wavelength was set at 640 nm, and emission at 670 nm, both using a 10 nm slit.

The concentration of AFR was determined using ESR spectroscopy. Spectra were obtained using a JEOL-RE2X spectrometer operating at 9.36 GHz with a 100 kHz modulation frequency, equipped with a TM_{110} cavity. Immediately after mixing, the samples were transferred to the quartz flat cell by means of a rapid sampling device, and a spectrum was recorded using a 2 min scan time. The area under the ESR absorption curve, as determined by double integration, is proportional to the amount of paramagnetic species in the sample, and this can be used as a quantitative assay. The concentration of AFR was determined by double integration

of the ESR spectra, using 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) as a standard. The spectra were obtained with the same instrument settings as used for collecting the experimental spectra, except for receiver gain. ESR spectrometer setting were as follows: microwave power, 40 mW; modulation amplitude, 0.63 G; time constant, 0.03 s; scan time, 2 min; scan width, 25 G.

The reduction of AFR was measured indirectly by monitoring the oxidation of 100 µM ascorbate (Aldrich, Zwijndrecht, The Netherlands) in a 10% suspension of erythrocytes (12). AFR was generated by oxidizing 100 µM ascorbate with 10 mU/ml of ascorbate oxidase. At 0 and 15 min, a sample of the suspension was centrifuged, and the amount of ascorbate remaining in the supernatant was measured immediately. The rate of ascorbate oxidation in the sample is determined from the difference in concentration between 0 and 15 min. Ascorbate concentrations were determined by measuring the absorption at 265 nm in a Beckman DU-65 spectrophotometer (ε = 14,500 $M^{-1}\,cm^{-1}$).

All experiments were performed at least three times, and error bars represent the standard deviation where applicable.

Results and discussion

The effect of AFR on the membrane potential

Addition of 1 µM $DiSC_3(5)$ to ascorbate-loaded or control erythrocytes induced a sharp increase in fluorescence, followed by equilibration at a lower level after the dye partitioned between the medium and the cells (Figure 2). Subsequently, 100 µM ascorbate and 42.5 mU/ml ascorbate oxidase were added in order to generate ascorbate radicals in the suspension. As shown in figure 2, AFR had no measurable effect on the fluorescence in the presence of control erythrocytes. However, in the presence of ascorbate-loaded erythrocytes the fluorescence increased, indicating a depolarization of the membrane. The addition of 100 µM ascorbate or 42.5 mU/ml ascorbate oxidase alone to a suspension of ascorbate-loaded erythrocytes did not affect the fluorescence. The membrane potential was only affected in presence of both agents, irrespective of the order in which they were added (not shown). Addition of DHA instead of ascorbate and ascorbate oxidase did not alter the fluorescence. Also, control experiments showed that none of the additions affected $DiSC_3(5)$ fluorescence in the absence of cells. The depolarization of the erythrocytes therefore depended on the formation of AFR in the suspension.

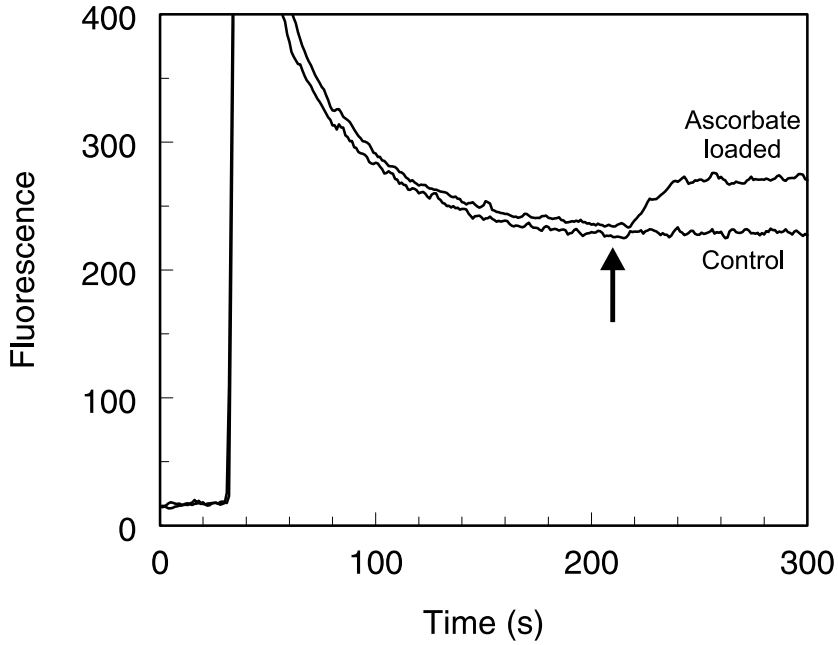

Figure 2: Depolarization of ascorbate-loaded erythrocytes in presence of AFR. 1 µM $DiSC_3(5)$ was added to a 1% suspension of either control or ascorbate-loaded erythrocytes at 30 s, and 100 µM ascorbate and 42.5 mU/ml ascorbate oxidase after equilibration of the dye at 210 s. After equilibration of the dye, an increase in fluorescence indicates a depolarization of the membrane.

To further study the effect of AFR on the membrane potential, different amounts of AFR were generated in a suspension of ascorbate-loaded erythrocytes. This was done by varying the amount of ascorbate oxidase in a suspension containing 100 µM ascorbate. Under similar conditions, the corresponding AFR concentrations were measured by ESR spectroscopy. As shown in figure 3, the extent of erythrocyte depolarization, expressed as the shift in $DiSC_3(5)$ fluorescence upon addition of AFR, depended on the concentration of extracellular AFR in the suspension.

Depolarization of ascorbate-loaded erythrocytes in the presence of extracellular AFR shows that the reduction of AFR must indeed be an electrogenic process. The transfer of charge over the membrane during the reduction of AFR agrees with the model of a transmembrane redox reaction with ascorbate as an intracellular electron donor.

Untreated erythrocytes can also reduce extracellular AFR, albeit at a lower rate than ascorbate-loaded cells (10, 12). Nevertheless, no changes in the membrane potential were observed upon exposure of control erythrocytes to AFR (Figure 2). We earlier showed that these cells can use NADH and endogenous ascorbate as electron donors for the reduction (12). Endogenous ascorbate is typically present

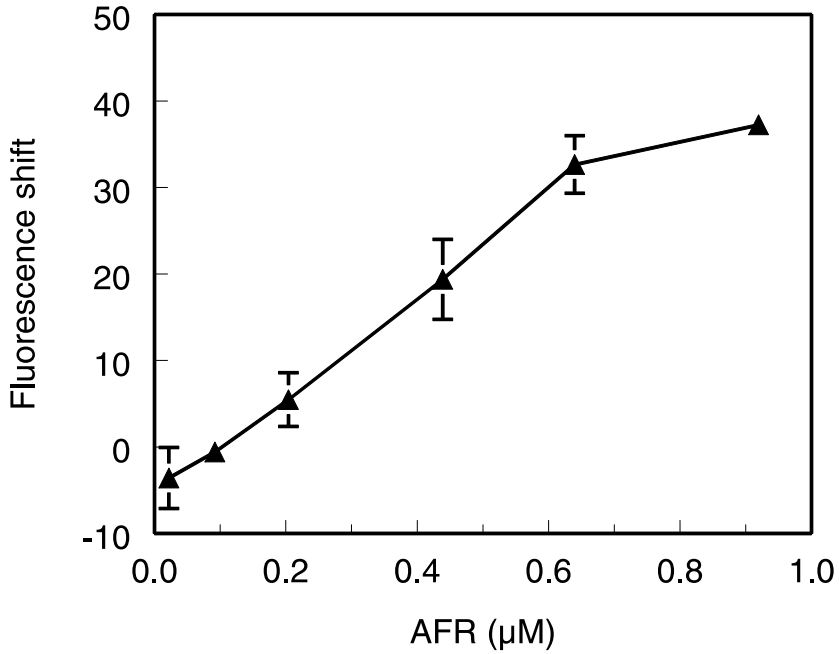

Figure 3. Correlation between erythrocyte depolarization and the concentration of AFR. 1 µM $DiSC_3(5)$ was added to a 1% suspension of ascorbate-loaded erythrocytes. After equilibration, 0, 1, 5, 20, 42.5 or 100 mU/ml ascorbate oxidase and 100 µM ascorbate were added to the suspension, and the subsequent increase in fluorescence was recorded. The data are the average of three experiments, and error bars represent the standard deviation. The corresponding AFR concentrations were established by ESR spectroscopy.

in the range of 20-50 µM, while ascorbate-loaded erythrocytes contained 1 mM ascorbate (28). The most likely explanation for the lack of response in the membrane potential would therefore be that the rate of charge transfer is too low to produce a detectable effect.

The effect of membrane potential on AFR reduction

It is conceivable that the membrane potential of a cell affects the transport of electrons over the membrane. In order to test whether this was the case, we studied the reductase activity by measuring the ascorbate stabilizing effect of cells (12). In this assay, ascorbate is oxidized to AFR by ascorbate oxidase. The reduction by the cells of AFR to ascorbate decreases the apparent rate of this reaction. For clarity, the difference between the ascorbate oxidation rate in each sample and the rate in buffer alone (3.68 µM/min) was defined as the apparent rate of AFR reduction. Several membrane potentials were established in erythrocytes by suspending them in buffers with different concentrations of potassium and 1 µM valinomycin. It was found that the rate of AFR reduction was dependent on

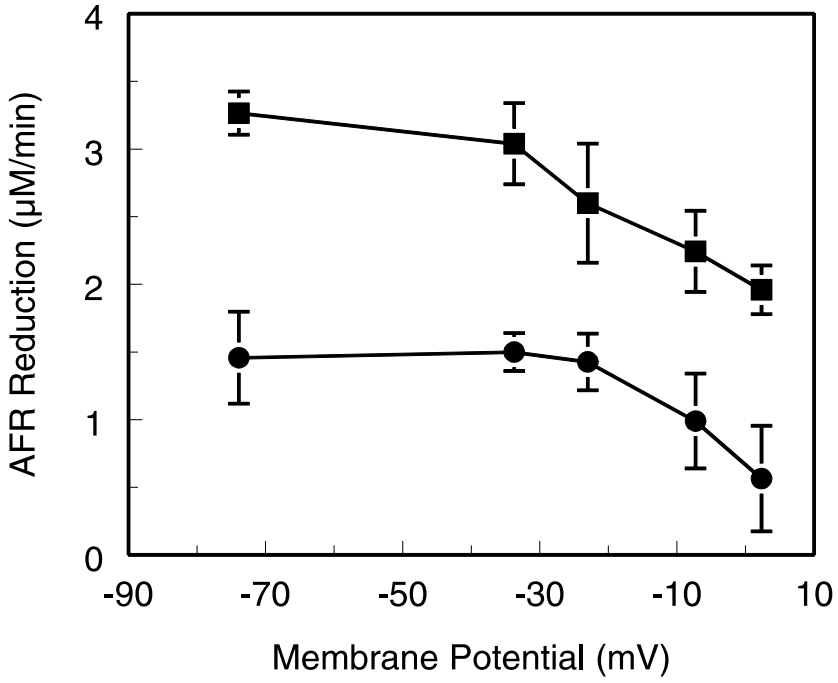

Figure 4. Effect of erythrocyte membrane potentials on the reduction of AFR. 100 µM ascorbate and 10 mU/ml ascorbate oxidase were added to a 10% suspension of control (●) or ascorbate-loaded (■) erythrocytes. The erythrocytes were suspended in buffers containing 0, 25, 50, 100 or 150 mM potassium and 1 µM valinomycin to establish different membrane potentials. Subsequently, the apparent rate of AFR reduction was established by measuring the rate of ascorbate oxidation, as described in the text. The data are the average of three experiments, and error bars represent the standard deviation. The membrane potentials in the figure were calculated using $V_m=58\cdot\log((\alpha\cdot[K]_o+[Cl]_i)/(\alpha\cdot[K]_i+[Cl]_o))$ (24). Constants were used as follows: α=20 (the ratio of the permeabilities of K and Cl in presence of valinomycin); $[K]_i$ =136 mM (intracellular K); $[Cl]_i$ =105 mM (intracellular Cl); $[Cl]_o$ =150 mM (extracellular Cl) (29). Normal resting erythrocytes have a membrane potential of about -9 mV (29)

the membrane potential of the erythrocytes. Hyperpolarization of the erythrocytes increased AFR reduction, whereas it was decreased by a depolarization of the cells (Figure 4). The clear correlation of the reduction rate with the membrane potential confirms the view that electrogenic transmembrane electron transport is involved.

The data presented in this chapter show that the reduction of AFR by erythrocytes causes depolarization, and that depolarization causes decreased reduction of AFR by the ascorbate-driven redox system. This could imply that this reduction of AFR is auto-inhibitory. However, it must be noted that the depolarization in our experiments required relatively high concentrations of AFR. The levels of the radical used in figure 4 were lower, and the levels in a physiological setting will probably

be even less, preventing significant changes in the membrane potential. It is therefore unlikely that a change in membrane potential is a limiting factor in the physiological reduction of AFR.

Conclusion

The data in this study provide evidence for the transmembrane nature of the ascorbate-dependent reduction of extracellular AFR by the erythrocyte. The model for the reduction of extracellular AFR that was suggested by earlier data now seems firmly established. Intracellular ascorbate donates a single electron to a system in the plasma membrane, which passes it on to reduce extracellular AFR (Figure 1). The electrogenic nature of the electron transfer provides information on the nature of the system in the plasma membrane. It has been suggested that small lipid soluble molecules like α-tocopherol and coenzyme Q can act as electron shuttles in the plasma membrane. Thus, they could transfer electrons from e.g. intracellular ascorbate to extracellular AFR. However, these electron shuttles bind a proton when accepting an electron, which means that a proton is transported together with the electron, and that no net charge crosses the membrane. However, our data show that the reduction of AFR is electrogenic, which implies that α-tocopherol and coenzyme Q do not play a major role in this process. Instead, it is more likely that one or more proteins in the erythrocyte membrane are involved in the electron transfer process.

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CHAPTER 6

The ascorbate:AFR oxidoreductase from the erythrocyte membrane is not cytochrome b_{561}

This chapter was modified from MM VanDuijn, J Van der Zee, and PJA Van den Broek, The ascorbate:AFR oxidoreductase from the erythrocyte membrane is not cytochrome b_{561}. Protoplasma, *In press*.

Summary

Erythrocytes contain a plasma membrane redox system that can reduce extracellular ascorbate radicals using intracellular ascorbate as an electron donor. In this study, the hypothesis was tested that cytochrome b_{561} was a component of this system. Spectroscopic analysis of erythrocyte membrane preparations revealed the presence of cytochrome b_5 and hemoglobin, but also of a cytochrome with properties similar to cytochrome b_{561}, reducible by ascorbate, and insensitive to CO. The presence of cytochrome b_{561} was studied further by RT-PCR analysis of erythrocyte progenitor cells, reticulocytes. However, no cytochrome b_{561} mRNA could be found. These results were corroborated by Western blot analysis with an anti-cytochrome b_{561} serum. No cytochrome b_{561} protein could be detected in extracts of erythrocyte membranes. It is therefore concluded that erythrocytes do not contain cytochrome b_{561} in their membranes. The possible involvement of other *b*-cytochromes in ascorbate-AFR oxido-reductase activity is discussed.

Introduction

Most cells contain a wide variety of systems to maintain their levels of anti-oxidants, such as ascorbate (vitamin C). These systems are especially important for primates and guinea pigs, which lack the capability for *de novo* synthesis of ascorbate. These species therefore rely on dietary intake of the vitamin and regeneration of oxidized ascorbate. Oxidation products of ascorbate include the ascorbate free radical (AFR) and dehydroascorbic acid (DHA). AFR is a relatively stable radical that is generated by the one-electron oxidation of ascorbate. In a second one-electron step AFR can be further oxidized to DHA. Alternatively, two molecules of AFR can disproportionate to form one ascorbate and one DHA molecule. Both AFR and DHA can be reduced back to ascorbate, but the fast and irreversible hydrolysis of DHA, with a half-life at 37 °C of approximately 10 min, asks for swift and efficient regeneration systems (1, 2).

Intracellularly, a number of reducing systems are available, such as glutathione (GSH), thioredoxin reductase, glutaredoxin, proteindisulfide isomerase and a mitochondrial NADH:AFR reductase (3-9). For the reduction of extracellular oxidation products of ascorbate, cells can transport DHA *via* the GLUT-1 glucose transporter for intracellular reduction to ascorbate (2, 10, 11). Also, a transmembrane NADH:AFR reductase has been described for the extracellular reduction of AFR, while another group reported the extracellular reduction of DHA (12, 13). Recently, our group identified a new pathway in the erythrocyte for the reduction of

extracellular AFR (14). It was found that, similar to NADH, intracellular ascorbate can serve as the electron donor for the reduction of extracellular AFR.

While there is good evidence for ascorbate-dependent electron transport across the membrane, it is still not clear what the functional components of this system are. The transfer of electrons from ascorbate to ascorbate radicals, over a membrane, is not a common process. Only one protein, most commonly found in chromaffin granules, has been shown to be involved in such a process. This protein, cytochrome b_{561}, has been isolated and the cDNA sequenced in a number of species, including humans (15). Its structure seems to be rather unique, because no homologues of the protein are known in genomic databases, other than the cytochrome b_{561} from different species. Cytochrome b_{561} is highly expressed in the membrane of granules inside chromaffin cells of the adrenal medulla. In these granules, synthesis of catecholamines by dopamine-β-hydroxylase oxidizes large amounts of ascorbate to AFR. Cytochrome b_{561} transfers electrons from cytoplasmic ascorbate to reduce the AFR in the granules back to ascorbate (16). Similar *b*-cytochromes have been identified in the plasma membrane of the plant *Phaseolus vulgaris*. They can reduce AFR and other substrates in the apoplast, using cytoplasmic ascorbate as an electron donor (17-19). Also, sequences have been identified in *Arabidopsis thaliana* with high homology to cytochrome b_{561} (e.g. Genbank #AF132115).

As cytochrome b_{561} has been found in several tissues and species, and as no other proteins have been described to transfer electrons from ascorbate to AFR, we hypothesized that cytochrome b_{561} might also be responsible for the transfer of electrons over the erythrocyte membrane. To investigate this, various techniques were used to gather evidence for the expression of cytochrome b_{561} in erythrocytes, and for its possible role in the reduction of extracellular ascorbate radicals.

Materials and Methods

Reticulocyte Isolation - Reticulocytes were isolated from EDTA anti-coagulated blood, freshly drawn by venipuncture from healthy human volunteers. Leukocytes were removed by filtration over a cellulose column with a bed of at least 1 cm (20). After elution from the column, erythrocytes were washed twice in sterile PBS, and once in PBS containing 0.1% BSA. Subsequently, 600 ml packed erythrocytes were mixed with 400 ml PBS/0.1% BSA and $8 \cdot 10^6$ Dynabeads M-450 coated with anti-CD71 antibody (Dynal, Oslo, Norway), and incubated for 45 minutes on a rolling mixer. After incubation, the beads were sedimented with a magnet,

and the pellet was rinsed with PBS/0.1% BSA until the supernatant remained clear. The beads were resuspended in PBS/0.1% BSA, and the procedure was repeated once. Analysis of the resulting pellet by staining with 0.5% New Methylene Blue in PBS showed that more than 99% of the remaining cells were reticulocytes.

Isolation of neutrophils - Human neutrophils were isolated from a buffy coat, obtained from the Bloodbank Leiden-Haaglanden, by starch sedimentation and Ficoll centrifugation (21). The neutrophils were suspended in 140 mM NaCl, 20 mM HEPES, 5 mM KCl and 10 mM glucose at pH 7.3.

K562 cells - K562 erythroleukemic cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 100 U/ml penicillin G, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 3 mM L-glutamine. Culture flasks were kept in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were harvested when the culture had reached a density of $\sim 7 \cdot 10^5$ cells/ml, and were washed twice in phosphate buffered saline before use.

RNA isolation and cDNA synthesis - All (RT)-PCR reagents were from Promega (Leiden, The Netherlands) unless stated otherwise. Total RNA was isolated from cells utilizing RNAzol B (Campro Scientific, Veenendaal, The Netherlands), according to the manufacturer's specifications. The RNA was found to be of sufficient quality, judged by its A_{260}/A_{280} ratio. The isolated RNA was used immediately for subsequent cDNA synthesis using 1 µg of RNA, 15 µg/ml oligo(dT_{15}), 1 mM dNTP's and 7000 U/ml M-MLV RNase H^- reverse transcriptase. A control without reverse transcriptase was always included.

PCR Analysis - PCR analysis was performed on an iCycler thermal cycler (BioRad, Veenendaal, The Netherlands) using 20 U/ml AmpliTaq DNA polymerase (Perkin Elmer, Nieuwerkerk a/d IJssel, The Netherlands), 2 µM of each primer (Life Technologies, Paisley, Scotland), 200 µM dNTP's and Perkin Elmer PCR buffer. Routinely, 32 cycles were run for K562 cells, and 40 cycles for all other cell-types, with 30 s denaturing at 95 °C, a 30 s annealing step, and 30 s extension at 72 °C, followed by a final extension of 7 min at 72 °C. Annealing temperatures, as well as other primer-specific conditions are indicated with the primers below. Two different primer-sets were used to detect cytochrome b_{561} cDNA. All primers were designed to span over introns, so the amplification of unspliced genomic DNA would result in longer products than the amplification of cDNA. The primers used were b561/1 (fw GGAACGAAGCTAAACGCAC, rev GGGAAGGAGGAAGATGGTAG, T_{anneal} 56 °C, 1.5 mM $MgCl_2$, product 287 bp) and b561/srv (fw CACAGCACTGCCTTACTACG, rev

CTGGGAGCCGGGGCTATCTC, T_{anneal} 57.5 °C , 10% DMSO, 1.0 mM $MgCl_2$, product 715 bp). The latter primers are identical to the ones used by Srivastava et al. (15). As a control for the quality of the cDNA, other primers were used for isotypes of the Na^+, K^+,-ATPase. These were β_1 (fw TGAACGAGGAGAGCGAAAG, rev AGTTGCCCAGTCCAAAATAC, T_{anneal} 58.5 °C, 2.0 mM $MgCl_2$, product 281 bp) and β_2 (fw TGATGATTCGCCCCAAGAC, rev CGACATTCTACATTCACCTCC, T_{anneal} 60.8 °C, 1.5 mM $MgCl_2$, product 541 bp). After the PCR reaction, the products were analyzed on a 1.5% agarose gel containing 0.3 µg/ml ethidiumbromide, and detected by transillumination using UV light.

Sequencing of PCR products was done by BaseClear (Leiden, The Netherlands).

Ghost preparation - White erythrocyte ghosts were prepared according to Weed et al. by hypotonic lysis of erythrocytes, obtained from the Bloodbank Leiden-Haaglanden (22). More densely packed membranes were obtained by ultrasonic treatment of the ghosts in a bath sonifier, and subsequent centrifugation. For blotting experiments, ghosts were prepared by hypotonic lysis of erythrocytes, followed by repeated washes of the membranes in 40 volumes of 5 mM potassium phosphate, pH 8.0, until white ghosts were obtained. Packed ghosts were dissolved in SDS sample buffer (0.1 M Tris-HCl pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 3% SDS, bromophenol blue), homogenized with a syringe, and incubated for 1 hour at 37 °C. Samples were not boiled, as this could lead to aggregation of the cytochrome (23).

Cytochrome b_{561} positive control - Bovine cytochrome b_{561} was isolated from fresh bovine adrenal medullae. Fresh adrenals were collected from the local slaughterhouse, after which the medullae were removed, chopped, and ground using a mortar, under liquid nitrogen. A sample of ground medullae was dissolved in SDS sample buffer, an further treated as above. A human positive control was prepared similarly from membranes obtained from a human pheochromocytoma (kindly provided by Dr D.K. Apps, University of Edinburgh).

Western Blotting - Samples were separated by SDS-PAGE using a 10% separating gel in a Mini-V 8.10 vertical gel apparatus (Life Technologies, Breda, The Netherlands) at 175 V. After electrophoresis, the gel was equilibrated in transfer buffer (10% methanol, 0.19 M glycine, 25 mM Tris), and proteins were transferred to an Immobilon-P membrane (Millipore, Etten-Leur, The Netherlands) at 200 V for one hour. The membrane was blocked overnight in 3% BSA in Tris Buffered Saline/0.25% Tween-20 (TBS/Tween), and incubated with anti-bovine b_{561} rabbit serum, and subsequently with HRP-linked anti-rabbit IgG, with intermittent washing

in TBS/Tween. Both antibodies were diluted in blocking buffer. The blot was detected by ECL using New-RX X-ray film (Fuji Photo Film, Tilburg, The Netherlands). The rabbit sera, a gift from Dr. D.K. Apps, were produced by immunizing rabbits with purified bovine cytochrome b_{561} (24).

Spectral characterization - Absorption spectra were recorded on an Aminco DW-2a spectrophotometer at 77 K using a low-temperature accessory (J4-9603, Aminco) in special cuvettes with a 2 mm pathlength (25). Spectra were accumulated from 9 consecutive scans of one sample. Sonified erythrocyte membranes were routinely used, but the spectral properties of normal ghosts were found to be very similar. Samples were incubated in an argon-flushed, stirred glass cuvette, and were reduced by either 500 mM ascorbate or 5 mM sodium dithionite. The sensitivity to CO was studied by flushing dithionite-reduced samples with CO for several minutes. Samples were subsequently transferred to a low temperature cuvette, and immediately frozen in liquid nitrogen.

Results and Discussion.

To assess the possible participation of cytochrome b_{561} in electron transport across the erythrocyte membrane, the presence of this cytochrome in erythrocytes was studied by a number of techniques. Cytochromes can be characterized by their specific absorption bands. Figure 1 shows that spectra recorded at 77K gave at

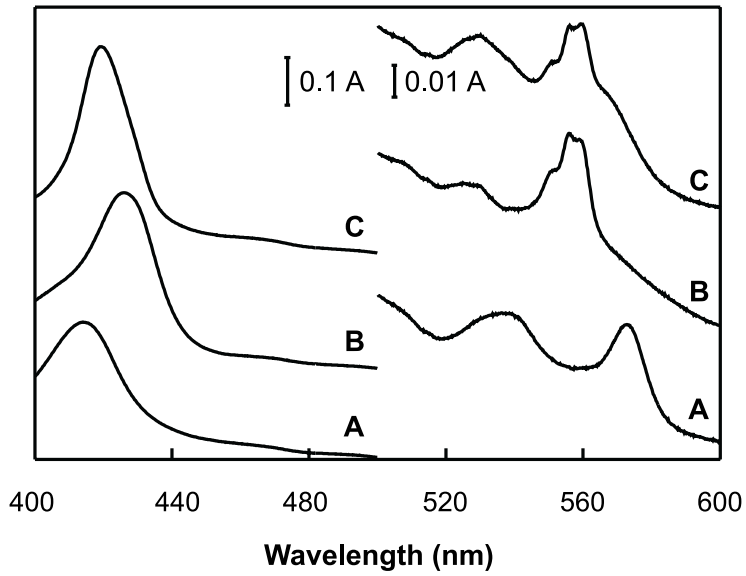

Figure 1. Absorption spectra of erythrocyte membranes. The spectra were recorded at 77K using membranes (5 mg/ml protein) that were oxidized (A), reduced with dithionite (B), or reduced with dithionite and treated with CO (C).

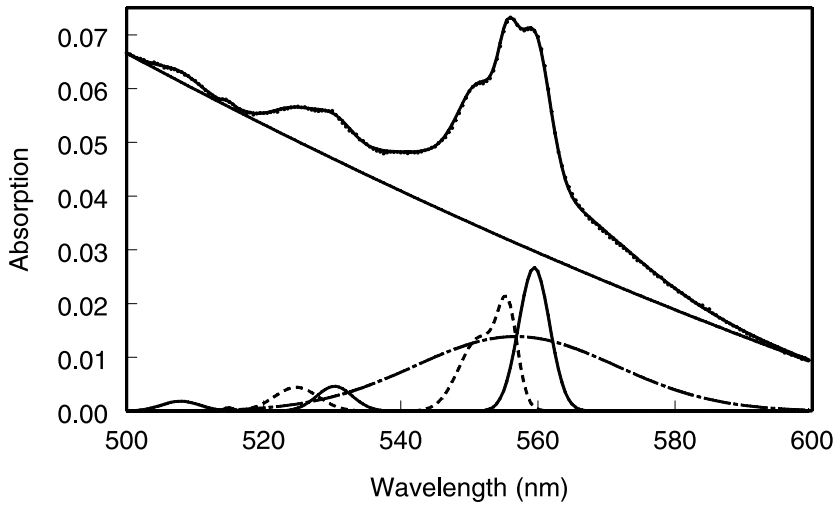

Figure 2. Deconvoluted absorption spectrum of reduced erythrocyte membranes. In the upper part of the graph, the dots represent original data, while the solid line shows the composite simulation. The baseline is shown below the spectra, while the Gauss peaks are shown at the bottom. The peaks were assigned as follows: cytochrome b_5 (- - -), hemoglobin (–·–), and remaining peaks (—). For clarity, two peaks from cytochrome b_5 at 552 and 556 nm are combined.

least four absorption peaks under oxidized conditions and even more when reduced conditions were employed. This suggests that the membrane preparations contained more than one cytochrome. The spectra were further analyzed by spectral deconvolution, a mathematical technique in which a set of (Gauss) curves and a baseline are used to fit the experimental data. In this way, complex spectra can be deconvoluted to the peaks of their components, which can subsequently be identified on basis of literature data of known heme proteins. Figure 2 shows a typical result of the deconvolution and the subsequent peak assignments of a spectrum obtained from membranes reduced with dithionite. This figure demonstrates that the curve fitting procedure resulted in spectra similar to the experimental data. Table 1 summarizes the peaks observed in the spectra of figure 1. Comparison of data recorded under oxidized and reduced conditions indicates that the membrane preparations contained hemoglobin and cytochrome b_5, as was already established in other studies (26). In addition, a cytochrome with absorption bands similar to that of cytochrome b_{561} (further referred to as cytochrome b_x) was found (27).

In subsequent experiments the CO sensitivity of the spectral components was measured. Addition of CO to dithionite-reduced samples induced changes in the spectrum, but the absorption peaks in the 550-560 nm region remained relatively unaffected (Fig. 1C). Moreover, it was found that the height of the peaks in this

Oxidized		Reduced		Reduced / CO	
Detected Peak	Origin (lit. value)	Detected Peak	Origin (lit. value)	Detected Peak	Origin (lit. value)
529	b_5 (530)	525	b_5 (525)	523	b_5 (525)
529	b_x	530	b_x	530	b_x (539)
540	Hb (540)	552	b_5 (552-3)	538	Hb (552-3)
564	b_5 (560)	556	b_5 (556-8)	552	b_5 (556-8)
564	b_x	557	Hb (555)	556	b_5
573	Hb (577)	559	b_x	560	b_x (569)
				565	Hb

Table 1: Assignment of absorption peaks. Absorption spectra of erythrocyte membranes, as shown in figure 1, were analyzed by mathematical deconvolution. Reduction was achieved by the addition of dithionite. The resulting peaks were compared to literature values of cytochrome b_5 and hemoglobin, known heme proteins of the erythrocyte (26, 28, 34, 35). Another component was found (indicated by b_x), resembling cytochrome b_{561} (27).

region was hardly affected by CO: the α-peaks of cytochrome b_5 remained at 122% ± 39 compared to the reduced situation, and cytochrome b_x at 79% ± 7 (n=4). As it is known that reduced hemoglobin binds CO, resulting in a shift of the spectral bands (28), while cytochromes b_5 and b_{561} do not, this confirmed our assignment of the peaks (Table 1).

If cytochrome b_x would be a component of an ascorbate-AFR transmembrane oxido-reductase, it can be expected that addition of ascorbate changes the oxidation state of the heme from oxidized to reduced. It was indeed observed that 500 µM ascorbate could reduce cytochrome b_x (data not shown), supporting the idea that it is involved in transmembrane electron transport. For both cytochrome b_x and b_5, reduction by ascorbate amounted to 50-60% of the reduction achieved by dithionite. For cytochrome b_{561}, a comparable level of reduction has been described using 100 µM ascorbate, whereas 85% was reached with ascorbate levels up to 10 mM (16, 29).

Our data show that erythrocyte membranes contain a CO-insensitive cytochrome with an α-peak at 559 nm in its reduced state. This cytochrome can also be reduced by ascorbate. Its maximum absorption is at a wavelength lower than described for cytochrome b_{561} (27), but spectra recorded at 77 K can be shifted compared to spectra at room temperature. It is therefore conceivable that cytochrome b_{561} is a component of erythrocyte membranes, but a *b*-cytochrome with similar spectral characteristics could also explain our results. It has been

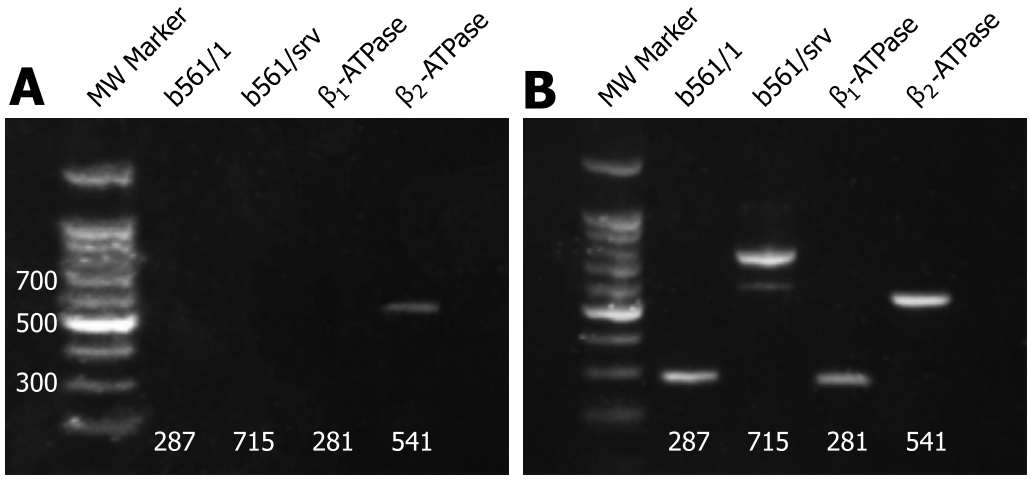

Figure 3. RT-PCR analysis. PCR-products prepared with different primers and cDNA obtained from reticulocytes (A) or K562 cells (B). The predicted lengths of products from the different primers are shown below each lane.

suggested that the heme protein with an absorption peak at 559 nm is cytochrome P420, a degradation product of cytochrome P450 (26). However, erythrocytes probably do not contain cytochrome P450, nor the P450-containing organelles (mitochondria and endoplasmic reticulum). Moreover, cytochrome P420 is expected to be CO sensitive, analogous to cytochrome P450. CO did not affect the 559-absorbing component, which excludes cytochrome P420 as a component of the red cell membrane.

To test the presence of cytochrome b_{561} in erythrocytes, a search for mRNA coding for this protein was performed. However, erythrocytes do not contain mRNA. If cytochrome b_{561} indeed is present in the erythrocyte membrane, it should be expressed during an earlier maturation stage in the hematopoietic pathway. It was therefore investigated whether cytochrome b_{561} mRNA was present in peripheral reticulocytes, the last erythrocyte progenitor cells still containing mRNA. For comparison, mRNA was isolated from human neutrophils, HL60 cells, and from K562 cells, a human erythroleukemic cell line that contains cytochrome b_{561} mRNA (15). cDNA was synthesized shortly after RNA isolation using oligo-dT_{15} primers and PCR analysis of the cDNA was performed. In all cases, PCR reactions without template, as well as reactions with template from cDNA synthesized in the absence of reverse transcriptase, remained without products. It is therefore unlikely that genomic DNA or other contaminants were present.

Two different primer sets were used for detection of cytochrome b_{561} mRNA, but reticulocyte cDNA did not give a PCR product with either set (Fig. 3). cDNA from

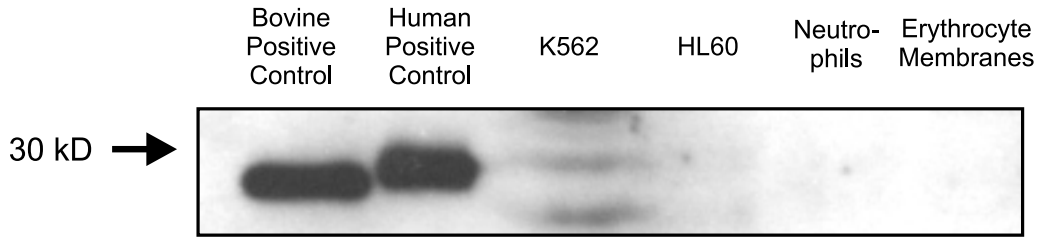

Figure 4. Western blot analysis. Extracts from K562 and HL60 cells, fresh human neutrophils, and erythrocyte membranes were separated by SDS-PAGE, blotted, and stained using an antiserum against bovine cytochrome b_{561}. As positive controls, extracts from bovine adrenal medulla, as well as extracts from human pheochromocytoma membranes were also included.

the K562 cell line, on the other hand, was positive for cytochrome b_{561}, showing that the PCR conditions were chosen appropriately. In addition, a PCR product obtained from b561/1 primers and K562 cDNA was sequenced to verify its origin. The sequence of the PCR product was found to correspond to the expected section of the cytochrome b_{561} coding sequence (not shown), confirming the presence of cytochrome b_{561} mRNA in K562 cells. To check the quality of the reticulocyte mRNA, the presence of mRNA coding for another protein was investigated. It has been described that, at least in peripheral blood, reticulocytes are the only cells containing mRNA for the β_2 isotype of the Na^+,K^+-ATP-ase, albeit in small amounts (30-32). Indeed, cDNA from reticulocytes produced a PCR product of the appropriate size when primers specific for the β_2-Na^+,K^+-ATPase were used (Fig. 3A). The band was not very strong, most likely due to the low abundance of the β_2 mRNA. Primers for the β_1-isotype, which is expressed in e.g. leukocytes but not in reticulocytes, did not result in a product. In K562 cells both isotypes were expressed (Fig. 3B). Human neutrophils and HL60 cells were positive for both cytochrome b_{561} and the β_2-Na^+,K^+-ATPase, but not for the β_1-isotype (results not shown). The results with HL60 cells are in contrast to earlier work, where cytochrome b_{561} was not found (15).

No cytochrome b_{561} mRNA could be detected in reticulocytes using RT-PCR. However, it is conceivable that the cytochrome would be transcribed at an earlier phase in erythrocyte development. In that case its mRNA could already have been degraded when the reticulocytes leave the bone marrow. Therefore, the presence of cytochrome b_{561} was studied at the protein level using Western blotting. As shown in figure 4, samples from human erythrocytes did not produce a band with a cytochrome b_{561}-specific antiserum. A positive control, i.e. cytochrome b_{561} from bovine adrenal medulla, gave a strong signal, as did a human pheochromocytoma

sample, indicating that the antisera cross-reacted with the human cytochrome b_{561}. Assuming that the erythrocyte component absorbing at 559 nm was indeed cytochrome b_{561}, the spectroscopic data indicated that at least 50 ng of the cytochrome should be present on the Western Blot (27). The human positive control contained 150 ng of pheochromocytoma membranes, in which up to 20% of the protein is cytochrome b_{561} (33). Thus, the amount of cytochrome from the erythrocyte sample loaded on the gel should have been well within the detection limit of the Western Blot. Nevertheless, no cytochrome b_{561} was detected by the anti-serum. K562 cells, which were positive for the cytochrome in the RT-PCR ((15) and Fig. 3), produced a faint band of the proper molecular weight (Fig. 4), showing that the b_{561} protein is present in these cells, albeit in small amounts. Cytochrome b_{561} could not be detected in extracts of HL60 or neutrophils, in spite of a positive RT-PCR result. Apparently, the levels are below the detection limit of Western blotting.

In conclusion, both RT-PCR and Western blotting experiments did not show the presence of cytochrome b_{561} in the erythrocyte. It is therefore concluded that the cytochrome is not the protein responsible for the ascorbate-dependent AFR reduction that was found in these cells. Apparently, another protein in the erythrocyte membrane is involved in this process. This protein may have strong similarities to cytochrome b_{561}, but homology searches on known sequences in genomic databases did not provide useful results. Alternative proteins could be expected to have transmembrane sections, and possibly high potential hemes as prosthetic groups. The unknown *b*-cytochrome found in our spectra could possibly play a role, but further investigations are necessary to elucidate its identity.

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CHAPTER 7

Analysis of transmembrane redox reactions: interaction of intra- and extracellular ascorbate species

Submitted for Publication in Methods in Enzymology.

Introduction

The pivotal role of ascorbate in the defense against oxidants, as well as in other physiological processes is generally recognized. To preserve ascorbate, it should stay in the reduced form. A number of systems inside the cell ensure the quick reduction of the oxidation products of ascorbate, which are ascorbate free radical (AFR) and dehydroascorbic acid (DHA). These reduction reactions are very efficient, and AFR and DHA will therefore be virtually absent in a healthy cell. Extracellular DHA can be regenerated by transport into the cell followed by intracellular reduction. Alternatively, it has been shown that AFR and DHA can be reduced on the extracellular face of the cell (1, 2). This reaction involves a redox system in the plasma membrane, which uses intracellular NADH as an electron source. We recently found that intracellular ascorbate can also be an electron donor for this reaction (3). However, the nature of the ascorbate-driven system in the plasma membrane remained uncertain. Most likely, the electron transfer is mediated by a protein in the plasma membrane. However, it has also been suggested that small lipid-soluble molecules like α-tocopherol and coenzyme Q can shuttle electrons from the intra- to the extracellular side of the membrane (4-6). Irrespective of the mechanism, this plasma membrane redox system efficiently helps to maintain the concentration of extracellular ascorbate, and is one of the main mediators in the interaction of intra- and extracellular ascorbate.

This chapter will highlight techniques that can be used to study ascorbate-related redox reactions across cell membranes. First, the quantification of intra- and extracellular ascorbate species will be discussed. Subsequently, we will present methods to establish the proper intra- or extracellular concentrations of some ascorbate species, as well as methods for the detection of redox reactions between these intra- and extracellular molecules.

Materials

Ascorbate – Ascorbate is stable as a solid, but should only be dissolved on the day of use because of its susceptibility to oxidative degradation. When kept on ice, stock solutions have sufficient stability for many hours, especially in acidic buffers. Ascorbate concentrations can be checked by measuring the absorbance at 265 nm (ε=14,500 $cm^{-1}M^{-1}$)

DHA – DHA is purchased as a solid. In solution it can degrade rapidly, especially at pH values above 5 (7). To avoid decomposition, solutions are made on ice and are used immediately. The purity of some commercial preparations has been

questioned. It may therefore be required to test the DHA. An easy and convenient way to do this is by reducing DHA to ascorbate with e.g. an excess amount of DTT (5 mM), and measuring the resulting absorbance at 265 nm. More than 95% of the DHA should be recovered as ascorbate. DHA can also be prepared in situ by mixing ascorbate and a large amount of ascorbate oxidase (> 0.5 U/ml). Alternatively, DHA can be prepared by oxidizing ascorbate with bromine. On ice, 5 µl bromine is added to 1 ml of 1 mM ascorbate. After mixing and 30 s reaction time, the solution is bubbled with nitrogen or argon to remove the bromine. After 10 min, when all bromine is lost, the solution should have lost its typical brown color.

Ascorbate oxidase – Ascorbate oxidase (EC 1.10.3.3) is a useful enzyme in the study of ascorbate and its free radical. Unfortunately, it does not retain activity in solution, and must be prepared freshly before use. The enzyme can be purchased as a lyophilized solid. However, we prefer to use ascorbate oxidase adsorbed to small spatulas (Roche Diagnostics, Almere, The Netherlands). The spatulas contain 17 U of ascorbate oxidase, and the enzyme can conveniently be dissolved in a buffer of choice.

$Ni(en)_3^{2+}$ – Tris-(ethylenediamine)-nickel(II) chloride 2-hydrate ($Ni(en)_3^{2+}$) can be prepared by dissolving 12 g $NiCl_2 \cdot 6H_2O$ in 60 ml H_2O, and subsequent addition of 14 ml of 70% ethylenediamine in water (v/v) (8). After reducing the volume to about 60% by evaporation in a boiling water bath, the solution is cooled, and purple crystals are formed. Crystallization can be promoted by the addition of ethanol. The crystals are obtained by filtration, two washes with ethanol, and air-drying.

Measurement of ascorbate species

Ascorbate

Ascorbate has a strong absorption band at 265 nm, allowing simple and convenient spectrophotometric quantification. However, a prerequisite for such assays is the absence of interfering compounds that scatter or absorb light at that wavelength. When direct spectroscopic measurements are not possible, HPLC can be used to separate ascorbate from other material absorbing at 265 nm. Many aspects of HPLC analysis of ascorbic acid were discussed in volumes of *Methods in Enzymology* (9, 10). HPLC analysis requires careful preparation of the samples to prevent oxidation of ascorbate. Precautions include cooling or freezing of samples,

acidification, chelation of metal ions by EDTA or DTPA, flushing vials with inert gas and deproteinizing the sample.

Both C-18 and SAX column packings have been employed for the separation of ascorbate species (10, 11). We have good experience with Partisil-SAX material, which yields a single ascorbate peak from cell extracts. The system comprises an (auto)-injector with a 100 µl loop, a Partisil SAX column (10 µm, 250 x 46 mm) with a 20 mm guard column, and a UV-detector set at 265 nm. A fraction collector or a radiochemical detector can be added for measurement of radio-labeled ascorbate or DHA. Ascorbate and DHA elute isocratically from the column with 7 mM potassium phosphate, 7 mM KCl pH 4.0. However, after a run with cell extracts, the column should be flushed with high salt (0.25 M potassium phosphate, 0.5 M KCl, pH 5.0) for 5 min to remove other cellular anions. Thus, the use of a gradient controller is recommended. For more demanding applications, UV-detection can be replaced by coulometric or amperometric electrochemical detection, which have a superior sensitivity. The HPLC analysis of multiple samples can take several hours. If an autosampler is used, cooling of the sample vials during analysis is required to prevent sample degradation pending analysis. In addition, vials may be flushed with an inert gas to prevent oxidation. When many samples are analyzed, it is recommended to check for degradation by adding standard samples at the beginning and end of a run.

HPLC analysis of ascorbate (and DHA) samples from cells requires techniques that preserve ascorbate and DHA. Due to the instability of these compounds, extraction of ascorbate from cells should be performed under conditions where minimal degradation occurs. Controls should be performed to check this. The following procedure was used for a leukemic cell line, but it can be applied to most cultured cells. After washing of the cells to remove extracellular ascorbate, about 10^6 cells are collected as a pellet in a microcentrifuge tube. Extraction and deproteinization are achieved by the addition of 600 µl methanol, dispersion of the pellet, and the subsequent addition of 400 µl water. To ensure the stability of ascorbate, EDTA and HCl are added to final concentrations of 50 µM and 50 mM, respectively. After centrifugation of the extract to remove precipitated proteins, the supernatant can be analyzed immediately by HPLC, or frozen for later analysis.

Analysis and sample pretreatment of ascorbate from erythrocytes differs from other cell types because of the presence of hemoglobin. It has been reported that denatured hemoglobin can catalyze the oxidation of ascorbate (12). Hemoglobin must therefore not be removed by precipitation with methanol. Instead, we lyse 200 µl packed erythrocytes in 3 volumes of 7 mM potassium phosphate pH 4.0,

removed membrane fragments by microcentrifugation and subsequently removed the hemoglobin from the supernatant by ultrafiltration. The reusable Millipore micropartition system with 30 kD cutoff membranes is suitable for this purpose. Ultrafiltration devices in a microcentrifuge format were found to clog during filtration. Apparently, the increased membrane surface of the Millipore unit prevents this problem, and yields adequate amounts of a clear colorless filtrate for further analysis. After filtration, methanol is added to the ultrafiltrate up to 60% v/v to precipitate any remaining small proteins, and 50 µM EDTA and 50 mM HCl (final concentrations) are added to stabilize ascorbate. After centrifugation, the sample can be frozen or analyzed directly by HPLC. When desired, it is also possible to rupture cells by freeze-thawing instead of hypotonic lysis (13).

Dehydroascorbic acid

Several different approaches are possible for the analysis of DHA. Four of them will be described here. Preparation of samples for DHA analysis needs even greater care than ascorbate samples. This is due to the swift decomposition of DHA, which has a half-life of about 10 min under physiological conditions. An acid pH and cold storage can slow down the hydrolysis reaction, but still, samples should be analyzed quickly (7). The use of autosamplers should therefore be avoided when DHA is not derivatized before e.g. HPLC analysis. Furthermore, it should be kept in mind that an assay for intracellular DHA may be of limited value. Although it is possible to extract DHA from cells, a part of it may be lost upon lysis of the cells, either by hydrolysis or by a redox reaction with a cellular component.

Reduction of DHA - Many protocols for the analysis of DHA are derived from methods for the determination of ascorbate. They involve the conversion of DHA to ascorbate by the addition of 5 mM reducing agent like DTT or β-mercaptoethanol to the sample. The difference in ascorbate content in samples with and without a reductant corresponds to the amount of DHA that was present. After a quick reduction, reduced and control (non-reduced) samples may be stored under ascorbate preserving conditions, such as an acid pH, low temperatures, and in the presence of EDTA.

Derivatization of DHA - DHA can be analyzed without a reduction step, when a suitable detection technique follows HPLC separation. DHA does not absorb light at useful wavelengths, nor can DHA be detected by electrochemical detection. It has been reported that pre- or post-column derivatization using *o*-phenylenediamine yields a stable fluorescent compound that is readily detectable (14). Other methods of derivatization allow separation and detection by GC-MS. However, it has been

suggested that derivatization reactions are prone to produce artifacts, indicating that this technique must be handled with great care (9).

Radioactive labeling of DHA - A convenient method to quantify unmodified DHA is the use of ^{14}C-labeled DHA. In the HPLC system described for ascorbate (see above), DHA elutes from the column before ascorbate. An inline radioactivity detector can be used for detection, or alternatively fractions can be collected for liquid scintillation counting. In the latter case, a sufficient number of fractions should be collected to allow peak separation.

NMR and ^{13}C-labeled DHA - An alternative that can be used for intact cells is [^{13}C]-NMR. This technique was described by Himmelreich *et al.* for erythrocyte suspensions containing [^{13}C]-ascorbate and [^{13}C]-DHA (2). The NMR spectra of these suspensions contain specific bands for ascorbate and DHA. Moreover, it was found that these bands have a shift that correlates with the concentration of hemoglobin in the solution. Thus, in erythrocytes, distinct peaks can be observed for intra-and extracellular ascorbate or DHA. Drawbacks of this method are that it requires relatively high concentrations of both erythrocytes and ascorbate or DHA. Moreover, the erythrocytes must be pretreated with carbon monoxide to produce carbonmonoxy-hemoglobin, which has a more stable diamagnetic nature than the oxy and deoxy forms. It is unclear whether the band-shift phenomenon can also be used in other cell-types.

Ascorbate Free Radical

AFR is an unstable molecule, with a lifetime of about one second. This labile nature requires its measurement in situ, without any sample pretreatment. Thus, the use of chromatographic techniques is excluded. Only spectroscopic techniques can offer real time measurement of the sample. In principle, AFR can be measured spectrophotometrically at 360 nm (ε= 4,900 $cm^{-1}M^{-1}$) (15). However, in biological samples, AFR only reaches concentrations in the nanomolar range, which results in absorption values that will usually not exceed the detection limit. Moreover, spectrophotometric measurements can not easily be performed in turbid cell suspensions.

Electron spin resonance (ESR) spectroscopy does not have these drawbacks, and allows the identification and quantification of paramagnetic species (such as free radicals) in turbid suspensions. However, the measurement of aqueous samples at room temperature requires the use of a flat quartz sample cell, and a special resonance cavity to accommodate this cell. The equipment has to be tuned after

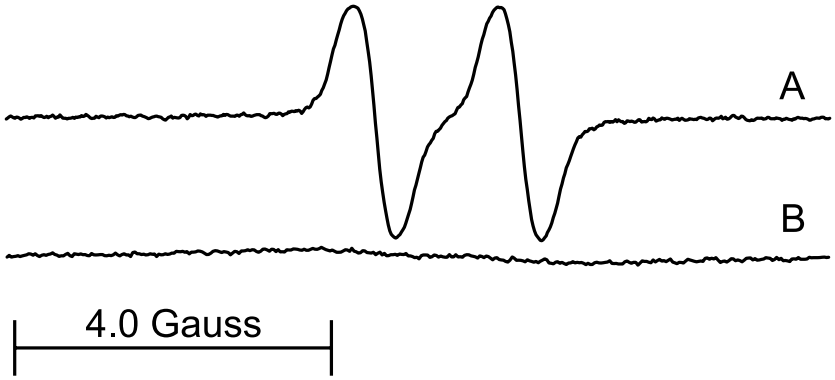

Figure 1: ESR spectra of ascorbate free radical. A, 100 µM ascorbate and 4 mU/ml ascorbate oxidase; B, same as A, but with 5 mM $Ni(en)_3^{2+}$.

the cell is positioned in the cavity. It is therefore recommended to use a sampling device to load samples into the cell while it is still positioned in the cavity, e.g. by aspiration (16). In this way, successive samples can be measured without having to adjust the position of the cell or the settings of the spectrometer. This also allows scanning within seconds after mixing of the samples. The equipment used for our experiments consisted of a JEOL RE2X X-band spectrometer operating at 9.36 GHz with a 100 KHz modulation frequency. Samples were transferred to a quartz flat cell in a TM_{110} cavity with a rapid sampling device. The ESR spectrometer setting were as follows: microwave power, 40 mW; modulation amplitude, 1 G; time constant, 0.3 s; scan time, 5 min; scan width, 15 G.

A typical AFR signal consists of a doublet with a hyperfine splitting a^{H4} = 1.8 G (Figure 1A). The ESR signal is proportional to the amount of paramagnetic species in the sample and this can be used as a quantitative assay. The concentrations of AFR can be determined by double integration of the ESR spectra, using the spectrum of a stable radical such as 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO), with known concentration, as a standard. It is important to use identical spectrometer settings while scanning the AFR spectrum and the TEMPO spectrum.

The scanning of an ESR spectrum can take several minutes, depending on e.g. signal intensity. When AFR concentrations need to be followed over a prolonged period of time or at a short timeframe, it is possible to lock the spectrometer to the Gauss value of the top of one of the peaks of the AFR doublet. Though this approach may result in a decrease in the signal-to-noise ratio, it allows the continuous tracking of AFR signal levels.

ESR spectroscopy does require expensive and specialized equipment, as well as a trained operator. However, it is the most powerful technique to study free radical molecules, like AFR, and their interaction with the living cell.

Interaction of intracellular and extracellular ascorbate

Several requirements should be met to study the interaction of intra- and extracellular ascorbate, DHA and AFR. First, cells have to be properly conditioned, resulting in the desired intracellular concentration of ascorbate. These cells can subsequently be used in an assay that can measure the interaction of the intra- and extracellular ascorbate forms.

Methods to modify intracellular ascorbate levels

The best way to modify ascorbate levels in the cell is by using the cell's transport systems in the membrane. Transport pathways exist for both ascorbate and DHA. However, many cells do not express the ascorbate transporter, but can quickly transport DHA through the GLUT-1 glucose transporter. Thus, loading with DHA is the preferred method. After transport, DHA is reduced to ascorbate, which only slowly leaks out of the cell. Good results have been obtained with an incubation period of 30 min at room temperature in a buffer containing DHA, although incubation at 37 °C might prove to be superior for some cell-types. After 30 min, most of the DHA will have been degraded, and no additional ascorbate will accumulate in the cells. The cells can then be washed and used for an experiment. For erythrocytes, we typically incubate a 20% suspension of washed cells in phosphate-buffered saline (PBS) containing up to 500 µM DHA and 2.5 mM adenosine at room temperature. Adenosine (or glucose) improves the accumulation of ascorbate in erythrocytes by supplying energy needed for the reduction of DHA. After 30 min of incubation under gentle mixing conditions, the cells are washed three times with PBS, and used for an experiment within an hour. The resulting intracellular ascorbate concentration depends on the concentration of DHA that is added. For example, erythrocytes require about 500 µM DHA to reach an intracellular concentration of 1 mM ascorbate. However, similar levels could be achieved in leukemic HL60 cells after incubation with only 25 µM DHA. It is, therefore, important to determine the result of the incubation by HPLC analysis.

Intracellular ascorbate can be removed by treatment with 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL). TEMPOL can freely diffuse into the cell and oxidize ascorbate to DHA, which subsequently diffuses out of the cell through

the GLUT-1 transporter. Three consecutive 5 min incubations of cells in buffer containing 1 mM TEMPOL removes over 90% of the ascorbate in the cells. It is important to subsequently wash the cells three times in regular buffer to remove all TEMPOL from the cells. It has been reported that intracellular reductants like NADH and NADPH were not affected by this treatment, but we observed a decrease in NADH levels under some conditions (3, 17). One should therefore check this in each cell-type that is used.

When studying ascorbate transport, it might be necessary to quickly separate cells from the incubation medium. For this purpose, cells can be spun down through a layer of dibutyl phtalate, or another oil of appropriate density. When a suspension is layered on top of the oil, centrifugation quickly sediments the cells to the bottom of the tube, while the medium remains on top. Thus, the cells are immediately separated from the medium, but remain intact. When radio-labeled substrates are used, it is convenient to use small microcentrifuge tubes. The bottom of the tube can be cut off after centrifugation to collect the pellet for liquid scintillation counting.

Methods to generate AFR

In several studies, AFR has been generated by mixing ascorbate and DHA. To calculate the resulting AFR concentration, the equilibrium constant of the disproportionation reaction (Reaction I) was used:

$$\text{Ascorbate} + \text{DHA} \longleftrightarrow 2\,\text{AFR} + H^+ \qquad \text{(Reaction I)}$$

$$\text{Ascorbate} + O_2 \xrightarrow[\text{Metal Ions}\ /\ \text{Ascorbate Oxidase}]{\text{Spontaneous}} \text{AFR} + O_2^{\bullet -} \qquad \text{(Reaction II)}$$

However, at micromolar or low-millimolar concentrations of ascorbate and DHA, this method is seriously hampered by redox active metals (Fe or Cu), present in the buffers that are used (18). Under these conditions, the AFR concentration is determined by metal-catalyzed reactions (Reaction II) and not by the equilibrium reaction. The most reliable and reproducible method to produce AFR is the incubation of ascorbate with moderate amounts of ascorbate oxidase. The concentration of ascorbate oxidase can be varied to generate different concentrations of AFR, which may be quantified by ESR spectroscopy. In our laboratory, 100 µM ascorbate and 1-50 mU/ml ascorbate oxidase gives a useful range of AFR concentrations for our experiments.

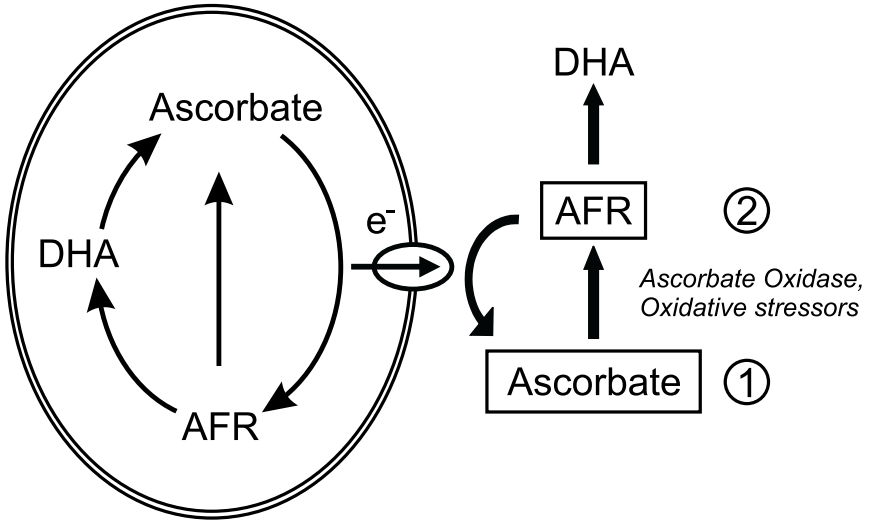

Figure 2: Principle of the AFR reduction assays. The reduction of extracellular AFR in a cell suspension can be measured by monitoring AFR using ESR spectroscopy [1], or by monitoring the decrease in concentration of extracellular ascorbate [2].

Methods to measure the ascorbate-dependent reduction of extracellular AFR

Extracellular ascorbate can be regenerated from AFR by a one-electron reduction step. This can be measured by two different methods. The first method monitors the oxidation of ascorbate in the suspension (Figure 2, [1]). Ascorbate is lost by oxidation when AFR is generated. The reduction of AFR regenerates ascorbate, and will thus appear to slow down the oxidation of ascorbate. The second method uses ESR spectroscopy to directly measure AFR in a cell suspension (Figure 2, [2]). Reduction of AFR should decrease its concentration in the suspension.

Measuring extracellular ascorbate oxidation by UV spectroscopy.

This assay measures the extracellular ascorbate concentration in a cell suspension where ascorbate is oxidized by ascorbate oxidase, thus directly measuring the cell's capacity to prevent the loss of ascorbate by oxidation. When AFR is reduced, the rate of ascorbate oxidation will appear to have decreased. The technique has therefore also been referred to as an ascorbate stabilization assay.

In each cell system, ascorbate and ascorbate oxidase concentrations, as well as cell densities, need to be optimized. The rate of AFR formation by ascorbate oxidase, and its corresponding steady state concentration, are critical when the ascorbate-dependent reduction of AFR has to be detected. When the rate is too high, the decrease in AFR concentration due to the ascorbate-dependent reductase will be relatively small, and hard to detect. On the other hand, a low rate of AFR

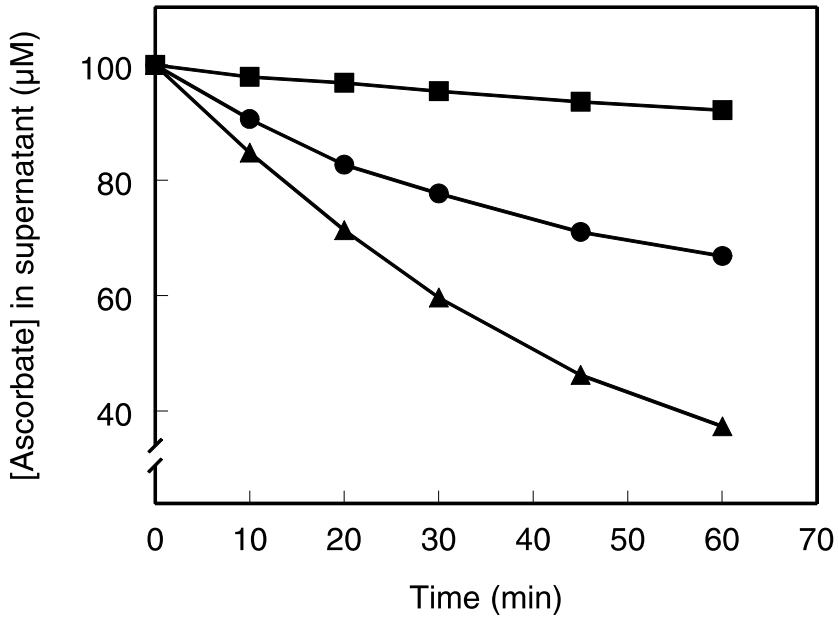

Figure 3: Oxidation of extracellular ascorbate in erythrocyte suspensions. 100 µM ascorbate and 4 mU/ml AO were incubated in 10% suspensions of ascorbate–loaded erythrocytes (■), control erythrocytes (●), or in the absence of cells (▲). Samples were centrifuged, and the absorption at 265 nm was measured in the supernatant at different timepoints.

formation will be overwhelmed by the reduction rate of the plasma membrane redox system. In this case, it is not possible to show the capacity of the ascorbate-dependent reductase activity.

For experiments with erythrocytes, ascorbate (100 µM) and ascorbate oxidase (4 mU/ml) are added to 8 ml of a 10% ascorbate-loaded cell suspension in a 10 ml screwcap tube, while rocking gently to prevent erythrocyte sedimentation. The oxidation of ascorbate by ascorbate oxidase proceeds in a linear fashion for more than 15 min (Fig. 3). Thus, the oxidation rate can be determined from samples taken after 0 and 15 min incubation. Duplicate samples (1.5 ml) are drawn from the tubes and centrifuged, and the supernatant is transferred to a quartz cuvette to measure the absorbance at 265 nm. An aliquot can also be used for HPLC analysis. Samples must be processed promptly, as the oxidation of ascorbate will continue in the supernatant. One should ensure that the activity of ascorbate oxidase is not affected by the cells, e.g. by verifying enzyme activity in the supernatant after an incubation with cells. In addition, control experiments with [^{14}C]-ascorbate-loaded cells should show that no intracellular ascorbate is leaking from the cells. The involvement of intracellular ascorbate in the reduction of AFR can be inferred from the comparison of cells with different internal concentrations of the vitamin. Figure 3 illustrates the effect of control and ascorbate-

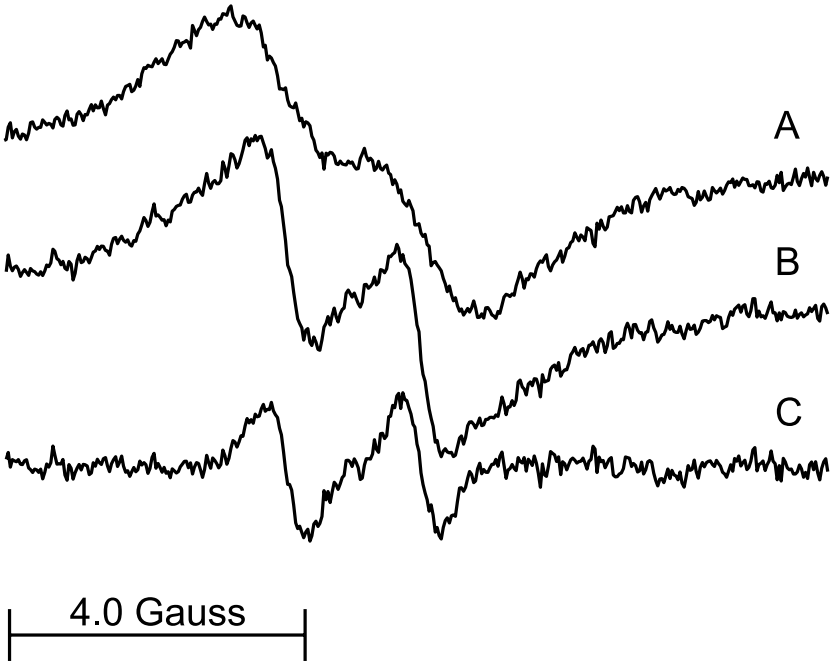

Figure 4: Detection of intracellular AFR using ESR. A, 1 mM ascorbate, 20 mU/ml AO and 5 mM $Ni(en)_3^{2+}$; B, same as A, but with 20% ascorbate-loaded erythrocytes; C, A subtracted from B. Erythrocytes were loaded with ascorbate, using 500 µM DHA, as described in the text.

loaded erythrocytes on the oxidation of ascorbate by ascorbate oxidase. Both affected the oxidation rate, but the effect was more pronounced in the presence of ascorbate-loaded cells. The protective effect of control erythrocytes can most likely be attributed to endogenous ascorbate, and a NADH-dependent redox system in the cells.

Measuring AFR reduction by ESR spectroscopy

The capacity of cells to reduce extracellular AFR and thus to prevent oxidation of ascorbate can also be measured by determining the AFR concentration by ESR spectroscopy. Ascorbate-loaded erythrocytes are resuspended at a 10% hematocrit in PBS and exposed to an AFR-generating system, consisting of 100 µM ascorbate and 4 mU/ml ascorbate oxidase. Immediately after mixing, the suspension is aspirated into a flat cell in an ESR spectrometer and the AFR signal intensity is determined. The AFR signal should be stable for the duration of the scan (5 min). By varying the intracellular ascorbate concentrations, the effect of various ascorbate concentrations on AFR signal intensity can be determined. It was found that, with e.g. 1 mM intracellular ascorbate, the extracellular AFR signal intensity decreased by 45% relative to control erythrocytes (3).

Discrimination between intracellular and extracellular AFR is possible with non-permeant line broadening agents. One potent and useful line broadening agent is the nickel ion. Due to its toxicity, it must be chelated when used in biological samples, e.g. with ethylenediamine, to $Ni(en)_3^{2+}$ (19). The chelate is not toxic, and does not affect the redox properties of AFR, while it preserves the line broadening properties. The addition of $Ni(en)_3^{2+}$ broadens a sharp ESR signal, resulting in a negligible amplitude compared to an unaffected signal (Figure 1B). When added to a suspension, it will only broaden extracellular AFR signals and leave intracellular signals unaffected. Figure 4 illustrates how 5 mM $Ni(en)_3^{2+}$ allows the detection of a small intracellular AFR signal in the presence of a large amount of extracellular AFR. The signal of extracellular AFR, generated by mixing 1 mM ascorbate with 20 mU/ml ascorbate oxidase, is broadened by the presence of 5 mM $Ni(en)_3^{2+}$, but is still visible due to the high concentration of AFR (Figure 4A). The same signal is observed in the presence of 20% control erythrocytes (not shown). However, the addition of ascorbate-loaded erythrocytes results in the signal given in figure 4B. Comparison of A and B shows that superimposed on the signal in figure 4A, a sharp signal can be observed that is unaffected by $Ni(en)_3^{2+}$. Subtraction of spectra 4A and 4B reveals that, indeed, a small AFR signal is present. As this signal is unaffected by $Ni(en)_3^{2+}$, it must be of intracellular origin, which nicely illustrates the formation of intracellular AFR as an intermediate in the reduction of extracellular AFR. Thus, the methods described in this chapter enabled us to show that intracellular ascorbate can be an electron donor for the reduction of extracellular AFR.

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Chapter 8

Summary and general discussion

Many cells can reduce oxidized molecules that are present at their extracellular surface. The reducing equivalents that are required for these reactions originate from the intracellular space of the cell. The intracellular reductants, or electron donors, do not necessarily leave the cell to reduce extracellular substrates. Instead, the reducing equivalents, i.e. electrons, cross the membrane through a system in the plasma membrane. This thesis describes a study on the properties of these plasma membrane redox systems, and in particular their interaction with ascorbate (vitamin C). This vitamin plays an important role, both as an intracellular electron donor and as an extracellular substrate.

Ascorbate chemistry and detection

Ascorbate oxidizes in two single electron steps, yielding ascorbate free radical (AFR) and dehydroascorbic acid (DHA), respectively. DHA is not stable under physiological conditions, and will hydrolyze irreversibly. AFR is also short-lived in solution. For instance, two molecules of AFR can disproportionate, yielding one molecule of ascorbate and one of DHA. This disproportionation reaction is reversible, and will reach an equilibrium state. Thus, AFR can also be generated by mixing ascorbate and DHA. This principle has been used in the past to generate AFR in experiments. The resulting concentration of AFR was calculated from the equilibrium constant of the disproportionation reaction. Chapter 2 of this thesis discusses some drawbacks of this approach. AFR is not only formed by the disproportionation reaction, but also from the spontaneous or metal-ion-mediated oxidation of ascorbate. Especially at low, physiologically relevant, concentrations of ascorbate, this oxidation will dominate the formation of AFR. Thus, a calculation of the concentration of AFR using the equilibrium constant of the disproportionation reaction will underestimate the actual concentration. Moreover, the generation of AFR from ascorbate and DHA will not result in the prolonged and stable generation of AFR, as DHA will quickly hydrolyze, thereby changing the equilibrium. It is concluded that other methods should be used to generate AFR. A good alternative is the oxidation of ascorbate by the enzyme ascorbate oxidase, which gives a more stable and predictable concentration of AFR. Nevertheless, the resulting AFR concentrations should always be measured experimentally.

The dynamic chemistry of ascorbate makes it a challenging molecule to study. Ascorbate is subject to oxidation, and its oxidation products are even more labile. Therefore, techniques are required that prevent the degradation of the sample, or measurements must be made *in situ*. Chapter 7 describes some problems and solutions related to the analysis of ascorbate, DHA and AFR, and their interactions

in a cellular environment. A variety of techniques, such as UV spectroscopy, electron spin resonance spectroscopy, HPLC and radioactive labeling can be used to track the different molecules as they react and move between cells.

Ascorbate and plasma membrane redox systems

Ascorbate is known as an indispensable component of the anti-oxidant defenses. Many of its anti-oxidant effects involve a direct reaction with an oxidant. However, for some reactions, the interaction of ascorbate with plasma membrane redox systems is required. The transfer, by these systems, of reducing equivalents across the membrane allows intracellular ascorbate to reduce extracellular molecules without leaving the cell. The intracellular space has an efficient regeneration system for the intracellular oxidation products of ascorbate, ensuring efficient reduction back to ascorbate. It is still not completely clear what the physiological substrates and functions of the plasma membrane redox systems are. The most important function of the ascorbate-dependent redox systems appears to be protection against oxidants. Various potentially harmful oxidants can be reduced directly at the cell surface by the redox system. However, the reduction of extracellular AFR, and therefore the preservation of extracellular ascorbate levels, may be of more importance. After extracellular ascorbate reacts with an oxidant, it can be regenerated from its product AFR by a reduction at the cell surface. The use of a plasma membrane redox system for this reduction is more efficient than the intracellular regeneration of ascorbate, as it circumvents different transport steps, which delay the availability of the regenerated ascorbate to the extracellular medium.

Two important electron acceptors were chosen for further study in this thesis. Ferricyanide is a non-physiological oxidant that has frequently been used for the study of plasma membrane redox systems. Cells are impermeable to this molecule, and its conversion can easily be quantified experimentally. The other substrate that was studied was the ascorbate free radical. The conversion of this substrate to ascorbate is more difficult to assess, but the reduction of AFR is physiologically more important.

The importance of ascorbate to plasma membrane redox systems was revealed by the earlier study of ferricyanide reduction by erythrocytes (1-4). Accumulation of ascorbate in the cells increased the rate at which extracellular ferricyanide could be reduced. However, various models were proposed to explain this effect of ascorbate. In chapter 3, the ascorbate-dependent reduction of ferricyanide was

studied in the human leukemic cell line HL60. Indeed, intracellular ascorbate stimulated ferricyanide reduction in a saturable dose-dependent manner. Our data excluded several models, but corroborated a model in which ferricyanide is reduced via a plasma membrane redox system, similar to the reduction of ferricyanide by erythrocytes as described by May et al. (4). The cells can also reduce ferricyanide in the absence of ascorbate, with NADH as the intracellular electron donor. However, it was unknown whether different redox systems were involved in the reduction of ferricyanide by either ascorbate or NADH. Experiments with the inhibitor *p*CMBS revealed that the reduction of ferricyanide in the absence of ascorbate was much more sensitive to the inhibitor than the reduction in the presence of ascorbate. This indicates that separate redox systems must be present in the plasma membrane of HL60 cells, one that uses NADH as its electron source, and one that uses ascorbate.

Though ferricyanide is a useful tool for the study of plasma membrane redox systems, it is a non-physiological molecule. The physiological relevance of ferricyanide reduction is therefore not always clear. It has previously been shown that cells could also reduce extracellular AFR using intracellular NADH (5, 6). AFR could be a physiological substrate for plasma membrane redox systems. Therefore, experiments were conducted to study whether ascorbate-dependent redox systems could also reduce AFR. As described in chapter 4, this was indeed the case in erythrocytes. The cells could also reduce AFR using NADH, but intracellular ascorbate significantly increased the reduction rate. Thus, AFR seems to be a physiological substrate for the ascorbate-dependent plasma membrane redox system of the erythrocyte. The effect of ascorbate on the reduction of AFR was quite similar to its effect on the reduction of ferricyanide, as found in HL60 cells. It is therefore conceivable that a single protein is responsible for the reduction of both AFR and of ferricyanide. However, further experiments are required to test this hypothesis.

Mechanisms and components of ascorbate-dependent redox systems

Extensive evidence showed that intracellular ascorbate can promote the reduction of extracellular substrates. However, the exact mechanism behind this phenomenon has remained unclear. The extracellular substrates AFR and ferricyanide both require one electron for their reduction. It is therefore most likely that intracellular ascorbate will also donate one electron, yielding intracellular AFR. Indeed, the formation of intracellular AFR has been observed while extracellular AFR or ferricyanide was reduced ((7), this thesis). The NADH-dependent reduction of AFR and ferricyanide

must have a more complex mechanism. NADH donates two electrons, while the acceptors can only accept one. Hence, the NADH-dependent plasma membrane redox system must contain a buffer system to accommodate both one- and two-electron interactions. This may involve the participation of quinone or flavin moieties in the electron transfer mechanism.

The electrons that are donated by the intracellular reductant must be transported across the plasma membrane by a redox system. It has been suggested that small lipid soluble molecules like coenzyme Q and α-tocopherol could act as an electron shuttle to cross the plasma membrane (8-10). However, our data indicate that it is more likely that protein factors in the membrane are involved. Ascorbate-dependent redox systems displayed saturable kinetics in both HL60 cells and erythrocytes. Also, ferricyanide reduction in HL60 cells could be inhibited by thiol reactive agents, and depletion or supplementation of the cells with α-tocopherol did not affect reductase activity, nor did the addition of analogues of coenzyme Q. In erythrocytes, the experiments described in chapter 5 showed that the ascorbate-dependent reduction of AFR affected the membrane potential. Thus, the reduction was electrogenic, causing the net transport of charge across the membrane during the reaction. Coenzyme Q and α-tocopherol could potentially shuttle electrons across the membrane, but can only accept an electron together with a proton. Thus, for e.g. coenzyme Q, the possible redox forms are the neutral molecules Q, $QH^{\bullet}$, and QH_2. A shuttle of coenzyme Q across the membrane can therefore not be electrogenic, whereas the electrogenicity of the reduction of AFR was clearly shown in chapter 5. For both erythrocytes and HL60 cells, a protein-mediated system is the best explanation for the data that were obtained on ascorbate-dependent redox systems. The involvement of a small electron carrier cannot be excluded for the NADH-dependent reduction of AFR or ferricyanide. Electrogenicity could not be shown under those conditions, whereas other groups provided evidence for a coenzyme Q shuttle that, driven by the NADH-dependent cytochrome b_5 reductase, could reduce extracellular AFR (9, 11). In spite of some opposing reports (12), the latter model seems a viable option to explain the NADH-dependent reduction of extracellular substrates.

The study of plasma membrane redox systems would be greatly facilitated by the identification of the putative proteins that drive the reaction. Comparison of expression levels in different tissues would be possible using molecular biological techniques, avoiding the problems that accompany functional assays. Also, it would be possible to resolve the questions that exist on the substrate specificity of the redox systems. At present, it is only possible to observe the conversion of substrates

by intact cells. It is therefore hard to establish whether multiple systems contribute to the conversion of a single substrate, or whether different substrates are converted by a single system. The ascorbate-dependent reduction of AFR in the erythrocyte strongly resembles a process in the adrenal gland. The adrenal medulla contains cells with chromaffin granules, in which large amounts of ascorbate are oxidized to AFR for the biosynthesis of norepinephrin. This AFR is subsequently reduced by a cytochrome b_{561} in the granule membrane, which uses cytoplasmic ascorbate as an electron donor. It was hypothesized that this cytochrome, which had never been found in the erythrocyte, might be responsible for the same reaction in the erythrocyte membrane. Therefore, the expression of cytochrome b_{561} was studied in erythrocyte membrane extracts with an antibody against cytochrome b_{561}, and by RT-PCR in mRNA from reticulocytes, erythrocyte progenitor cells that still contain nucleic acids (Chapter 6). However, no evidence could be found for cytochrome b_{561} using either technique. Thus, cytochrome b_{561} cannot be the ascorbate:AFR reductase of the erythrocyte. It is thought that another, possibly similar, protein is present in the erythrocyte for the ascorbate-dependent reduction of AFR.

Future attempts to identify the erythrocyte protein could start on the presumption that it has structural similarity to cytochrome b_{561}. Though this is by no means certain, it allows for a number of practical search strategies that have a good chance of success. The spectral properties of cytochromes could be exploited to find unknown cytochromes in the erythrocyte membrane, that have a midpoint potential which is appropriate for redox reactions with ascorbate. Alternatively, it could be attempted to find genes or proteins that have a sequence homology with cytochrome b_{561}. If the erythrocyte protein is related to cytochrome b_{561}, crucial segments of its structure, like heme or ascorbate binding sites, are likely to be conserved. Similar domains can be sought in the human genomic databases that are rapidly being developed by private and public institutes. Subsequently, the expression of candidate genes could be tested in the erythrocyte. The identification of a plasma membrane reductase will be more difficult when it is unrelated to cytochrome b_{561}. Possibly, a cDNA library of erythrocyte precursor cells can be screened for features of other membrane redox proteins. However, it is not certain that the ascorbate:AFR reductase of the erythrocyte is still transcribed in the reticulocyte stage. It appears difficult to screen isolated protein fractions from erythrocytes for the desired activity, as this would require the proper reconstitution of the protein in a sealed membrane environment. With any of the strategies that were mentioned, it seems that considerable effort is still required to identify the ascorbate-dependent reductase(s) in the plasma membrane.

Physiological relevance and distribution

The experiments described in this thesis unavoidably do not reflect the physiological conditions that can be expected in vivo. Ascorbate loading yielded a concentration of 1 mM in erythrocytes, whereas 50 µM is the normal level. Extracellular AFR concentrations were probably higher than under normal oxidative stress, while ferricyanide is not physiological at any concentration. High concentrations were required to reveal the properties of a system, also due to the limitations of the techniques that were used. Nevertheless, the results do bear a significant relevance to the physiological situation. Systems that are characterized using high levels of substrate are likely to function at lower concentrations as well. Indeed, the data indicated that ascorbate-dependent redox systems must still have significant effects at an intracellular concentration of 50 µM. Also, it must be noted that other cells, such as neutrophils, can contain ascorbate up to the millimolar range. However, little is known about plasma membrane redox systems in such cells.

Throughout this thesis, blood-borne cells were used as a model. Blood is exposed to relatively high oxidative stress, among others due to its high oxygenation. Assuming that plasma membrane redox systems are involved in the protection against extracellular oxidants, blood is therefore a likely place to find these systems. Ascorbate-dependent redox systems have not been studied in many other tissues. Evidence has been found in endothelial cells of the pulmonary artery and in perfused rat liver, but also e.g. in plants (13-15). Thus, the evidence for an ascorbate-dependent redox system is available for only a limited number of cell-types.

In summary, the results that were presented show that both erythrocytes and HL60 cells contain plasma membrane redox systems that can reduce extracellular substrates. Intracellularly, either NADH or ascorbate can donate electrons to drive these reactions. It was found in HL60 cells that, though both NADH and ascorbate could drive the reduction of extracellular ferricyanide, separate systems must be present in the plasma membrane of the cells to transfer the reducing equivalents. Also, the results showed that is unlikely that α-tocopherol or coenzyme Q are involved in these systems. A physiological extracellular substrate of plasma membrane redox systems is AFR. This substrate was reduced by erythrocytes, depending on either intracellular NADH or intracellular ascorbate. The ascorbate-dependent reduction of AFR was electrogenic, confirming vectorial electron transport across the plasma membrane. Also, the electrogenicity of the reaction implies that ascorbate-dependent electron transfer is not mediated by the diffusion of small electron carriers like coenzyme Q. Instead, it is likely that a membrane protein is

involved. Cytochrome b_{561} could potentially explain the effect, but was found to be absent in the erythrocyte. Instead, it is possible that homologues of this cytochrome in the erythrocyte membrane are responsible for the ascorbate-dependent redox reactions of this cell.

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Samenvatting voor de leek

In dit proefschrift wordt een redoxsysteem beschreven dat in de plasmamembraan (buitenwand) van de meeste cellen aanwezig lijkt te zijn. Het systeem zorgt voor het omzetten van stoffen aan de buitenkant van de cel via een chemische reaktie waarbij electronen worden overgedragen, een zogenaamde redox reaktie. De voor deze reaktie benodigde elektronen worden aan de binnenkant van de cel afgegeven door een donor. Het redoxsysteem zorgt ervoor dat de electronen over de plasmamembraan worden vervoerd naar de buitenkant van de cel, en daar worden gebruikt om een acceptormolecuul om te zetten. Hoewel er verschillende ideeën bestaan over het doel van deze reakties, lijken ze belangrijk te zijn bij het tegengaan van oxidatieve stoffen. Deze stoffen -onder meer radikalen- zijn erg reaktief, en kunnen essentiële onderdelen van de cel beschadigen. Dit kan worden voorkomen door de oxidatieve stoffen snel onschadelijk te maken, voordat ze iets beschadigen. Mogelijk spelen de redoxsystemen in de plasmamembraan hierbij een rol.

Ook ascorbaat, beter bekend als vitamine C, maakt oxidatieve verbindingen onschadelijk met een redoxreaktie. In deze studie is gebleken dat ascorbaat en de redoxsystemen kunnen samenwerken om dit nog beter te doen. Een belangrijke reden voor de samenwerking is de regeneratie van ascorbaat. Deze vitamine kan worden vergeleken met een oplaadbare batterij. Na de reaktie met een oxidatieve verbinding is de batterij leeg, en nutteloos voor het lichaam. Het is dan ook belangrijk dat de batterij snel weer opgeladen wordt. Het opladen van de batterij gaat het beste aan de binnenkant van de cel. Het redoxsysteem kan electronen van ascorbaat binnen de cel doorgeven aan oxidatieve verbindingen buiten de cel. Het dient zo als het ware als een verlengsnoer om de electronen van ascorbaat naar buiten te brengen. Op deze manier helpt ascorbaat bij het omzetten van stoffen buiten de cel, maar kan zelf binnen blijven en efficiënt geregenereerd worden.

Toch is de aanwezigheid van ascorbaat ook buiten de cel noodzakelijk, en moet het ook daar na een reaktie geregenereerd kunnen worden. We hebben gevonden dat het regenereren buiten de cel plaats kan vinden met behulp van het redoxsysteem in de plasmamembraan, en andere ascorbaat moleculen die aan de binnenkant van de cel aanwezig zijn. Dit systeem kan vergeleken worden met een lege batterij aan de buitenkant van de cel die, via een verlengsnoer, opgeladen wordt met behulp van een volle batterij aan de binnenkant van de cel. Zo wordt er voor gezorgd dat er, ook aan de buitenkant van de cel, altijd voldoende ascorbaat aanwezig is om het lichaam te beschermen.

We hebben ook geprobeerd uit te vinden hoe het systeem in de celwand is opgebouwd. De reakties aan de buitenkant van de cel lijken veel op die van een speciaal eiwit dat van een andere plaats in het lichaam bekend was. Er is getest of dit eiwit ook ook voorkwam in de plasmamembraan van de cellen die wij bestudeerd hebben. Dit bleek echter niet het geval te zijn. Wel bleek uit veel kenmerken van de reakties van het systeem dat er waarschijnlijk een ander, nog onbekend eiwit bij betrokken moet zijn. Het is goed mogelijk dat het nieuwe eiwit een beetje op het reeds bekende eiwit lijkt. Dat laatste zou kunnen helpen om het later te kunnen vinden.

List of publications

M.M. Van Duijn, J. Van der Zee, J. VanSteveninck and P.J.A. Van den Broek. Ascorbate stimulates ferricyanide reduction in HL-60 cells through a mechanism distinct from the NADH-dependent plasma membrane reductase. *J Biol Chem* 1998; 273:13415-13420.

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M.M. VanDuijn, J. Van der Zee and P.J.A. Van den Broek. Interaction of extracellular and intracellular ascorbate: detection and characterization. Submitted for publication in *Methods Enzymol*.

Curriculum Vitae

Martijn Maurice van Duijn werd op 16 augustus 1972 geboren in Katwijk. Na het behalen van het diploma in het Voorbereidend Wetenschappelijk Onderwijs aan het Pieter Groen College in Katwijk, werd in 1990 aan de Universiteit Leiden aangevangen met de studie scheikunde. Nadat een jaar later de propaedeuse was behaald, is de studie voortgezet in de bovenbouwstudie Bio-Farmaceutische Wetenschappen. Deze studie werd afgerond met een tweetal stages. De eerste betrof een onderzoek aan de foto-isomerisatie van urocaanzuur, een proces dat samenhangt met UV-geïnduceerde immuunsuppressie. Dit onderzoek aan de afdeling Medicinale Fotochemie, onder Prof. Dr. G.M.J. Beijersbergen van Henegouwen, werd na een jaar voltooid, en werd gevolgd door acht maanden onderzoek aan de afdeling Dermatology, van het Massachusetts General Hospital in Boston, USA. Onder Dr. T. Hasan werd het effect van fotodynamische therapie op signaal-transductie door de EGF receptor onderzocht. Na deze stages werd in september 1996 het doctoraal diploma in de Bio-Farmaceutische Wetenschappen behaald.

In december 1996 is begonnen met het promotieproject over plasma membraan redox systemen in de vakgroep Moleculaire Cel Biologie van het Leids Universitair Medisch Centrum. Dit onderzoek werd begeleid door Dr. P.J.A. van den Broek en Dr. J. van der Zee, onder supervisie van Prof. Dr. J. van Steveninck. Na diens overlijden in 1998 is de supervisie overgenomen door Prof. Dr. H.J. Tanke. Tot december 2000 zijn in deze groep de werkzaamheden uitgevoerd die hebben geleid tot dit proefschrift.

Na zijn promotieonderzoek heeft de auteur een post-doctorale onderzoeksfunctie aanvaard aan het FOM instituut voor Atoom- en Molekuulfysica in Amsterdam.

Nawoord

Hoewel mijn naam als enige voor op dit boekje staat, is het natuurlijk absoluut onjuist dat de totstandkoming ervan de verdienste van één persoon zou zijn. Vele mensen hebben een bijdrage geleverd in de afgelopen jaren, en dit is de plek om hen daarvoor te bedanken.

Om te beginnen is er Karmi, die tijdens het gehele project hulp heeft geboden als analiste, en daarbij veel geduld heeft getoond. Ook Laurence heeft me enige tijd als analiste geholpen, maar werkt inmiddels als AIO aan haar eigen promotie. Verder hebben Verena, Marissa en Jeroen zich als stage-studenten enige maanden willen inspannen voor mijn project. Ook zij waren een grote hulp, zowel door hun praktische werk, als door de discussie over het werk die een stage-project met zich meebrengt. Helaas hebben niet al hun projecten kunnen uitgroeien tot een afgerond geheel in dit proefschrift, maar dat heeft zeker niet aan hun inzet gelegen. Ook dank aan mijn familie, voor de basis die ze voor mij hebben gelegd, en voor de niet-aflatende steun die ze bieden. Verder wil ik alle andere vrienden en collega's die mij in woord en daad hebben bijgestaan de afgelopen jaren bedanken voor hun onmisbare bijdrage.

De laatste vermelding is altijd voor de belangrijkste persoon. Lieffie, in de jaren dat dit proefschrift tot stand is gekomen hebben we nog heel wat meer gebouwd, en je hebt je op die manier aardig onmisbaar weten te maken. Bedankt voor alles!

Martijn

Stellingen

Deze stellingen behoren bij het proefschrift "Ascorbate and its interaction with plasma membrane redox systems"

1. De reductie van extracellulaire ascorbaat radicalen door ascorbaat-afhankelijke plasmamembraan redoxsystemen levert een fysiologisch belangrijke bijdrage aan de instandhouding van de extracellulaire ascorbaat concentratie.

   Dit proefschrift.

2. Aangezien ascorbaat en NADH een verschillend aantal electronen overdragen in een redoxreactie, is te verwachten dat de plasmamembraan redoxsystemen waaraan zij electronen doneren volgens verschillende mechanismen werken, en dat de betrokken eiwitten weinig homologie zullen vertonen.

   Dit proefschrift.

3. Het feit dat de zogenaamde disproportioneringsreactie resulteert in een evenwicht tussen ascorbaat radicalen en ascorbaat en dehydroascorbaat, maakt het experimenteel aantonen van interconversie tussen ascorbaat en dehydroascorbaat een voorbeeld van het opnieuw uitvinden van het wiel.

   Dit proefschrift,

   Nishikawa Y, Kurata T. Interconversion between dehydro-L-ascorbic acid and L-ascorbic acid. *Biosci Biotechnol Biochem* 2000; 64:476-483.

4. Het nalaten van een bepaling van de NADH-concentratie in de cel na depletie van ascorbaat met behulp van TEMPOL belemmert het trekken van eenduidige conclusies over de betrokkenheid van ascorbaat bij cellulaire redoxreacties.

   Dit proefschrift,

   May JM, Qu ZC et al. Protection and recycling of α-tocopherol in human erythrocytes by intracellular ascorbic acid. *Arch Biochem Biophys* 1998; 349:281-289,

   Winkler BS, Orselli SM et al. The redox couple between glutathione and ascorbic acid: a chemical and physiological perspective. *Free Radic Biol Med* 1994; 17:333-349).

5. De aanname dat remmers van glucosetransport de opname van dehydroascorbaat kunnen voorkomen is niet van toepassing op een experiment waarbij erythrocyten worden gebruikt.

   Dit proefschrift,

   Himmelreich U, Kuchel PW. C-13-NMR studies of transmembrane electron transfer to extra-cellular ferricyanide in human erythrocytes. *Eur J Biochem* 1997; 246:638-645.

6. Aangezien de consumptie van hoge doses ascorbaat resulteert in een snelle uitscheiding in de urine, is van het gebruik van dergelijke doses weinig extra bescherming te verwachten.

7. Hoewel het vaststellen van de aminozuurvolgorde van eiwitten over het algemeen weinig problemen oplevert, zijn voorspellingen over de ruimtelijke structuur, wanneer niet ondersteund door technieken als röntgen-diffractie of cryo-EM, zodanig afhankelijk van de interpretatie van de onderzoeker dat er vaak sprake is van niet meer dan een 'educated guess'.

   Fleming PJ, Kent UM. Cytochrome b_{561}, ascorbic acid, and transmembrane electron transfer. *Am J Clin Nutr* 1991; 54:1173s-1178s,

   Srivastava M, Gibson KR et al. Human cytochrome b_{561}: a revised hypothesis for conformation in membranes which reconciles sequence and functional information. *Biochem J* 1994; 303:915-921.

8. De aanwezigheid van evenveel publicaties over stimulerende dan wel remmende effecten van ascorbaat op de celproliferatie suggereert dat er geen direct verband is tussen ascorbaat en proliferatie.

   Alcain FJ, Buron MI et al. Ascorbate free radical stimulates the growth of a human promyelocytic leukemia cell line. *Cancer Res* 1990; 50:5887-5891,

   Park CH, Kimler BF. Growth modulation of human leukemic, preleukemic, and myeloma progenitor cells by L-ascorbic acid. *Am J Clin Nutr* 1991; 54:1241s-1246s.

9. Omdat reactieve zuurstofvormen belangrijke functies vervullen in normale fysiologische processen, kan overmatige consumptie van anti-oxidanten, bijvoorbeeld in de vorm van voedingssupplementen, leiden tot ongewenste neveneffecten.

10. Doordat werkzaamheden in toenemende mate afhankelijk zijn van informatietechnologie, is het noodzakelijk geworden dat er in de werkomgeving minstens één werknemer aanwezig is, die in staat is de dagelijkse computerproblemen van de rest op te lossen.

11. De prestaties van de Nederlandse en andere spoorwegen doen het gezegde 'Het loopt als een trein' aan betekenis inboeten.

12. Analyses met een HPLC-systeem geven de wetenschapper een hernieuwde waardering voor het oude ambacht van loodgieter.

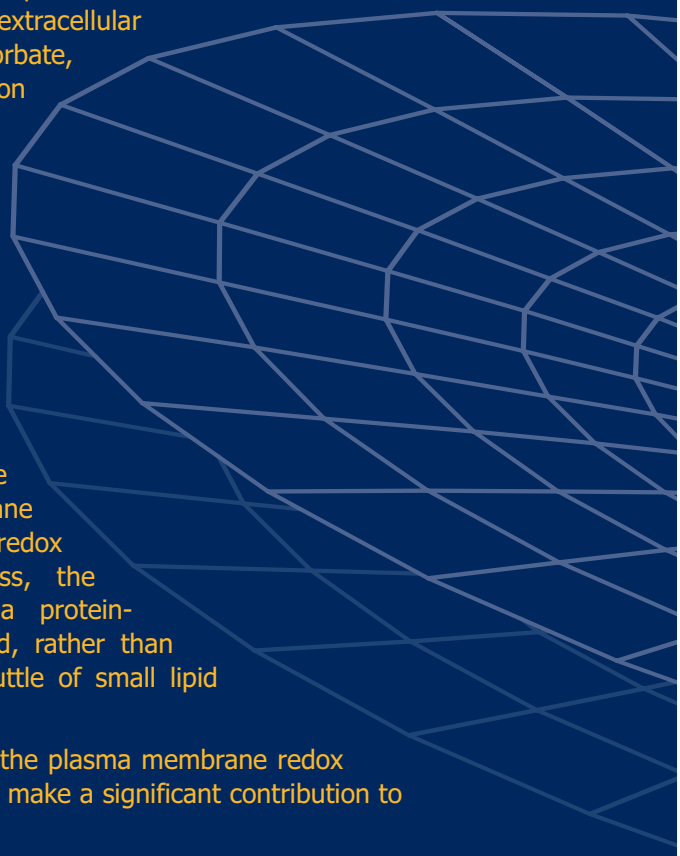
13. Het wereldwijde milieubeleid doet vermoeden dat de uitstoot van CO_2 pas significant zal gaan dalen wanneer de voorraad fossiele brandstof uitgeput raakt.

Martijn van Duijn

Ascorbate and its interaction with plasma membrane redox systems

Many cells contain a redox system in their plasma membrane that can mediate the transfer of electrons from intracellular donors to extracellular acceptors. Several functions have been attributed to the system, but its most important function seems to be the protection against oxidants. This is also reflected in its interaction with the important anti-oxidant molecule ascorbate, or vitamin C.

This thesis describes several studies on ascorbate chemistry, plasma membrane redox systems, and, in particular, their interaction with ascorbate. It was found that separate redox systems exist in the plasma membrane, which can use either NADH or ascorbate as intracellular electron donors for the reduction of extracellular substrates. Other experiments showed that plasma membrane redox systems also interact with extracellular ascorbate. An oxidation product of ascorbate, the ascorbate free radical, was reduced on the extracellular face of the cell by a redox system that uses intracellular ascorbate as an electron donor. Thus, extracellular ascorbate is regenerated, and loss of the vitamin is prevented. The ascorbate free radical is believed to be an important physiological substrate for plasma membrane redox systems.



The components of plasma membrane redox systems are still unknown. The involvement of one candidate membrane protein in the ascorbate-dependent redox reactions was excluded. Nevertheless, the experiments strongly suggest that a protein-mediated mechanism must be involved, rather than alternative mechanisms, such as a shuttle of small lipid soluble molecules like α-tocopherol.

Regardless of the mechanism involved, the plasma membrane redox systems described in this thesis seem to make a significant contribution to the anti-oxidant effects of ascorbate.