SULFHYDRYL GROUPS, DISULFIDE BRIDGES AND THE STATE OF COPPER IN THREE BLUE OXIDASES

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1. Introduction

Out of the four copper atoms per molecule of laccase two copper atoms, Type 3, have an undetermined redox state, are not detectable by EPR and are non-paramagnetic. However, these oxidases can accept four electron equivalents [1]. Redox titrations have shown [1,2] that a 330 nm chromophore is not associated with Type 1 or Type 2 Cu^{2+} but with a unit accepting two electrons. During reduction of this chromophore no new EPR signals are formed. It has been suggested that this chromophore is associated with Type 3 copper, which may be present in the divalent state as a binuclear complex [3]. In an alternative proposal, it is not the copper that accepts the two electrons, but a disulfide bridge closely associated with a copper pair [4]. The aim of this study was to present additional evidence about the state of copper in relation to sulfhydryl groups and disulfide bridges in order to choose a model for the Type 3 copper.

We studied fungal laccase A and B from *Polyporus versicolor* and tree laccase from *Rhus vernicifera*. From determinations of the redox state of both the copper and the cysteine residues in the proteins we can conclude that all copper is in the divalent state and that consequently the Type 3 copper is present as a binuclear divalent complex.

**Abbreviations:*

DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
NBD-Cl	7-chloro-4-nitro-2-oxa-1,3-diazole
MNNP	2-chloromercuri-4,6-dinitrophenol
Gd-HCl	guanidine hydrochloride
MES	2 (*N*-morpholino) ethane sulfonic acid

2. Experimental

Visible and ultraviolet spectra and EPR spectra at 9 GHz were recorded and evaluated as previously described [5].

Protein concentrations were determined from the extinction coefficients at 280 and at about 610 nm [6–8] and also with amino acid analysis [9], using the composition of tree laccase [6], fungal laccase A [10] and fungal laccase B (Briving, C. and Deinum, J., unpublished).

The reagents NBD-Cl* [11], DTNB [12] and iodoacetate [13] were used in a 50- to 100-fold excess over the protein (10 to 30 μM). The protein was added anaerobically to the reagent in Gd-HCl. The modified proteins were isolated by gelfiltration through a column of Sephadex G-25. In the EPR determinations the total copper concentration was about 600 μM.

Denaturation of the proteins and sulfhydryl group determinations were always performed under strictly anaerobic conditions, using the technique and equipment as described earlier [14].

3. Results and discussion

As is shown in table 1 all copper becomes detectable by EPR upon anaerobic denaturation of the laccases in 6 M Gd-HCl containing 25 mM EDTA. EPR spectra of the denatured proteins are identical with the spectrum of the copper standard in the same medium. Upon anaerobic denaturation there is only one sulfhydryl group detectable in all three laccases (see table 1). The results obtained with the three reagents are con-

Table 1
Number of sulfhydryl groups per molecule and the state of copper in three laccases

	Total ½ cys after performic acid oxidation[a]	Reactive sulfhydryl groups in the anaerobically denatured proteins			Total Cu[e]	EPR detectable Cu^{2+} in the anaerobically denatured proteins at pH 5.2 (1 M Na acetate) or pH 7.5 (0.3 M Tris-HCl)
		iodoacetate[b] pH 8.6 or 10	NBD-Cl[c] pH 7.5	DTNB[d] pH 7.5		
Tree laccase	6.9	0.85	1.0	1.0	4	3.85
Fungal laccase B	5	1.0	1.0	0.9	4	3.9
Fungal laccase A	5	–	0.98	0.9	4	3.9

The sulfhydryl group determinations were done with the proteins which were untreated, 'oxidized' or reduced with 4 electron equivalents of ascorbate or Fe^{2+}. The redox state of the copper in the native enzyme did *not* have an effect on the number of detectable sulfhydryl groups in the denatured enzymes. This number was calculated as the mean value for several experiments. The proteins were denatured anaerobically with 6 M Gd–HCl with 25 mM EDTA in 0.3 M Tris–HCl buffer.

a) Measured as cysteic acid [15].
b) Measured as *S*-carboxymethyl cystein [13].
c) Measured as ΔA_{240} using E=13 $mM^{-1} \cdot cm^{-1}$ [11] directly and after isolation of the modified proteins.
d) Measured as ΔA_{412} using E=13,6 $mM^{-1} \cdot cm^{-1}$ [12] directly and after isolation of the product and reduction with an excess of mercaptoethanol.
e) Determined chemically with 2,2′-biquinoline [16] and by EPR after denaturation and oxidation with H_2O_2.

sistent. No differences were found in the number of detectable sulfhydryl groups in the oxidized denatured proteins compared with proteins which had been reduced with four electron equivalents of ascorbate before denaturation. From the difference between the total number of half cystine residues per molecule determined after performic acid oxidation (see table 1) and the free sulfhydryl groups one can calculate the number of disulfide bridges. Thus, tree laccase contains three disulfide bridges and the fungal laccases two. The two peptides containing the disulfide bridges in fungal laccase B have been isolated (Briving, C. and Deinum, J., unpublished). The conversion of free sulfhydryl groups to a disulfide bridge is a redox reaction for which one needs an electron acceptor with a high redox potential, e.g. Cu^{2+} or O_2. As O_2 is not present during the determinations and all Cu is still present as Cu^{2+} we can exclude the possibility that sulfhydryl groups could become oxidized upon denaturation. Thus, all types of copper must be present already in the native protein as Cu^{2+} as the only possible electron acceptors, the disulfide bridges, are still present in their oxidized form. However, these proteins contain several potential electron donors, e.g. the carbohydrates or tyrosine residues which might be able to reduce the copper under the appropriate conditions. We found indications for such donors in denaturations with bathocuproine disulfonic acid. However, other electron donors do not affect our conclusion about the number of sulfhydryl groups and the redox state of the copper.

In discussing the state of Type 3 copper it is useful to compare the proposed models as illustrated in fig.1. Both model 1 and 2 are based on the existence of a copper pair. Model 2 was proposed by Byers et al. [4]. In this model a disulfide is the two-electron accepting

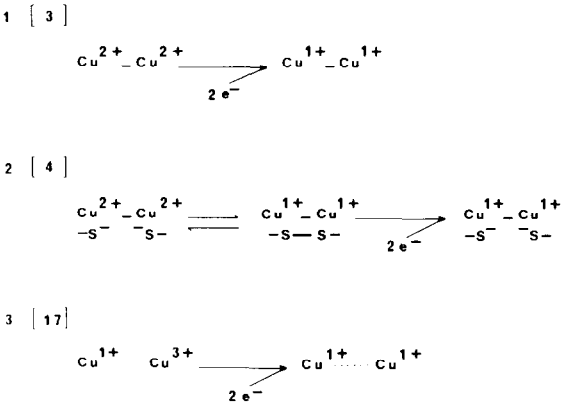

Fig.1. Models for the EPR non-detectable copper in blue oxidase.

system. In model 3 it is low-spin Cu^{3+} which can accept two electrons [17]. However, one must have very special conditions in the native enzyme to reduce Cu^{3+} in one step to Cu^{1+} without a Cu^{2+} intermediate. The copper that is Cu^{1+} in the oxidized enzyme would have to be exceptionally stable. Furthermore, Levitski et al. [18] found that a Cu^{3+} peptide rapidly degraded. Thus, model 3 is very unlikely. Model 2 is attractive in that the reduced form has a potential four-electron unit from which it should be easy to transfer four electrons to oxygen. However, model 2 is directly inconsistent with our data. All copper is Cu^{2+} in the denatured proteins and there is only one free sulfhydryl group, whereas for model 2 one would expect more such groups. We also find no more sulfhydryl groups after reduction. The conclusion is that only model 1 is acceptable. However, a disulfide bridge can still be associated with such a Cu^{2+}–Cu^{2+} pair.

Titration of *native* tree laccase with MNNP [19] (see fig.2) revealed the interaction of one sulfhydryl group per molecule with this mercurial. However, this reagent did not have an effect on the EPR spectrum,

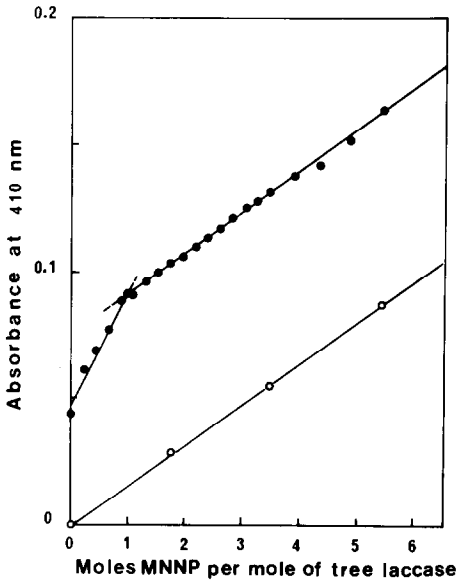

Fig.2. Optical titration with MNNP [19] in 0.1 M MES with 0.1 M EDTA at pH 5.5. Aliquots of 1 mM MNNP were added with a microsyringe to a 23 μM tree laccase solution (•), or to a blank without protein (○). The absorbance at 410 nm was corrected for changes in the volume. The absorbance became constant within the mixing time.

the optical absorption at 610 nm or the enzymatic activity of the laccase. The free sulfhydryl group in native tree laccase reacted less with DTNB and not at all with iodoacetate. The fungal laccases have no reactive sulfhydryl groups in the native protein in the reduced or oxidized form. For several small 'blue' proteins containing only Type 1 Cu^{2+} it has been proposed [20] that a sulfhydryl group is near this Cu^{2+} centre. The single sulfhydryl group in tree laccase is reactive even in the native enzyme. This means that at least in tree laccase neither Type 1 nor Type 2 Cu^{2+} can be directly coordinated to a sulfhydryl group. Further studies are in progress with proteins only containing Type 1 Cu^{2+}.

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