Cu(I) analysis of blue copper proteins

Phillip M. HANNA, Ramasamy TAMILARASAN and David R. McMILLIN*

Department of Chemistry, Purdue University, West Lafayette, IN 47906, U.S.A.

A simple colorimetric test for the Cu(I) content in blue copper proteins is described. The procedure is based on the formation of a complex between Cu(I) and 2,2'-biquinoline in an acetic acid medium. Analyses of spinach plastocyanin, *Pseudomonas aeruginosa* azurin and *Rhus vernicifera* stellacyanin show that the cysteine residue in the type 1 site does not induce Cu(II) reduction under our conditions. There is evidence in laccase samples for the presence of an endogenous reductant that can reduce 0.14 ± 0.04 mol of Cu(II)/mol of protein; however, the addition of EDTA eliminates the interference. The analysis shows that $25 \pm 2\%$ of the type 3 copper ions are in the reduced form in the resting enzyme and that $80 \pm 15\%$ of the type 3 copper ions are reduced in preparations of type-2-depleted laccase. There is growing interest in the development of chemically modified forms of laccase, and our method should be very useful for establishing the valence state of the metal centres in the various derivatives.

INTRODUCTION

The Cu(I) ion occurs naturally in some proteins. Both copper thionein (Weser & Hartmann, 1984) and deoxyhaemocyanin (Préaux & Gielens, 1984) have been shown to contain the Cu(I) ion in the resting state. Laccase contains four copper ions, but only two are paramagnetic. Earlier, this observation was taken as evidence that half of the copper ions occur in the cuprous state (Broman *et al.*, 1962). However, this view was altered when redox titrations demonstrated that laccase will accept about 4 mol of electrons/mol of enzyme (Malkin *et al.*, 1969; Fee *et al.*, 1969). Nowadays the redox and the magnetic properties of laccase are understood in terms of a binuclear binding site that contains a pair of anti-ferromagnetically coupled Cu(II) ions. This so-called type 3 copper site is also responsible for a shoulder at around 330 nm in the absorption spectrum of the resting enzyme (Malkin *et al.*, 1969). E.p.r.-active copper ions are found in the type 1 and the type 2 binding sites, and the type 1 copper is also responsible for a strong visible absorption that maximizes at 614 nm. Since the development of a procedure for selective removal of the type 2 copper from laccase (Graziani *et al.*, 1976; Morpurgo *et al.*, 1980b), a controversy has developed concerning the valence state of the type 3 copper ions in the type-2-depleted form of the enzyme (Reinhammar & Oda, 1979; Morpurgo *et al.*, 1980a,b; LuBien *et al.*, 1981; Frank *et al.*, 1983). The conflicting results of various investigators have led Frank & Pecht (1986) to propose that there are three different forms (isotypes) of type-2-depleted *Rhus* laccase. They propose that in the α form the type 3 site is oxidized though the absorbance shoulder at 330 nm is missing, that in the β form the type 3 site is oxidized and the 330 nm absorbance is fully developed, and that in the γ form the type 3 site is reduced. This interpretation is hard to accept because virtually the same procedure has been used in all work to date. Nevertheless, the results are confusing. Frank & Pecht (1986) have reported that their preparation has an oxidized binuclear site since it takes 3.0 electron equivalents of hydroquinone to reduce the type 1 copper. On

the other hand, Solomon and co-workers (Kau *et al.*, 1987) have argued strongly that the type-2-depleted form lacks the 330 nm absorption band because the type 3 copper ions are essentially fully reduced. The latter results seem definitive because the X-ray absorption edge is a direct probe of oxidation state. In order to provide a more accessible method for characterizing type-2-depleted laccase as well as other chemically modified forms of the enzyme, we have developed a spectrophotometric assay that is specific for Cu(I) in laccase.

The method is based on the studies by Breckenridge *et al.* (1939), who reported that the Cu(I) ion formed a deep-purple complex with 2,2'-biquinoline and that the complex was useful for colorimetric determination of Cu(I) in solution. Hoste (1950) subsequently demonstrated that the analysis was specific for Cu(I) in an alcoholic medium. Klotz & Klotz (1955) modified the method for the analysis of selected copper proteins in a medium containing glacial acetic acid. They reported that glacial acetic acid labilized copper from haemocyanin and that it dissolved excess 2,2'-biquinoline as well as the denatured protein. Moreover, they showed that Cu(II) ions did not interfere, a point more clearly demonstrated by Kertész (1957). In subsequent work with *Limulus polyphemus* haemocyanin, Felsenfeld (1960) found that the presence of protein thiol groups could confuse the analysis by inducing the reduction of Cu(II) to Cu(I). He went on to show that the total copper concentration could be determined if a few crystals of cysteine hydrochloride were added to the protein before the biquinoline reagent. On the other hand, he showed that the addition of mercurial reagents or EDTA minimized the side reaction with protein thiol groups. In the present paper we show how the biquinoline method can be adapted to the determination of the Cu(I) content of blue copper proteins, including laccase.

EXPERIMENTAL

Materials

The bacterial type *Pseudomonas aeruginosa* (strain no.

* To whom correspondence should be addressed.

10145, American Type Culture Collection, Baltimore, MD, U.S.A.) was cultured according to the procedure described by Lenhoff & Kaplan (1956). Azurin was isolated from the cell paste and purified by the method of Ambler & Brown (1967) to an absorbance ratio A_{626}/A_{280} of 0.46. Plastocyanin was extracted from spinach leaves (DeHahn Produce Co., Lafayette, IN, U.S.A.) and purified by the method of Markley *et al.* (1975) to an absorbance ratio A_{278}/A_{597} of 1.2. Both stellacyanin and laccase were extracted by the method of Reinhammar (1970) from the acetone-dried powder of *Rhus vernicifera* latex, which was harvested near Chu Shi, China, and supplied by Saito and Co., Osaka, Japan. The absorbance ratios A_{280}/A_{604} and A_{280}/A_{614} of 8.6 and 16.4 were obtained for stellacyanin and laccase respectively. Some experiments were carried out with stellacyanin, obtained analogously from the latex of the Japanese lacquer tree, with an absorbance ratio of 6.2.

All reagent-grade chemicals were used without further purification. L-Ascorbic acid (sodium salt) and L-cysteine hydrochloride were obtained from the Sigma Chemical Co., and 2,2'-biquinoline (98%) was obtained from Aldrich Chemical Co. KCN was a product of the J. T. Baker Chemical Co., and the disodium salt of EDTA was purchased from the Fisher Scientific Co.

Methods

All buffers were prepared from deionized water and were passed through columns of (Bio-Rad) Chelex 100 resin (pre-equilibrated with the same buffer) as needed in order to remove trace metal ions. Glassware was soaked for at least 12 h in strong acid and plasticware was soaked for at least 24 h in an EDTA solution to avoid contamination by adventitious metal ions.

The completely demetallated proteins were prepared by dialysis against KCN according to previously published methods (Morpurgo *et al.*, 1972; McMillin *et al.*, 1974; Morris *et al.*, 1983). The apo-azurin preparation (McMillin *et al.*, 1974) was modified such that the 50 mM-cyanide solution was in 0.1 M-imidazolium acetate with a final pH of 7 rather than the prescribed 25 M-Tris buffer at pH 8. In some samples the free thiol group in apo-laccase was complexed with Hg(II) as described previously (Morie-Bebel *et al.*, 1984). Type-2-depleted laccase was prepared by the procedure of Morpurgo *et al.* (1980b) and by the modification of Frank *et al.* (1983). Cu(I)-azurin was produced by reducing the native protein with excess $\text{Na}_2\text{S}_2\text{O}_4$ and purified by anaerobic dialysis. The reduced protein was stable to air oxidation over the time frame of our experiments.

Before analysis all protein samples were dialysed against 0.1 M-phosphate buffer, pH 6.0. Protein concentrations for laccase or apo-azurin were determined by the biuret method (Goa, 1953), calibrated on the assumptions that native laccase contains exactly four copper ions and native azurin contains exactly one copper ion. With the assumption of 1:1 stoichiometry, the protein concentrations of solutions containing native plastocyanin, stellacyanin or azurin were determined by analysis for total copper by using the method of Felsenfeld (1960). The apo-stellacyanin concentration was calculated from $\epsilon_{280} = 24000 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

The Cu(I) analysis was determined with a modified form of the procedure described by Felsenfeld (1960). In our method a suitable amount of the protein sample is first diluted to 2.00 ml in 0.1 M-phosphate buffer, pH 6.0,

and then, with the aid of a volumetric flask, is further diluted to 5 ml with a 2,2'-biquinoline solution (0.5 mg/ml) in glacial acetic acid. A blank solution is prepared in the same way with 2.00 ml of the buffer solution. After 10 min the absorbances at 546 nm were measured, and the Cu(I) concentration was calculated as:

$$[\text{Cu(I)}] = \frac{A_{546}^S - A_{546}^P}{(6300 \text{ M}^{-1} \cdot \text{cm}^{-1})b} \cdot D \quad (1)$$

where A_{546}^S is the blank-corrected analyte absorbance at 546 nm, b is the pathlength, D is the dilution factor, and the factor $6300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ represents the molar absorptivity of the bis(biquinoline)Cu(I) complex. The term A_{546}^P represents a small absorbance/scattering correction due to the presence of the macromolecules. The A_{546}^P value could be measured directly with a solution of apoprotein, but this procedure is inconvenient. We have found that the following expression:

$$A_{546}^P = (280 \text{ M}^{-1} \cdot \text{cm}^{-1})b \cdot C/D \quad (2)$$

where C is the protein concentration, also gives a reliable estimate. Here the apparent molar absorptivity of $280 \text{ M}^{-1} \cdot \text{cm}^{-1}$ represents an average value obtained for the various apoproteins. If EDTA was introduced during analysis, it was added to the protein sample before the addition of the biquinoline reagent.

RESULTS AND DISCUSSION

Control experiments

In order to establish the method, control experiments have been carried out with a number of systems. The results given in Table 1 reveal that for three relatively small blue copper proteins, containing only type I copper, no Cu(I) ion is detected in the assay of oxidized protein. Moreover, the data in Fig. 1 demonstrate that the analysis is specific for Cu(I) ion when mixtures of Cu(I)- and Cu(II)-azurin are used.

These results establish two important points. The first

Table 1. Results of Cu(I) assays

Ascorbate is not present except where noted. Cu(I) content is expressed per mol of laccase when present.

Sample	Cu(I) content (mol/mol of protein)
Azurin	Not detected*
Plastocyanin	Not detected*
Stellacyanin	Not detected*
Apo-laccase with stellacyanin	$0.14 \pm 0.04^\dagger$
Apo-laccase with Hg(II) and stellacyanin	$0.12 \pm 0.06^\ddagger$
Native laccase	$0.49 \pm 0.02^\S$ $0.48^\parallel$ $4.0 \pm 0.08^\P$

* Protein was oxidized with ferricyanide before analysis.

† Average for nine trials.

‡ Average for three trials.

§ In the presence of 63 mol of EDTA/mol of copper; average for two trials.

|| In the presence of 5300 mol of EDTA/mol of copper.

¶ Excess ascorbate added; average for two trials.

Fig. 1. Spectrophotometrically determined amounts of Cu(I) plotted versus the analytically prepared amounts for various mixtures of Cu(I)- and Cu(II)-azurin in 0.1 M-sodium phosphate buffer, pH 6.0

is that the cysteine thiol group of the type 1 binding site does not reduce Cu(II) to Cu(I) under the conditions of our assay. Secondly, protein carbohydrate residues have no effect either. Spinach plastocyanin has a molecular mass of 10900 Da and contains about 5.4% by wt. carbohydrate (Kato *et al.*, 1962), though no carbohydrate residues were identified in the crystal structure of poplar plastocyanin (Colman *et al.*, 1978). On the other hand, stellacyanin has a molecular mass of about 20000 Da, 20% of which represents carbohydrate (Peisach *et al.*, 1967). In neither case is there any evidence for Cu(I) formation. The same is true if stellacyanin is assayed in the presence of aqueous Cu^{2+} .

Application to laccase

Laccase is a much larger protein with two other copper-binding sites and a molecular mass of approx. 110000 Da, 45% of which is due to carbohydrate (Reinhammar, 1970). In light of these differences, further control experiments were carried out, and here we find evidence of some type of endogenous reductant (Table 1). Thus in the presence of apo-laccase we detect 0.14 ± 0.04 mol of Cu(I)/mol of apo-laccase added to stellacyanin solutions. The rate of reaction was not measured, but the same results were obtained for 5 min and 15 min incubations with 2,2'-biquinoline. Fig. 2 shows conclusively that Cu(I) is formed in these experiments and justifies the application of eqn. (1) to the results. The chemical nature of the endogenous reducing agent is unknown, but it is probably not the free cysteine since the presence of Hg(II) ion has no effect on the analysis. This is not surprising in view of the results discussed above and the fact that a non-stoichiometric amount of reductant is involved.

When we analyse solutions of native laccase we find 0.70 ± 0.09 mol of Cu(I)/mol of enzyme whether the protein has been freshly extracted or stored for several months at -20°C . However, part of the Cu(I) detected can be attributed to the action of the aforementioned endogenous reductant. To counteract this effect, we carried out analyses in the presence of EDTA, since

Fig. 2. Absorption spectrum of bis-(2,2'-biquinoline)Cu(I) generated from Cu(II)-stellacyanin in the presence of apo-laccase

Contributions from both stellacyanin and apo-laccase have been subtracted out with the blank.

Felsenfeld (1960) has shown that EDTA suppresses reduction. When EDTA was added in equimolar amounts compared with 2,2'-biquinoline, or if an even larger excess was added, native laccase analysed for 12–13% Cu(I) [0.49 mol of Cu(I)/mol of laccase; Table 1]. If, on the basis of the discussion below, we assume that the Cu(I) centres are found in the type 3 site, this means that $25 \pm 2\%$ of the sites are reduced. These results agree quite nicely with earlier results obtained by X-ray absorption, which indicates $22 \pm 3\%$ of the type 3 coppers are reduced (Hahn *et al.*, 1983; Kau *et al.*, 1987). Finally, if we pre-treat the native enzyme with 5–10 mol equivalents of H_2O_2 , the Cu(I) content decreases such that less than 3% of the type 3 copper remains in the reduced state, also in accord with the results obtained by Solomon and co-workers (Kau *et al.*, 1987).

Type-2-depleted laccase

Independent samples of type-2-depleted laccase were prepared by following as closely as possible the procedures published by Morpurgo *et al.* (1980b) and by Frank *et al.* (1983). In the resulting derivatives we have found 2.8 and 2.6 copper ions present per molecule of enzyme respectively, with corresponding A_{280}/A_{614} absorbance ratios of 19.0 and 18.6. Since the absorbance data reveal that the type 1 site is essentially intact and oxidized, some of the type 3 copper as well as the type 2 copper has evidently been removed. In accord with the results of Morpurgo *et al.* (1980b) and Kau *et al.* (1987), as well as Frank & Pecht (1986), there is little or no indication of a 330 nm absorbance feature in either spectrum. The derivatives analysed for 1.2 and 1.5 mol of Cu(I)/mol of protein respectively.

We conclude that $80 \pm 15\%$ of the type 3 copper in type 2-depleted laccase is found in the reduced state even when the procedure of Frank & Pecht (1986) is used and the protein is exhaustively dialysed against ferricyanide. In separate experiments we have confirmed that the 330 nm absorbance grows upon treatment with H_2O_2 and that the absorbance increase correlates with a decrease in Cu(I) content. Thus addition of 2–3 mol of H_2O_2 /mol of protein produces 0.22 mol of

Cu(II)/mol of type 3 site when a molar absorptivity of $\Delta\epsilon_{333} = 2650\text{M}^{-1}\cdot\text{cm}^{-1}$ (calculated from the difference spectrum between oxidized and reduced native protein) is used. The corresponding Cu(I) analysis shows a decrease of 0.21 mol worth of the Cu(I) ion. Similarly, addition of 10 mol equivalents of H_2O_2 produces another 0.38 mol of Cu(II)/mol of type 3 sites, and the biquinoline assay reveals a corresponding decrease of 0.33 mol of Cu(I)/mol.

In summary, we have shown that a simple spectrophotometric assay can be used to determine the amount of Cu(I) in laccase samples. The presence of spurious reductants can confuse the results, but this effect is suppressed by the addition of EDTA. Our results strongly suggest that the published procedures for removing the type 2 copper from laccase yield a form in which the type 3 copper ions are essentially fully reduced. It is of course conceivable that a subtle change in the experimental procedure during the removal of the type 2 copper could lead to another form of the protein. Indeed, Reinhammar & Oda (1979) report that they have prepared type-2-depleted laccase that retains the 330 nm absorbance band that is a characteristic of the oxidized type 3 site. The important point is that the Cu(I) assay that we have developed will provide for better characterization of type-2-depleted preparations in the future as well as for the characterization of other chemically modified forms.

Caveat regarding the use of EDTA

Although EDTA is recommended for the Cu(I) analysis of laccase, it should not be used when the total copper analysis is desired, i.e. when a reducing agent is deliberately added. EDTA has no effect on the copper analysis of stellacyanin so long as excess ascorbate is added before the addition of the biquinoline reagent. However, the presence of EDTA strongly interferes with the total copper analysis of native laccase. This result may be explained by the fact that laccase is an oxidase. As a consequence, a steady-state content of Cu(II) can be expected to persist in the enzyme, and the cupric copper that is released during denaturation is subject to scavenging by EDTA.

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