
CHAPTER 7

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

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 ($\epsilon=14,500 \text{ cm}^{-1}\text{M}^{-1}$)

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 μ l 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)₃²⁺ – Tris-(ethylenediamine)-nickel(II) chloride 2-hydrate (Ni(en)_3^{2+}) can be prepared by dissolving 12 g $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 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 μ l loop, a Partisil SAX column (10 μ m, 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 μ l methanol, dispersion of the pellet, and the subsequent addition of 400 μ l water. To ensure the stability of ascorbate, EDTA and HCl are added to final concentrations of 50 μ M 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 μ l 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 μ M 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 ($\epsilon = 4,900 \text{ cm}^{-1}\text{M}^{-1}$) (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

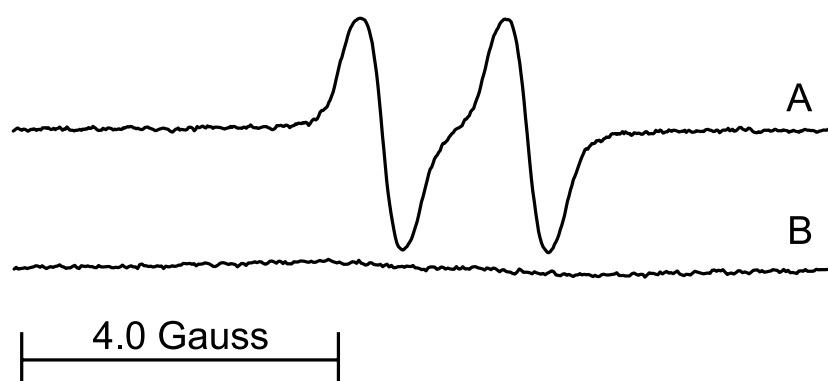


Figure 1: ESR spectra of ascorbate free radical. A, 100 μ M ascorbate and 4 mU/ml ascorbate oxidase; B, same as A, but with 5 mM Ni(en)_3^{2+} .

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 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.

A typical AFR signal consists of a doublet with a hyperfine splitting $a^{\text{H4}} = 1.8$ 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 μ M 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 μ M 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 μ M DHA. It is, therefore, important to determine the result of the incubation by HPLC analysis.

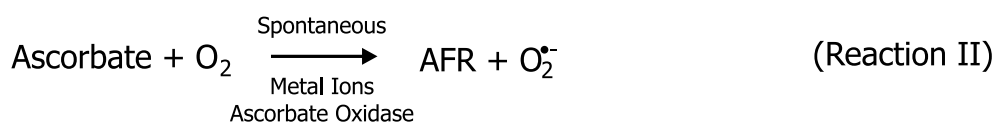
Intracellular ascorbate can be removed by treatment with 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL). TEMPOL can freely diffuse into the cell and oxidize ascorbate to DHA, which subsequently diffuses out of the cell through

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 phthalate, 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:



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.

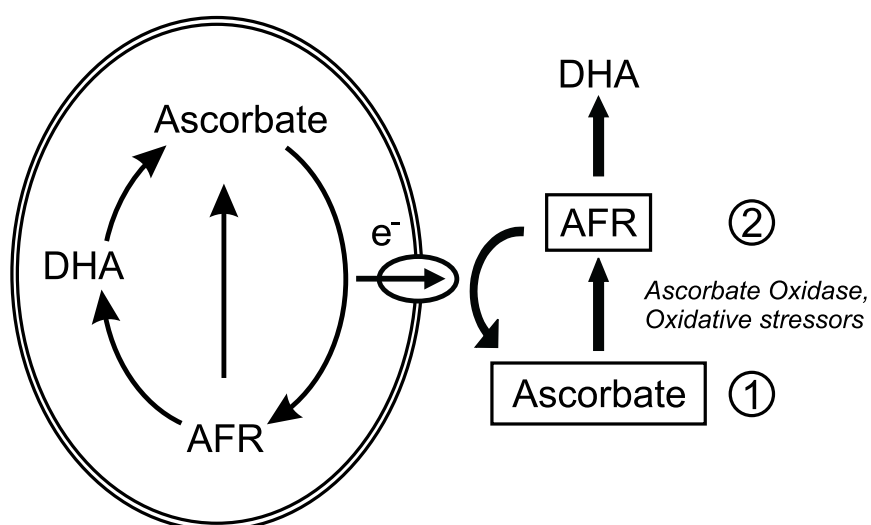


Figure 2: Principle of the AFR reduction assays. The reduction of extracellular AFR in a cell suspension can be measured by monitoring AFR using ESR spectroscopy [1], or by monitoring the decrease in concentration of extracellular ascorbate [2].

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

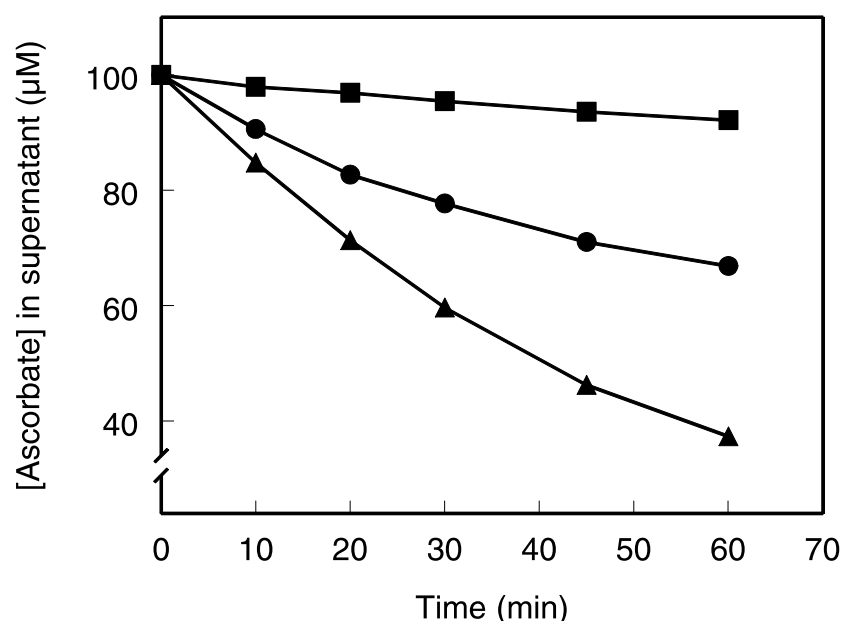


Figure 3: Oxidation of extracellular ascorbate in erythrocyte suspensions. 100 μM ascorbate and 4 mU/ml AO were incubated in 10% suspensions of ascorbate-loaded erythrocytes (■), control erythrocytes (●), or in the absence of cells (▲). Samples were centrifuged, and the absorption at 265 nm was measured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

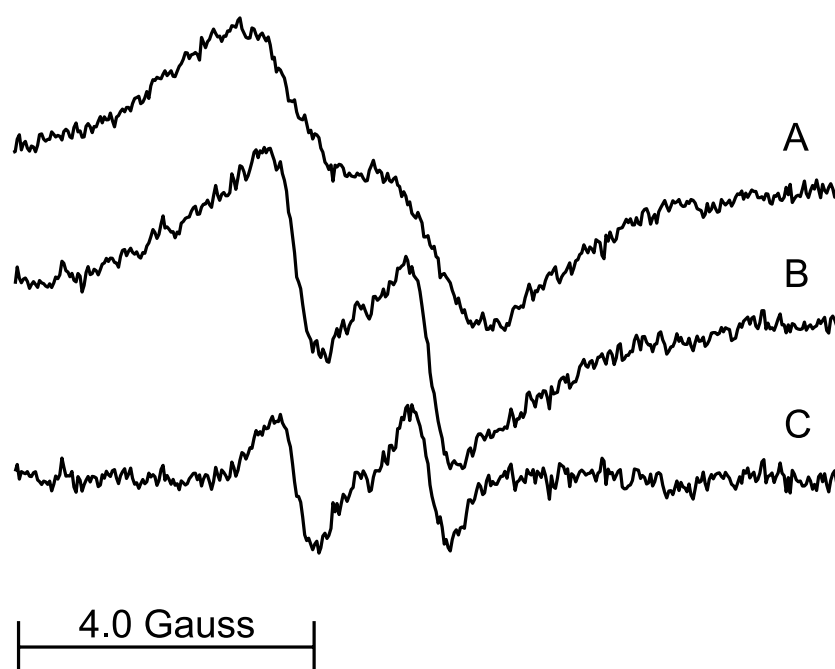


Figure 4: Detection of intracellular AFR using ESR. A, 1 mM ascorbate, 20 mU/ml AO and 5 mM Ni(en)_3^{2+} ; B, same as A, but with 20% ascorbate-loaded erythrocytes; C, A subtracted from B. Erythrocytes were loaded with ascorbate, using 500 μM DHA, as described in the text.

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.

References

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