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Binding of copper to porcine apoceruloplasmin

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Ceruloplasmin (Cp, ferroxidase iron (II): oxygen oxidoreductase, EC 1.16.3.1) is a blue multifunctional copper protein (M_r 130000) found in the α_2-globulin fraction of vertebrate plasma. Its molecule contains six to seven copper atoms of three spectroscopically distinguishable types: 1, 2 and 3. Three copper atoms are e.p.r. detectable: two of type 1 and one of type 2 [1, 2]. The other three to four copper atoms are e.p.r. nondetectable and two of them form a spin-coupled pair of Cu(II) (type 3).

Only type 1 Cu(II) (1a, 1b) is responsible for the intense blue color of ceruloplasmins ($A^{1\%}_{cm,610}$ 0.69 - 0.75) [3, 4]. It is generally accepted that only four out of the six (or seven) Cu(II) atoms take part in the Cp catalytic activity: one of the blue type (1a), readily reoxidizable, one non-blue (type 2), and a pair of coupled copper atoms of type 3 [3, 5].

The copper atom transported by Cp is incorporated efficiently into other copper containing proteins such as cytochrome *c* oxidase or superoxide dismutase [5]. The reversible binding of copper by Cp is fundamental for the physiological role of Cp. The reduction of Cu(II) to Cu(I) by physiological substrates is the first step in the release of copper from Cp. Cu(I), characterized by high exchangeability could be incorporated into apoprotein to form the holoenzyme with copper fixed in the Cu(II) state with the aid of oxygen [5].

So far, in only two papers [6, 7] it has been reported that copper can be reversibly dissociated from human Cp in the presence of ascorbic acid or cysteine. However, biochemical analyses of regenerated ceruloplasmin indicated that the blue color, the content of tightly bound copper in Cp and enzymatic activity were restored only partially. Therefore it seemed reasonable to reinvestigate this problem using other species of Cp.

Nine preparations of porcine apoceruloplasmin from Cp obtained after [8], were reduced with ascorbic acid (apoCp A) according to Morell & Scheinberg [6].

Alternatively porcine apoceruloplasmin was reduced with cysteine (apoCp B) according to Morell *et al.* [7] except that a higher concentration of Cp crystalline cysteine hydrochloride (2 mg/mg Cp) was used.

In Cp and apoCp preparations protein concentration was determined by the biuret method, and the content of tightly bound copper in ceruloplasmin, according to the modified method of Gubler *et al.* [9], with sodium diethyldithiocarbamate. The oxidase activity was measured spectrophotometrically as described by Ravin [10] at pH 5.8 in 0.4 M acetate buffer with *p*-phenylenediamine as a substrate. Specific activity of Cp (μmol/min per mg of Cp) was calculated using the molar absorption coefficient, $\varepsilon = 1910\ M^{-1} \times cm^{-1}$ of Bandrowski's base (oxidation product of *p*-phenylenediamine) [11].

The isolated porcine Cp preparations were of high chemical purity and biological activity as evidenced by the absorbance ratio of A_{610}/A_{280} 0.0522 ± 0.00055, copper content 3.09 ± 0.098 μg Cu/mg Cp and specific oxidase activity 5.5 ± 0.20 U/mg Cp. ApoCp preparations obtained in the presence of either ascorbic acid (apoCp A) or cysteine (apoCp B) had only about 3% of the initial copper content in Cp and they lacked both enzymatic activity and blue color.

Experiments on binding of copper from $CuSO_4$ to apoceruloplasmin were carried out with both apoCp A and apoCp B in 0.3 M

acetate buffer, pH 5.3, at the Cu/apoCp molar ratio from 2:1 to 16:1 in the presence of either ascorbic acid (20 μg/ml) or cysteine (2 μmoles/μmol Cu) as reducing agent. After 20 h the excess of reducing agent and copper ions was removed by either (1) exhaustive dialysis against 0.9% NaCl, or (2) passing through a column with ion exchanger Chelex-100 at the maximal flow rate, followed by dialysis against 0.9% NaCl.

When procedure (2) was applied, binding of copper to apoCp A in the presence of ascorbic acid even at $CuSO_4$/apoCp molar ratio as high as 16:1, was very low, i.e. 1.59 Cu atom/Cp molecule, enzymatic activity was restored only in 1% and blue color in 5% (absorbance ratio A_{610}/A_{280}). When the excess of unbound copper was removed, exclusively by dialysis (procedure 1), the number of bound copper atoms was much higher: 6.39 Cu atom/Cp molecule, i.e. it reached the initial number of copper atoms associated with the native Cp molecule. However, it seems that not all of these copper atoms were bound to prosthetic sites in the Cp molecule, since both the enzymatic activity and the intensity of blue color remained practically unchanged i.e. 0.8% and about 4%, respectively. These results may be explained by the data of Zgirski & Frieden [12] who showed that porcine Cp could bind besides 7 prosthetic copper atoms, the additional 3 copper atoms which are readily removed by Chelex-100 but are non-dialysable; these ions have no effect on the intensity of blue color and enzymatic activity. Lack of even one copper atom of the four which are necessary for enzymatic activity makes such a molecule inactive. This explains discrepancies between the relatively large number of bound copper atoms (even bound in prosthetic sites) and relatively low restoration of both enzymatic activity and blue color.

Somewhat better results were obtained when binding of copper atoms to apoCp A was carried out in the presence of cysteine. After removal of the excess of unbound Cu by Chelex 100 or exclusively dialysis the following values were obtained: 2.66 and 4.79 Cu/Cp, 22.6% and 23.4% for restored blue color, and 8.16% and 8.28% for enzymatic activity, respectively.

Much better biochemical parameters for reconstitution of Cp were obtained when apoCp B, instead of apoCp A, was incubated with Cu(II) ions in the presence of cysteine when either the ion exchanger or dialysis was applied (64.2% or 70.3% recovery of catalytic activity and 50.3% or 59.2% of the blue color), respectively. However, in the presence of ascorbic acid only 5.3% or 6.5% of the initial catalytic activity of porcine Cp and 11.6% or 12.9% of blue color were recovered, respectively.

These observations suggest that cysteine is a much better reducing reagent for reconstitution of Cp in comparison with ascorbic acid, at least in the case of porcine Cp.

Experiments in which apoCp B was incubated with Cu(II) ions at the Cu/apoCp molar

Table 1

Biochemical properties of nine reconstituted Cp preparations obtained from apoCp B[a] after the incubation with Cu(II) in the presence of cysteine.

The excess of copper ions unbound to apoCp was removed by passing through Chelex 100 column and dialysis. Mean ± S.D. of experiments are given

Cu/apoCp molar ratio	Copper content			Specific activity		Absorbance ratio	
	μg/mg	%[b]	$\frac{\text{mol Cu}}{\text{mol Cp}}$	U/mg	%[b]	A_{610}/A_{280}	%[b]
2:1	0.73 ± 0.13	23.7 ± 3.19	1.49 ± 0.27	0.74 ± 0.14	13.5 ± 2.66	0.0149 ± 0.0015	28.7 ± 2.94
4:1	1.02 ± 0.12	32.9 ± 3.91	2.06 ± 0.25	1.26 ± 0.17	22.9 ± 3.17	0.0206 ± 0.0021	39.6 ± 4.13
8:1	1.65 ± 0.12	53.3 ± 3.99	3.35 ± 0.25	3.35 ± 0.27	60.9 ± 4.36	0.0247 ± 0.0038	47.4 ± 7.35

[a]ApoCp obtained in the presence of cysteine;

[b]calculated in percent of native Cp

ratio of 2:1, 4:1 and 8:1 in the presence of cysteine (Table 1) suggest that first blue copper atoms are bound at the prosthetic sites responsible for blue color, i.e. type 1a and 1b atoms (relatively high restoration of blue color in comparison with specific activity). Only at the Cu/apoCp molar ratio 8:1, the number of copper atoms bound in prosthetic sites of type 2 or type 3 increased and, subsequently, the restored enzymatic activity increased up to approx. 60% of the initial activity.

Our results with porcine Cp generally support the data of Morell *et al.* [6, 7] on reconstitution of human Cp. The incomplete reconstitution of Cp from either source suggests that some unidentified agents take part in the reversible dissociation of copper ions from Cp.

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