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THE RATIO OF TYPE-1 AND -2 Cu(II) IN HUMAN CERULOPLASMIN

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SUMMARY

1. Spectroscopic titrations of ceruloplasmin with NADH, in the presence of trace amounts of phenazine methosulphate, demonstrate that at pH 7.0 the protein is capable of accepting only four electron equivalents. Changing the ambient pH to 5.2 or preincubation with neocuproin or azide at pH 7.0 does not alter the number of equivalents that can be accepted.

2. Titration curves at pH 5.2 show a different character from those obtained at pH 7.0 and this difference is due to the species being reduced, namely the Cu(II) atoms.

3. Nernst plots of the titration data demonstrate an equilibrium between type-1 and type-2 Cu and suggest that ceruloplasmin contains one type-1 Cu and three type-2 Cu atoms.

4. The concave character of the titration curve at pH 5.2 and 25 °C is due to a difference in redox potential of 36 mV between type-1 and type-2 Cu, together with the lack of absorbance of type-2 Cu. At pH 7.0 there is no difference in redox potential.

5. Neocuproin and azide both cause a difference in redox potential between the two types at pH 7.0. The data also support the idea that ceruloplasmin contains one type-1 and three type-2 Cu atoms.

6. Increasing concentrations of azide produce an increasing difference in redox potential between the two types of Cu(II).

7. Titrations followed at 330 nm suggest that the changes observed at this wavelength are induced by a conformational change of the protein.

INTRODUCTION

Electron paramagnetic resonance[1,2], magnetic susceptibility[3], and titration with ascorbate[4,5] or NADH in the presence of trace amounts of phenazine metho-

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sulphate[6] have demonstrated that of the eight copper atoms per molecule of ceruloplasmin[7] four are Cu(II). In contrast to this earlier work, Carrico *et al.*[8] have recently published data showing that ceruloplasmin accepts one electron for every copper atom.

EPR data have shown that the copper atoms in ceruloplasmin are not all equivalent[9], in fact that three different types of copper are present, namely the paramagnetic type-1 ('blue') and type-2 ('non-blue')[9,10], and the EPR non-detectable copper. It has been suggested[6,10–12] that of the four paramagnetic copper atoms present, two are in the form of type-1 and two are in the form of type-2. A comparison has also been made between ceruloplasmin and the various forms of laccase which have been reported to have equal amounts of the two forms of Cu(II) (refs 10 12). However, there is some disagreement in the literature concerning the relative amounts of the two types of Cu(II) in *Rhus*-laccase. Makino and Ogura[13] have evidence suggesting that this protein contains one type-1 and three type-2 Cu(II).

In this work optical titrations of ceruloplasmin with NADH in the presence of trace amounts of phenazine methosulphate are described. The use of phenazine methosulphate as an intermediary in electron transfer to cytochrome *c* was introduced by Massey[14]. The technique of the titration was further developed in our laboratory using NADH as the electron donor in investigations on cytochrome *c* and cytochrome aa_3 (refs 15–17).

The present investigations were initiated in order to obtain further information on the form of the titration curve under different conditions and about the distinction between type-1 and type-2 Cu under reducing conditions. Part of this work has been published in a preliminary form[6].

METHODS

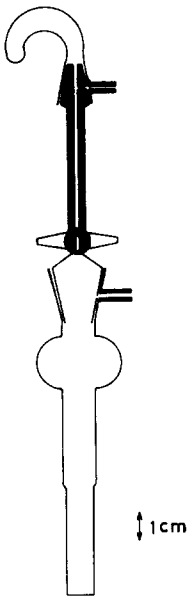
Human ceruloplasmin was isolated from Cohn plasma fraction IV-1 by a modification of the method of Deutsch *et al.*[18]. The essential alterations consisted of a shorter extraction (5 h) at low pH, the use of DEAE-Sephadex during the whole procedure and the application of the batch method in all but the last chromatography steps.

An absorbance coefficient of $10.9 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ at 610 nm, calculated from data given in ref. 18 was used. Copper assays according to Felsenfeld[19] and to Peterson and Bollier[20] demonstrated the presence of 8 g atoms of Cu per mole ceruloplasmin when the concentration was determined from $A_{610\ nm}$.

Titrations were carried out in Thunberg cuvettes. Initially every point in the titration curve necessitated the use of a separate cuvette. Later titrations were carried out using the special Thunberg cuvette shown in Fig. 1, which enables several additions to be made to the reaction vessel without loss of anaerobiosis. Discrete quanta of NADH could be added to the lower compartment, containing ceruloplasmin and phenazine methosulphate, by turning the "cul-de-sac" stopcock.

Anaerobiosis was achieved by repeated evacuation and flushing with purified nitrogen or argon gas.

Spectroscopic measurements were carried out on Cary spectrophotometers; Models 14, 15 and 17.



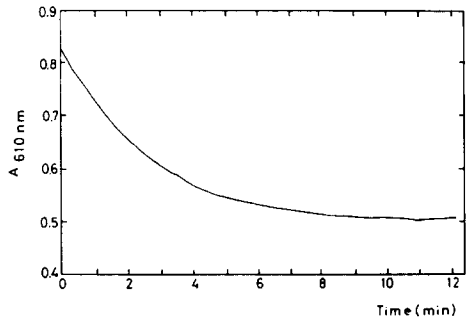


Fig. 1. Special Thunberg cuvette allowing several additions to be made to the reaction cuvette without loss of anaerobiosis. The graded burette and the "cul de sac"-stopcock have approximately the same bore (1.2 mm).

Fig. 2. Typical time course of the absorbance at 610 nm upon reduction of ceruloplasmin by addition of NADH and trace amounts of phenazine methosulphate under anaerobic conditions. Experimental conditions: 2.25 ml ceruloplasmin, final concentration 74.5 μM, in 0.3 M sodium acetate (pH 7.0), was mixed anaerobically with 0.2 ml NADH, final concentration 90 μM, in 0.1 M Tris–HCl buffer (pH 8.0) and 0.05 ml phenazine methosulphate, final concentration 6.0 μM.

Cohn fraction IV-I was a gift from the Bloedtransfusiedienst (Blood bank) of the Netherlands Red Cross.

NADH, phenazine methosulphate, *p*-chloromercurisulphonate and Tris were purchased from Sigma, neocuproin was obtained from Fluka and sodium azide and sodium acetate were from B.D.H. DEAE-Sephadex was purchased from Pharmacia.

RESULTS

Optical titrations at pH 7.0

When ceruloplasmin is mixed anaerobically with limited amounts of NADH, in the presence of catalytic amounts of phenazine methosulphate the absorbance at 610 nm decreases gradually until an equilibrium is reached (Fig. 2). The decline of absorbance in the equilibrium state is directly proportional to the amount of NADH added, until 2 moles of NADH, or four electron equivalents per mole of ceruloplasmin have been added (Fig. 3). Addition of more NADH does not result in any further transfer of electrons to ceruloplasmin.

In an experiment carried out under the same conditions, except for a higher concentration of phenazine methosulphate (30 μM), the absorbance at 610, 330 and 340 nm were recorded, as well as the absorbance difference between 387 and 365 nm (Fig. 4). The latter, which is a measure of the oxidized phenazine methosulphate still

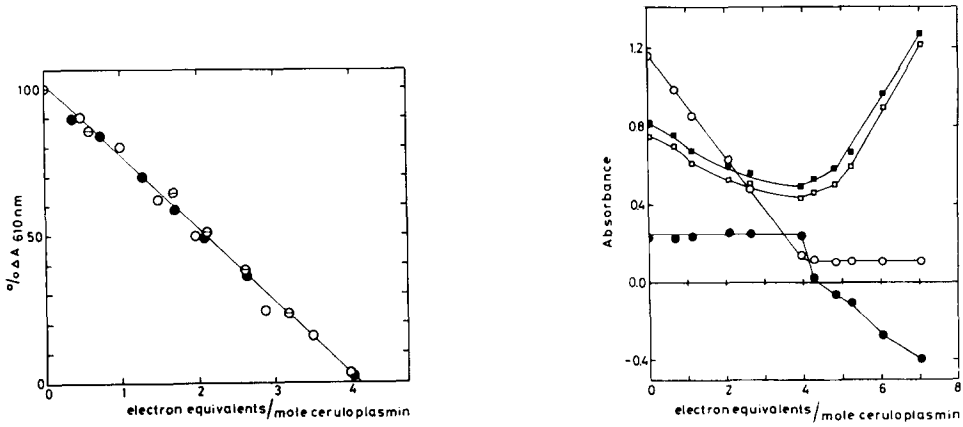


Fig. 3. Anaerobic titration of ceruloplasmin with NADH in the presence of phenazine methosulphate. Results of three different titrations are shown, all normalised to the same $\Delta A_{610\ nm}$ (oxidized *minus* reduced) (100%). Final concentrations of ceruloplasmin were 55 (○), 110 (⊖) and 137 (●) μM. A 1-cm Thunberg cuvette contained 2.25 ml of the protein solution in 0.3 M sodium acetate (pH 7.0) in the reaction cuvette. One sidearm contained various amounts of NADH in a volume of 0.2 ml and the other 0.05 ml of phenazine methosulphate giving a final concentration of 6 μM. After anaerobiosis was achieved, the reaction was started by tipping in NADH and phenazine methosulphate. The decrease in absorbance was followed until no further change was seen.

Fig. 4. Changes of $A_{610\ nm}$ (○), $A_{330\ nm}$ (■), $A_{340\ nm}$ (□) and the absorbance of oxidized phenazine methosulphate (measured as $A_{387\ nm} - A_{365\ nm}$) (●), caused by increasing amounts of NADH. A 1-cm modified Thunberg cuvette contained 3.0 ml of 108 μM ceruloplasmin in 0.3 M sodium acetate (pH 7.0) and 0.35 ml 0.01% phenazine methosulphate (final concentration 30 μM). The burette part contained an NADH solution in 0.3 M sodium acetate (pH 7.0). After anaerobiosis was achieved discrete quanta of NADH were added to the ceruloplasmin solution. Spectra were drawn on a Cary 17 spectrophotometer after the absorbance change had come to a standstill. The absorbances given in the figure are all normalised to a volume of 3.0 ml.

present, remains unchanged, until four electron equivalents per mole of ceruloplasmin are added, whereas the absorbances at 330 and 340 nm show a sigmoidal decrease. The difference in absorbance between 330 and 340 nm remains unchanged throughout the whole titration. When more than four electrons are added $A_{340\ nm}$ again increases. The increase divided by the extra amount of NADH added agrees well with the absorbance coefficient used for calculation of the NADH concentration ($6.22 \cdot 10^3\ M^{-1} \cdot cm^{-1}$) (ref. 15).

The absorbance of oxidized phenazine methosulphate abruptly declines after addition of more than four electron equivalents. The absorbance difference between 387 and 365 nm becomes increasingly more negative because of the greater contribution of the absorbance of the excess NADH at 365 nm relative to that at 387 nm.

From the constant level of oxidation of phenazine methosulphate we can conclude that when not more than four electrons have been added, all of these actually reach ceruloplasmin. This also means that the sigmoidal decrease of the absorbance at 330 nm is a phenomenon of the reduction of ceruloplasmin itself.

These results are in agreement with other titration data found in the literature[4,5] and fit the established theory[12,21], namely that of a total of eight copper atoms in ceruloplasmin, four are normally in the Cu(II) state and can be reduced.

A small overshoot of the decline of $A_{610\ nm}$, lasting 10–15 min was sometimes, but not usually observed. A large overshoot was found after preincubation with 5 mM *p*-chloromercurisulphonate and equilibrium was reached in approx. 20 min. Mercurial-treated ceruloplasmin is able to accept 8 electrons. No effect was found on the absorbance at 610 nm after addition of the mercurial to ceruloplasmin. With respect to both the number of electron equivalents accepted and the degree of overshoot, but not the time needed to reach equilibrium, mercurial-treated ceruloplasmin behaves in the same way as reported by Carrico *et al.*[8] in their titrations at low ionic strength.

Optical titrations at pH 5.2

A number of titrations have also been carried out at pH 5.2 in order to make a comparison with some titrations in which a different reductant, Fe(II), is used (Veldsema, A., unpublished). It should be added that under anaerobic conditions and in the absence of phosphate ions NADH is sufficiently stable to allow these experiments to be carried out.

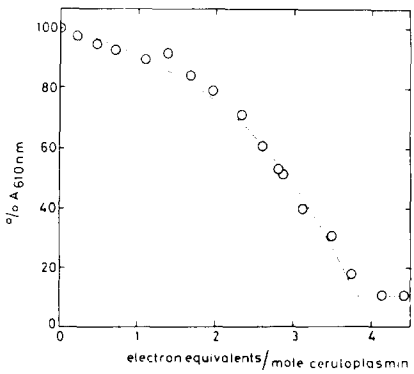


Fig. 5. Anaerobic titrations of ceruloplasmin at pH 5.2. The final concentration of ceruloplasmin was 142 μM in 0.3 M sodium acetate buffer (pH 5.2). NADH was dissolved in 0.1 M sodium acetate (pH 7.0). Phenazine methosulphate was added as 0.05 ml of an 0.01% solution. The procedure was as described in the legend to Fig. 4. — — —, shows the theoretical curve calculated on the basis of the presence of one type-1 and three type-2 Cu atoms, with a difference in redox potential between the two types of 36 mV.

The titration curve obtained at pH 5.2 (Fig. 5) shows that, also under these conditions, ceruloplasmin is capable of accepting four electrons per molecule, but the titration curve is concave with respect to the abscissa in contrast to the straight line obtained at pH 7.0.

Experiments (not shown) similar to that previously shown in Fig. 4, but carried out at pH 5.2, showed that phenazine methosulphate remains oxidized until four electron equivalents of NADH are added, and that the decline of $A_{340\ nm}$ followed the same course at pH 5.2 as at pH 7.0. Thus any explanation of the concave shape of the titration curve at pH 5.2 has to be based upon the species being reduced, that is the Cu(II) atoms in ceruloplasmin.

Malmström and co-workers[1,22,23] have demonstrated that although different types of Cu(II) are present in ceruloplasmin, the absorbance at 610 nm is almost

entirely due to the type-1 Cu. Thus we have to withdraw the opinion expressed earlier[6], that both type-1 and type-2 Cu contribute to the absorbance at 610 nm. Also there is evidence that in "blue oxidases" type 1 is reduced much faster than type 2 (refs 10, 23, 24). In particular, anaerobic titrations of ceruloplasmin with Fe(II), measured with the EPR technique (Veldsema, A., unpublished), show that type 1 is reduced initially, followed by a redistribution of electrons over both types of Cu(II). Thus, there should be a redox equilibrium between type-1 and type-2 Cu. To test this, a Nernst plot can be made relating the redox levels of type-1 and type-2 Cu.

During the entire course of the titration the number of electrons accepted by ceruloplasmin is known (see Fig. 4), and $A_{610\ nm}$ is a measure of the concentration of type-1 Cu since this is known to be the sole contributor to the absorbance at this wavelength[1,24]. However, since the total number of reducible Cu(II) atoms, type 1 and type 2, is four, the number of type-1 atoms can only be one, two or three. Thus one can calculate the fraction of type 1 in the oxidized and the reduced states at every point of the titration curve, for each possible combination of type-1 and type-2 atoms present. From the total amount of electrons added and the amount of electrons accumulated on type-1 Cu the fraction of type-2 in the oxidized and the reduced state can also be calculated.

The Nernst plots arising from combination of one type-1 with three type-2 copper atoms and of two type-1 and two type-2 copper atoms are given in Figs 6A and 6B. For the third combination, *i.e.* three type-1 and one type-2 copper atoms, the amount of electrons actually added to the system is soon insufficient to reduce three atoms of type-1 configuration to the redox state indicated by the titration curve.

A sequential transfer between a Cu(I), *i.e.* a reduced type-1 Cu and a type-2 Cu(II) atom in ceruloplasmin should result in a Nernst plot with a slope of one. From Figs 6A and 6B it can be seen that the combination that gives the result closest to that expected is one in which one atom of type-1 configuration is combined with three atoms of type-2 configuration (Fig. 6A). The difference in redox potential between type-1 and type-2 Cu at pH 5.2, calculated from the Nernst plot (Fig. 6A),

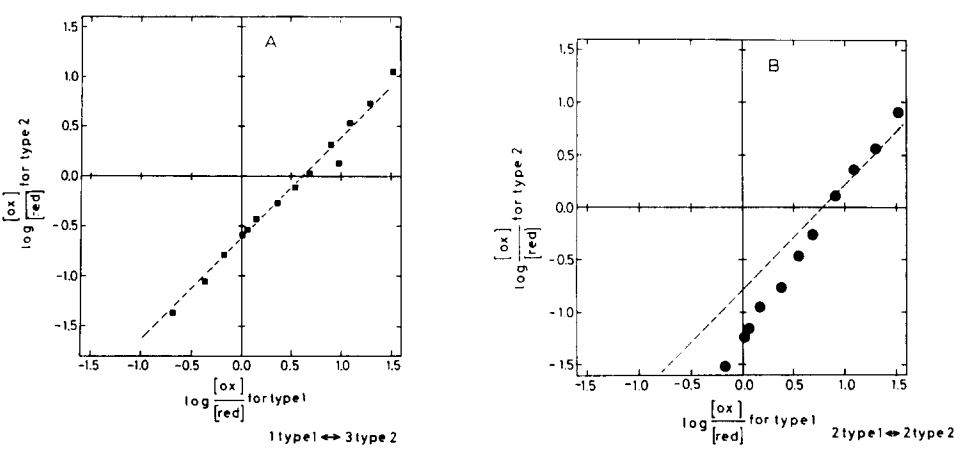


Fig. 6. Nernst plots of two possible combinations of type-1 and type-2 Cu. Data used in the calculation of these figures are taken from the titration curve at pH 5.2, as shown in Fig. 5. A. One type-1 and three type-2 Cu. B. Two type-1 and two type-2 Cu.

was found to be 36 mV with type 1 as the component with the lower potential.

An explanation of the results assuming equal amounts of type-1 and type-2 Cu atoms (Fig. 6B) is the presence of two type-1 Cu atoms differing in redox potential. But since there is no experimental indication available to support this, this explanation seems less attractive to us.

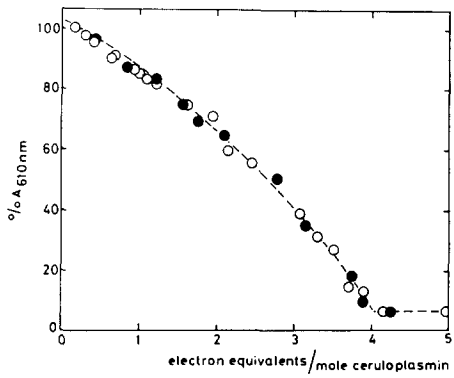


Fig. 7. Anaerobic titration of ceruloplasmin preincubated with neocuproin. Two experiments are shown in this figure. In the first (●), the cuvettes contained 0.3 M sodium acetate, 50 μM ceruloplasmin and 800 μM neocuproin in a volume of 2.25 ml. Otherwise this experiment was carried out as described in the legend to Fig. 3. In the second experiment (○), the cuvette contained 0.3 M sodium acetate, 168 μM ceruloplasmin, 2 mM neocuproin and 6 μM phenazine methosulphate in a volume of 3.0 ml. NADH dissolved in 0.3 M sodium acetate (pH 7.0) was added by turning the stopcock. Absorbance decreases were followed after every addition, until a constant level was reached. The results of both experiments are normalised to the same $\Delta A_{610\ nm}$ (oxidized *minus* reduced) (100%).

Optical titrations in the presence of neocuproin

When ceruloplasmin is incubated with neocuproin at pH 7.0 no significant effect on the visible spectrum is seen. Titration of ceruloplasmin, after incubation with neocuproin, resulted in the curve shown in Fig. 7. This concave titration curve resembles that obtained after titration of untreated ceruloplasmin at pH 5.2 (Fig. 5). The Nernst plot shown in Fig. 8A reveals that the combination of one type-1 with three type-2 Cu atoms again gives a slope approaching one (1.08). Neocuproin at pH 7.0 causes a small change in redox potential (17 mV) between the two types of copper atoms. The combination of two type-1 with two type-2 Cu atoms (Fig. 8B) results in a slope close to 1.5 for the midpart of the S-shaped curve. Although this result can be explained on the basis of two type-1 Cu atoms differing in redox potential and absorbance contribution at 610 nm, we prefer a ratio of one type-1 to three type-2 Cu atoms since no additional assumptions have to be introduced.

The effect of azide on the titration curve

When azide is mixed with ceruloplasmin, aerobically or anaerobically, a small decrease in absorbance at 610 nm is observed (see ref. 25), the decline in absorbance becoming constant after about 5 min.

The results of the titrations at the three different azide concentrations (4, 10 and 40 mM) are shown in Fig. 9. The titration curves previously presented in this paper have been calculated with reference to the absorbance of the ceruloplasmin

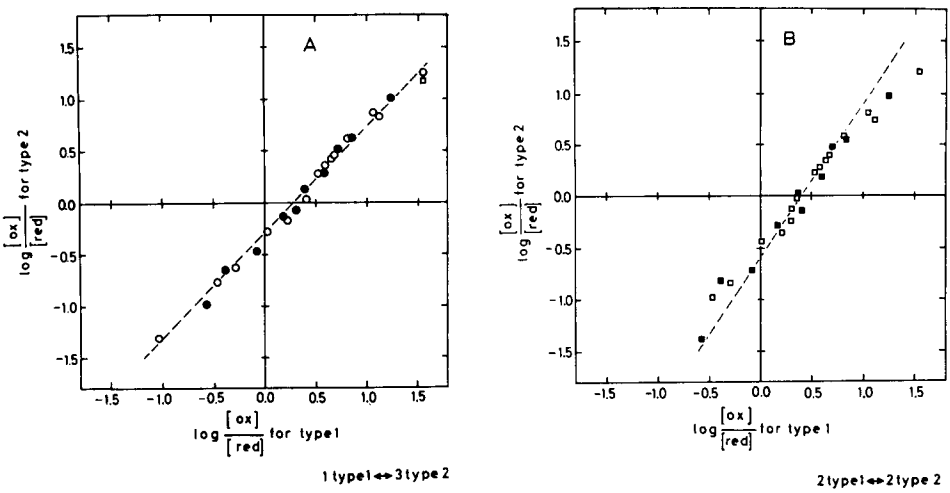


Fig. 8. Nernst plots of titration data from Fig. 7. Ceruloplasmin preincubated with neocuproin. A. One type-1 and three type-2 Cu. B. Two type-1 and two type-2 Cu.

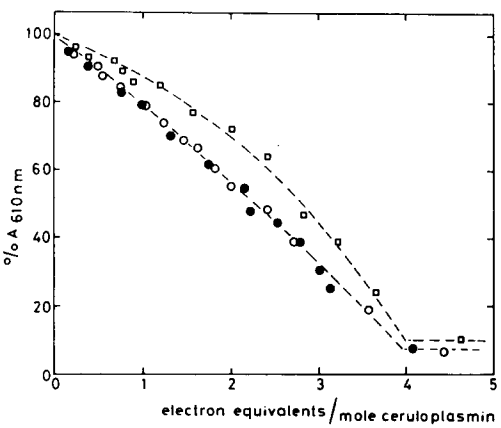


Fig. 9. Anaerobic titrations of ceruloplasmin preincubated with azide. The final concentrations of azide were 4 mM (●), 10 mM (○) and 40 mM (□). The final concentrations of ceruloplasmin were, respectively, 48, 44 and 58 μM. The appropriate concentrations of ceruloplasmin were mixed with those of azide in a volume of 2.25 ml. Otherwise the titrations were carried out exactly as described in Fig. 3. Absorbance changes are given in relation to the absorbance obtained after incubation with azide.

sample before treatment. In this case where preincubation of ceruloplasmin with azide itself causes a drop in absorbance the value obtained after preincubation with the appropriate azide concentration has been used as the reference.

The titration curves with 4 and 10 mM azide are slightly concave, and that with 40 mM markedly concave. As was the case at pH 5.2 or in the presence of neocuproin, the concave titration curve is due to a difference in redox potential between the type-1 and type-2 Cu atoms (see Figs 10A and 10B). Small differences in redox potential are found even with the lower concentrations of azide (3 and 6 mV, with 4 and 10 mM azide, respectively). With 40 mM azide the difference in redox potential is found to be 24 mV.

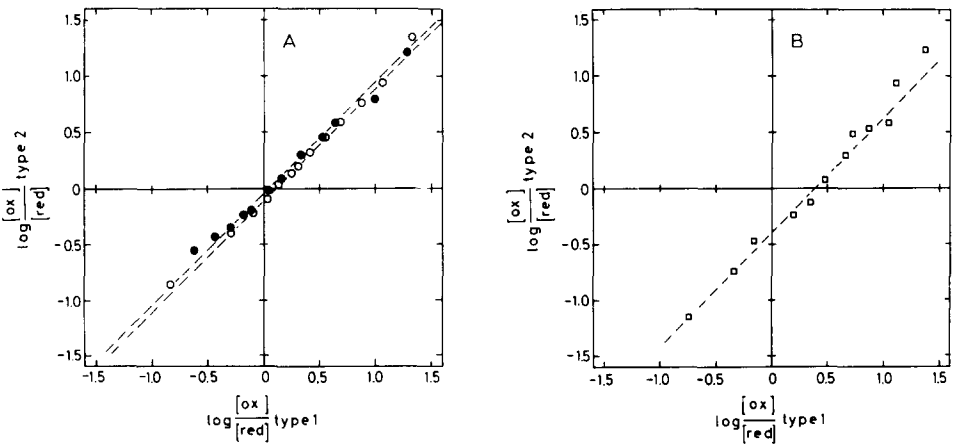


Fig. 10. Nernst plots of the reduction of ceruloplasmin preincubated with three different concentrations of azide. A. 4 mM (●) and 10 mM (○) azide. B. 40 mM azide. The ratio of type-2 to type-1 Cu used here is 3.

The 330-nm band

Fig. 11 shows that the titration curve followed at 330 nm does not parallel that at 610 nm, the former being distinctly sigmoidal (see also ref. 8). This is different from that observed in comparable titrations with either polyporus laccase[26,27] or with *Rhus vernicifera* laccase[13,27]. The difference from laccase is even more apparent when

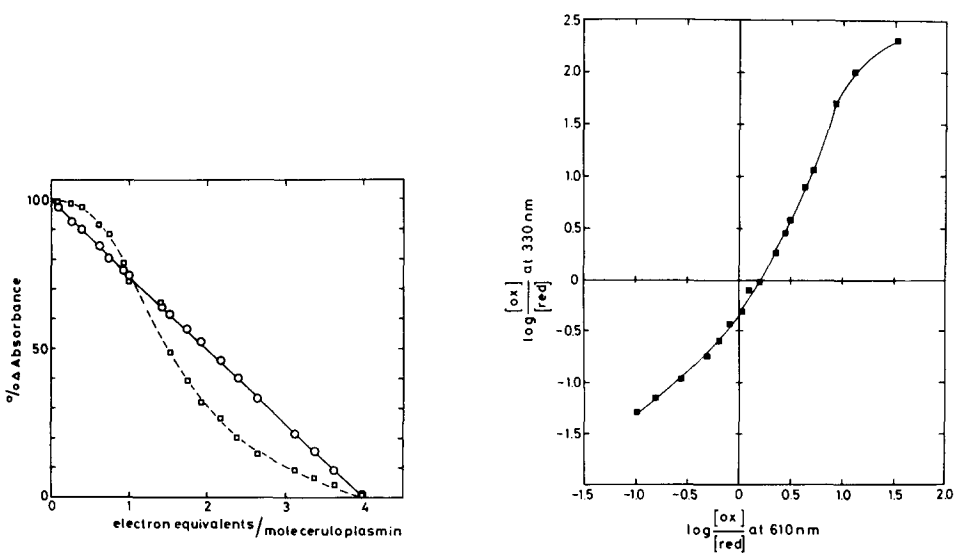


Fig. 11. Titration curve of the titration of ceruloplasmin with NADH and trace amounts of phenazine methosulphate measured at 610 (○) and 330 nm (□) at pH 7.0. The absorbances are expressed as percentage oxidized, the difference between fully oxidized and fully reduced being taken as 100%. The ceruloplasmin concentration used was 135 μM. Otherwise the titration was carried out as described in the legend to Fig. 4. Readings were made after the absorbance change at 610 nm had stopped.

Fig. 12. Nernst plots relating the redox states at 610 and 330 nm. Data are taken from the titration presented in Fig. 11.

the data are compared in a Nernst plot, relating the redox states at 330 nm to those at 610 nm (Fig. 12). This Nernst plot could not be resolved into two one-electron step components by the procedure of Dutton *et al.*[28], indicating that the chromophore at 330 nm does not take part in a straightforward redox equilibrium with type-1 Cu. We may also exclude the possibility of a direct redox equilibrium with type-2 Cu because the straight titration line at 610 nm shows that there is no difference in redox potential between the two types of Cu(II) at pH 7.0.

In our view the absorbance changes at 330 nm are probably a reflection of a conformational change around the EPR non-detectable copper atoms induced by the state of the protein. It is unlikely that the 330-nm chromophore is a separate electron acceptor, since all the electrons added to ceruloplasmin are accounted for by the four reducible Cu(II) atoms. Clearly more experiments are needed to establish the true nature of this band.

DISCUSSION

Our results demonstrate that under almost all conditions tested, four electrons are needed to abolish the optical and magnetic properties of the prosthetic copper. In the presence of *p*-chloromercurisulphonate more than four electrons are accepted. In contrast to our findings, this has been found by Carrico *et al.*[8] in the absence of *p*-chloromercurisulphonate. However, they obtained results similar to those normally found by us, *i.e.* abolition of both optical and magnetic properties by a total of four electrons, when using a medium containing a high concentration of phosphate. This might indicate that either in the absence of phosphate or at low ionic strength[8], a reversible conformational change in ceruloplasmin may be induced by prolonged treatment under anaerobic and reducing conditions. This view may be substantiated by the experiments of Marriot and Perkins[29], who demonstrated that 75% of the prosthetic copper of ceruloplasmin is exchangeable with isotopically-labelled copper within 1 h, under reducing conditions, compared with 10% under aerobic conditions. In earlier experiments, Sternlieb *et al.*[30] could not demonstrate any exchange of ^{64}Cu, *in vivo*. The reconstruction of ceruloplasmin from the apoprotein and added ionic copper by Aisen and Morell[31] was also carried out under reducing conditions.

The unfolding of proteins is not uncommon under reducing circumstances[32,33]. Thus it is not unlikely that unfolding of ceruloplasmin, followed by a reversible loss of copper, could occur upon prolonged maintainance of the reduced state, and that these changes would be facilitated at low ionic strength[8].

Erickson *et al.*[34] observed that the apoprotein contains four more –SH groups per mole of enzyme than the native protein. This might provide the basis for an explanation for the effect of *p*-chloromercurisulphonate found in the present work, namely that the mercurial competes favourably with some of the copper atoms for their binding sites, allowing a series of changes that could lead to a redistribution of the added electrons.

Model studies also suggest copper to sulphur binding in copper proteins and indicate that the valence of copper atoms is obscured by 'valence mixing'[35]. This is also consistent with the low amount of EPR-detectable copper found in ceruloplasmin.

Some of our ceruloplasmin preparations have shown the same titration characteristics as the *p*-chloromercurisulphonate-treated protein, although the time for reaching equilibrium is much shorter in the case of the *p*-chloromercurisulphonate-treated enzyme. The results that we have obtained with the mercurial-treated enzyme are similar to those obtained by Carrico *et al.*[8] with a crystallised preparation of ceruloplasmin. Poulik[36] has made a distinction between native and three times crystallised ceruloplasmin, the latter containing extra components.

In conclusion it can be said that when a difference in redox potential exists between the two types of Cu(II) in ceruloplasmin, a non-linear titration curve at 610 nm is found. This difference can be visualised in Nernst plots (Figs 6, 8 and 10) which show a slope equal to one, only when based on the presence of one type-1 and three type-2 Cu atoms per molecule of ceruloplasmin. This is not similar to results obtained from simulated EPR spectra by Andréasson and Vänngård[10], and the values quoted in the review by Malkin and Malmström[12] for ceruloplasmin. Pending direct measurement of the redox potential during the titration where a definite choice can be made we feel that the presence of two type-1 Cu atoms differing in absorbance contribution and redox potential is less attractive. Results comparable to ours with ceruloplasmin have been reported for *Rhus* laccase by Makino and Ogura[13].

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