Efficiency of Nitrilotriacetate in the Removal of Type 2 Copper from Laccase and Ascorbate Oxidase

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(Received October 24, 1989; revised March 6, 1990)

Abstract

The powerful chelating agent nitrilotriacetate was found to be quite efficient in the removal of type 2 Cu, under reducing conditions, from the blue oxidases ascorbate oxidase and *Rhus vernicifera* laccase. While the effect of this substance on ascorbate oxidase was comparable to that of other reagents, as for instance EDTA, the effect on laccase was substantially larger than that of any other previously tested chelator, in particular EDTA which is by itself ineffective. This result confirms that the size of the chelator strongly affects its reactivity with laccase type 2 Cu, suggesting that the latter ion is more deeply buried in the protein matrix than the copper of ascorbate oxidase.

Introduction

Blue oxidases catalyze the oxidation of a variety of substrates (ascorbate, polyphenols, aromatic polyamines) by molecular oxygen, which is reduced to water [1]. They are widespread in nature, both in plants (laccase and ascorbate oxidase) and animals (ceruloplasmin). The catalytic function is carried out by several Cu ions present in each molecule, four in laccase [2, 3], eight in ascorbate oxidase [4] and at least five in ceruloplasmin [5]. The coordination environment is not identical for all Cu ions, but it is of three well characterized different types, called type 1, 2 and 3 [1]. Type 1 Cu shows an intense visible absorption spectrum, with maximum intensity at about 600 nm (ϵ_M = 5–6000) and an EPR spectrum with narrow hyperfine splitting ($A_\parallel$ = 4–6 mT); type 2 Cu has a broader $A_\parallel$ (15–20 mT) in the usual range of low molecular weight Cu(II) complexes; type 3 Cu has an intense optical absorption band at 330 nm and consists of a pair of EPR silent Cu(II) ions, due to antiferromagnetic coupling.

The type 2 Cu site is exposed to interaction with solvent molecules and in particular with chelating agents, from which it can be selectively removed with loss of catalytic activity [6]. Comparative studies were carried out on ascorbate oxidase from zucchini peelings and laccase from the latex of the Japanese lacquer tree *Rhus vernicifera,* with reagents such as EDTA, dimethyl glyoxime (DMG), and *N,N*-diethyldithiocarbamate (DDC) [7]. Ascorbate oxidase was found to be more reactive than laccase, i.e. its type 2 Cu could be completely removed by any of the above reagents in appropriate conditions, while only partial removal was obtained from laccase using DMG or DDC and no removal at all with EDTA. The latter reagent showed, however, the peculiar property of enhancing the capacity of Cu removal of DMG and DDC. These effects were explained with a lesser accessibility of the type 2 Cu site of laccase, only available to the smaller molecules, and with the ability of the latter ones to function as Cu carriers from inside the protein to the bulk solution, where they were bound by EDTA.

In the case of ascorbate oxidase the reaction with DDC proved very helpful in the re-determination of the stoichiometry of the three Cu types. Type 1, 2 and 3 Cu were found to be in a 2:2:4 ratio, respectively [8], as in laccase (1:1:2). X-ray studies showed furthermore [9] that the crystalline protein consists of two identical subunits, each binding four Cu ions. Three ions form a triangular cluster located at the interface between two domains, which contribute four histidyls each as copper ligands. The three Cu ions are not equivalent since one is bound to two

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0020-1693/90/$3.50

histidyl residues and two are bound to three histidyls each.

In the present paper we report the results obtained upon treatment of laccase and ascorbate oxidase with the powerful chelating agent nitrilotriacetate (NTA), which was shown to be very active in iron mobilization from ferritin [10]. The comparable reactivity against NTA of the two proteins may indicate similar coordination of the copper sites, lending further support to previous suggestions of the proximity of type 2 and type 3 Cu sites based on spectroscopic data [11–14]. The interest in NTA, on the other hand, was raised by its occasional introduction in detergents of domestic use, as a builder in place of phosphates.

Experimental

Laccase was purified from the acetone powder of the latex from the Japanese lacquer tree *Rhus vernicifera* supplied by Saito & Co. (Osaka, Japan) according to Reinhammar [15]. Ascorbate oxidase was purified by the method of Avigliano *et al.* [16]. In both cases a single band was present in SDS gel electrophoresis. The protein concentration was measured by the 280 nm absorbance, by using an extinction coefficient of 93.5 mM^{-1} cm^{-1} for laccase [17] and 240 mM^{-1} cm^{-1} for ascorbate oxidase [4]. The total content of copper was measured by reaction with 2,2′-biquinolyl [18] and by atomic absorption spectrophotometry, with a Perkin-Elmer 3030 apparatus equipped with an HGA-400 graphite furnace. EPR detectable copper was measured by double integration of EPR spectra using Cu-EDTA as the standard solution. The blue copper content was measured from the visible absorbance by using the extinction coefficients of 5700 M^{-1} cm^{-1} at 614 nm for laccase [17] and 5000 M^{-1} cm^{-1} at 610 nm for ascorbate oxidase monomer [8], both in the native and type 2 Cu depleted samples. The oxidase activity was measured spectrophotometrically at 25 °C, from the decrease of absorbance of sodium ascorbate at 265 nm for ascorbate oxidase and the increase of absorbance of hexacyanoferrate(II) at 425 nm for laccase. All reagent-grade chemicals were purchased from Merck and were used without further purification. The optical spectra were recorded with a Beckman UV 5230 spectrophotometer and the EPR spectra at X-band (9.15 GHz) were recorded with a Varian E-9 spectrometer. When necessary, the samples were concentrated by a microconcentrator (Amicon Centricon 30) or by ultradialysis.

Results and Discussion

Solutions of ascorbate oxidase (30 μM) or laccase (50 μM) were dialyzed for about 4 h against a 1.0–10.0 mM solution of NTA in 0.1 M acetate buffer pH 5.2. At the same time the solutions were deaerated by a stream of bubbling nitrogen. After addition of solid hexacyanoferrate(II) up to a final concentration of 1–2 mM, the dialysis was continued for about 15 h in the case of ascorbate oxidase and about 15 or 48 h in the case of laccase. With this treatment the solutions became pale yellow because of reduction of blue Cu ions. Subsequent dialysis against several changes of aerated 0.1 M phosphate buffer pH 6.0, restored the blue colour. The analytical data referring to laccase and ascorbate oxidase samples are reported in Table 1. Optical and EPR spectra of native and NTA-treated laccase samples are reported in Figs. 1–3.

From Table 1 it is immediately apparent that at 2.0 mM NTA and 48 h dialysis time the protein was inactivated by NTA with a loss of total copper content approaching 25% of the native protein value and a loss of paramagnetic copper of about 50% the native value. Less satisfactory results were obtained at shorter dialysis times even in the presence of larger NTA concentrations, since the treated protein samples display a relatively high activity and high copper content. The EPR spectra of Fig. 3 also show the presence, after the latter treatment, of a non-native type 2 Cu signal. When the dialysis time was increased to 48 h at 10.0 mM NTA, the protein was inactivated as in the presence of 2.0 mM NTA, but a loss of blue copper was obtained and moreover all Cu types were affected. It appears that a low NTA

TABLE 1. Analytical data relating to native *Rhus vernicifera* laccase and ascorbate oxidase and to samples depleted of copper under different conditions (time of dialysis in parentheses)

Sample	Copper content[a]			Percent activity
	Type 1	EPR detectable	Total	
Laccase				
Native	1.1	2.0	4.2	100
2 mM NTA (48 h)	1.0	1.1	2.8	15
Reconstituted	1.1	2.0	3.6	90
2 mM NTA + 2 mM EDTA (48 h)	0.9	1.0	2.7	10
5 mM NTA (15 h)	1.0	1.5	3.0	56
10 mM NTA (15 h)	1.0	1.5	3.0	33
10 mM NTA (48 h)	0.7	1.1	3.1	12
Ascorbate oxidase				
Native	1.9	3.8	7.4	100
2 mM NTA (15 h)	1.7	2.4	4.9	0
2 mM NTA (48 h)	1.6	2.1	3.7	0

[a]The values were normalized to the protein absorbance at 280 nm.

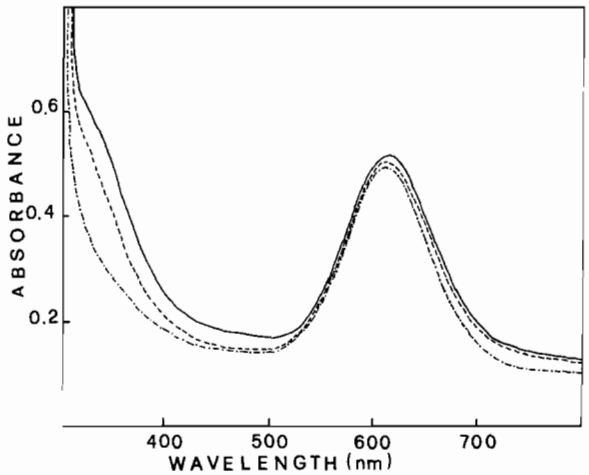

Fig. 1. Optical spectra of native and type 2 Cu depleted laccase: 90 μM native enzyme (solid line); treated with 2.0 mM NTA for 48 h (dot-dashed line); recombined (dashed line).

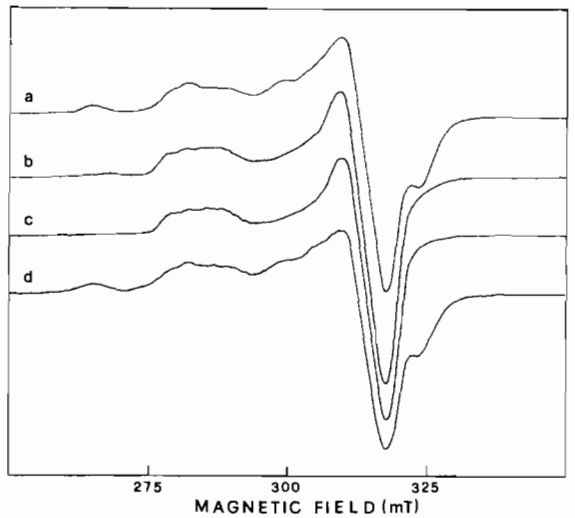

Fig. 2. X-band EPR spectra of native and type 2 Cu depleted laccase: (a) 0.16 mM native enzyme; (b) treated with 2.0 mM NTA for 48 h; (c) treated with 2.0 mM NTA and 1.0 mM EDTA for 48 h; (d) recombined. Instrumental conditions were: microwave frequency 9.15 GHz; microwave power 20 mW; modulation amplitude 1.0 mT; temperature 100 K.

concentration and long dialysis time provide best conditions for type 2 Cu removal. The recovery of activity, as reported in Table 1, was obtained by reconstitution of the type 2 Cu depleted laccase under reducing conditions, with the procedure previously described [6].

The removal of type 2 Cu from ascorbate oxidase was much easier than from laccase (Table 1), as previously found with other chelating agents. When the reaction was carried out by overnight dialysis against 1.0–10.0 mM NTA and 1.0 mM hexacyanoferrate(II), the type 2 Cu loss was comparable to that obtained using EDTA in the same conditions [7]. A longer dialysis time caused more extensive copper removal but also precipitation of the protein, showing that the larger NTA concentration had a more pronounced denaturing action on this protein than on laccase.

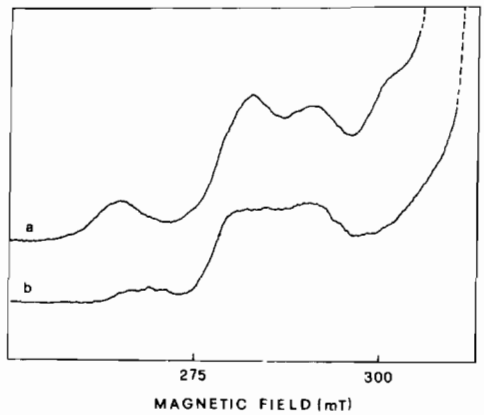

Fig. 3. Low field portion of X-band EPR spectra of native and excess NTA treated laccase: (a) 0.16 mM native laccase; (b) treated with 10 mM NTA for 48 h. Instrumental conditions as in Fig. 2 at fourfold larger gain.

In the optical spectra of both laccase and ascorbate oxidase the shoulder at 330 nm was substantially decreased after the treatment with NTA. An average decrease of 2200 M^{-1} cm^{-1} was obtained for laccase and of 1700 M^{-1} cm^{-1} for ascorbate oxidase monomer. In air and in the absence of the reducing agent the copper content and the enzymatic activity were unaffected by NTA in both proteins.

The novel result of this research is that NTA is able to remove type 2 Cu from the Japanese laccase without the assistance of EDTA, as from ascorbate oxidase, confirming a previous suggestion that the reactivity of chelating agents with laccase is related to their size. The type 2 Cu depleted form of *Polyporus versicolor* laccase was also recently prepared by procedures which use small molecules such as cyanide and thiourea in the presence of ascorbate [19].

The unsuccessful attempts to decrease the time required for the preparation of type 2 Cu depleted laccase by increasing the concentration of NTA in the dialysis medium (Table 1) also confirm the idea that the metal site is less accessible to chelating agents than it is in ascorbate oxidase and that a molecular rearrangement independent of the ligand concentration is required for release of the metal. The large carbohydrate component of laccase may play a role in the stabilization of copper into the catalytical site, but a difference in the protein matrix cannot be excluded. In general, however, the two proteins display a very similar behaviour towards NTA.

The efficiency of NTA in metal ion mobilization indicates that it may have quite dangerous effects when introduced in large amounts in ambient life, leading to inactivation of metallo-enzymes.

Acknowledgements

The skilful technical assistance of Mr A. Ballini is gratefully acknowledged. This work was in part carried out with funds provided by the Italian Ministero della Pubblica Istruzione.

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