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An X-ray structural study of human ceruloplasmin in relation to ferroxidase activity

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Abstract The role of ceruloplasmin as a ferroxidase in the blood, mediating the release of iron from cells and its subsequent incorporation into serum transferrin, has long been the subject of speculation and debate. However, a recent X-ray crystal structure determination of human ceruloplasmin at a resolution of around 3.0 Å, in conjunction with studies associating mutations in the ceruloplasmin gene with systemic haemosiderosis in humans, has added considerable weight to the argument in favour of a ferroxidase role for this enzyme. Further X-ray studies have now been undertaken involving the binding of the cations Co(II), Fe(II), Fe(III), and Cu(II) to ceruloplasmin. These results give insights into a mechanism for ferroxidase activity in ceruloplasmin. The residues and sites involved in ferroxidation are similar to those proposed for the heavy chains of human ferritin. The nature of the ferroxidase activity of human ceruloplasmin is described in terms of its three-dimensional molecular structure.

Key words Ceruloplasmin · Ferroxidase · Iron Metabolism · Copper transport

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Introduction

It has long been recognised that ceruloplasmin is an oxidase, the oxidation of *p*–phenylenediamine is a classic assay for the enzyme, and similarities between the structures of ceruloplasmin [1] and ascorbate oxidase [2] give strong support for this oxidase function. In particular, it has been recognised that *in vitro* the enzyme can act as a ferroxidase, and the substrate that shows the lowest K_m and highest V_{max} appears to be Fe(II) [3]. However, the outstanding question is whether this ferroxidase activity is one of the enzyme's main biological functions in the blood. In 1968 Lee, Nacht, Lukens and Cartwright [4] reported that pigs made copper-deficient by dietary restrictions were unable to release iron to the plasma and had low levels of ceruloplasmin. A series of articles (see for example [5–7]) have suggested a multi-functional role for the enzyme in the blood including a ferroxidase activity whereby ceruloplasmin is involved in mediating the release of iron from cells, oxidation of the iron to the Fe(III) state, and its subsequent incorporation into *apo*–transferrin. The presence of free Fe(II) in the environment is dangerous, since it can participate in Fenton and Haber-Weiss-type reactions to yield the biologically deleterious $OH^{\bullet}$ radical. On the other hand, free Fe(III) at physiological pH is almost insoluble. Little is known about the mechanisms whereby iron is released from cells and in what form. There is no firm evidence that transferrin interacts directly with ceruloplasmin or that ceruloplasmin oxidises Fe(II) to Fe(III) which is then taken up by transferrin. Indeed, in vitro, transferrin has such a great avidity for Fe(III) that it will act as a ferroxidase, and a standard method of loading iron into *apo*–transferrin is by ad-

ding ferrous sulfate. However, in vivo, ceruloplasmin could be required to assist transferrin in preventing the release of Fe(II) ions into the environment, particularly during the onset of infection or some other acute phase such as bodily injury. Alternatively, in tissues where there are low levels of oxygen (such as parts of the liver) then the enzymatic activity of ceruloplasmin may be essential for ferroxidation. This article will address the putative role of ceruloplasmin as a ferroxidase in vivo. Firstly, the metal centres in ascorbate oxidase and ceruloplasmin will be compared. Secondly, mutations in ceruloplasmin associated with systemic haemosiderosis will be discussed in terms of the molecular structure. Finally, cation soaking experiments will be discussed from which mechanisms of ferroxidase and other substrate oxidation can be hypothesised.

Experimental

X-ray crystallography

Crystals of human ceruloplasmin were obtained as previously described [1] and some preliminary cation-soaking experiments undertaken. The conditions for which diffraction data were usefully collected are listed in Table 1. Considerably longer soaks and/or higher concentrations of reagents caused deterioration in the crystallinity so that only low resolution data (worse than 3.5–4.0 Å) could be collected, whereas much shorter soaks and/or lower reagent concentrations gave smaller or insignificant changes in the native diffraction pattern. The experiments described in Table 1 are fully reproducible, but other soaking conditions and their associated effects require investigation in order to achieve a comprehensive analysis of cation binding to ceruloplasmin; such studies are subject to the future availibility of synchrotron resources and will be reported elsewhere. Diffraction data were collected in each case at 276 K on station 9.6 at the SRS, CCLRC Daresbury Laboratory using the oscillation method and wavelengths in the range 0.87–0.92 Å. The detector was a 30-cm diameter Mar-Research image plate system, and the principal statistics of the data collection are given in Table 1. In the case of the ferrous sulfate soaking experiment, a further data set was also collected on station 9.5 using a wavelength of 1.4 Å. All the oscillation images were processed using the MOSFLM [8] suite of programs, and final scaling and data reduction were achieved with ROTAVATA and AGROVATA (CCP4 program suite [9]).

For each soaking experiment, the data were scaled against the data for the native protein using SCALEIT (CCP4 program suite [9]) and a difference Fourier synthesis was computed using coefficients $|F_{\text{native+cation}} - F_{\text{native}}|$ and phase information calculated from the native structure and also with the original phase information derived by the program DM [10]. Within experimental error, both sets of phases gave the same difference electron density features which were interpreted in terms of cation-binding sites. No attempts have been made to refine the cation binding sites due to the limitation in the resolution of the diffraction patterns.

EPR and X-ray fluorescence analysis

EPR spectra of the native ceruloplasmin crystals were recorded with a Brücker ER 200D-SRC spectrometer at 9.41 GHz and 77 K. Metal analyses were performed by the energy-dispersive X-ray fluorescence spectrometry (EDXRF) method described by Viksna, Mwiruki, Jagner and Selin [11]. The crystals were analysed with a secondary target energy-dispersive X-ray fluorescence spectrometer. Elemental concentrations were calculated from the intensities of characteristic X-ray lines when the samples were irradiated with monochromatic X-rays from a secondary molybdenum target.

Results and discussion

Throughout the following discussion it should be clearly remembered that the resolution of the data is limited to around 3.0 Å and that precise details of the structure cannot be described. Details of the overall organisation

Table 1 Crystal soaking conditions and X-ray data collection

	Native hCP	Co(II)	Fe(II)	Fe(III)	Cu(II)
Soaking conditions					
Reagent		$CoCl_2$	$FeSO_4$	$FeCl_3$	$CuSO_4$
Concentration		1 mM	1 mM	1 mM	1 mM
Time (h)		72	3	21	60
Data collection[b]					
Number of crystals	7[a]	3	1	1	1
Total number of measurements	312967	105098	67707	69801	55324
Number of unique hkl	44533	34053	34496	26643	29197
Resolution (Å)	3.0	3.2	3.2	3.2	3.5
Overall R_{merge}	0.072	0.072	0.049	0.055	0.059
Number of measurements contributing to R_{merge}	311591	100749	55317	67407	46689
Multiplicity	7.0	3.1	2.0	2.6	1.9
R_{merge} for highest resolution shell	0.276	0.236	0.206	0.228	0.243
Overall % completeness	98.5	93.4	91.4	71.7	97.4
% completeness in highest resolution shell	93.6	93.1	93.3	74.8	93.1
Overall % of hkl with $I > 3\sigma(I)$	83.3	80.3	82.8	84.4	80.3
% of hkl with $I > 3\sigma(I)$ in highest resolution shell	51.2	51.2	53.3	57.2	47.6

[a] The data for the native protein results from two data collection runs, the first involving two crystals with an effective resolution of 3.1 Å and 94.7% complete and a second involving five crystals to an effective resolution of 3.0 Å and 95.8% completeness

[b] In each case data were collected on station 9.6 at the SRS using wavelengths in the range 0.87–0.92 Å

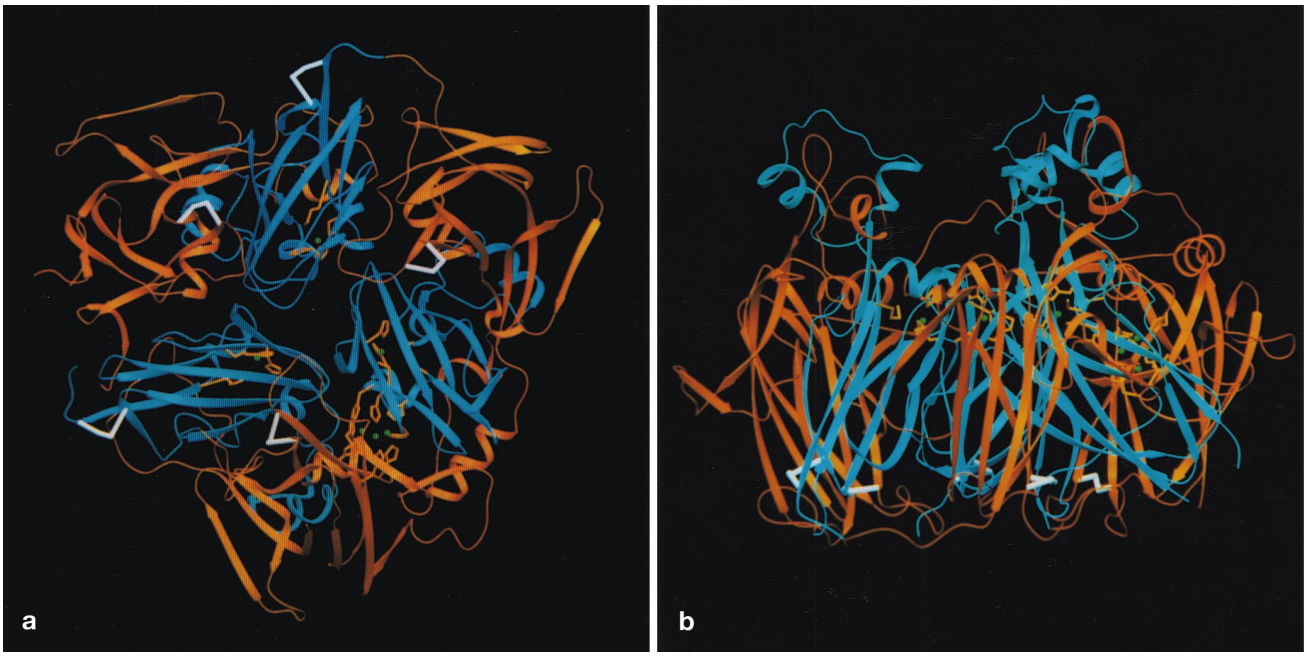

Fig. 1a,b The overall tertiary structure of human ceruloplasmin colour coded to highlight the sequence and structural triplication, **a** parallel and **b** perpendicular to the pseudo-threefold axis. The 6 domains have cupredoxin-type folds [19] with large loop insertions, particularly between the first and second strands of each domain. Domains 2, 4 and 6 contain mononuclear copper atoms (coloured *green*), whereas a trinuclear copper centre is located at the interface of domains 1 and 6. The configuration and location of the trinuclear copper centre and the nearest mononuclear copper in domain 6 is essentially that found in ascorbate oxidase. The disulfide bridges, coloured *white* and located in the first five domains are at the bottom (almost planar) surface of the molecule

of the enzyme and the six integral copper-binding sites have been described previously [1], but Fig. 1 shows backbone traces of the polypeptide chain viewed along and perpendicular to the pseudo-threefold axis of the molecule respectively and colour coded to emphasise the threefold repeat in the structure.

The metal centres in ascorbate oxidase and ceruloplasmin

In ascorbate oxidase [2], the substrate binds close to the mononuclear copper site and donates an electron. This electron is then transferred through a cysteine residue to one of two histidine residues bound to the trinuclear copper cluster. Dioxygen binds to the cluster and is reduced to two molecules of water after a four-electron transfer. Ceruloplasmin has an almost identical arrangement of copper atoms involving the domain 6 copper and the trinuclear cluster, but in addition there are two further mononuclear copper atoms in domains 2 and 4. The disposition of the mononuclear coppers, with interatomic separations of around 18 Å, is intriguing; had nature organised the molecule so that the copper atoms were bound in the odd-numbered domains, these inter-mononuclear copper distances would increase substantially, since these domains point outwards from the pseudo-threefold axis. Langen et al. [12] have studied the networks for coupling the internal redox centres in azurin and cytochrome *c* with the surface of these proteins. A distance of 18 Å between metal centres is well within the range for effective electron transfer, and, as shown in Fig. 2, there are clear pathways linking the mononuclear copper atoms in ceruloplasmin. At one end of the pathway is a hydrogen bond interaction between an N atom of a histidine residue, bound to the first copper atom by its N^{δ} atom, and the side chain of a conserved glutamate. The pathway then passes through an X−D−X sequence, where X is a hydrophobic residue, to a second histidine attached to the second copper atom. The presence of the aspartic acid probably indicates that transfer can take place through the main chain atoms and/or a combination of main chain and aspartate and histidine side chains. The pathways linking the three mononuclear copper atoms can be summarised as follows;

Cu2-H276	V275	D274	V273	E272	H-bond …				H1026-Cu6
Cu4-H685	… H-bond …			E971	I972	D973	L974	H975	-Cu6
Cu2-H324	… H-bond …			E633	A632	D635	V636	H637	-Cu4

In the electron density synthesis at 3.0 Å resolution, the H324 N ..... O^{δ} E633 interaction in the Cu2 to Cu4 pathway is long at 3.9 Å, compared with values of 3.0 Å for the other two pathways. Whether this is artefactual because of the limitation in resolution or a real difference implying that transfer of electrons between Cu2 and Cu4 is not significant must await a higher resolution structure. In practice, as an oxidase, the transfer of electrons through Cu6 to the trinuclear cluster would be of prime importance, since the trinuclear centre

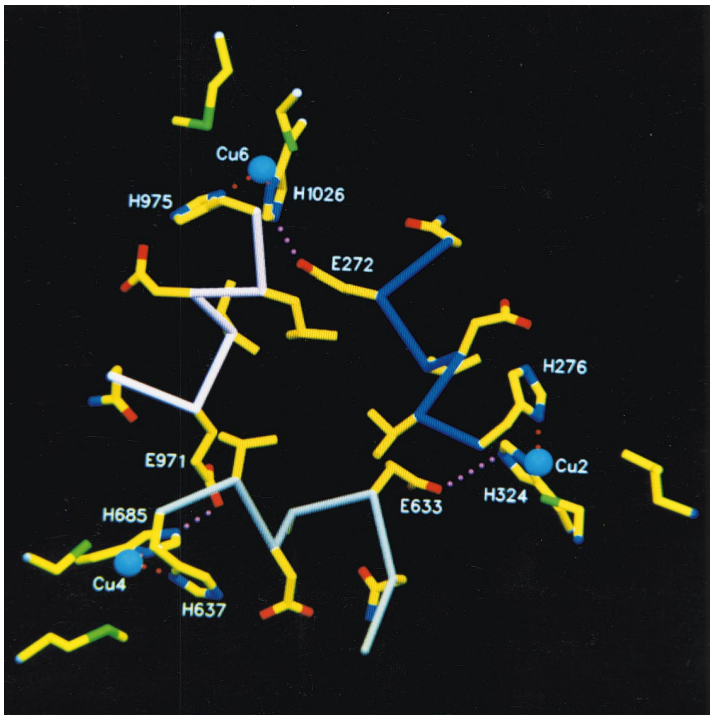

Fig. 2 Electron transfer pathways between the mononuclear "integral" copper atoms in ceruloplasmin

would be the site where oxygen is reduced to water. The electron transfer would normally be expected to be slower by the routes involving the coppers in domains 2 and 4 than by that involving Cu6 because of the extra distance that the electron has to travel, and this may imply that the enzyme can oxidise more than one substrate molecule at a time.

In ascorbate oxidase, two molecules of azide can bind at one of the type III copper atoms in the trinuclear centre [13], and this is also thought to be the site of oxygen binding. In the case of ceruloplasmin a gold cyanide derivative was used in the structure solution. For this derivative, a linear NC-Au-CN molecule, the gold atom was found to bind at 5.6 Å and 6.3 Å from the two type III copper atoms of the trinuclear cluster respectively, in a channel leading to the surface of the protein and with no chemically sensible Au - protein interactions. Assuming Au-C and C≡N distances of 2.0 Å and 1.15 Å respectively, the closest approach of a CN nitrogen to a type III copper would be 2.45 Å. An explanation of this observation is that the derivative is binding through one of the cyanide ligands (as opposed to the gold atom itself) in a similar way to that of azide to ascorbate oxidase. This may suggest that oxygen also binds to ceruloplasmin in a similar way to that for ascorbate oxidase.

Systemic haemosiderosis

Recent papers [14–17] associating mutations in the ceruloplasmin gene with aceruloplasminemia and neurovisceral haemosiderosis have also focused attention on the putative role of ceruloplasmin as a ferroxidase. In certain cases, systemic haemosiderosis appears to be caused by incomplete expression of the ceruloplasmin molecule, with the polypeptide chain being prematurely terminated. These observations gives credence to the proposition that the enzyme may be involved in facilitating the release of intracellular iron and its oxidation prior to uptake and transport by transferrin. Further, the X-ray structure of human ceruloplasmin provides structural explanations as to why, if the truncated molecules are actually secreted into the bloodstream, they will have their oxidase function severely impaired and may be highly susceptible to proteolytic cleavage. Thus, Fig. 3 shows the trace of the α-carbon polypeptide backbone for domains 1 and 6 of human ceruloplasmin only; domain 1 is shaded red on the left hand side of the figure. The blue and magenta structures on the right hand side of the figure represent domain 6, and, in particular, the blue trace is the portion of the polypeptide chain, residues 991 to 1046 inclusive, which would be missing if incorrect expression terminated the chain at 991 [14]. The copper-binding ligands that would be concomitantly missing comprise three of the ligands that bind to the domain 6 mononuclear copper (C1021, H1026 and M1031). In the absence of these ligands, it can be concluded that domain 6 is very unlikely to bind copper, and this should have a profound effect on the oxidase efficiency of the enzyme. In addition, two of the histidine residues that bind to the trinuclear cluster (H1020 and H1022) are also absent, which gives rise to the possibilities that this cluster may not bind in the same way as in the intact enzyme or that it may not bind at all; in either case this will affect the oxidase activity of the enzyme. Further, the amino acid residues on the missing peptide contribute over 50% of the hy-

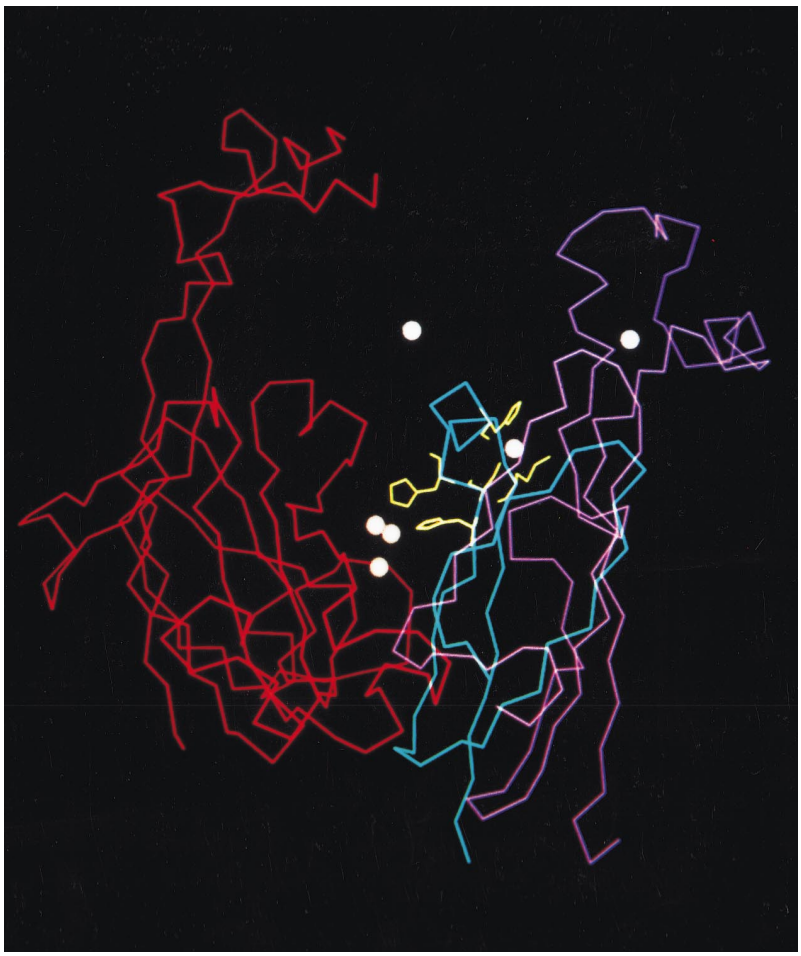

Fig. 3 Traces of the α-carbon polypeptide backbone of domains 1 and 6 in the human ceruloplasmin structure. On the left hand side, domain 1 is shown in red; this domain contributes four histidine residues to the trinuclear cluster (copper atoms are depicted as white spheres). Domain 6 is on the right hand side of the figure and also contributes four histidine residues to the cluster. The portion of the polypeptide chain coloured *blue* is that which would be missing in the truncated enzyme. This polypeptide, residues 991 to 1046 inclusive, includes two histidine residues bound to the trinuclear centre and three residues bound to the mononuclear copper in domain 6; these residues are depicted in *yellow*. The absence of this carboxyl-terminal polypeptide would also remove over 50% of the interdomain hydrogen-bond and ion-pair interactions observed in the intact enzyme

drogen-bond and ion-pair interactions between domains 1 and 6 in the intact enzyme. It is therefore highly probable that the incomplete enzyme will adopt a different organisation of the tertiary structure and may well exist in an open configuration. Such a configuration will be even more susceptible to proteolytic cleavage than the correctly folded enzyme.

The net effect of the missing C-terminal portion of the polypeptide chain is that the enzyme may not fold into the organisational tertiary structure depicted in Fig. 1 and that its ability to act as an oxidase will be severely impaired with respect to the intact enzyme. Incomplete folding and increased susceptibility to proteolytic cleavage would be consistent with the observed aceruloplasminemia such as the failure to find ceruloplasmin in liver extracts by the Western blot and ELISA methods [14]. An impairment of the ferroxidase activity would be consistent with a gradual accumulation of intracellular iron leading to systemic haemosiderosis; the truncated enzyme would not mediate the release of iron from cells nor its oxidation prior to uptake by transferrin. Truncation of the ceruloplasmin chain at residue 858 [16, 17], that is, with the whole of domain 6 and a portion of domain 5 missing, would be even more catastrophic. The secretion of this protein into the bloodstream would result in complete loss of ferroxidase activity through the mechanisms proposed above, and, since the molecular fold would probably be far more open than as shown in Fig. 1, the protein would be even more susceptible to proteolytic degradation.

Putative mechanism for the biological ferroxidase activity of ceruloplasmin

In this section, various items of circumstantial evidence will be presented which enable rational speculation regarding the mechanism of substrate oxidation.

Surface charge distribution

Figure 4 shows charge distributions, computed by the GRASP program incorporating the Del Phi algorithm [18], over the bottom and top surfaces of the ceruloplasmin molecule (see Fig. 1b). Whereas the almost smooth bottom surface is comparatively featureless, the top surface shows a pronounced distribution of negative charge in between the large loops, connecting strands 1 and 2 of each domain. Such a charge distribution appears to be designed for attracting positively charged metal ions towards the mononuclear copper

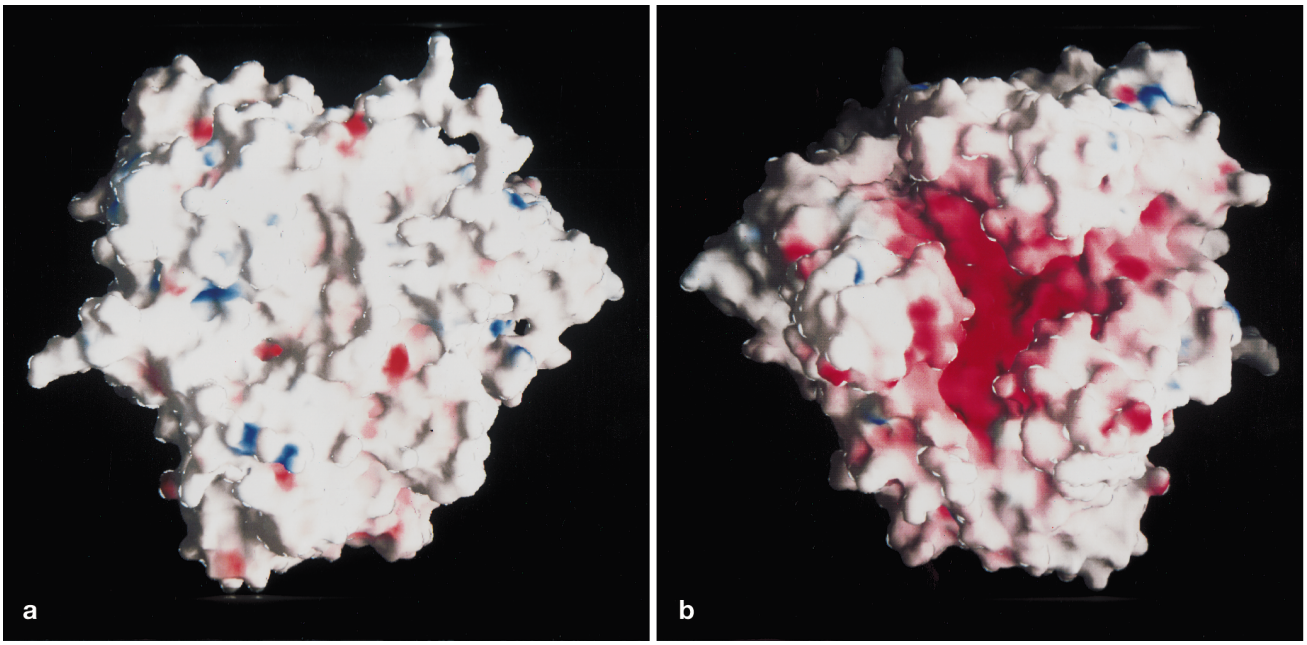

Fig. 4a,b Charge distributions on the surface of the ceruloplasmin molecule, computed using the GRASP program [18] and including the Del Phi algorithm, showing **a** the bottom, almost planar surface of the molecule, and **b** the negatively charged regions on the top of the molecule amongst the large loops formed by insertions between the first and second strands in the six domains. The negatively charged regions may attract positively charged cations chelated by small ligands, while the loops may restrict the access of large macromolecules

centres. However, the size of the cations and their local environments will be restricted by steric effects caused by the loops, and this can be contrasted with the case of nitrite reductase [19]. In the latter structure, the type I mononuclear copper atoms are located in the odd domains towards the outside of the trimeric molecule, consistent with the requirement for electron transfer between nitrite reductase and pseudo-azurin, a molecule comprising some 130 amino acid residues.

The "labile" cation-binding sites in domains 4 and 6

After refinement of the structure at 3.0 Å resolution [1], difference Fourier syntheses revealed two well-defined electron density maxima some 9.0–10.0 Å distant from the integral mononuclear copper sites in domains 4 and 6. Indeed, a re-examination of the original density-modified electron density synthesis prior to refinement also showed maxima in these positions at roughly half the peak height of the copper atoms. Initially these peaks were thought to be solvent molecules, but, in the light of the cation-binding experiments described in Table 1, it is possible to interpret these maxima as two additional metal binding sites, now termed the "labile" cation sites. For the domain 6 labile site the ligating residues are H940, E935 and D1025 from domain 6 and E272 from domain 2 (see Fig. 5). The corresponding ligands for the domain 4 site are H602, E597 and D684 from domain 4 and and E971 from domain 6, although the relative diffuseness of the peaks indicates less order than for the domain 6 labile site. No peak is found in domain 2 where the equivalent histidine residue has been replaced by Y241 and the remaining domain 2 residues are E236, N323 with E633 from domain 4.

In ascorbate oxidase, the organic substrate is believed to bind in the vicinity of the mononuclear copper centre. It has been proposed that one of the histidine residues bound to the copper by its N^{δ} atom has the N atom available for substrate interaction and that the site is bounded by two tryptophan residues [2]. Neither of these tryptophan residues is conserved in any of the mononuclear copper binding domains in ceruloplasmin, but H940 in domain 6 and H602 in domain 4 are in roughly equivalent positions to one of these tryptophan residues.

The nature of the metals in these "labile" sites in the native protein cannot be determined unambiguously from the electron density alone, but X-ray fluorescence analysis on the native crystals indicates that only copper is present to any significant extent, so that this metal must occupy both the labile sites and the integral sites. Other metals are also detected with this very sensitive analytical method, but their concentrations in the crystal are much lower than the copper content. Thus Fe and Ni are present at less than 1%, Zn less than 2% and Ca less than 4% of the copper content. Furthermore the EPR spectrum of the native crystals, Fig. 6 spectrum A, confirms that there are copper atoms bound to sites other than the integral copper sites. Figure 6, spectrum B, shows a spectrum of a ceruloplasmin preparation that has been dialysed against 0.05 M acetate buffer at pH 5.5 and containing 0.5 mM EDTA as a chelating agent [20]. In spectrum A there are extra in-

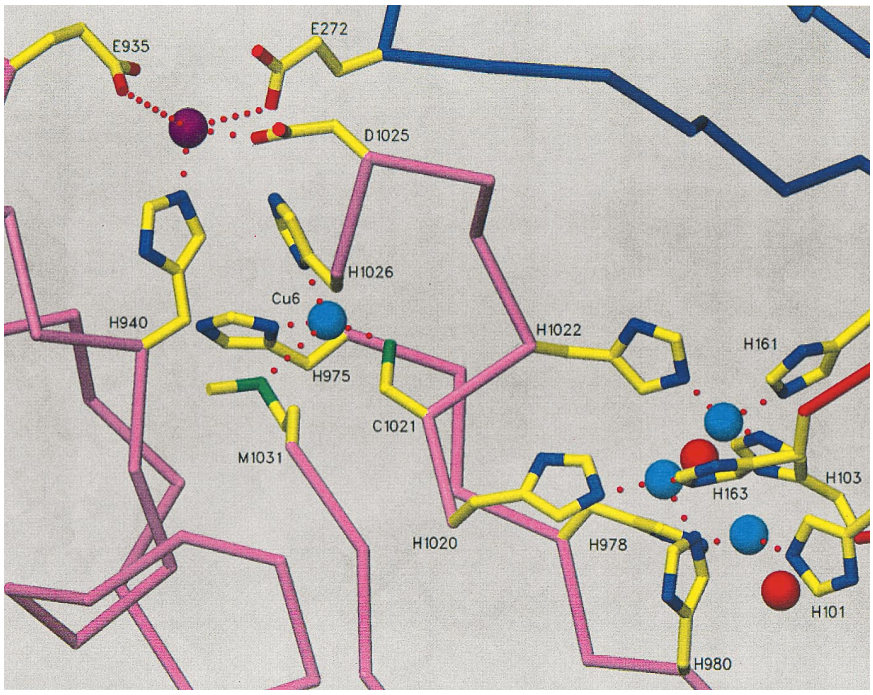

Fig. 5 The labile cation-binding site in domain 6. This site is surrounded by three negatively charged residues and a histidine and could possibly accommodate more than one cation. The histidine is some 3–4 Å away from one of the histidines bound to the mononuclear integral copper in domain 6, thus facilitating transfer of electrons between these two residues. In the native structure the labile sites are partially occupied by copper ions

tensities at about 2670 and 2810 G and these lines may represent the –3/2 and –1/2 hyperfine lines of a new copper species. If so, the EPR parameters $g_{\|}$ and $A_{\|}$ would be about 2.33 and 140 G respectively, indicating that the metal is preferentially coordinated to oxygen ligands. The integrated intensity of all species between 2600 and 2780 G is about 40–50% of the whole spectrum, and this should be compared to the type II copper intensity of the native protein, which is about 30% of the total integrated spectrum. In the crystal structure, the heights of the electron density maxima in the labile sites are approximately 50% of the main copper peaks, indicating incomplete site occupancy and a total copper content of around seven atoms per ceruloplasmin molecule, six integral copper sites fully occupied and two labile sites roughly 50% occupied. Such a number compares favourably with values obtained by other workers who purify ceruloplasmin in the absence of chelators, e.g. 6.6(2) by Erhenwald and Fox [21].

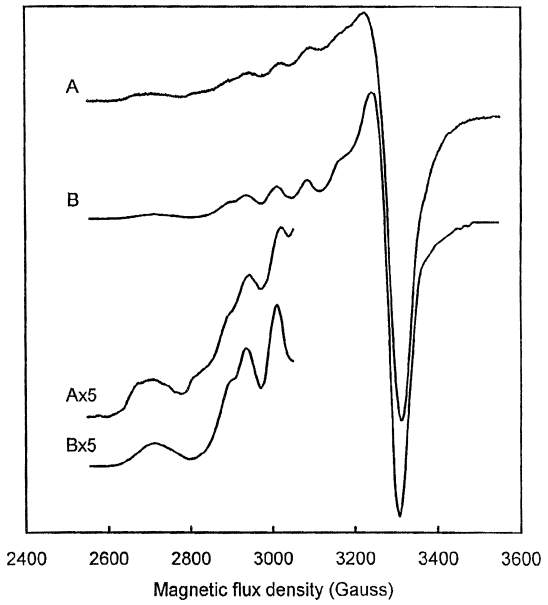

Fig. 6 EPR spectra of the native crystals of ceruloplasmin (A) and previously reported ceruloplasmin in solution (B); spectra were recorded at 9.41 Ghz and at 77 K. Parts of the spectra are also shown with a five-fold magnification

When crystals of native ceruloplasmin are soaked with $CuCl_2$ then additional copper appears in the labile metal-binding sites (Table 2). There are at least two additional sites under the soaking conditions used. One of these additional sites is at the top of the molecule, partially protected by the large domain loops (nearest residues H737, H1028, Q740 & Q742) whilst the second appears more accessible in the vicinity of H988 towards the planar bottom side of the molecule. Whether these additional sites are physiologically important is not yet known, but the labile cation binding sites in domains 4 and 6 could clearly be utilised for copper transport in a manner independent of protein turnover.

Cation-binding experiments

The labile metal-binding sites in domains 4 and 6 are also accessible by other metal cations as indicated in Table 2, when crystals of the native enzyme are soaked in buffers containing various metal cations. The soaking conditions are only representative. A prolonged soak of the native crystals with both Fe(II) and Fe(III) cations produced interesting colour changes [from sky blue to bright orange in the case of $Fe(II)SO_4$], but the crystallinity, and hence resolution of the corresponding

Table 2 Difference electron density syntheses and cation binding sites. For each cation, a difference synthesis was computed using coefficients $|F_{\text{native+cation}}| - |F_{\text{native}}|$ and phase information derived from the structure of the native protein and also with the original phase information derived using the program DM [21]; in each case the difference syntheses showed the same features at the cation binding sites

Metal cation	Peak height (rms)				Comment
	Domain 4 sites		Domain 6 sites		
	Labile	Holding	Labile	Holding	
Native	+ 4	–	+ 6	–	Determined as Cu(II) by EPR and X-ray fluorescence analysis
Cu(II)	+10	–	+ 6	–	Cu(II) fills up labile sites. Weak additional Cu(II) sites also observed – see text.
Co(II)	+20	–	+14	–	Co(II) either fills up labile sites or replaces labile Cu(II) at these sites.
Fe(II)	− 4	+6	−10	+13	Fe(II) replaces labile Cu(II) and after oxidation to Fe(III) is relocated to the holding sites. At both holding sites the electron density is diffuse indicating multiple sites and/or disorder.
Fe(III)	–	–	−12	+ 8	Fe(III) replaces Cu(II) at the labile site in domain 6 and translocates to the holding site in this domain. The holding site electron density is diffuse – see above. No significant effect in domain 4 under the soaking conditions used.

diffraction patterns, deteriorated dramatically, making any structural changes impossible to interpret. An exploration of other soaking conditions is in progress.

Co(II). Soaking the native crystals with $CoCl_2$ produced large positive peaks at the labile binding sites in both domains 4 and 6 (Fig. 7a). It is not clear whether the labile copper has been replaced by the Co or if the Co has just filled up the partially occupied labile sites, although from the peak heights and other soaking experiments (see below) the former is more likely. Further, the X-ray data alone cannot determine the oxidation state of the metal. The site is rich in oxygen donors consistent with the presence of Co(II), but an alternative scenario could be that the Co(II) has been oxidised to the kinetically inert Co(III), which then remains in the labile sites; future experiments will employ epr and X-ray absorption spectroscopy to attempt to resolve this issue. In the case of metal oxidation at the domain 6 site, electron transfer could presumably proceed through a number of pathways. H940 and H975 are within 3.5 Å of one another, and a transfer of an electron from the cation through them to the mononuclear copper atom, Cu6, would be followed by transfer to the trinuclear centre through C1021 and H1020 and/or H1022. For domain 4 a similar pathway is available from H602 to H685 and Cu4, and then to Cu6 and the trinuclear cluster. However, electron transfer from the cation could also involve D1025, either followed by transfer to H1026 and Cu6 and then to the trinuclear centre or directly through the main chain to H1022 and this centre.

Fe(II). Fe(II) cations also bind to ceruloplasmin, but in a different manner to Co(II). For Fe(II) binding, difference electron density syntheses revealed negative peaks at the labile positions in both domains 4 and 6, indicating loss of labile copper. Concomitantly, positive electron density peaks some 9–10 Å on the solvent side of the labile positions are observed; these peaks are provisionally termed the "holding" sites and their environment is defined by the four negatively charged residues depicted in Fig. 7b. The difference Fourier maps also indicate a movement of side chains E935 and E597 in domains 6 and 4 respectively, away from the labile positions towards the holding sites. Further proof of these peaks being due to the presence of iron was provided when the soaking experiments were repeated and data were collected at a wavelength of 1.4 Å, designed to exploit the anomalous scattering of iron. (Optimisation of the wavelength to around 1.74 Å at the iron Kα edge was not possible for technical reasons). An anomalous difference Fourier synthesis revealed peaks at the holding positions as indicated in Fig. 7b; details of this and other anomalous dispersion experiments will be described elswhere. The existence of binding sites for Fe(II) in both domains 4 and 6 is consistent with studies undertaken by McKee and Frieden in 1971 [22].

An interpretation of these observations is that Fe(II) cation displaces labile Cu(II) and releases an electron. The oxidised Fe(III) is then released from the labile site (causing the negative peak observed in the electron density systhesis) and ligated in the holding site by the negatively charged residues, thus accounting for the positive peaks seen in the normal and anomalous difference Fourier syntheses. E935 in domain 6 (E597 in domain 4) may play an important role in the translocation process, since negative density is observed corresponding to its position in the native protein and a positive peak in a position in which it is orientated towards the holding site. The released electron is transferred to the trinuclear copper centre causing partial reduction of a molecule of dioxygen. Iteration of this procedure would lead to further electrons being transferred to the trinuclear copper centre and

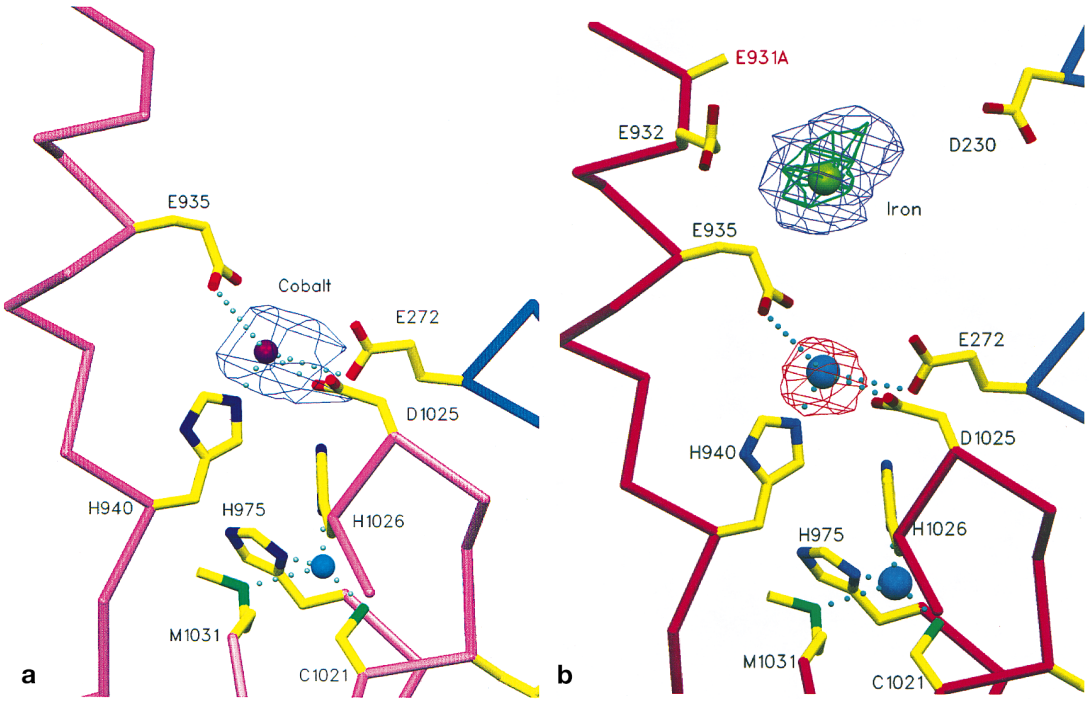

Fig. 7 Cation binding to ceruloplasmin by **a** Co(II) and **b** Fe(II). In **b**, the *red* electron density is negative (at the 8 rms level) indicating loss of the labile Cu(II) cations. The *blue* density is positive (at the 8 rms level) indicating acquisition of cations at the holding sites. The *green* density at the holding site results from an anomalous difference Fourier synthesis using the data set collected with a wavelenght of 1.4 Å and confirms the assignment of this posiure peak to iron cations soaked into the crystal. The holding site electron density maxima in both domains 4 and 6 are rather diffuse, typical of one or more cations occupying a population of slightly different sites. The case for Fe(III) is essentially similar to that for Fe(II) in domain 6; there is no evidence under the soaking conditions used that the domain 4 holding (or labile) sites are occupied

the possibility of more than one ferric iron being stored at the holding site. Domain 4 provides a second, albeit slower, electron transfer route and a second holding site near the outside of the molecule. As in the case of cobalt binding, epr methods will be employed to characterise the oxidation state of the Fe and thus substantiate this proposition.

Whether the holding sites are accessible by small chelating molecules for Fe(III) prior to the uptake of the iron by *apo*–transferrin, or by *apo*–transferrin directly, remains an important question. However, there are similarities between the residues at the holding sites and those proposed for the heavy chains of ferritin [23]. H-type ferritins from humans, *Escherichia coli* ferritin and haem-containing bacterioferritin from *E. coli* have been found to bind a variety of metals at a dinuclear site situated within the subunit four-helix bundle. X-ray analysis of the *E. coli* ferritin has shown that two iron atoms are bound at this centre, 3.8 Å apart, and that the ligands include a histidine and three glutamate residues; additional glutamate residues bind a third iron atom on the inner surface of the protein shell at about 7.0 Å from the iron pair. In ferritin, Fe(II) is oxidised for storage as ferrihydrite or hydrous ferric phosphate, and Fe(III) must be able to move from the ferroxidase centre into the large internal cavity in the ferritin molecule. Higher-resolution studies are required to establish whether the labile sites in ceruloplasmin bind one or two iron atoms. Pending such studies, the presence of side chains similar to those at the ferroxidase centre of ferritin suggests that Nature uses similar environments and mechanisms for ferroxidase activity in the intracellular storage of iron in ferritin and in the removal of ferrous ions in the blood.

Fe(III). The results of Fe(III) cations binding to ceruloplasmin are essentially the same as those observed for Fe(II) cations. A negative peak is observed at the "labile" cation site in domain 6, corresponding to loss of labile copper, and a diffuse positive peak in the vicinity of the "holding" site. Presumably, in this case the Fe(III) cations displace the labile copper and then translocate to the holding site, but without electron transfer. Under the soaking conditions used there are no significant signs of Fe(III) cations binding at the domain 4 holding (or labile) sites.

Summary

A more detailed examination of the ceruloplasmin structure has revealed feasible electron transfer pathways between the three mononuclear copper atoms and the trinuclear centre. The X-ray structure also provides

a rational explanation for the loss of ferroxidase activity in the blood when the enzyme is expressed in a truncated form. Further, two labile cation-binding sites have been identified in addition to the six integral copper atoms. The labile sites are close to the two blue type I copper atoms in domains 4 and 6 and could be utilised for metal oxidation. In the case of cation binding at the labile site in domain 6 an electron could be transferred to the type I copper and then directly to the trinuclear centre through the cysteine-histidine linkages. For cations binding at domain 4, oxidation would be expected to proceed via electron transfer to the type I copper and then indirectly to the trinuclear centre by the transfer pathway linking Cu4 and Cu6. This longer pathway indicates slower electron transfer. There is no equivalent labile site in domain 2 where the methionine residue is substituted by a leucine. There is a similarity between the putative ferroxidase centres in ceruloplasmin and those identified in various ferritins such as the H-type chain in humans. Various cations have been shown either to bind in the labile sites (Co and Cu) or to empty these sites and bind to holding sites towards the outside of the molecule. This series of cation-binding X-ray structures allows a creditable hypothesis for a mechanism of ferroxidase activity by ceruloplasmin. However, this need not imply that ceruloplasmin plays a dominant role as a ferroxidase in the blood, but the enzyme may provide a secondary mechanism to be used in conditions of crisis or in tissues where the oxygen tension is low and other ferroxidases such as transferrin cannot operate efficiently. It is to be hoped that some of these speculations will stimulate a new look at the biochemistry and physiology of ceruloplasmin. Ceruloplasmin is a large and complicated molecule and may play a multifunctional role in the bloodstream. Further structure-function relationship studies should provide more insights into the roles and modes of action of ceruloplasmin.

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