The Interaction of Ascorbate Oxidase with L-Dopa, L-Tyrosine and 3,4-Dihydroxycinnamic Acid. Evidence for Irreversible Damage of the Enzyme During Catechol Oxidase Activity

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Received November 9, 1984

Abstract

The aerobic interaction between ascorbate oxidase and L-tyrosine, L-3,4-dihydroxyphenylalanine or 3,4-dihydroxycinnamic acid in 1:10 molar ratio was followed by optical absorption, CD and EPR spectroscopy in 0.1 M phosphate buffer at pH 5.0. While the spectra of the system ascorbate oxidase–L-tyrosine remain practically unaffected after several hours, indicating that no oxidation of the amino acid occurs in the conditions employed, rather drastic changes can be observed in the spectra of the ascorbate oxidase-catechol systems. In particular, while the optical absorption below 500 nm increases markedly due to the formation of the substrate oxidation products, an irreversible decrease in intensity of the absorption, CD and EPR spectral features associated with the blue copper(II) chromophores indicates that a partial loss of Type 1 copper by ascorbate oxidase has occurred during this secondary catechol oxidase activity. A copper species characterized by weak positive CD activity at 370 nm and EPR signal at intermediate field between those of the Type 2 and Type 1 coppers can be detected in the early stages of the reaction. The irreversible damage undergone by the protein during catechol oxidase activity may have biological significance and accounts for the low yield of purified enzyme obtained when the crude enzyme extract is left in prolonged contact with low molecular weight cell components, rich in *o*-diphenolic compounds.

Introduction

Ascorbate oxidase (L-ascorbate: oxygen oxidoreductase, EC 1.10.3.3) is a copper enzyme found in plant tissues that belongs to the small group of enzymes usually referred to as 'blue oxidases' [1–3]. The enzyme contains the three types of biological copper, classified as Type 1, Type 2 and Type 3 according to an established terminology [4], in the stoichiometry of three Type 1, one Type 2 and four Type 3 copper atoms per protein molecule, M_r = 140000 [5–7]. The molecular properties and catalytic activity of ascorbate oxidase have been reviewed recently [8–10]. Although this enzyme has been considered strictly specific for the aerobic oxidation of L-ascorbic acid, it is also able to oxidize a variety of natural and synthetic catechol derivatives [11]. This secondary catechol oxidase activity is related to the well known process of darkening undergone by fruits and vegetables containing ascorbate oxidase and *ortho*-diphenolic compounds either during their senescence stage or after damaging their cellular structure, for instance upon cutting. Since nothing is known about the fate of the enzyme after this secondary activity, we thought it of interest to follow the spectroscopic changes undergone by ascorbate oxidase in the presence of potential substrate molecules. This paper reports the EPR, optical absorption and CD spectral investigation of the aerobic interaction between ascorbate oxidase and L-3,4-dihydroxyphenylalanine (L-dopa), L-tyrosine or 3,4-dihydroxycinnamic acid (caffeic acid).

Material and Methods

All reagents were of the highest grade commercially available and used as received. Ascorbate oxidase was extracted from green zucchini (*Cucurbita pepo medullosa*) and purified according to the most recently published procedure [6]. The protein had optical indices A_{280}/A_{610} = 24.7 and A_{330}/A_{610} = 0.90 (0.1 M phosphate buffer, pH 6.8); these values remained unchanged after preparative gel electrophoresis on polyacrylamide gel under the conditions

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0020-1693/85/$3.30

described previously [6]. Protein concentration was determined assuming ϵ_{610} = 9700 M^{-1} cm^{-1} in 0.1 M phosphate buffer at pH 6.8 and M_r = 140000. The copper content determined by flame atomic absorption was 8.2 ± 0.2 g-atoms per mol of protein. The enzymic activity measured spectrophotometrically at 23 °C by monitoring the change in absorbance at 265 nm due to the oxidation of L-ascorbate corresponded to 3850 Dawson units [12].

All experiments were done in the air with protein dissolved in 0.1 M phosphate buffer at pH 5.0. A concentrated protein sample, obtained by use of a Sartorius collodion bag, was diluted to approximately 10^{-4} M with buffer and divided into four equal parts. The first one was examined as such, while the appropriate amount of 0.1 M aqueous solution of L-dopa hydrochloride, L-tyrosine hydrochloride or sodium 3,4-dihydroxycinnamate (10 μl) was added to each of the remaining solutions. A 1:10 molar ratio of enzyme:substrate was used for each phenolic compound.

The absorption spectra were measured with a Perkin-Elmer Lambda 5 spectrophotometer. Circular dichroism spectra were recorded on a Jobin Yvonne Mark III dichrograph, calibrated with a solution of isoandrosterone in dioxane. The CD data are expressed in terms of the differential molar extinction coefficient $\Delta\epsilon = \epsilon_l - \epsilon_r$ (M^{-1} cm^{-1}). The EPR spectra were obtained on a Varian E-109 spectrometer operating at X-band frequencies. Sample solutions as used for the absorption and CD spectra were placed in quartz EPR tubes and frozen in liquid nitrogen; spectra were then routinely measured at −140 °C by employing a V-4000 variable temperature control apparatus. The pH measurements were made at room temperature with an Amel model 328 pH-meter.

Results and Discussion

The amino acids L-tyrosine and L-dopa were chosen as potential phenolic substrates in this investigation for their structural similarity, sufficient solubility in aqueous medium and the presence of a similar centre of chirality within the molecule. Caffeic acid was taken as an optically inactive reference substrate as it has a carbon chain of the same length as that of the two phenolic amino acids. The reactions were followed by optical absorption, CD and EPR spectroscopy in 0.1 M phosphate buffer at pH 5.0, since at this pH the rate of substrate oxidation by ascorbate oxidase is significantly lower than that at pH ~ 7 [11]. While L-dopa and caffeic acid are oxidized by ascorbate oxidase to give complex mixtures of products [11], L-tyrosine is not expected to react with the enzyme, especially in the conditions employed here. In fact a solution of L-tyrosine in 0.1 M phosphate buffer at pH 6.8 (1 mg/ml) remains completely unaffected in the presence of ascorbate oxidase (1 μg/ml) in conditions similar to those employed to test the catechol oxidase activity of the enzyme [11]. Therefore, the system ascorbate oxidase–L-tyrosine should provide an appropriate reference for monitoring the spectral changes occurring in the system ascorbate oxidase–L-dopa during enzymic activity.

The absorption and CD spectra of native ascorbate oxidase recorded at pH 5.0 are not significantly different from those reported near pH 7.0 [6, 7, 13]. The CD extrema for the present preparation are located at 730 ($\Delta\epsilon$ = −16.85), 610 (+6.83), 550sh (+3.64), 475 (−5.46), 420 (+0.50) and 330 nm (−2.73) (Fig. 1). The appearance of the EPR

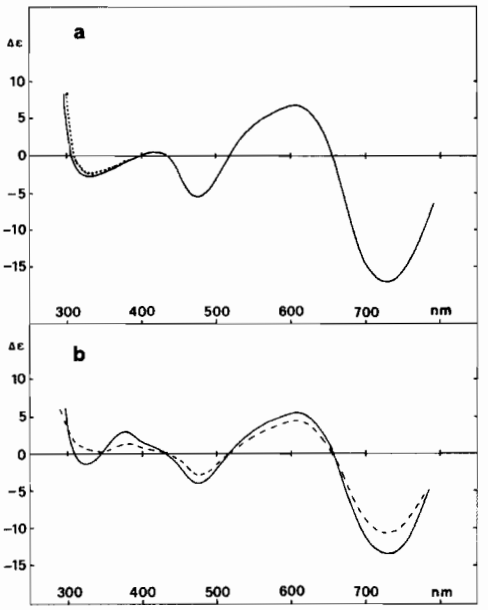

Fig. 1. (a) Circular dichroism spectra of native ascorbate oxidase in 0.1 M phosphate buffer at pH 5.0 (——), and of the system ascorbate oxidase–L-tyrosine at pH 5.0 two h after the addition of the amino acid (·····). (b) Circular dichroism spectra of the system ascorbate oxidase–L-dopa recorded 5–10 min (——), and two h (— — —) after the addition of substrate.

spectrum of ascorbate oxidase in frozen solution at pH 5.0 is also very similar to that at pH ~ 7 [5–7], though accurate g and A values for the copper centres can only be obtained with appropriate simulation of the spectrum. Upon addition of L-tyrosine the visible absorption and CD spectra of ascorbate oxidase are practically unaffected (Fig. 1). The EPR spectrum of the mixture is also very similar, although not exactly superimposable, to that of the free enzyme, but this most probably results from a slight difference in the phasing of the EPR signals. The spectra of the mixture do not undergo appreciable change within several h, indicating that negligible modification of the

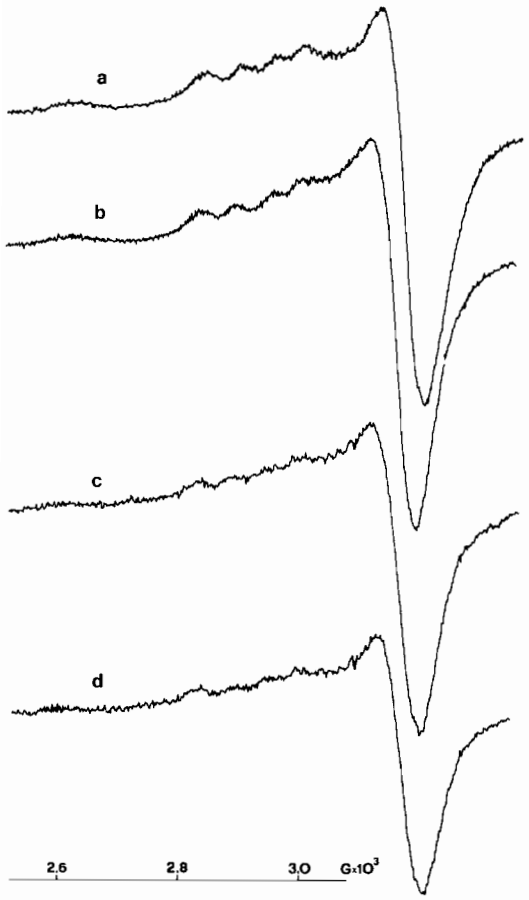

Fig. 2. Frozen solution EPR spectra at −140 °C in 0.1 M phosphate buffer at pH 5.0 of: (a) native ascorbate oxidase (ν = 9.074 GHz); (b) the system of ascorbate oxidase–L-tyrosine two h after the addition of the amino acid (ν = 9.072 GHz); (c) the system of ascorbate oxidase–L-dopa 5–10 min after the addition of substrate (ν = 9.075 GHz); (d) the same system as in (c) two h after the addition of substrate (ν = 9.075 GHz). All recordings were made with the following spectrometer setting: modulation frequency 100 kHz; power 10 mW; gain 6.3 × 10^3; modulation amplitude 5 G.

protein occurs in these conditions (Figs. 1 and 2). A decrease of about 20% in the specific activity of the enzyme, however, suggests that L-tyrosine may act as an inhibitor of ascorbate oxidase.

The addition of L-dopa to ascorbate oxidase at pH 5.0 causes rather drastic changes in the absorption, CD and EPR spectra of the protein even within a few min of the reaction at room temperature. In particular, a marked decrease in intensity of the absorption, CD and EPR features related to the Type 1 copper indicates progressive bleaching of the blue chromophore. The decrease in absorbance at 610 nm amounts to about 20% after 5–10 min from the addition of L-dopa, with a corresponding decrease in CD activity at 730, 610 and 475 nm (Fig. 1). After longer reaction times the negative CD activity near 330 nm is lost and possibly replaced by a weak band of positive sign. These changes are flanked by a marked increase of optical absorption below 500 nm, due to the formation of the quinone and of more complex oxidation products, and the appearance of a positive CD feature centred at 370 nm. The colour of the solution turns from blue to brown. As shown in Fig. 1, approximately two h after the addition of substrate the residual CD activity at 730 and 610 nm corresponds to 65% of the original enzyme intensity. Assuming that only the Type 1 copper(II) ions contribute to the optical activity at these wavelengths and that equal contributions arise from each of the three Type 1 sites, the decrease in CD activity at 730 and 610 nm accounts for the loss of approximately one blue copper(II) centre per protein molecule. We obviously have no indication that this loss is specific but it can be noted that further loss of CD activity occurs at a much slower rate. For instance, the residual CD activity at 730 and 610 nm for the reaction mixture after further storage at 4 °C for one week is approximately 50% of the original intensity. But with the same treatment the samples of native ascorbate oxidase and the mixture of ascorbate oxidase and L-tyrosine also lose approximately 10% of the original enzyme CD activity at 730 and 610 nm. Bleaching of Type 1 copper of ascorbate oxidase on addition of L-dopa is also evident in the EPR spectra in Fig. 2. Even though the resolution of the spectra is not very good, a very weak new EPR feature can be detected in the spectrum recorded 5–10 min after the addition of substrate near 2730 G, between the $m_I = -3/2$ hyperfine lines of the Type 2 and Type 1 coppers. The overall result, however, is an apparent reduction in intensity of the EPR signal, as is clearly shown by the spectrum recorded two h after the addition of L-dopa. The quantitative evaluation of the overall decrease of EPR detectable copper and of the ratio between Type 1 and Type 2 copper requires accurate simulation and double integration of the spectrum, which may be prohibitively difficult in the present case where more than two EPR detectable species are apparently present. However, a qualitative estimation based on the peak-to-peak amplitude of the high-field component of the EPR signal gives about 80% of residual EPR intensity in the spectrum recorded after 5–10 min and about 65% in that after two h of the reaction. If the reduction of EPR intensity were entirely due to bleaching of the Type 1 copper, these data would be in perfect agreement with the figures deduced from the optical and CD spectra. The specific activity of the sample drops to about 20% of that of the free enzyme. After longer reaction times the intensity of the EPR signal increases to approximately the original level of the free enzyme but the appearance of the resulting spectrum resembles that of aged [6] or impure [14]

preparations of ascorbate oxidase, indicating that a structural modification of the protein with irreversible loss of Type 1 copper has occurred during the enzymic activity.

The system ascorbate oxidase–caffeic acid at pH 5.0 undergoes spectral changes that are practically identical with but perhaps somewhat less dramatic than those shown in Figs. 1 and 2 for the system ascorbate oxidase–L-dopa. Thus the loss of CD activity at 730 and 610 nm three h after the addition of substrate amounts to approximately 30% of the original enzyme intensity, while the weak positive CD activity appearing in the 300–400 nm region is slightly better resolved and shows two peaks at 350 and 375 nm. The EPR spectrum of the mixture at the same reaction time is very similar to that reported in Fig. 2d. Although these results are only qualitative, the presence of an amino group in the substrate molecule clearly has a minor effect on the oxidation reaction.

The appearance of CD activity at 350–400 nm in both systems of ascorbate oxidase–L-dopa and ascorbate oxidase–caffeic acid shows that it is not due to the complex mixture of quinone products formed in the reactions. On the other hand this spectral feature can not be related to the accumulation of enzyme–substrate complexes containing the catechol derivative directly bound to enzyme copper(II) centres, since chiral copper(II)–catecholate complexes display CD bands near 450 nm [15], but the amount at pH 5.0 must be negligible [16]. Species absorbing at about 360 nm [1], or exhibiting positive CD activity near 370 nm [17], have been detected in reoxidation studies of reduced laccase with dioxygen and attributed to some oxygen intermediate. However, we believe that in the present case the CD activity at 350–400 nm, together with the weak EPR signal at intermediate field between those of the Type 1 and Type 2 coppers, originates from an irreversible modification of part of the Type 1 copper sites during the enzymic activity, since similar changes occur, albeit on a much longer time scale, during the ageing process undergone by ascorbate oxidase when isolated [7]. It is obviously impossible to relate this modification of Type 1 sites to any step of the catalytic cycle of the enzyme, which is probably very complex; though, after complete oxidation of the substrate the fully reoxidized enzyme contains a significant portion of copper bound nonspecifically and is clearly irreversibly damaged.

Spectral changes similar to those described here at pH 5.0 occur at a faster rate also during the enzymic oxidation of the catechols at pH 7.0, while it should be noted that no such modification of ascorbate oxidase is apparent in the enzymic oxidation of L-ascorbic or reductic acid under physiological conditions [6, 9]. The structural alteration and loss of activity of ascorbate oxidase during catechol oxidase activity is related to the observation that in the course of the isolation and purification of the enzyme, prolonged contact of the crude enzyme extract with low molecular weight cell components, rich in *o*-diphenolic compounds, results in progressive darkening of the mixture and lowers significantly the yield of purified enzyme. This observation may have biological significance, since the irreversible damage undergone by the protein during catechol oxidase activity may represent a mechanism for the progressive inactivation and elimination of ascorbate oxidase in plants when the activity of this enzyme in the plant growing process becomes unnecessary or is replaced by that of more efficient enzymes.

Acknowledgements

This work was supported by a grant from the Italian MPI. The authors thank the Italian CNR for instrumentation facilities.

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