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THE INTERACTION OF NITRIC OXIDE WITH ASCORBATE OXIDASE

F.X.R. VAN LEEUWEN, R. WEVER, B.F. VAN GELDER, L. AVIGLIANO* and B. MONDOVI*

*Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, Amsterdam (The Netherlands) and *Institutes of Biological Chemistry and Applied Biochemistry, University of Rome and Centre for Molecular Biology, National Research Council, Rome (Italy)*

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Summary

1. The reaction of nitric oxide with oxidized and reduced ascorbate oxidase (L-ascorbate : oxygen oxidoreductase, EC 1.10.3.3) has been investigated by optical absorption measurements and electron paramagnetic resonance, and the results are compared with those of ceruloplasmin.

2. Upon anaerobic incubation of oxidized ascorbate oxidase with nitric oxide a decrease of the absorbance at 610 nm is found, which is due to an electron transfer from nitric oxide to Type-1 copper.

3. In the presence of nitric oxide the EPR absorbance of ascorbate oxidase decreases and shows predominantly a signal with characteristics of Type-2 copper ($g_{\parallel}$ = 2.248; $A_{\parallel}$ = 188 G), whereas the Type-1 copper signal has vanished.

4. Comparison of the intensities of the EPR signals before and after NO-treatment points to the presence of one Type-2 and three Type-1 copper atoms per molecule of ascorbate oxidase.

5. It is shown that the changes in the optical and the EPR spectrum of ascorbate oxidase induced by nitric oxide are reversible. No difference in enzymic activity is found between the native enzyme and the NO-treated enzyme after removal of nitric oxide.

Introduction

Ascorbate oxidase (EC 1.10.3.3) isolated from green zucchini squash, *Cucurbita pepo medullosa*, belongs together with laccase and ceruloplasmin to the blue copper-containing oxidases. Ascorbate oxidase has a molecular weight of approximately 140 000 [1,2]. The copper content of the enzyme has not yet been established, since several investigators [2—5] have reported values

ranging from six to twelve copper atoms per molecule of enzyme. Like in laccase and ceruloplasmin, in ascorbate oxidase the various types of copper have been distinguished on basis of their EPR characteristics into paramagnetic Type-1 copper, with a narrow hyperfine splitting, paramagnetic Type-2 copper, with a broad hyperfine splitting and diamagnetic copper. The latter two copper species have no distinct absorbance in the visible region of the absorption spectrum, whereas Type-1 copper causes the blue colour of the enzyme (A_{max} at 610 nm) [6].

In previous papers we have reported on the effect of nitric oxide on the EPR and optical spectra of oxidized and reduced ceruloplasmin [7,8]. It was found that in oxidized ceruloplasmin nitric oxide reacts with Type-1 copper, whereas Type-2 copper is unaffected. From these results it was concluded that dependent on the number of copper atoms per molecule (seven or eight), ceruloplasmin contains one Type-2 and two or three Type-1 copper atoms per molecule. Although the enzymic activities of ascorbate oxidase and ceruloplasmin are quite different, there exists a great correspondence in optical and EPR properties of both proteins. Therefore, it is of interest to study the reaction of nitric oxide with ascorbate oxidase.

In the present paper the stoichiometry and some properties of the various types of copper in ascorbate oxidase have been determined and the results are compared with the corresponding data obtained with ceruloplasmin [7,8].

Materials and Methods

Ascorbate oxidase was purified from green zucchini squash, *Cucurbita pepo medullosa*, according to Avigliano et al. [1]. Two preparations were used: Sample-1, conserved in liquid nitrogen, and Sample 2, stored at −20 °C, with copper contents of 5.4—6.5 and 7.5—8.0 atoms per molecule of enzyme, respectively. The purity of the preparations was determined from the ratio of A_{280}/A_{610nm}, which was approximately 25 for both preparations.

The reaction with nitric oxide was carried out in Thunberg-type optical and EPR cuvettes. Anaerobiosis was achieved as reported previously [7]. Nitric oxide was obtained either directly from a gas holder (Matheson gas products) or generated by the reaction of ascorbic acid and sodium nitrate (1 M, British Drug Houses, Analar Grade) in a specially developed device on top of the EPR cuvette. Absorption spectra were measured with a Cary-17 spectrophotometer. EPR spectra were recorded using a Varian E-9 EPR spectrometer. Samples were cooled by a liquid-nitrogen flow system to 88 °K or to lower temperatures by a helium transfer system (Air Products Inc. model LTD-3-100) with automatic temperature controller. The microwave frequency was determined with a Hewlett-Packard frequency counter (5246L) with frequency converter (5255A). The magnetic field was calibrated using an AEG magnetic field meter (GA 11-22.2).

Activities were determined at 23 °C by measuring the initial rate of the oxygen consumption, using a Gilson oxygraph, which is equipped with a Clark electrode. Ascorbate oxidase (0.5 μM) was diluted in 100 mM potassium phosphate, pH 7.0. The reaction was started by adding ascorbic acid to a final concentration of 1 mM.

Results

Anaerobic incubation of oxidized ascorbate oxidase with nitric oxide at 20°C (Fig. 1) causes a decrease in the absorbance at 610 nm and 800 nm and an increase in the 330 nm absorbance. Increasing the nitric oxide pressure from 0.5 to 1.0 atmosphere and lowering the temperature from 20°C to 11°C at a fixed nitric oxide pressure (1.0 atmosphere) brings about an enhancement of the changes in optical absorption spectrum. Since the absorbance at 610 nm originates from Type-1 copper it is concluded that nitric oxide interacts with this type of copper. The decrease of the 610 nm absorbance in the presence of nitric oxide, which is indicative of an enlarged electron density on the Type-1 copper, can be restored (not shown) by repeated evacuation and flushing of the cuvette with nitrogen gas. Hence, binding of nitric oxide to the enzyme is reversible and brings about an electron transfer from the ligand to the Type-1 copper. Furthermore, from the experiment of Fig. 1 the dissociation constant of the nitric oxide complex of ascorbate oxidase can be estimated and is found to be approximately 2 atmospheres at room temperature.

Fig. 2 illustrates the effect of nitric oxide on the EPR spectra of two preparations of ascorbate oxidase, Sample 1 and Sample 2, with copper contents of 5.4—6.5 and 7.5—8.0 copper atoms per molecule of enzyme, respectively. In the presence of nitric oxide (0.5 atmosphere) the EPR spectra of both preparations (trace C) show predominantly the characteristics of Type-2 copper ($g_{\parallel}$ = 2.248; $A_{\parallel}$ = 188 G) whereas the hyperfine resonances of the Type-1 copper ($g_{\parallel}$ = 2.229; $A_{\parallel}$ = 58 G) have vanished. The EPR spectra of both nitric oxide-treated preparations of ascorbate oxidase, although differing in shape of the $g_{\perp}$ signal, are considerably reduced in intensity when compared to the untreated preparations. This observation and the disappearance of the Type-1 copper hyperfine point to the formation of an EPR-undetectable complex

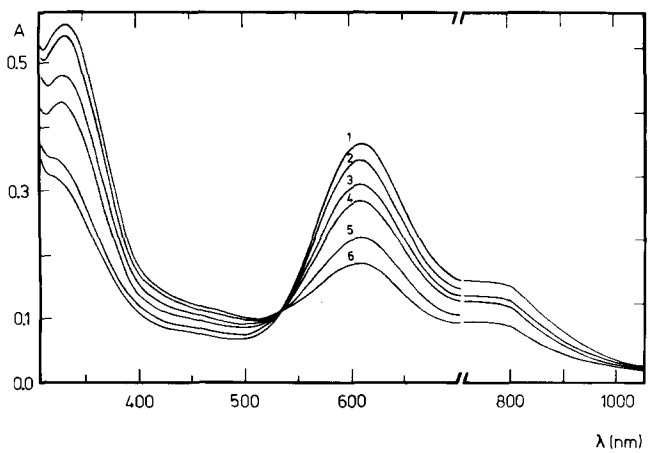

Fig. 1. The effect of nitric oxide on the absorbance spectrum of ascorbate oxidase. 1, native enzyme (Sample 1); 2—4, equilibrated with approximately 0.2, 0.5 and 1.0 atm nitric oxide, respectively, at 20°C; 5 and 6 equilibrated with 1.0 atm nitric oxide at 15°C, and 11°C, respectively. The reaction medium, 100 mM potassium phosphate buffer (pH 7.0), contained 34 μM ascorbate oxidase.

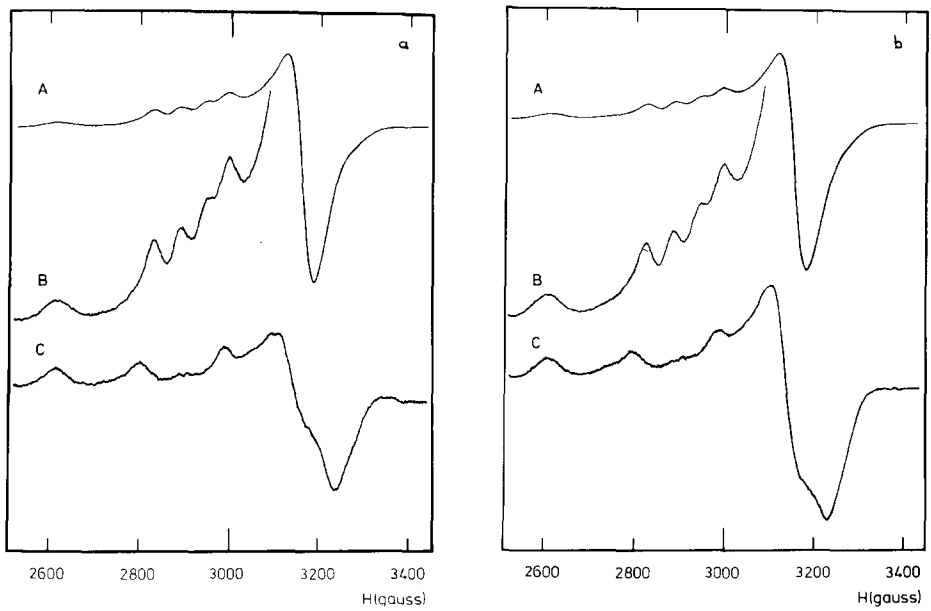

Fig. 2. The effect of nitric oxide on the EPR spectrum of ascorbate oxidase. a, Sample 1 (130 μM enzyme in 100 mM potassium phosphate, pH 7.0) and b, Sample 2 (170 μM enzyme in 100 mM potassium phosphate, pH 7.0) in the absence (A,B) and in the presence (C) of approximately 0.5 atm NO. Spectra B and C were recorded at a 5-fold higher receiver gain. EPR conditions: frequency, 9.087 GHz; microwave power, 10 mW; modulation amplitude, 10 G; scanning rate, 62.5 G · min^{-1}; time constant, 1.0 s; temperature, 88°K.

between nitric oxide and Type-1 copper, whereas the Type-2 copper in ascorbate oxidase does not react with nitric oxide.

The EPR spectra of both oxidized ascorbate oxidase preparations are quite similar (Fig. 2, upper spectra). Double integration of these spectra and comparison of the data with a 1 mM Cu-EDTA standard, reveals that in Sample 1 and Sample 2 of oxidized ascorbate oxidase 59% and 48%, respectively, of the copper atoms are EPR-detectable. Since in Sample 1 about six copper atoms and in Sample 2 about eight copper atoms per molecule are found, it is calculated that in both preparations nearly four copper atoms per molecule are paramagnetic. Consequently, the difference in copper content between the samples is due to the presence of varying amounts of diamagnetic copper in the enzyme.

Upon incubation with nitric oxide, the EPR signals of both preparations of ascorbate oxidase account for 33% (Sample 1) and 42% (Sample 2) of the spins present in the corresponding preparations of oxidized ascorbate oxidase. This difference in EPR-detectable spins between the nitric oxide-treated preparations is clearly illustrated in Fig. 2, traces C, where the shape of the $g_{\perp}$ signals differs distinctly. Since this indicates that a resonance, additional to that of Type-2 copper, contributes to the EPR spectrum of ascorbate oxidase incubated with nitric oxide, the contribution of Type-2 copper to the EPR spectrum of both preparations was calculated from the area of the low field hyperfine line, according to Vänngård [9]. This calculation, which has also been carried out by Deinum et al. [5] for oxidized ascorbate oxidase, shows that

83% (Sample 1) and 64% (Sample 2) of the intensity of the EPR signals of nitric oxide-treated enzyme corresponds with Type-2 copper. Thus it is concluded that in both preparations Type-2 copper contributes 27% (0.33 × 0.83 and 0.42 × 0.64) to the intensity of the EPR signal of oxidized ascorbate oxidase.

Upon anaerobic reduction of both samples of ascorbate oxidase with an excess of ascorbic acid, a weak EPR signal in the $g = 2$ region with an intensity of 2—2.5% of that of oxidized ascorbate oxidase is observed (not shown). Incubation of these reduced samples with nitric oxide causes a 3—4-fold increase in intensity of the signal around $g = 2$, but at half field ($g = 4$) no distinct EPR absorption can be detected. Thus in reduced ascorbate oxidase there is no evidence that a similar complex is formed between nitric oxide and monovalent copper pairs, as was found with reduced ceruloplasmin [8].

In line with the optical data it is also found by EPR, that nitric oxide binds reversibly to the oxidized as well as to the reduced enzyme. The reversibility of the reaction of nitric oxide with oxidized ascorbate oxidase is also tested by measuring the enzymic activity (see Materials and Methods). The specific activity of Sample 1 and Sample 2 is found to be 1360 and 820 μmol oxygen consumed · min^{-1} · mg $protein^{-1}$, respectively. As expected from the spectroscopic data, no difference in activity is detected between the native enzyme and the enzyme of which nitric oxide is removed by evacuation after an incubation with this ligand for 15 min at room temperature.

Discussion

The absorption spectrum of oxidized ascorbate oxidase shows a striking resemblance with that of ceruloplasmin. Consistent with the work of Deinum et al. [5], but contrary to Lee and Dawson [4], in our oxidized ascorbate oxidase preparation no absorption band or shoulder is found at 880 nm. This difference in observation can be due to the fact that the preparation of Lee and Dawson contains more copper atoms per molecule of enzyme than those of Deinum et al. [5] and ours.

The effect of nitric oxide on the absorption spectrum of oxidized ascorbate oxidase is similar to that observed with ceruloplasmin, except that no distinct absorption band at 400 nm is found in nitric oxide-treated ascorbate oxidase. However, it is conceivable that the 400 nm band is small and obscured by the absorbance around 330 nm.

Similar to ceruloplasmin, in the EPR spectrum of NO-treated ascorbate oxidase the Type-1 copper signal has vanished, whereas in the absorption spectrum at room temperature only part of the 610 nm absorbance has disappeared. For NO-treated ceruloplasmin it was found that the 610 nm absorbance disappears when the absorption spectrum of the same sample is recorded at liquid nitrogen temperature (Van Leeuwen, F.X.R., unpublished). These observations and the temperature dependence of the decrease of the 610 nm absorbance in the presence of nitric oxide can be explained by an increase in solubility of nitric oxide in the medium or a higher affinity of the enzyme for nitric oxide at lower temperature.

From the EPR data, obtained with NO-treated ascorbate oxidase, it is

concluded that the enzyme contains one Type-2 copper atom (27%) out of four paramagnetic copper ions. Then, if only two types of copper contribute to the EPR spectrum of oxidized ascorbate oxidase, the other three copper atoms are Type-1. This stoichiometry, also reported by Deinum et al. [5] for ascorbate oxidase, corresponds with that previously found by us for ceruloplasmin [7]. On the other hand, it has recently been reported [10] that each type of paramagnetic copper contributes 50% to the EPR spectrum of ascorbate oxidase. However, this result was obtained from computer simulations in which EPR parameters for Type-2 copper ($g_{\parallel}$ = 2.22 and $A_{\parallel}$ = 209 gauss) were used, differing from those found experimentally in the presence of nitric oxide ($g_{\parallel}$ = 2.248 and $A_{\parallel}$ = 188 gauss).

In the NO-treated ascorbate oxidase preparations, the contribution of the EPR resonance additional to that of Type-2 copper is twice as great in Sample 2 as in Sample 1. Since the number of diamagnetic copper atoms in Sample 2 is also twice that of Sample 1, it is proposed that the additional resonance stems from 'diamagnetic' copper atoms in NO-treated ascorbate oxidase. It is obvious that this resonance is only present in the EPR spectrum of the NO-treated enzyme, since no significant difference in shape of the EPR spectra of the untreated, oxidized samples of ascorbate oxidase could be detected.

Although upon addition of nitric oxide to reduced ascorbate oxidase a small increase in intensity of the EPR spectra is observed, no EPR signals are found near $g = 2$ and $g = 4$ resembling those of NO-treated hemocyanine [11—13], tyrosinase [13] and reduced ceruloplasmin [8]. In these proteins the signals near $g = 2$ and $g = 4$ have been attributed to dipole-dipole coupled Cu(II)-ions, which are formed by an interaction between Cu(I) and nitric oxide. The absence of these typical signals in ascorbate oxidase can be explained either by a lack of reactivity of nitric oxide for the Cu(I)-ions in the reduced enzyme or by a greater distance between the copper ions in reduced ascorbate oxidase than in ceruloplasmin [8,13].

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