Copper(I) transfer into apo-stellacyanin using copper(I)-thiourea as a copper-thionein model

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The direct incorporation of Cu(I) from $[\text{Cu(I)}(\text{thiourea})_3]\text{Cl}$, a structural analogue of Cu-thionein, into apo-stellacyanin, was successful both aerobically and anaerobically. A characteristic c.d. band of Cu(I)-stellacyanin at 270 nm ($\theta = -12.5 \times 10^3$ degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$) was seen. On oxidation with hexacyanoferrate(III) or by air, the correct Cu(II) binding into the active centre of this 'Type 1' Cu-protein was deduced from chiroptical measurements which were supported by e.p.r. data. Thus Cu-thiourea turned out to be an excellent Cu(I)-donor in aqueous systems for the complete reconstitution of mononuclear Blue copper proteins.

The molecular mechanism of copper transfer from possible copper proteins is unknown. Both Cu(II) and Cu(I) are co-ordinated more-or-less specifically to proteins. For example Cu_2Zn_2 -superoxide dismutase is a well-characterized and good candidate for Cu(II). The exclusive co-ordination of Cu(I) is found in Cu(I)-thionein (Weser *et al.*, 1977). This protein is discussed as an important case for Cu(I) transfer (Hartmann *et al.*, 1983; Morpurgo *et al.*, 1983). A direct Cu(I) transfer seemed highly desirable to avoid many uncontrolled side reactions.

The apoprotein of stellacyanin, a well-known representative of the Type 1 Cu-protein, was shown to be successfully reconstituted both aerobically and anaerobically in the presence of yeast Cu-thionein (Hartmann *et al.*, 1983). On oxidation by air or hexacyanoferrate(III), e.p.r. signals were indicative of the specific binding of Cu(II) into the active centre. No spurious copper was detectable.

During the course of the structure elucidation of Cu-thionein, Cu-thiourea proved to be a prominent model for the copper thiolate chromophore. $[\text{Cu(I)}(\text{thiourea})_3]\text{Cl}$ is well known to form polymeric corner-linked tetrahedra (Okaya & Knobler, 1964). One double-bonded sulphur atom serves as the bridging ligand. In Cu-thionein the stoichiometry is 8 thiolate sulphur atoms per 4 copper atoms. Essentially identical e.x.a.f.s. spectra were obtained, suggesting the same tetrahedral arrangements of the sulphur atoms around each copper

atom. By assuming a more closely packed structure in Cu-thionein, the best guess for arranging 4 Cu-thiolate tetrahedra fitted the cubane type of many known Cu(I) compounds (Bordas *et al.*, 1982). The stability of Cu-thiourea in aqueous solution prompted a functional study on its possible Cu(I)-thionein-like reactivity. In addition to e.p.r. spectroscopy, c.d. measurements were included for the control of the reconstitution experiments with stellacyanin. Fortunately no chiroptical interference of the characteristic Cotton bands of stellacyanin were seen when Cu-thiourea was used.

Experimental

Stellacyanin was purified as described by Reinhammar (1970) from the acetone-dried powder of the latex of the Japanese lacquer tree (*Rhus vernicifera*) (supplied by Saito and Co., Osaka Japan). The apoprotein was obtained as described previously (Morpurgo *et al.*, 1974) by dialysis against 20 mM-KCN/20 mM- Na_2CO_3 , pH 11. It contained less than 5% residual copper and could be reconstituted with CuSO_4 up to 90% of the original Cu-content.

Crystalline $[\text{Cu(I)}(\text{thiourea})_3]\text{Cl}$ was synthesized as described by Okaya & Knobler (1964). Copper was quantified by atomic-absorption spectroscopy on a Perkin-Elmer 400 S spectrometer equipped with a HGA-768 unit. E.p.r. spectra were run on a Varian E-9 spectrometer at -160°C , and c.d. measurements were performed on a Jasco 500 spectropolarimeter.

Abbreviations used: e.x.a.f.s., extended X-ray absorption fine structure.

Anaerobiosis was obtained by repeated cycles of evacuation-flushing of the samples with purified argon in a Thunberg apparatus sealed to an e.p.r. tube or to a quartz spectropolarimeter cuvette.

Results and discussion

The direct incorporation of Cu(I) from Cu(II)-thiourea into apostellacyanin was successful within 5 min, both aerobically and anaerobically. The Cu(I) binding into the active centre was clearly monitored by c.d. Under anaerobiosis a c.d. spectrum different to that of the apoprotein and Cu(II)-stellacyanin appeared (Fig. 1). A negative Cotton band was observed at 270 nm, which was assigned to the Cu(I)-protein ($\theta = -12.5 \times 10^3$ degrees $\cdot$ cm 2 $\cdot$ dmol $^{-1}$). On converting Cu(II)-stellacyanin with ascorbate into Cu(I)-stellacyanin, exactly the same spectrum was seen. Reconstitution of apostellacyanin with Cu-thiourea at 20°C in the presence of air resulted in the formation of the blue chromophore within 20 h. The chiroptical properties were typical for the Cu(II)-stellacyanin as deduced from the characteristic Cotton extrema at 450, 305 and 265 nm. When hexacyanoferrate(III) was added as the oxidizing agent after Cu-thiourea, the blue colour was recovered immediately. Thus the slow process requiring 20 h was reoxidation by air.

Supporting evidence of the direct Cu(I)-transfer into apostellacyanin using Cu-thiourea was obtained by e.p.r. spectrometry after oxidation by air or hexacyanoferrate(III). No spurious Cu(II) was observed throughout the reconstitution reaction. All stellacyanin-Cu(II) was fully detectable (Fig. 2).

In general, the initial Cu(I) incorporation proceeds much faster compared with the direct addition of Cu(II). This was demonstrated by the anaerobic oxidation of Cu(I)-stellacyanin by hexacyanoferrate(III). Thus Cu(I) only binds to the native site, showing a higher specificity than Cu(II), which is initially bound to non-native ligands (Morpurgo *et al.*, 1974). In comparing the Cu(I) transport from Cu-thionein and Cu-thiourea into apo-stellacyanin, the Cu(I) transfer of the latter compound proceeds considerably faster. This could either be assigned to the lower stability and/or the better accessibility of this complex to the copper-binding site of the protein. Gassing of aqueous solutions of Cu-thionein and Cu-thiourea with H $_2$ S resulted in the spontaneous precipitation of Cu $_2$ S from the model complex. By contrast, Cu-thionein remained stable when the sample was held under H $_2$ S for more than 10 h. Nevertheless, Cu(I)-thiourea survives treatment with aqueous buffers for more than 24 h. It should be preferably used for Cu(I)-reconstitution studies. In contrast,

Fig. 1. C.d. spectra of apo-, Cu(I)-recombined or reduced stellacyanin, reoxidized or native stellacyanin. Apo-stellacyanin (0.04 mM) in 20 mM-Tris/HCl buffer, pH 8.0 (—); plus 0.2 mM-Cu(I)-thiourea or 0.04 mM native stellacyanin, in the same buffer, anaerobically reduced with 0.08 mM-ascorbate (---); apo-stellacyanin plus Cu(I)-thiourea after 24 h incubation in air, or native stellacyanin (----).

Fig. 2. E.p.r. spectra of apo-, Cu(I)-recombined, reoxidized, and native stellacyanin

A, 0.15 mM-apo-stellacyanin in 20 mM-Tris/HCl buffer, pH 8.0; B, plus 0.15 mM-Cu(I)-thiourea; C, plus 0.15 mM-hexacyanoferrate(III); D, 0.15 mM native stellacyanin in the same buffer. Instrument conditions were: microwave frequency, 9.15 GHz; microwave power, 20 mW; modulation amplitude, 1.0 mT; temperature, 100 K.

when [Cu(I)(CH $_3$ CN) $_4$]ClO $_4$ is employed as a Cu(I)-donor, high concentrations of acetonitrile are required for stabilization of Cu(I) (Hemmerich & Sichert, 1963).

The copper co-ordinated sulphur deserves special attention, as thiourea alone is known to act as a scavenger of oxygen radicals. For example, lipid peroxidation is inhibited in the presence of

thiourea (Koster & Slee, 1983). In the course of reconstitution experiments, possible copper-driven Haber-Weiss reactions are minimized (Weser & Hartmann, 1984). Thus this Cu(I) complex is specially suited for reconstitution studies on copper-binding centres where uncontrolled oxidation reactions can take place.

In conclusion, Cu-thiourea can be considered not only as a structural model but also as a convenient functional analogue for Cu-thionein of both microbial and vertebrate origin.

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