Interaction of Ascorbic Acid with Disulfides

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Electron spin resonance (EPR) spectra demonstrate the formation of a complex between the ascorbate ion and disulfides such as oxidized glutathione, cystine, gamma globulin, bovine serum albumin, 4-4′ dithiopyridine, and dithiobutyric acid. However, except in the case of 4-4′ dithiopyridine, ascorbic acid (AH_2) is not capable of reducing the disulfide bond in these compounds. These results can be explained in terms of the redox potential of ascorbic acid and its radical semidehydroascorbic acid (SDA). These compounds can reduce the S–S bond of 4-4′ dithiopyridine, the redox potential of which is $E_0' = -0.1$ V and, thus, more positive than the ones determined for the ascorbic acid system.

Introduction

Recent studies have shown the appearance of a characteristic electron spin resonance (EPR) signal when ascorbic acid (AH_2) reacts with a variety of biological molecules [1]. This signal has been assigned to the ascorbate radical (AH·) formed by binding electrostatically to compounds containing both, disulfides and amines. Two questions have been left unanswered by these studies, namely the role of sulfur in this reaction and the ability of AH_2 to reduce disulfide bonds. Theoretical aspects of the latter question have been discussed previously [2]. Since the redox potential for the reaction

$$2\,AH_2 \rightarrow 2\,AH\cdot + 2e + 2\,H^+, \quad E_0' = -0.33\ V \quad (1), (13)$$

is about the same as that for

$$RSSR + 2e + 2\,H^+ \rightarrow 2\,RSH, \quad E_0' = -0.33\ V \quad (2)$$

it has been postulated that the generation of high concentrations of AH· from AH_2 would probably reduce most disulfides [2]. As has been reported, the E_0' for S–S groups is dependent upon the specific disulfide, and lies in the range of -0.20 V to -0.40 V (for example, cystine is -0.33 V, glutathione is -0.33 V, thiophenol is -0.31 V, whereas albumin is -0.24 V, and insulin is -0.38 V) [4]. In addition, the second step in the oxidation of AH_2, *e.g.* the conversion of AH· to dehydroascorbic acid (A), has also been thought to possibly participate in the reduction of disulfides. The redox potential of this reaction, $2\,AH\cdot \rightarrow 2\,A + 2e + 2\,H^+$, is $E_0' = -0.2$ V at pH 7 [13]. There were certain indications that AH_2 supplementation increases the SH to SS ratio in various tissues [9] and that it might cleave the disulfide bond in 4-4′ dithiopyridine [1]. For this reason, the interaction of AH_2 with a number of biological molecules containing S–S groups but no amino groups as well as with 4-4′ dithiopyridine has been investigated in regard to the AH· formation and to reduction of S–S bonds.

Materials and Methods

All chemicals were of reagent grade and were used without further purification. Unless otherwise specified, all reactions were carried out in 0.067 M potassium phosphate buffer (pH 7.4).

Generation of the AH· radical was accomplished by irradiating the reaction mixtures in open petri dishes with 100 μW/cm² of short wave ultraviolet light for up to 90 min. UV irradiation under these conditions is sufficient to continually generate large amounts of AH· [2].

Attempts to reduce dithiobutyric acid (DTA), 4-4′ dithiopyridine (DTP), cystine (CySS), oxidized glutathione (GSS), and bovine serum albumin (BSA) consisted of reacting various concentrations of AH_2 ranging from 2×10^{-5} M to 2×10^{-1} M with 4×10^{-4} M of either DTA, DTP, CySS, or GSS and with 2×10^{-6} M of BSA with and without UV irradiation. Repeated determination of free sulfhydryl groups was carried out according to the method of Grassetti [3].

Optical absorption spectra were obtained on all compounds, both before and after irradiation by using a Cary Model 219 spectrophotometer; particular attention was given to 230 nm (HS^-), 190 nm (cysteine, CySH), 236 nm (cys^-), and to 335 nm (RSS^-) [4].

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Attempts to reduce gamma globulin or insulin (each 2×10^{-5} M) were carried out at pH 7.4 by using 2×10^{-3} to 2×10^{-1} M of AH_2. The gamma globulin reaction mixtures were analyzed by polyacrylamide gel electrophoresis (5.6% acrylamide) containing 1% SDS according to the method of Fairbanks [5]. Several control samples were reduced with 50 mM of dithiothreitol. Insulin samples were analysed with the aid of 30% polyacrylamide slab gels according to the methods developed by Pantazis for analyzing small polypeptides [6]. Disulfide reduction of gamma globulin or insulin results in peptides of lower molecular weight since the subunits are held together by disulfide bonds.

The measurement of the redox potential of DTP was performed on a Metrohm Polarecord E 261 Polarograph equipped with a calomel standard electrode in concentrated KCl. Electron spin resonance studies were carried out as described previously with a Varian E9, 100 kHz spectrometer with a modulation amplitude of 0.2 mT and microwave power of 5 mW at the X-band frequency [1].

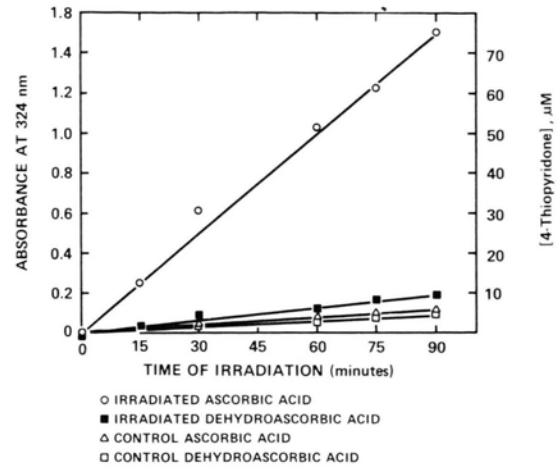

Fig. 1. Time course for the reduction of 4-4′ dithiopyridine to thiopyridone as a function of ascorbic acid oxidation. Thiopyridone (reduction product of 4-4′ dithiopyridine) generation can be followed at 324 nm (molar extinction coefficient = 1.98×10^4 [3]) since neither DTP nor ascorbic acid absorb at this wavelength. The level of AH· in this reaction is equivalent to the concentration of thiopyridone generated since 1 mol of AH· yields 1 mol of thiopyridone [3]. $2AH\cdot + DTP = 2A + 2TP$.

Results and Discussion

As shown in Table I, none of the biological compounds were reduced by ascorbic acid undergoing oxidation. Only in the case of DTP a cleavage of the disulfide bond could be observed. This finding supports observations by Karasch *et al.* [7] according to which aromatic disulfides are more easily cleaved than aliphatic disulfides. Fig. 1 shows the rate of reduction of 4-4′ dithiopyridine by ascorbic acid. The results suggest that cleavage of the S–S bond in DTP is relatively rapid. In contrast, dehydroascorbic acid under the same experimental conditions does not result in any appreciable reduction of DTP.

Table I. Summary of reactions between ascorbic acid and and several disulfide containing compounds.

Compound	Reduced by ascorbic acid oxidation	Generation of EPR signal at $g = 2.005$
4-4′ dithiopyridine [a]	+	+
cystine [b,c]	–	+
oxidized glutathione [b,c]	–	+
dithiobutyric acid [b,c]	–	+
bovine serum albumin [b,c]	–	+
gamma globulin [b,d]	–	+
insulin [b,d]	–	+

[a] Reduction of 4-4′ dithiopyridine was measured by following the production of 4-thiopyridine spectrophotometrically [3].
[b] Free SH groups were assayed according to the method of D. R. Grassetti and S. F. Murray, Jr., Arch. Biochem. Biophys. **119**, 41–49 (1967).
[c] Reduction products were monitored by measuring UV absorbance at specific wavelengths, see text.
[d] Reduction analyzed by SDS polyacrylamide gel electrophoresis, see text.

This result can be explained by the redox potentials of both of these compounds: the redox potential of DTP was determined to be $E_0' = -0.1$ V and is, thus, more positive than the ones determined for the ascorbic acid system. Thus, DTP can be reduced by AH· whose redox potential has been determined to be -0.2 V. The biological disulfides investigated cannot be reduced, however, by AH· due to their unfavourable redox potentials (-0.4 to -0.2 V). In this context, it should be pointed out that the aliphatic disulfide dithiobutyric acid behaved like biological disulfides in that it was not reduced by ascorbic acid.

AH_2 oxidation is a two step process in which the intermediate form (AH·) is either oxidized further to dehydroascorbic acid (A) or reduced back to ascorbic acid. This system can, therefore, act either

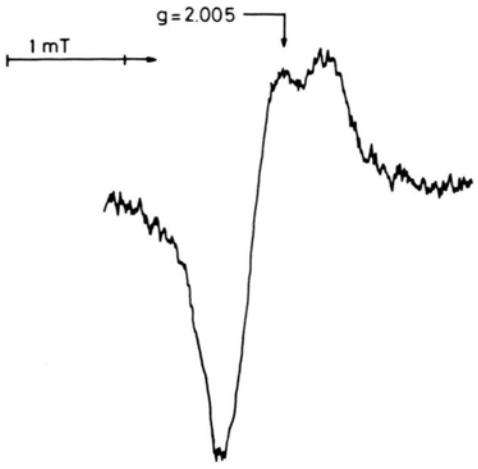

Fig. 2. EPR signal of a lyophilized sample of an aqueous mixture of dithiobutyric acid and ascorbic acid in a molar ratio of 1:1. The EPR detection conditions were: 0.2 mT modulation amplitude at 100 kHz modulation and 5 mW microwave power at 9.4 GHz.

as pro-oxidant or antioxidant depending on the relative redox potentials [8].

Another aim of this study was to determine whether AH_2 binds to S–S containing molecules. In a previous EPR study, it was shown that AH_2 forms ionic bonds with a variety of biological molecules. The question of the participation of sulfur in this binding process remained open [1]. Using ultrafiltration techniques with [^{14}C]ascorbic acid, we observed binding of several molecules of AH_2 to each BSA molecule; SH blocking agents (*e.g. p*-chloromercuribenzoate) prevented this. Using EPR methodology, a spectrum was obtained with dithiobutyric acid (Fig. 2) which was identical to that generated by the binding of AH_2 with certain biological molecules [1] and with the other disulfides investigated. Since nitrogen is not present in dithiobutyric acid, it is reasonable to assign the S–S group a role in the intermolecular bond formation. This bond may be an intermediate complex similar in type to that formed between A and reduced glutathione, cysteine or thioglycolic acid [10]. In this case, Drake *et al.* had shown that when dehydroascorbic acid is reduced to ascorbic acid in the presence of SH groups, concomitant oxidation of these SH groups occurs with the formation of intermolecular complexes.

The reverse of this reaction, *i.e.* reduction of biological disulfides by AH_2 or $AH\cdot$, apparently does not take place under the conditions employed in this study, because of the redox potentials involved. However, the EPR measurements indicate that an intermediate complex between the $AH\cdot$ and R–S–S–R might be formed and complex may represent an important binding site for ascorbic acid *in vivo*. Such binding of ascorbic acid to serum albumin or cell membrane proteins may play an important role in transport and prevention of autooxidation of this vitamin [11].

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