
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 (**Reduction Oxidation**) 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)
$\text{HO}^\bullet, \text{H}^+ / \text{H}_2\text{O}$	2310
$\text{O}_2^{\bullet-}, 2 \text{H}^+ / \text{H}_2\text{O}_2$	940
$\alpha\text{-tocopheroxyl}^\bullet / \alpha\text{-tocopherol}$	500
Ferricyanide / Ferrocyanide	360
$\text{H}_2\text{O}_2, \text{H}^+ / \text{H}_2\text{O}, \text{HO}^\bullet$	320
AFR, $\text{H}^+ / \text{ascorbate}$	282
CoenzymeQH $^\bullet$ / CoenzymeQH $_2$	200
CoenzymeQ / CoenzymeQH $^\bullet$	-36
DHA / AFR	-174
$\text{NAD}^+ / \text{NADH}$	-320
$\text{NADP}^+ / \text{NADPH}$	-324
$\text{O}_2 / \text{O}_2^{\bullet-}$	-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,

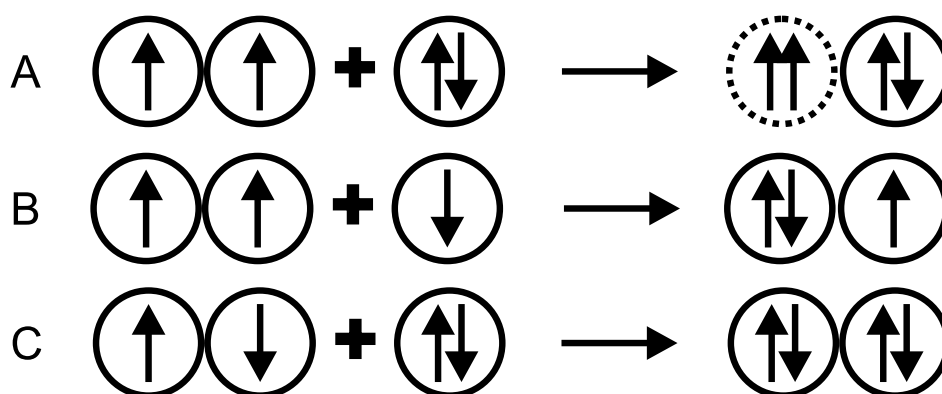


Figure 1. Spin-state and reactivity of oxygen. Parallel spins are not allowed in a single orbital, represented by the circles in the figure. Normal (triplet state) oxygen has two single parallel electrons in separate orbitals. A two-electron interaction (A) would result in parallel spins, and is therefore 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 ($\bullet\text{O}-\text{O}\bullet$). 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

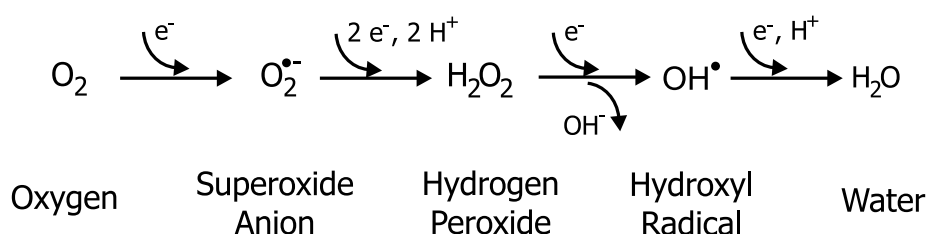


Figure 2. Redox states of oxygen. Four electrons (and four protons) are needed to reduce O_2 to two molecules of water.

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

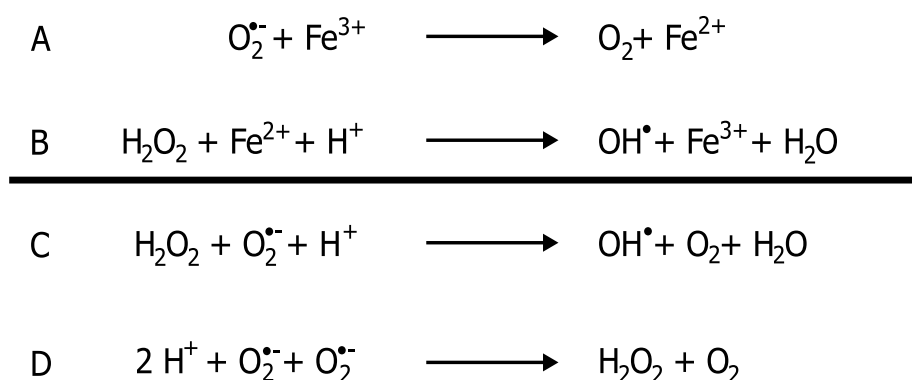


Figure 3: The Haber-Weiss reaction. The Haber-Weiss reaction (C) is catalyzed by metal ions. This is due to reactions A and B, the latter being the classical Fenton reaction (11). The superoxide anion is often released, e.g. by mitochondria. Hydrogen peroxide can be formed from the superoxide anion by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

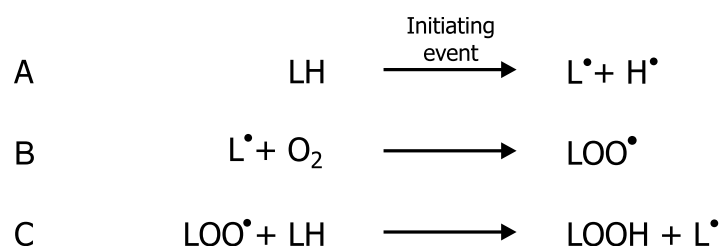


Figure 4. Lipid Peroxidation chain reaction. The reactions above illustrate how a single initiation reaction (A) in a polyunsaturated lipid LH can result in a cascade of propagation reactions (B and C) that produce lipid hydroperoxides. α -Tocopherol can break the propagation chain by reacting with a lipid peroxy 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 O₂ 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 A₂ 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-gulonolactone 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

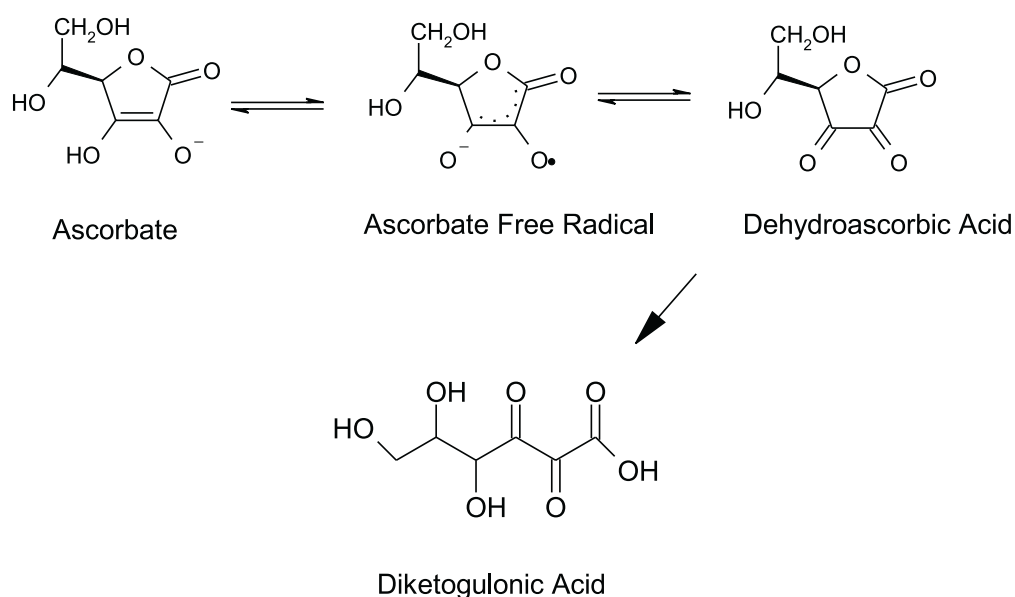


Figure 5. Structure of ascorbate and its derivatives. Ascorbate can be oxidized in two successive one-electron steps to ascorbate free radical and dehydroascorbic acid. The unpaired electron in ascorbate free radical is distributed over the ring structure, stabilizing the molecule. The ring-structure 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 C₂ and C₃ 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 C₂ and C₃ 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-transporters 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 3 α -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

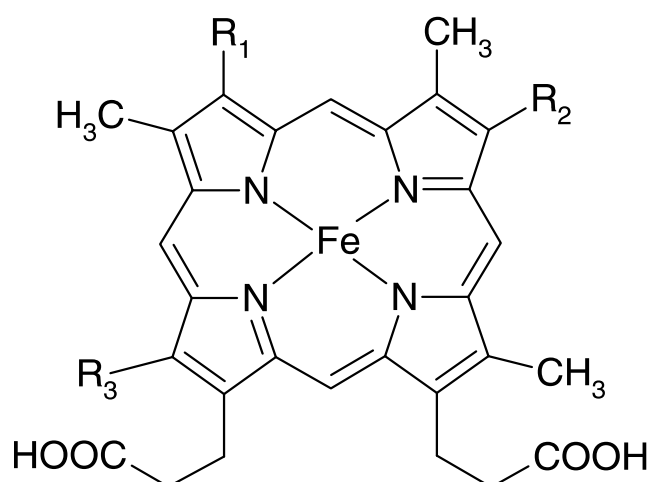
Ascorbate is generally presented as a powerful anti-oxidant molecule, implicated in the protection against numerous diseases (44). Health authorities therefore recommend a minimum dietary intake of ascorbate, known as the Recommended Dietary Allowance (RDA, approximately 60 mg/day). However, many others 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 <i>a</i>	-C ₁₇ H ₂₉ O	-C=CH ₂	-CH=O
Heme <i>b</i>	-C=CH ₂	--	-CH ₃
Heme <i>c</i>	-CH-CH ₃ S-Protein	-CH-CH ₃ S-Protein	--
Heme <i>d</i>	-CH-CH ₃ OH	-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 ligation, 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

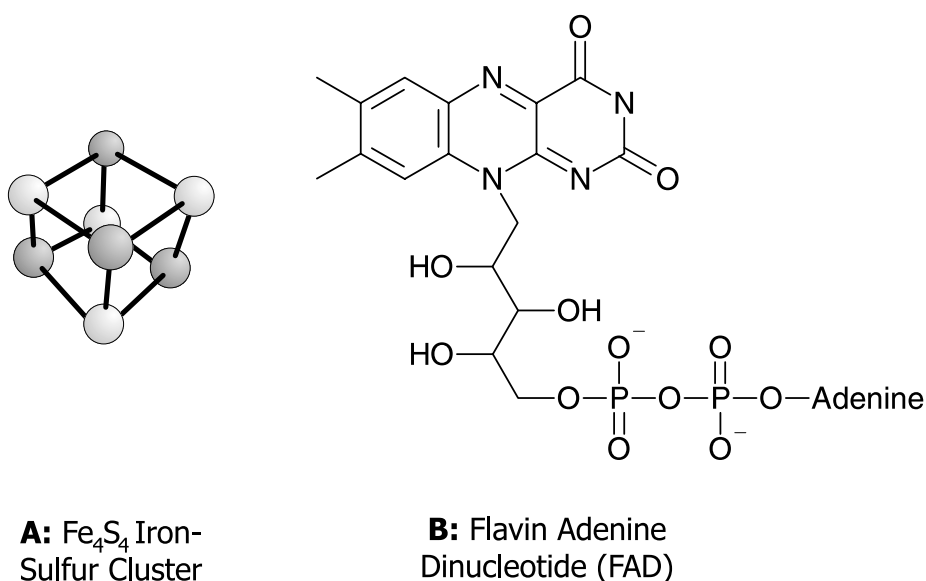


Figure 7. Iron-sulfur clusters and flavins. Iron-sulfur clusters can be found in many configurations, the smallest being two sulfur and two iron atoms. Shown is a cluster of four iron and sulfur atoms, represented by dark and light spheres (A). Flavins can be found as Flavin Adenine Dinucleotide (FAD) (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 ($\text{FH}^\bullet$), 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: $\text{Fe}(\text{CN})_6^{3-}$. 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 ($\text{Fe}(\text{CN})_6^{4-}$) 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 (*glyco-protein*, estimated weight 91 kDa, from *phagocyte oxidase*) 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

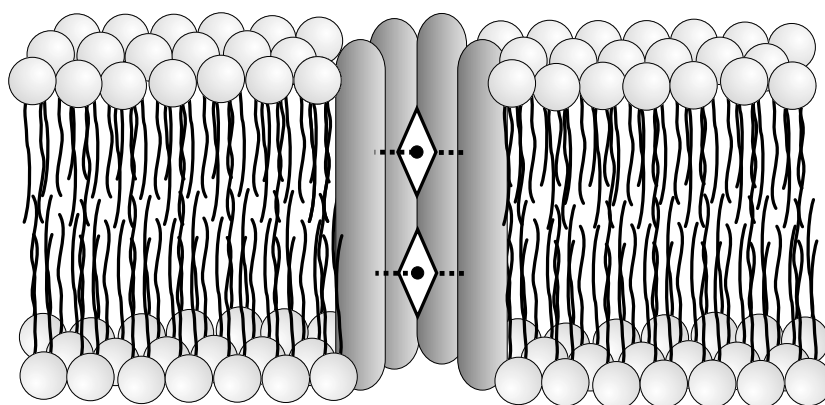


Figure 8. Bis-heme motif. Both cytochrome b_{561} and gp91phox share a structural similarity. They have two b -type hemes bound to histidine residues on their transmembrane helices. These heme groups are important in the transfer of electrons over the membrane.

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 $O_2^{\cdot -}$ (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 $O_2^{\cdot -}$ 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 $O_2^{\cdot -}$ (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 (H₂O₂) with signal transduction pathways. It was found that H₂O₂, which freely crosses the membrane, causes inactivation of protein tyrosine phosphatases (PTPs) (123, 139). Thus, an increased tyrosine phosphorylation level can result from H₂O₂, and the activation of several mitogenic pathways. The stress-related transcription factor NF κ B is also strongly associated with the effects of H₂O₂ (140). These signaling events could be responsible for the increase in cell proliferation that has been found after treatment of cells with H₂O₂ (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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