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Selective Modification of the Two Type I Copper Sites in Human and Bovine Ceruloplasmin with the Action of Azide and L-Cysteine

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The two type I copper sites of human and bovine ceruloplasmin (CP) were indirectly modified through the binding of N_3^- to types II and III copper ions. Based on the fact that the activities of CP's modified with N_3^- were 85 and 47% for human and bovine CP, respectively, it was disclosed that the electronic and steric states of the "fast" type I Cu or "CuA" which is coupled with the types II and III copper ions was changed reversibly, whilst the "slow" type I Cu or "CuB" which is not coupled with the types II and III copper ions was changed to a type II copper ion and/or irreversibly reduced. On the other hand, the reaction of a limited amount of L-cysteine with CP gave the absorption and electron paramagnetic resonance spectra of CP whose CuB was selectively reduced. The treatment of this CP with 4,7-diphenyl-2,9-dimethyl-1,10-phenanthroline-4′,4″-disulfonate did not result in the selective removal of CuB.

Ceruloplasmin (CP) is one of the serum globulins possessing oxidase activity towards Fe(II) and some aromatic amines and phenols.[1] Like the other multicopper oxidases, laccase,[2] and ascorbate oxidase,[3] CP contains three kinds of copper(II) ions besides one or more copper(I) ions, i.e. two type I coppers characterized by the intense absorption at 610 nm and very small hyperfine coupling constants in the electron paramagnetic resonance (EPR) spectrum, one type II copper with EPR hyperfine parameter similar to that found in usual tetragonal copper(II) complexes and a pair of type III copper ions which are antiferromagnetically coupled and exhibit relatively strong absorption at around 330 nm.[1] It has been revealed that the two type I coppers of human and bovine CP's are oxidized at different rates:[4,5] The "fast" one or "CuA" has been elucidated to be coupled with the types II and III copper ions, constituting the active site, whilst the "slow" one or "CuB" is isolated from the active site.[5] Many studies on the effect of anions like N_3^-, SCN^-, OCN^-, F^- on human CP(HCP) have reached the conclusion that only one of the two type I coppers is indirectly affected by the anions in the light of the absorption change at 610 nm.[6,7] However, in the previous work on bovine CP(BCP) we disclosed both the type I coppers are affected by these anions: The steric and electronic structure of one of the two type I coppers is reversibly changed, whereas the other is irreversibly reduced and/or changed to a type II copper.[8] In the present paper, we report the discrimination of the two type I coppers both of which are indirectly perturbed by N_3^-. Further, we furnish the absorption and EPR spectra of CP whose CuB is selectively reduced.

Experimental

HCP was purchased from Green Cross Corp. and BCP was prepared from bovine serum as described previously.[5,8] The absorption ratio of A_{610}/A_{280} was 0.041 and 0.044 for HCP and BCP, respectively. All reagents used were of analytical grade. Absorption spectra were obtained on a Union Giken SM-401 high sensitivity spectrophotometer and circular dichroic (CD) spectra on a JASCO J-500A spectropolarimeter. EPR spectra were recorded on a JEOL FE-1X spectrometer at 77 K. The amount of EPR detectable Cu^{2+} was estimated based on an EPR signal intensity of ethylenediaminetetraacetatocopper(II) as a standard. Total copper content of CP was determined using a Jarrell-Ash AA-1 atomic absorption spectrometer.

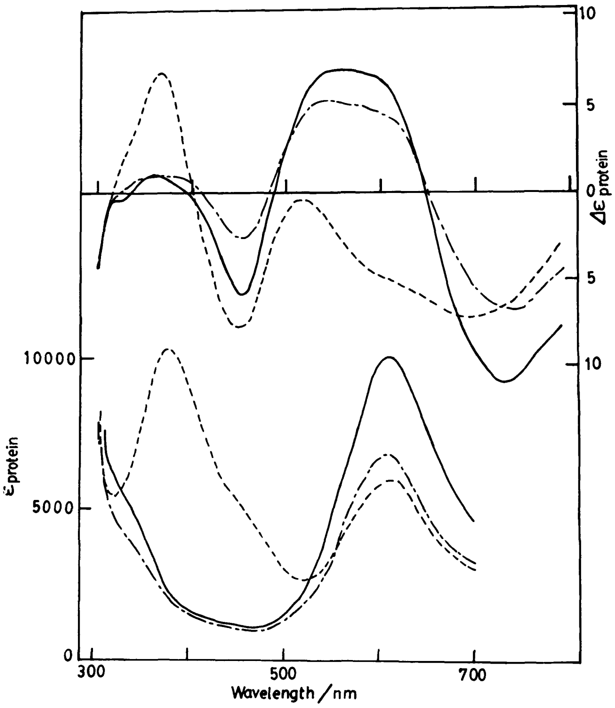

Fig. 1. Absorption and CD spectra of native (——), N_3^--treated (×1720, incubated for 2 d) (----), and dialyzed (— — —) human ceruloplasmin (protein concentration 2.9×10^{-5} mol dm^{-3}, pH 6.5, 0.1 mol dm^{-3} Na-phosphate buffer).

Results and Discussion

The absorbance at 610 nm of HCP treated with 1720 fold excess of N_3^- for 2 days decreased to 60% of the original intensity. Concomitantly, new band appeared at 381 and at 450 nm (Fig. 1). In the CD spectra, the sign of the band at about 660 nm arising from the charge transfer, S^-(cysteine)→Cu inverted from positive to negative because of the change of the steric and electronic states of one type I copper: The steric structure has been supposed to change from tetrahedrally distorted tetragon to trigonal pyramid.[9] The ellipticity of the negative CD band at 448 nm coming from the charge transfer, N(histidine)→Cu became greater, being accompanied by a development of a positive band at 370 nm due to the charge transfer, N_3^-→Cu (types II and III coppers). Simultaneously, the band at about 330 nm arising from the type III coppers almost lost its intensity. However, the CD spectral pattern returned to the original one after N_3^- was removed by dialysis against buffer solution. Each band showed a smaller intensity (Fig. 1) in accordance with the absorbance decrease of the band centered at 610 nm, although 9% recovery of the absorption intensity was observed. In the EPR spectra, the type II copper signal changed significantly: $g_{\parallel}=2.26$, $A_{\parallel}=0.019$ cm^{-1} for native HCP and $g_{\parallel}=2.26$, $A_{\parallel}=0.015$ cm^{-1} for N_3^--treated HCP (Fig. 2). The spin Hamiltonian parameters of one type I copper also changed slightly: $g_{\parallel}=2.21$, $A_{\parallel}=0.0072$ cm^{-1} for native HCP and $g_{\parallel}=2.18$, $A_{\parallel}=0.0076$ cm^{-1} for N_3^--treated HCP. Another possibility is that the type I copper which has the former parameter was reduced and/or changed to a type II copper. However, in the simulation study the following parameters were given for the two type I coppers of HCP: $g_{\parallel}=2.21$, $A_{\parallel}=0.0074$ cm^{-1} and $g_{\parallel}=2.22$ and $A_{\parallel}=0.0095$ cm^{-1}.[10] The former set of values are similar to those observed in our study. But the signal containing the latter parameters have not been observed in the present study. The whole feature of EPR spectrum returned to that similar to the native HCP after dialysis, while the increase of the type II copper signal(s) was noticeable. The amount of EPR detectable Cu^{2+} was estimated to be 3.0, 2.7, and 2.9 for native, N_3^--treated, and N_3^--treated and succeedingly dialyzed HCP. This result indicates that one type I copper was changed mostly to a type II copper through the binding of N_3^- to the type II and III copper ions even taking into consideration that the estimation error of EPR detectable Cu^{2+} is 10% or even more.

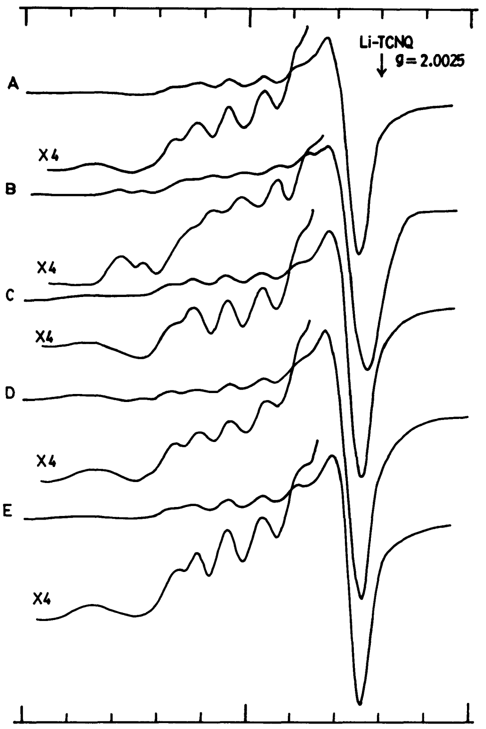

Fig. 2. X-band EPR spectra of native(A), N_3^-(×1720)-treated(B), dialyzed as (B)(C), DDP-treated after cysteine-treatment(D), and dialyzed as (D)(E) human ceruloplasmin; microwave frequency 9.21 GHz, microwave power 5 mW, modulation 0.8 mT at 100 kHz, amplitude 630 or 1000.

The activity[11] of the dialyzed HCP after N_3^--treatment for oxidizing 1,4-benzenediamine was 85%

Table 1. Characterization of Modified HCP and BCP

State of CP	Total Cu	EPR detectable Cu^{2+}	Relative oxidase activity/%	Relative absorbance at 610 nm/%
Native HCP	6.8	3.0	100	100
N_3^--treated HCP	—	2.7	—	60
Dialyzed HCP after N_3^--treatment	6.8	2.9	85	69
Cysteine-treated HCP	—	2.2	88	66
Dialyzed HCP after cysteine-treatment	6.9	2.7	87	77
Native BCP	6.7	3.0	100	100
N_3^--treated BCP	—	3.1	—	59
Dialyzed BCP after N_3^--treatment	6.7	3.7	47	45
Cysteine-treated BCP	—	2.0	98	54
Dialyzed BCP after cysteine-treatment	6.3	2.6	74	—

of that of the native HCP in spite of the 31% decrease of the absorbance at 610 nm (Table 1). The two type I coppers have been differentiated from the different rate of reoxidation, i.e. the "fast" one or "CuA" and the "slow" one or "CuB".[5] The former has been believed to be coupled with the types II and III coppers to constitute the active site, while the latter is not.[6,7] In addition, CuA has been supposed to be affected irreversibly by several anions such as N_3^-, since this anion binds with types II and III coppers to exert the fatal perturbation on protein structure. However, the fact that the dialyzed HCP after N_3^--treatment still reserved the activity indicated that CuA is the type I copper which is reversibly modified by the treatment with N_3^-. On the other hand, CuB is supposed to be changed mainly to a type II copper irreversibly owing to its reduction by the S^- group of the cysteine residue, the succeeding disruption of the Cu–S (cysteine) bond and the final automatic reoxidation.

A similar behavior was also observed for BCP (spectra are not shown). Although BCP is especially unstable, the dialyzed BCP after N_3^--treatment showed 45% activity as compared with that of the native enzyme (Table 1). The conservation of the oxidase activity indicates again that CuA was reversibly modified by N_3^- as was observed in the case of HCP. The amount of EPR detectable Cu^{2+} increased from 3 to 3.7 probably because the destruction of the active site including the type III site had occurred during the treatment of this enzyme with N_3^-.

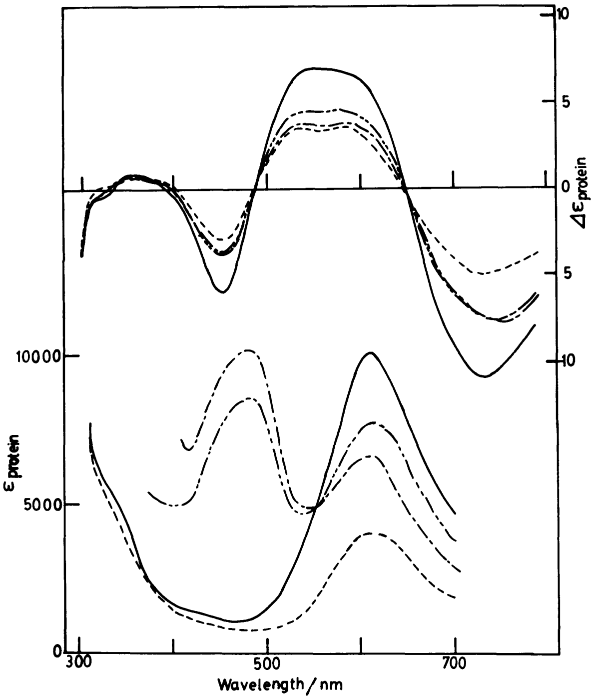

Fig. 3. Absorption and CD spectra of native (——), cysteine-treated (----), DDP-treated after cysteine-treatment (——), and dialyzed (———) human ceruloplasmin.

Native HCP was treated in three steps with two moles of L-cysteine. The resulting HCP gave absorption and CD spectra whose all bands were reduced to almost half of the native enzyme. Since CuA is coupled with the types II and III coppers, electrons afforded to this copper site were intramolecularly transferred to the type II copper and finally to the type III coppers where O_2 received them to give $2H_2O$. This intramolecular electron-transfer process is so rapid that electrons received by CuA are consumed in a few minutes.[12] However, the electron received by CuB is not transferred to other coppers because this type I copper is isolated from the active site. Accordingly CuB is autooxidized rather slowly, although the rate is not so slow as the autooxidation rate of the type I copper of simple blue copper proteins like plastocyanin, azurin, stellacyanin, plantacyanin, nitrite reductase, and amicyanin.[13] Thus the resulting absorption, CD and EPR spectra of the cysteine-treated HCP was not affected by CuB. This means that those spectra correspond to those of laccase which has the intrinsic one type I, one type II, and a pair of type III copper ions. It is not unexpected that the whole feature of the EPR spectrum was rather similar to that of the native enzyme (Fig. 2), even if the amount of EPR detectable Cu^{2+} was really decreased from 3.0 to 2.0. A rigorous EPR simulation study for the native enzyme should be performed again very carefully, since the former simulation study[10] suggested that the two type I coppers gave apparently different EPR parameters (vide supra).

In order to remove CuB selectively, 4,7-diphenyl-2,9-dimethyl-1,10-phenanthroline-4′,4″-disulfonate (DDP) was acted on a cysteine treated-HCP. A strong new band, which does not contribute to the CD spectrum, appeared at 485 nm. The absorption did not become pale appreciably after dialysis against buffer solution, since the Cu(I)-complex was firmly bound on the protein surface. The EPR detectable amount of Cu^{2+} increased from 2.3 for the cysteine-treated state to 2.7 for the dialyzed state. The corresponding absorptions at 610 nm were 66 and 77% of that of the native HCP, respectively, indicating that CuB was mainly changed to a type II copper irreversibly and was partly reduced and succeedingly autooxidized during dialysis. The net decrease of 0.3 Cu^{2+} may arise from the exclusion of CuB by the chelating reagent. Alternatively, since the experimental error in estimating the EPR detectable amount of Cu^{2+} is usually 10% or more, CuB may not be removed at all and the copper source of the 485 nm band might be copper(I) ions bound on protein surface. The activity of cysteine treated HCP did not change appreciably (Table 1),[14] indicating that CuB does not contribute to the appearance of the oxidase activity of

HCP. A similar result was also obtained in the case of BCP (Table 1)(spectra are not shown).

As a conclusion, it was confirmed that the type I CuA is assembled together with the types II and III copper ions to form the active site, but the type I CuB is isolated from the active site. Both the type I coppers are affected indirectly by the action of N_3^- on CP. Then, unexpectedly, CuB is mainly altered to a type II copper irreversibly, whereas CuA is reversibly modified in its steric and electronic states.

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