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BINDING OF COPPER TO PORCINE APOCERULOPLASMIN: PARTIAL RECONSTITUTION OF THE ENZYME

Apoceruloplasmin was obtained from porcine ceruloplasmin (Cp) preparations according to the method of Morell and Scheinberg in which ascorbic acid (apoCp A) or cysteine (apoCp B) were used as a reducing agent. Copper free Cp preparations (apoCp) were incubated with copper ions in the presence of reducing agents: ascorbic acid or cysteine.

ApoCp A preparations possessed very good biochemical parameters alike apoCp B but in contrast to latter ones apoCp A more badly bound copper ions and showed very low ability to recover enzymatic activity and blue color following incubation with copper ions (~ 1% of restored enzymatic activity and ~ 4% – blue color, in the presence of ascorbic acid or ~ 8% of restored enzymatic activity and ~ 23% – blue color, in the presence of cysteine). Much better parameters were found in reconstituted Cp obtained from apoCp B following incubation with copper ions in the presence of cysteine (64.2% of restored enzymatic activity and 50.3% – blue color) but not in the presence of ascorbic acid (5.3% and 11.6%, respectively). As we can see independently of the kind of apoCp better results have always been achieved in the presence of cysteine.

The cysteine used as a reducing agent during both obtaining of apoCp preparations and reconstitution of Cp by incubation with cupric ions became a much better reagent in comparison with ascorbic acid.

Ceruloplasmin (Cp, ferroxidase iron (II) : oxygen oxidoreductase, EC 1.16.3.1) is a blue multifunctional copper protein (M_r = 130 000) found in the α_2–globulin fraction of vertebrate plasma. Its molecule contains six to seven copper atoms present in three spectroscopically (EPR) distinguishable types of binding sites: types 1, 2 and 3. Three copper atoms are paramagnetic and EPR detectable: two of type 1 and one of type 2 [2, 6, 14]. Three to four copper atoms are EPR nondetectable. Two of these form a spin-coupled pair of Cu(II) (type 3) and others form a hypothetical type 4 the properties of which are unknown. Only type 1 Cu(II) is responsible for the intense blue color of ceruloplasmins ($A^{1\%}_{1cm}$ at 610 nm = 0.69 – 0.75 for mammalian ones), ie. la and 1b copper atoms [7, 8, 16].

It is generally agreed that only four of six (seven) Cu(II) atoms take part in catalytic activity. For the blue oxidases there is a strong experimental support for the idea that the active site is made up of one blue type 1 copper atom, ie. type 1a (reoxidized very fast), one nonblue type 2 and a pair of magnetically coupled type 3 coppers [4, 8]. These are situated in such a way that electrons can be accepted from the substrates by type 1 and 2 coppers and transferred to the copper pair which most likely binds the oxygen molecule and reduces it [1, 3, 15].

The role of Cp as a copper transport protein is physiologically very important, because only this form of copper is incorporated very well into other copper containing proteins such as cytochrome c oxidase or superoxide dismutase [4]. Greater lability of Cu(I) strongly suggests the presence of a reductive step in copper release. The reduction of Cu(II) of Cp by physiological substrates would be the first step. In its Cu(I) form which is characterized by the high exchange-ability, copper would be added to an apoprotein where it would be fixed into the holoenzyme in the Cu(II) state with the aid of oxygen [4]. The possibility of reversible binding and release of copper by the protein may be the basis for its physiological role. It would be very interesting to obtain in vitro some evidences supporting such a possibility.

So far there are only two papers in literature [10, 11] which show in case of human Cp that copper can be reversibly dissociated under certain conditions in the presence of ascorbic acid or cysteine but biochemical analyses of regenerated ceruloplasmin indicated that blue color, tightly bound Cp copper and enzymatic activity were restored only partially. Therefore it seemed to us reasonable to carry out similar experiments also with other species of Cp. This paper presents conditions for preparing a porcine apoprotein which would be capable of recombining with copper to form ceruloplasmin.

MATERIAL AND METHODS

Fresh porcine blood was obtained from a slaughterhouse and collected in the presence of both 0.02M sodium citrate and 6-aminocaproic acid. Plasma was used directly for the preparation of ceruloplasmin according to our own method [9].

Procedure for obtaining of apoceruloplasmin was carried out in an acetate buffer and in the presence of sufficient ascorbic acid or cysteine to keep the ceruloplasmin copper in the monovalent state throughout its removal from the protein. Advantage was taken of the fact that the diethyldithiocarbamate ion combines tightly with copper to form a colloidal suspension. This made it

possible to remove by ultracentrifugation the copper which had been separated from Cp.

Preparation of porcine apoceruloplasmin with ascorbic acid as a reducing agent (apoCp A) according to modified method of Morell and Scheinberg [10]: To Cp preparation was added 1/4 vol of 6M acetate buffer, pH 5.2, and 2 mg of crystalline ascorbic acid per 1 mg of Cp which instantly decolorized the blue solution. During the next 10 h the solution was kept at 4°C and stirred while 1% sodium diethyldithiocarbamate (1/4 vol of initial Cp volume) was added, dropwise. After 3 h the final golden brown mixture was centrifuged in a VAC 601 ultracentrifuge for 3 h at 40 000 rpm at 0°C. The colorless supernatant was dialyzed for 20 h against 0.9% NaCl solution.

Preparation of porcine apoceruloplasmin with cysteine as a reducing agent (apoCp B) according to modified method of Morell et al. [11]: Following dialysis of Cp solution for 20 h against 0.05M phosphate buffer, pH 7.3, containing 0.17M NaCl, 1/4 vol. of 2.4M acetate buffer, pH 5.1, was added. Crystalline cysteine hydrochloride (2 mg/mg Cp) was then dissolved in the protein solution. After 5 h to the decolorized Cp solution was added dropwise 1% aqueous solution of sodium diethyldithiocarbamate with continuous stirring and the mixture was set aside for overnight at 4°C. The resulting deep brown suspension was dialyzed for 20 h against 10 l of 1M sodium acetate containing 1% NaCl and next dialyzed overnight against 5 l of 0.05M sodium acetate containing 1% NaCl. The insoluble Cu-diethyldithiocarbamate complex was then removed by centrifugation for 3 h at 40 000 rpm. Colorless supernatant contained apoceruloplasmin (apoCp B).

Binding of copper to apoceruloplasmin: These experiments were carried out with apoCp A and apoCp B in 0.3M acetate buffer, pH 5.3, at molar ratio Cu/apoCp from 2:1 to 16:1 in the presence of either ascorbic acid (20 μg/ml) or cysteine (2 μmoles/μmol Cu) as reducing agents. The presence of reducing agent was necessary because addition of cupric ions without a reducing agent to a solution of apoCp does not result in the formation of blue reconstituted ceruloplasmin. After 20 h excess of reducing agent and copper was removed by either 1) exhausting dialysis against 0.9% NaCl or 2) passing through column with ion exchanger Chelex-100 with maximal flow rate and then dialysis against 0.9% NaCl.

Protein concentration was determined by the biuret method with bovine serum albumin as a standard. Tightly bound ceruloplasmin copper content determinations were performed according to a modified method of Gubler et al. [5] with sodium diethyldithiocarbamate in couvettes with 5 cm path length. Oxidase activity was measured spectrophotometrically as described by Ravin [12] at pH 5.8 in 0.4M acetate buffer with p-phenylenediamine (PPD) as a substrate. Specific activity (μmol/min/mg of Cp) was calculated employing the molar absorption coefficient of Bandrowski's base, 1910 [13].

RESULTS

Nine porcine Cp preparations possessed a high degree of both chemical purity and biological activity. These parameters were evaluated by absorbance ration of A_{610nm}/A_{280nm}(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 by us in the presence of both ascorbic acid (apoCp A) and cysteine (apoCp B) possessed very good biochemical parameters as shown below in the Tab. 1.

Table 1

Some biochemical parameters
of apoceruloplasmin preparations obtained
in the presence of ascorbic acid (apoCp A) or cysteine (apoCp B)

Preparation	Copper content		Absorbance ratio A_{610}/A_{280}	Specific activity
	μg/mg apoCp	%[a]		
ApoCp A	0.086[b] ±0.029	2.79 ±0.96	0	0
ApoCp B	0.104 ±0.082	3.38 ±2.74	0	0

[a] – Copper content calculated as percentage of that in Cp preparation which was assumed as 100%.

[b] – Mean value ± standard deviation.

Results in the Tab. 2 and the Tab. 3 present the data from experiments carried out to evaluate the recombining ability of apoCp preparation and copper to form ceruloplasmin.

DISCUSSION

The possibility of reversible binding and release of copper by ceruloplasmin (Cp) may be the basis of its physiological role as a copper transport protein. Morell et al. [10, 11] were first to obtain some evidences for such a suggestion. They showed in case of human Cp that copper could be reversibly dissociated from human Cp molecule in the presence of ascorbic acid or cysteine but biochemical analyses of regenerated Cp indicated that blue color, tightly bound Cp copper and enzymatic activity were restored only in 57.5%, 58.4% and 86%, respectively. Our paper presents further data concerning conditions for both obtaining of porcine apoCp, and reconstitution of Cp after incubation of apoCp with copper ions.

Table 2

Some biochemical properties of reconstituted Cp preparations obtained from apoCp A[a] or apoCp B[a] following the incubation with Cu(II) at molar ratio Cu/apoCp 16:1 in the presence of ascorbic acid (I) or cysteine (II). Excess of copper unbound with apoCp was removed by passing through Chelex-100 column (1) or by dialysis (2)

Preparation			Copper content			Specific activity		Absorbance ratio	
			μg/mg	%[b]	$\frac{\text{mol Cu}}{\text{mol Cp}}$	U/mg	%[b]	A_{610}/A_{280}	%[b]
ApoCp A	ascorbic acid (I)	Chelex (1)	0.78[c] ±0.08	26.1 ±2.71	1.59 ±0.16	0.054 ±0.010	0.98 ±0.18	0.0026 ±0.00021	5.04 ±0.41
		dialysis (2)	3.15 ±0.26	104.9 ±8.69	6.39 ±0.53	0.044 ±0.006	0.80 ±0.11	0.0020 ±0.00019	3.88 ±0.37
	cysteine (II)	Chelex (1)	1.31 ±0.12	42.2 ±3.82	2.66 ±0.24	0.449 ±0.072	8.16 ±1.31	0.0117 ±0.0012	22.6 ±2.32
		dialysis (2)	2.36 ±0.19	78.7 ±6.26	4.79 ±0.38	0.455 ±0.078	8.28 ±1.43	0.0122 ±0.0016	23.4 ±3.01
ApoCp B	ascorbic acid (1)	Chelex (1)	1.41 ±0.14	45.6 ±4.47	2.86 ±0.28	0.291 ±0.097	5.30 ±1.77	0.0060 ±0.00046	11.6 ±0.89
		dialysis (2)	3.30 ±0.36	112.9 ±12.09	6.69 ±0.72	0.356 ±0.105	6.48 ±1.91	0.0067 ±0.00057	12.9 ±1.10
	cysteine (II)	Chelex (1)	1.75 ±0.11	56.6 ±3.44	3.55 ±0.21	3.53 ±0.26	64.2 ±4.73	0.0262 ±0.0025	50.3 ±4.83
		dialysis (2)	2.27 ±0.68	77.2 ±24.2	4.61 ±1.44	3.87 ±0.49	70.3 ±8.93	0.0308 ±0.0032	59.2 ±6.13

[a] – ApoCp obtained in the presence of ascorbic acid (A) or cysteine (B).
[b] – Calculated as percentage in relation to value for native Cp which was assumed as 100%.
[c] – Mean value ± standard deviation.

Table 3

Some biochemical properties of reconstituted Cp preparations obtained from apoCp B[a] after the incubation with Cu(II) in the presence of cysteine at molar ratio Cu/apoCp 2:1 (I), 4:1 (II) and 8:1 (III). Excess of copper unbound with apoCp was removed by passing through Chelex-100 column

Preparation	Copper content			Specific activity		Absorbance ratio	
	μg/mg	%[b]	mol Cu / mol Cp	U/mg	%[b]	A_{610}/A_{280}	%[b]
I	0.73[c] ±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
II	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
III	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 as percentage in relation to value for native Cp which was assumed as 100%.
[c] – Mean value ± standard deviation.

As we can see from Tab. 1 porcine apoCp preparations in the presence of both ascorbic acid (apoCp A) and cysteine (apoCp B) possessed very good biochemical parameters even better than apoCp reported by Morell et al [10], which showed still 5.7% of the enzymatic activity, 2.48% of the color and ~7% of the initial value of copper.

Experiments with both proteins, ie. apoCp A and apoCp B for binding of copper were carried out in the presence of ascorbic acid or cysteine. As mentioned in Material and Methods the presence of reducing agent was necessary because addition of cupric ions without such a reagent to a solution of apoCp does not result in the formation of blue reconstituted ceruloplasmin.

As we can see from Tab. 2 binding of copper to apoCp A even at high molar ratio Cu/apoCp = 16:1 in the presence of ascorbic acid was very low: 1.59 Cu at./ Cp molecule, only ~ 1% of restored enzymatic activity and ~ 5% – blue color. When excess of unbound copper was removed, not with Chelex-100 as above but by dialysis, number of bound copper atoms was much higher: 6.39 Cu at./ Cp molecule, ie. approximately 100% of initial number of copper atoms associated with the Cp molecule. However, these copper atoms were not bound to prosthetic sites in Cp molecule, because despite a much higher amount of bound copper atoms, restored enzymatic activity and blue color were very low and practically the same as previously when Chelex-100 was used, ie. 0.8% and ~ 4%, respectively. These results may well be

explained by the data of Zgirski and Frieden [17] who showed, that porcine Cp could bind besides 7 prosthetic copper atoms, additional 3 copper atoms. They were readily removed by Chelex-100 but were nondialysable and they influenced neither blue color nor enzymatic activity. Moreover we have to remember that of 6–7 tightly bound prosthetic copper atoms in Cp molecule only two are responsible for blue color (type la and 1b) and four – for catalytic activity (type 1a, 2 and 2 atoms of type 3). Lack of even one copper atom of these four which are necessary for enzymatic activity makes such a molecule inactive. This fact explains that there exist great discrepancies between relatively large number of bound copper atoms (even bound in prosthetic sites) and relatively low regenerated both enzymatic activity and blue color (absorbance ratio A_{610nm} / A_{280nm}).

A little better results were obtained when binding of copper atoms to apoCp A was carried out in the presence of cysteine instead of ascorbic acid (see Tab. 2). Also in this case we can see a big difference between the amount of bound copper atoms depending on the technique of removing of copper excess (Chelex-100 or dialysis): 2.66 and 4.79 Cu/Cp, respectively and practically no difference in restoring of blue color (22.6% and 23.4%) or enzymatic activity (8.16% and 8.28%) what can be explained as above.

Much better biochemical parameters have been stated for reconstituted Cp from apoCp B which was achieved from porcine Cp in the presence of cysteine not ascorbic acid. However, these good results could be seen only when apoCp B has been incubated with Cu^{2+} ions in the presence of cysteine (64.2% or 70.3% recovered catalytic activity and 50.3% or 59.2% – blue color) but not in the presence of ascorbic acid (only 5.3% or 6.5% recovered catalytic activity and 11.6% or 12.9% – blue color) (see Tab. 2).

These observations suggest that cysteine used as a reducing agent during both obtaining of apoCp preparation and incubation with cupric ions for reconstitution of Cp is much better reagent in comparison with ascorbic acid (at least in case of porcine Cp).

Very interesting data have been reported for reconstituted Cp preparations obtained from apoCp B after the incubation with Cu^{2+} ions in the presence of cysteine at molar ratio Cu/apoCp 2:1, 4:1 and 8:1 (Tab. 3). From data listed in Tab. 3 we can see that first of all blue copper atoms are bound in prosthetic sites, ie. type 1a and 1b (relatively high value of % restored blue color) and probably others but not all necessary for catalytic activity (lower value of % restored specific activity in comparison with % restored blue color or copper content). Only at molar ratio Cu/apoCp 8:1 number of copper atoms bound in prosthetic sites type 2 or type 3 increases and therefore % of restored enzymatic activity also increases up to level ~ 60%.

Though our data, generally supporting Morell et al. observations, still require further experiments on Cp from different sources, the reverse ability of copper to recombine with both human and porcine Cp seems to reflect common physiological function of these kinds of oxidoreductases.

REFERENCES

[1] Branden R., Deinum J. (1978), FEBS Lett., **89**, 180.
[2] Deinum J., Vanngard T. (1973), Biochim. Biophys. Acta, **310**, 321.
[3] Farver O., Goldberg M., Pecht J. (1980), Eur. J. Biochem., **104**, 71.
[4] Frieden E., Hsieh H. S. (1976), Adv. in Enzymol. and Related Areas of Mol. Biol., **44**, 187.
[5] Gubler C. J., Lahey M. E., Ashenbrucker H., Cartwright G. E., Wintrobe M. (1952), J. Biol. Chem., **196**, 209.
[6] Herve M., Garnier A., Tosi L., Steinbuch M. (1976), Biochim. Biophys. Acta, **439**, 432.
[7] Herve M., Garnier A., Tosi L., Steinbuch M. (1978), Biochem. Biophys. Res. Commun., **80**, 797.
[8] Herve M., Garnier A., Tosi L., Steinbuch M. (1981), Eur. J. Biochem., **116**, 177.
[9] Hilewicz-Grabska M., Zgirski A., Krajewski T., Płonka A. (1988), Arch. Biochem. Biophys., **260**, 18.
[10] Morell A. G., Scheinberg J. H. (1958), Science, **127**, 588.
[11] Morell A. G., Aisen P., Blumberg W. E., Scheinberg J. H. (1964), J. Biol. Chem., **239**, 1042.
[12] Ravin H. (1961), J. Lab. Clin. Med., **58**, 161.
[13] Rice E. W. (1962), Anal. Biochem., **3**, 452.
[14] Ryden L., Bjork J. (1976), Biochemistry, **15**, 3411.
[15] Ryden L. (1982), Proc. Natl. Acad. Sci. USA, **79**, 6767.
[16] Zgirski A. (1986), *Ceruloplazmina człowieka, świni i krowy. Stabilność i alternatywna droga działania*, Acta Univ. Lodz., Łódź.
[17] Zgirski A., Frieden E. (1990), J. Inorg. Biochem., **39**, 137.

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WIĄZANIE MIEDZI PRZEZ APOCERULOPLAZMINĘ ŚWINI: CZĘŚCIOWA REKONSTRUKCJA ENZYMU

Z preparatów ceruloplazminy (Cp) świni otrzymano apoceruloplazminę wg metody Morella i Scheinberga, w której jako czynnika redukującego miedź w ceruloplazminie używano kwasu askorbinowego (apoCp A) lub cysteiny (apoCp B). Pozbawione miedzi preparaty ceruloplazminy (apoCp) inkubowano z jonami miedzi w obecności reduktorów: kwasu askorbinowego lub cysteiny.

Preparaty apoCp A charakteryzowały się bardzo dobrymi parametrami biochemicznymi, podobnie jak apoCp B, ale w przeciwieństwie do tych ostatnich, gorzej przyłączały miedź i w bardzo małym stopniu były zdolne do przywracania aktywności i zabarwienia po inkubacji z jonami miedziowymi (~ 1% powrotu aktywności i ~ 4% – barwy, w obecności kwasu askorbinowego oraz ~ 8% powrotu aktywności i ~ 23% – barwy, w obecności cysteiny). Znacznie lepsze parametry dla odtworzonej Cp uzyskiwano po inkubacji apoCp B z jonami miedziowymi w obecności cysteiny (64.2% powrotu aktywności i 50.3% barwy), ale nie w obecności kwasu askorbinowego (5.3% powrotu aktywności i 11.6% barwy). Jak widać niezależnie od rodzaju apoCp, zawsze korzystniejsze rezultaty otrzymywano w obecności cysteiny.

Cysteina stosowana jako reduktor, zarówno podczas otrzymywania preparatów apoCp, jak i podczas inkubacji apoCp z jonami miedziowymi w celu odtworzenia ceruloplazminy, okazała się znacznie lepszym odczynnikiem w porównaniu z kwasem askorbinowym.