Sequential Reconstitution of Copper Sites in Caeruloplasmin

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Abstract

Titration of apo-caeruloplasmin employing substoichiometric concentrations of [Cu(I)–(thiourea)$_3$]Cl was performed to elucidate possible sequential incorporation of copper into the different specific binding sites. The successful reconstitution was monitored by A_{610} absorption, EPR spectroscopy and oxidase activity. Maximum activity and final absorption at 610 nm were reached after 20 min. When both A_{610}, indicative for type 1 copper, and oxidase activity were expressed per g-atom of copper, a sequential insertion was found. Owing to the specific data at the beginning, some type 3 copper appeared to be preferentially incorporated. After 3–4 g-atoms (including most of type 1 and type 2 copper), both absorption and oxidase activity surpassed transient maxima. Then type 3 and 4 copper were further bound to reach the known stoichiometry of six copper atoms per mole of protein.

Introduction

Caeruloplasmin, the blue copper oxidase of vertebrate plasma, is found to bind essentially all of the plasma copper [1]. Six to seven copper atoms are reported to be coordinated at specific binding sites. In general, two type 1, one type 2, one pair of type 3 and one type 4 copper atoms are present [2]. Type 1 copper exhibits intensive A_{610} absorption ($\epsilon_{Cu} \sim 5000$ cm^{-1} M^{-1}) and an unusually small hyperfine coupling constant ($A_{\parallel} \sim 0.006$ cm^{-1}). The ϵ_{Cu} of type 2 copper in the 600 nm region is only 50–150 cm^{-1} M^{-1} and the EPR properties are similar to those exhibited in distorted tetragonal environments. The type 3 copper is a magnetically coupled binuclear copper centre. Unlike the normal electronic absorption of Cu(II) near 600 nm, it absorbs at 330 nm and is EPR silent. A type 4 copper was proposed by Rydén [2] which displays neither electronic absorption nor EPR characteristics. It is possibly a mononuclear metallothionein-like unit or a partially degraded type 3 copper. At the same time, the glycoprotein proved to be an important extracellular antioxidant *in vivo* [3]. It can inhibit both lipid peroxidation and the Fenton reaction by catalysing the oxidation of ferrous ions to the ferric state. Due to the subsequent incorporation of Fe(III) into apo-transferrin, no empty coordination sites at the iron are available [4–6]. Furthermore, caeruloplasmin is able to scavenge activated oxygen intermediates $\cdot O_2^-$, H_2O_2 and OH$\cdot$, probably by stoichiometric reactions. The inhibition of leucocyte-induced degradation of hyaluronic acid by caeruloplasmin supports the possible biological role of caeruloplasmin in controlling the activity of leucocytes during systemic inflammation [7].

Copper transport and homoeostasis are proposed to be further important functions of caeruloplasmin [8]. The molecular side of copper transport is still unknown; in this context the reconstitution of caeruloplasmin deserves special attention. One important question remains; is there a sequential incorporation into the apo-protein or is the earlier reported 'all-or-nothing' process the prevalent mode [9]?

The copper binding to apo-caeruloplasmin is of marked biological significance. As copper is known to be incorporated in the reduced form only [10], intracellular Cu(I)–thiolate-rich proteins, called copper–thionein, are considered to be potent copper donors for the biosynthesis of caeruloplasmin in the liver. Reconstitution experiments using copper–thionein actually demonstrated the transfer of copper into apo-caeruloplasmin [11]. Unfortunately, the system used was rather too complex to see further details of copper incorporation. The use of the structural analogue [Cu(I)–(thiourea)$_3$]Cl for Cu–thionein promised to shed some light on the question of a possible sequential copper insertion into the apo-protein. The different stages of reconstitution were followed by EPR spectroscopy, electronic absorption and oxidase activity measurements.

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Experimental

Initially caeruloplasmin was prepared from porcine, bovine and human sera [12, 13], respectively. Human caeruloplasmin proved to be the most suitable and stable protein for the planned experiments. Due to limitations in obtaining large quantities of fresh human serum, commercial human caeruloplasmin was subjected to anion-exchange (Mono Q column, Pharmacia) and additionally to gel-filtration (Superose 12, Pharmacia) employing fast protein liquid chromatography (FPLC). There were no detectable differences between the purified commercial sample and caeruloplasmin isolated from fresh human serum. Therefore human caeruloplasmin (type X) was obtained from Sigma (Heidelberg) and purified using the former procedures. The apo-protein was prepared using the modified procedures of ref. 14. The holoprotein was dialysed against 500 volumes of a 50 mM sodium acetate buffer (pH 5.5) containing 50 mM KCN, 5 mM EDTA and 150 mM NaCl (final pH 9.8) for 5 h. Removal of both the formed copper–cyanide complex and excessive cyanide from the apo-protein was successful after dialysis of four changes each of 500 volumes of 150 mM NaCl in 50 mM sodium acetate buffer (pH 5.5) for 4 h. The copper content was less than 2%. No detectable EPR spectrum or oxidase activity were noticed.

Oxidase activity was determined photometrically using *N,N*-dimethyl-*p*-phenylenediamine as substrate [15]. One unit is arbitrarily defined as the amount of caeruloplasmin that will cause a ΔA_{550} of 0.01/min under the following conditions. The reaction mixture was composed of 440 µl of 200 mM sodium acetate buffer (pH 5.5) and 50 µl of the same buffer, containing 2 mM *N,N*-dimethyl-*p*-phenylenediamine and 0.5 mM EDTA, and then incubated at 37 °C for 2 min. The reaction was started by addition of 10 µl of the diluted samples. Oxidase activity was measured for 10 min against a blank with buffer instead of the test samples.

[Cu(I)–(thiourea)$_3$]Cl was prepared as described earlier [16]. This Cu(I) complex was most suitable for reconstitution experiments as the Cu(I) remained stable in aqueous solution for more than 24 h [17]. Reconstitution was achieved after incubation of the apo-protein (50 µM) in the presence of the appropriate amount of [Cu(I)–(thiourea)$_3$]Cl. Possible unspecifically bound copper was removed by treatment with 0.1 g cation chelating resin (Chelex 100, Biorad).

EPR spectra at 100 K were run on a Varian E-109 spectrometer. Measurements at 70 K were performed on a Varian E4 unit with an Oxford Instruments' helium-flow cryostat. Signal intensities were determined by double integration with correction for baseline, and were compared with a Cu–EDTA standard [18]. The contribution of the type 2 copper was substracted from the integrated spectra in the super-hyperfine region to assess the amount of type 1 copper and type 2 copper, respectively. Electronic absorption was recorded on a Beckman DU-40 spectrophotometer. Copper was analysed on a Perkin-Elmer 400 S atomic absorption spectrometer equipped with a HGA 76B unit.

Results and Discussion

Electronic Absorption and Oxidase Activity

Freshly prepared apo-caeruloplasmin was successfully reconstituted using stoichiometric concentrations, *i.e.* six moles of [Cu(I)–(thiourea)$_3$]Cl per mole of protein, and in the presence of air. Both oxidase activity and electronic absorption at 610 nm rose sharply during the first 10 min. After 20 min maximal values were obtained.

Titration of apo-caeruloplasmin with substoichiometric concentrations of the Cu(I) complex was carried out to reveal possible sequential incorporation into the different specific copper binding sites. In contrast to stoichiometric copper concentrations, up to 2 h were needed to achieve constant A_{610} absorption and oxidase activity. All samples were additionally treated with cation chelating resin to remove unspecifically bound Cu(II). The uptake of copper was monitored by atomic absorption spectrometry and proceeded linearly as expected. Reappearance of the type 1 copper centre was separately followed by electronic absorption. As the molar absorption A_{610} of the type 1 copper was near 5000 $cm^{-1}\ M^{-1}$, the minimal contribution from the type 2 centre ($\epsilon_{Cu} \sim 100\ cm^{-1}\ M^{-1}$) could be neglected. The rise of the A_{610} absorption was highest up to 3–4 g-atoms of copper, which was followed by a reduced rate up to six moles of [Cu(I)–(thiourea)$_3$]Cl. The oxidase activity essentially paralleled the reappearance of the type 1 chromophore (Fig. 1).

Unfortunately it is not possible to assess the exact stoichiometry of the specific copper centres known for maximal enzymic activity from the data of Fig. 1. It was proposed that one type 1 copper, one type 2 copper and a pair of type 3 coppers are the four copper centres responsible for oxidase activity [2]. That type 2 copper was essential was further confirmed by inhibitor studies using fluoride and azide [19]. In addition to the active copper species, it is not surprising to realize that copper incorporation still continues under conditions where activity is already optimal.

An approach to elucidate the overall stoichiometry looked promising when A_{610} and oxidase activity were expressed per g-atom of incorporated copper, respectively. Provided the 'all-or-nothing' process has occurred, all specific parameters ought to remain constant from the very beginning onwards. However,

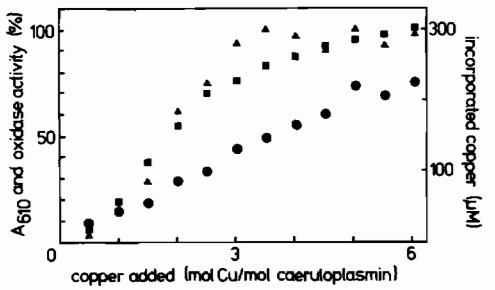

Fig. 1. Titration of apo-caeruloplasmin with substoichiometric concentrations of [Cu(I)–(thiourea)$_3$]Cl. Relationship between recovered type 1 copper, oxidase activity and inserted copper: ■ A_{610}; ▲ oxidase activity; ● incorporated copper. For comparative reasons the results were expressed as percent of the maximum values. Reconstitution was carried out in 50 mM sodium acetate buffer (pH 5.5) + 150 mM NaCl for 2 h; apo-caeruloplasmin, 50 μM, [Cu(I)–(thiourea)$_3$]Cl, 25–300 μM; one unit is arbitrarily defined as the amount of caeruloplasmin that will cause a ΔA_{550} value of 0.01/min; the reproduceability was better than ±10%. Note: No further copper binding was measured in the presence of excessive copper. All spurious copper was removed by Chelex treatment.

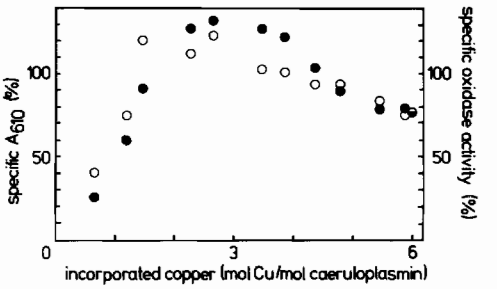

Fig. 2. Reconstituted caeruloplasmin: specific absorption and oxidase activity per g-atom of incorporated copper. ○ specific A_{610}; ● specific oxidase activity expressed as percent of native protein values. Incubation conditions were as in the legend to Fig. 1.

there is a substantial delay in the type 1 copper formation and oxidase activity until two moles of copper are bound in the apo-protein. When three copper moles are inserted, the specific data were about 30% higher compared to those of the native enzyme. From four copper moles onwards a progressive decrease down to the specific values of the holoprotein was noticed (Fig. 2).

Electron Paramagnetic Resonance

Unlike electronic absorption, EPR measurements allow the separate assignment of type 1 and type 2 copper. A gradual increase in the intensity of the EPR spectra is apparent (Fig. 3). Quantification by double integration, which should detect all 3 d^9 copper, was expected to show differences in the incorporation rates. The total paramagnetic copper increased linearly (Fig. 4a) and no significant differences

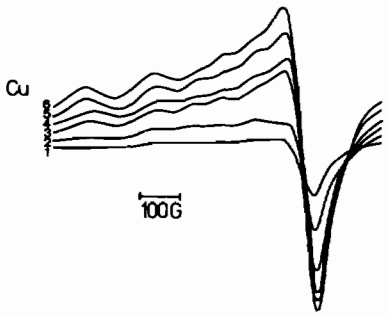

Fig. 3. EPR spectra of caeruloplasmin titrated with Cu(I)–(thiourea)$_3$. Caeruloplasmin was incubated with [Cu(I)–(thiourea)$_3$]Cl (1–6 mol) for 2 h and oxidised with equimolar H_2O_2. Spectra were recorded on a Varian E-109 spectrometer at 100 K with the following instrument settings: microwave power, 20 mW; frequency 9.24 GHz; modulation amplitude, 1 mT. Incubation conditions were as in the legend to Fig. 1.

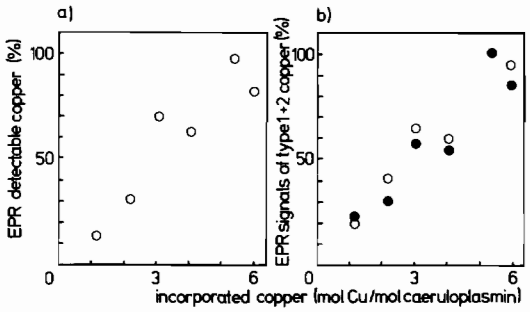

Fig. 4. (a) Integrated EPR signals of reconstituted caeruloplasmin. EPR spectra were run at 70 K on a Varian E-4 spectrometer. Signal intensities were determined by double integration with correction for the baseline and tube diameter; total EPR detectable copper was expressed as percent of the maximum values. Instrument settings: microwave power, 20 mW; frequency, 9.18 GHz; modulation amplitude, 1 mT. Incubation conditions were as in the legend to Fig. 1. (b) EPR signals of reconstituted caeruloplasmin. ○ Type 1 copper; ● type 2 copper; expressed in percent of maximum values. Instrumental and incubation conditions were as in the legend to Fig. 4a.

between the incorporation rates of type 1 and type 2 copper were measured (Fig. 4b). Reduced caeruloplasmin was fully reoxidised in the presence of hydrogen peroxide [20]. Prolonged incubation and addition of a two-fold molar excess of H_2O_2 did not increase any of the above specific parameters. Thus, the initial low type 1 copper formation cannot be attributed to the presence of reduced caeruloplasmin. On the basis of these data there is no evidence for selective incorporation into one site.

The present results allow the conclusion that there is indeed a sequential incorporation of Cu(I) into the empty characteristic binding sites of caeruloplasmin. The diminished specific A_{610} absorption is indicative

that at the beginning considerable amounts of type 3 (and/or type 4) copper are preferentially incorporated. After 3–4 g-atoms (including most of the type 1 and type 2 copper), both absorption and oxidase activity surpass transient maxima. Then the type 3/4 copper are further incorporated to reach the known stoichiometry of six copper moles per mole of protein.

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