

The Reversibility of the Vitamin C Redox System: Electrochemical Reasons and Biological Aspects

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The biological efficacy of vitamin C depends on its redox abilities as given by the relations between ascorbic acid, semidehydroascorbic acid, and dehydroascorbic acid. It is shown by means of proton magnetic resonance spectroscopy that the enzymatic (by ascorbate oxidase) as well as non-enzymatic (by iodine) oxidation of ascorbic acid is, in principle, reversible despite the hydration and structural changes during the formation of dehydroascorbic acid. The strong redox activity of semidehydroascorbic acid which results in a fast disproportionation to ascorbic acid and dehydroascorbic acid is inferred from an inversion of the electrochemical potentials of the vitamin C redox system. The capacity of this is maintained by a fast reduction of dehydroascorbic acid *e.g.* by reduced glutathione, preventing its delactonization and further degradation.

Introduction

Ascorbic acid is known as an ubiquitous and essential substance in the biochemistry of living cells [1]. It serves not only as a reductant or cofactor of certain enzymatic reactions but is also proposed as a component of a general redox buffer system due to the equilibria with its half (by a one electron step) and fully (by a two electron step) oxidized forms semidehydroascorbic acid and dehydroascorbic acid. The molecular details of oxidation and reduction, however, including structural changes and non-reversible degradation products, have not been completely elucidated yet [2].

In the blood of normal human subjects the stability of the equilibrium between ascorbic acid and dehydroascorbic acid seems to be maintained by certain enzymatic reactions. Thus, *e.g.* (GSH)-dehydroascorbate oxidoreductase (EC 1.8.5.1) has been supposed to be responsible for the reduction of dehydroascorbic acid [3] and NADH-semidehydroascorbate oxidoreductase (EC 1.6.5.4) for the reduction of semidehydroascorbic acid to ascorbic acid [4]. The oxidation of ascorbic acid on the other hand seems to be controlled by copper proteins [5–7], *e.g.* ascorbate oxidase (EC 1.10.3.3) in plant and dopamin- β -hydroxylase (EC 1.14.17.1) or ceruloplasmin in animal tissue. It might be suggested that any unbalance of this complex redox mecha-

nism is a consequence or acts even as a promotor of a great variety of diseases. Thus, an increased concentration of copper proteins and dehydroascorbic acid have been found *e.g.* in tumour tissue [7, 8] and the blood of diabetic patients [9] concomitantly with a decreased level of ascorbic acid.

Especially the semidehydroascorbic acid concentration as detected *e.g.* in the blood of patients with acute lymphatic leukemia [7] seems to be very important in connection with the etiology and diagnosis of cancer. The concentration of the vitamin C radical is controlled by the electrochemical potentials of the redox equilibria of vitamin C. Since there is some doubt on the existence of these equilibria [10] and on the importance of the vitamin C side chain for biological processes, the enzymatic and non-enzymatic oxidation and the reduction of vitamin C in its reduced (ascorbic acid) and oxidized form (dehydroascorbic acid) has been reinvestigated by means of the NMR (nuclear magnetic resonance) technique.

Materials and Methods

L(+)-Sodium ascorbate (ASC), iodine, and the buffer components have been purchased from Merck, Darmstadt, red. L-glutathione (GSH) from Fluka, Buchs, Switzerland. Dehydro-L(+)-ascorbic (DHA) was crystallized and purified according to Staudinger and Weis [11]. D₂O was supplied by Merck, Sharp and Dohme. Ascorbate oxidase (EC 1.10.3.3) has been purchased from Boehringer,

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Mannheim, and tested for its activity by measuring the decrease of the optical density of ASC at 265 nm using citrate-phosphate buffer and serum albumine for stabilization.

All experiments were performed in deuterated acetate buffer (0.1 M, pD 5.6 = pH-meter reading + 0.4). Pure oxygen was bubbled through the solution in the studies with ascorbate oxidase.

The ^1H -nuclear magnetic resonance (NMR) spectra were taken with a Varian XL-100-15 FT spectrometer operating at 100 MHz with a 5 mm insert and a probe temperature of 26 °C. Internal D_2O provided the field-frequency lock. 200 scans with an acquisition time of 2 or 4 s have been accumulated for all spectra. In some cases (Figs. 2 and 3) the water resonance has been suppressed by an inversion-recovery pulse sequence.

Results and Discussion

As has been shown recently, dehydroascorbic acid exists in aqueous solution as a hydrated bicyclic monomer [12] (Fig. 1). This structure can be identified by comparing the ^1H -NMR spectra of ascorbic acid (ASC) (Fig. 2a) and dehydroascorbic acid (DHA), freshly dissolved (Fig. 2b) or produced in solution by the oxidation of ascorbic acid with iodine (Fig. 2c).

All resonances of DHA are downfield with respect to those of ASC, an effect which may be

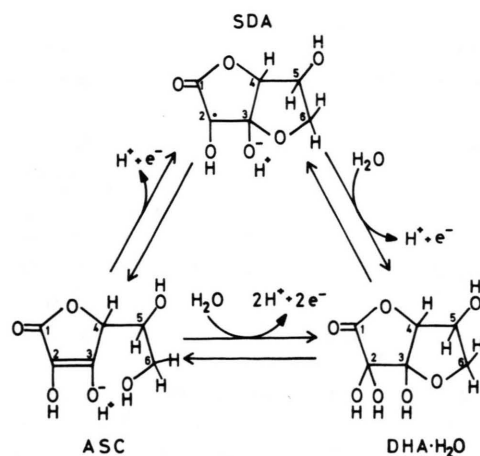


Fig. 1. Redox equilibria and structures of vitamin C in its reduced (ASC: ascorbic acid) and oxidized (DHA: dehydroascorbic acid) forms with the semidehydroascorbic acid radical (SDA) as an intermediate.

explained by the reduced charge density of the oxidized form. The downfield shift of the $\text{CH}_2\text{-6}$ resonances, however, is larger than would be anticipated from the inductive charge effect along a free side chain. This supports the assumption of a cyclic side chain structure of DHA in which case the $\text{CH}_2\text{-6}$ is treated adjacent to O-3 and is more sensitive to a change in charge density of the C-2/C-3 region of the furanose ring. Furthermore, the magnetic non-equivalency of the $\text{CH}_2\text{-6}$ -protons [12] and their unexpected strong coupling to the unpaired electron in the SDA radical [13] confirms the participation of the side chain during oxidation.

The mixture of DHA with reduced glutathione (GSH) gives the ^1H -NMR-spectrum as shown in Fig. 3a. The coupling patterns of H-4 and H-5 (near 4.9 and 4.4 ppm, resp.) clearly demonstrate the free side chain structure known for ASC (Fig. 2a)

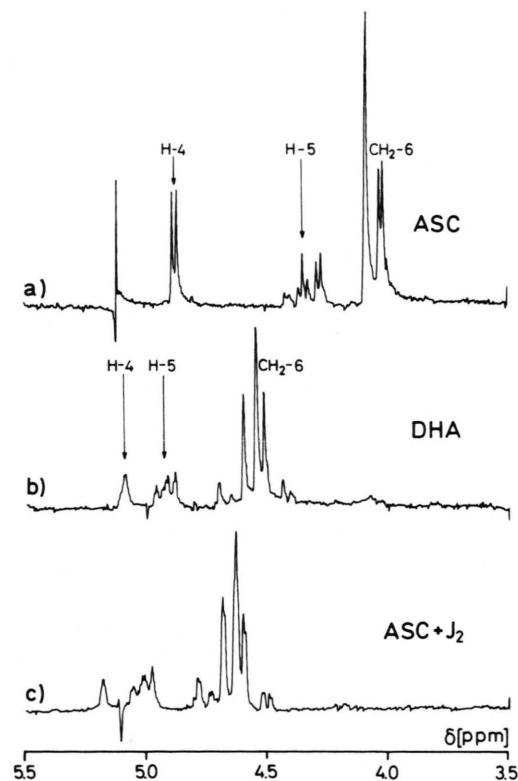


Fig. 2. ^1H -NMR spectra in acetate buffer (pD 5.6) of ascorbic acid (ASC) (a) and dehydroascorbic acid (DHA), dissolved after crystallisation and purification (b) and produced in solution by the oxidation of ascorbic acid with iodine (c). The signals are shifted downfield in spectrum (c) due to the decreased pD value (pD 4.0 by the production of JH). Concentrations: 50 mM each.

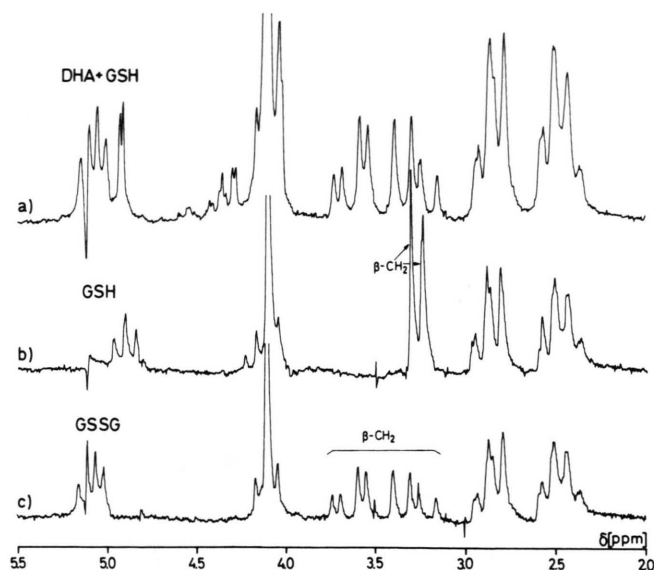


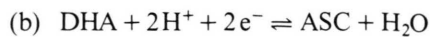
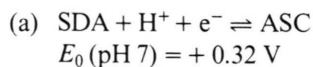
Fig. 3. ^1H -NMR spectra (pD 5.6) of dehydroascorbic acid (DHA, 50 mM) reduced by GSH (100 mM) (a) and of glutathione in its reduced (GSH, 50 mM) (b) and oxidized forms (GSSG, 50 mM) (c).

whereas the CH_2 -6-resonances (near 4.1 ppm) are superimposed by resonances of oxidized glutathione (Fig. 3c).

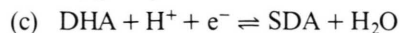
As can be seen, all reduced glutathione (Fig. 3b) added to DHA in an equimolar mixture is oxidized, and the integral intensities of the ASC resonances observed represent almost 100% of the original value of DHA before its reduction.

Hence, despite the instability and hydrated bicyclic structure of DHA, it is shown that, in principle, the oxidation of ASC is reversible as long as its γ -lactone ring structure is maintained.

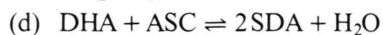
If the concentrations of reduced and oxidized vitamin C can be interpreted in terms of a redox equilibrium, it is evident that iodine (standard redox potential $E_0 = +0.535\text{ V}$) oxidizes ASC to DHA (Fig. 2c) and GSH ($E_0(\text{pH } 7) = -0.33\text{ V}$) (Fig. 3a) reduces DHA to ASC ($E_0(\text{pH } 7) = +0.06\text{ V}$ as measured potentiometrically [15]). To understand the biological importance of vitamin C, however, the two electron reaction from ASC to DHA and vice versa has to be interpreted in more detail as a sequence of two one electron steps with SDA as a radical intermediate (Fig. 1 and Eqns. a–c):



$$E_0(\text{pH } 7) = +0.06\text{ V}$$



$$E_0(\text{pH } 7) = -0.20\text{ V}$$



$$K_d(\text{pH } 7; 25^\circ\text{C}) = 1.5 \cdot 10^{-9}\text{ M.}$$

The potential of the ASC/SDA-system (+0.32 V, Eqn. (a)) was measured in an aqueous solution (pH 7) using cytochrom b_5 [16]; it agrees well with values of +0.30 and +0.33 V as calculated [17] and found by cyclic voltammetry in dimethylformamide [18]. Hence, the two electron reaction from ASC to DHA (Eqn. (b)) is lower in its potential than the one electron step to SDA, and for the potential of the reverse reaction (Eqn. (c)) one obtains a theoretical value of $2(0.06) - 0.32 = -0.20\text{ V}$ (pH 7) in good accordance with values measured polarographically [19].

This inverted correlation of the potentials, similar to that known, *e.g.* for the Cu^{2+} - Cu^+ - Cu^0 -system [20], means that SDA is a strong reducing agent, more efficient than ASC, and also an oxidizing agent, more potent than DHA. It results in an instability of the redox-amphoteric system with the consequence that the concentration of free SDA in solution is normally very low. The disproportionation constant K_d of the reaction Eqn. (d) was measured to be $K_d = 5.1 \times 10^{-9}\text{ M}$ (pH 6.4; 25°C) [21] by means of electron spin resonance (ESR) spectroscopy (stopped flow method). This agrees rather well with the theoretical value ($K_d(\text{pH } 7; 25^\circ\text{C}) = 1.5 \times 10^{-9}\text{ M}$) estimated by the application of Nernst's law on Eqns. (a) and (c) in combination with the equilibrium condition according to Eqn. (d).

It should be noted, however, that energy added to the system, *e.g.* by exergonic reactions or by irradiation, will result in an increased concentration of SDA produced by molecular oxygen [2]; this is due to a short unbalancing of the equilibria. In any case, the immediate relaxation to the equilibrium state produces both DHA and ASC by the disproportionation of SDA (Eqn. (d)).

In the biological environment most molecular processes are controlled by enzymatic reactions. Thus, the oxidation of ASC may be seen as being catalyzed by ascorbate oxidase or other oxidative enzymes which act directly on ASC or use it as a cofactor. It is supposed, that ascorbate oxidase

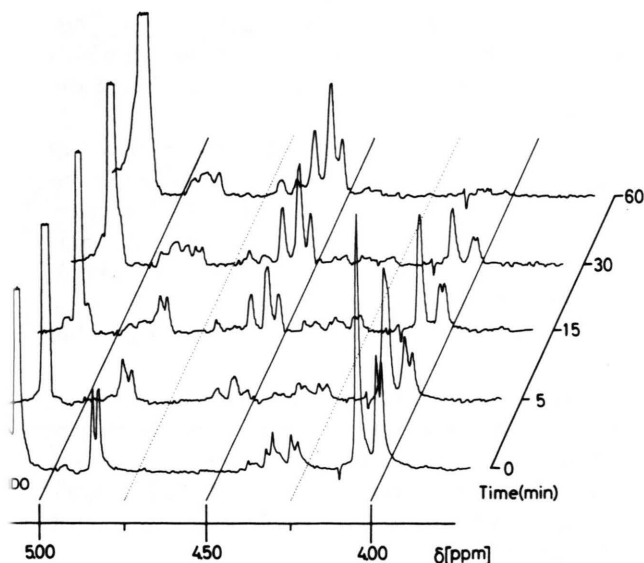


Fig. 4. Oxidation of ascorbic acid (25 mM) by ascorbate oxidase (25 $\mu\text{g}/\text{ml}$) in acetate buffer (pD 5.6) followed by the time dependence of the proton magnetic resonance intensities of ascorbic acid (decreasing) and dehydroascorbic acid (increasing).

oxidizes ASC to SDA in a very specific way, using oxygen as reactivator [22–24]. This enzymatic oxidation is usually observed by the decrease of the optical absorption of ASC at 265 nm. In this way it is impossible to determine the amount and structure of DHA produced by the disproportionation mechanism discussed above.

In the ^1H -NMR spectrum, however, the increase of specific resonances of DHA observed concomitantly with the decrease of those of ASC (Fig. 4) undoubtedly indicates the production of DHA, with the same bicyclic structure as demonstrated (compare with Fig. 2b; the H-4 resonances are hidden by the water lines in Fig. 4). It is seen, that the time dependence of the optical absorption of ASC (Fig. 5a) corresponds to that observed in the NMR spectra (Fig. 5b). Furthermore, the parallelism between the decrease of the ASC and the increase of the DHA resonances indicates the fast disproportionation of the SDA radicals.

Oxidation and reduction are generally balanced in living cells by oxidative and reductive enzymes. Beyond this, the chemical instability of DHA requires a protection of fast reduction mechanism also

due to economical reasons, *e.g.* to reduce the daily need of vitamin C in man. Glutathione with glutathione reductase [25] may be responsible for this in human blood.

The turnover of GSH to GSSG can be followed by means of ^1H -NMR spectroscopy using the cysteinyl $\beta\text{-CH}_2$ resonance intensities which are different for the reduced (Fig. 3b) and oxidized state (Fig. 3c). As ascorbate oxidase is highly specified for ASC and exerts no activity on GSH, the oxidation of GSH in a mixture with ASC and the enzyme (Fig. 5c) can be explained only via the oxidation of ASC. The nearly constant resonance intensities observed for ASC and DHA indicate indeed a reduction of DHA by GSH. Thus, besides a short time delay, vitamin C is obviously protected against degradation by GSH also after enzymatic oxidation.

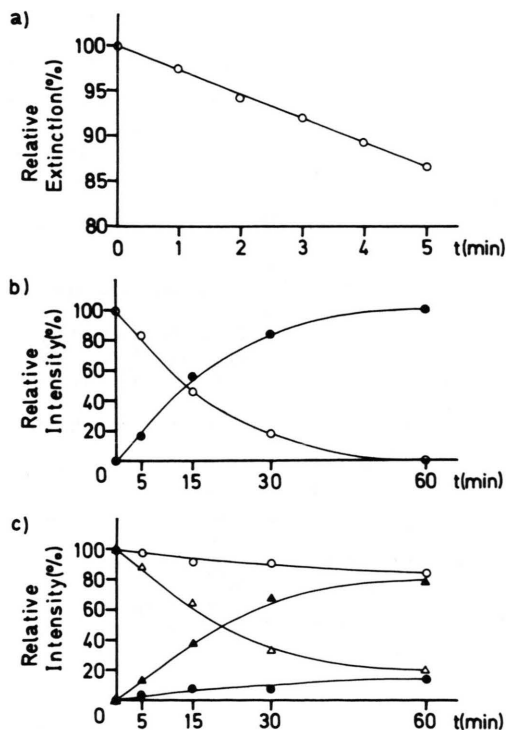


Fig. 5. Activity of ascorbate oxidase in acetate buffer (pD 5.6) measured by the decrease of the optical absorption of ascorbic acid at 265 nm (0.1 mM ascorbic acid, 0.13 $\mu\text{g}/\text{ml}$ ascorbate oxidase) (a), by the decrease/increase of the $\text{CH}_2\text{-6}$ proton magnetic resonance intensities of ascorbic/dehydroascorbic acid (s. Fig. 4) (b), and by the turnover of glutathione in a mixture with ascorbic acid (concentrations: 25 mM) (c). Symbols used: ascorbic acid (\circ), dehydroascorbic acid (\bullet), GSH (Δ), and GSSG (\blacktriangle).

Conclusions

The experiments demonstrate that the oxidation of ascorbic acid is, in principle, reversible, *i.e.* that vitamin C forms a reversible redox-system consisting of its reduced (ascorbic acid), radical (semidehydroascorbic acid), and oxidized (dehydroascorbic acid) forms. The inversion of the electrochemical potentials reveals semidehydroascorbic acid as a very potent redox agent; its usually small concentration and fast disproportionation make it difficult, to discriminate the activities from those of ascorbic acid and dehydroascorbic acid in the equilibrium state.

In combination, the vitamin C redox system may function and can be described as a redox buffer system *e.g.* in blood [8] or, more general, in all living cells. However, because of the chemical instability of dehydroascorbic acid, normally the free concen-

tration in that form should be relatively low. This, indeed, has been verified in human blood, in which case this concentration does not exceed 10% of the value of total vitamin C [5, 8, 9].

An enhanced level of ascorbic acid (compared at a constant total vitamin C concentration) might be prevalently caused by the predominance of reducing agents (*e.g.* GSH or other substances with SH-groups) or by the lack or suppression of oxidase activities. A lowered level of ascorbic acid may express an excessive oxidation to dehydroascorbic acid followed by a fast degradation.

Thus, in both directions, an altered level of ascorbic acid may serve as an indicator of malignancies. However, as vitamin C is stable only in its reduced form (*i.e.* ascorbic acid), its redox equilibrium is maintained most easily by an uptake of the vitamin in this form.

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