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An X-ray crystallographic study of the binding sites of the azide inhibitor and organic substrates to ceruloplasmin, a multi-copper oxidase in the plasma

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Abstract Ceruloplasmin is a multi-copper oxidase, which contains most of the copper present in the plasma. It is an acute-phase reactant that exhibits a two- to three-fold increase over the normal concentration of 300 μg/ml in adult plasma. However, the precise physiological role(s) of ceruloplasmin has been the subject of intensive debate and it is likely that the enzyme has a multi-functional role, including iron oxidase activity and the oxidation of biogenic amines. The three-dimensional X-ray structure of the human enzyme was elucidated in 1996 and showed that the molecule was composed of six cupredoxin-type domains arranged in a triangular array. There are six integral copper atoms per molecule (mononuclear sites in domains 2, 4 and 6 and a trinuclear site between domains 1 and 6) and two labile sites with roughly 50% occupancy. Further structural studies on the binding of metal cations by the enzyme indicated a putative mechanism for ferroxidase activity. In this paper we report medium-resolution X-ray studies (3.0–3.5 Å) which locate the binding sites for an inhibitor (azide) and various substrates [aromatic diamines, biogenic amines and (+)-lysergic acid diethylamide, LSD]. The binding site of the azide moiety is topologically equivalent to one of the sites reported for ascorbate oxidase. However, there are two distinct binding sites for amine substrates: aromatic diamines bind on the bottom of domain 4 remote from the mononuclear copper site, whereas the biogenic amine series typified by serotonin, epinephrine and dopa bind in close vicinity to that utilised by cations in domain 6 and close to the mononuclear copper. These binding sites are discussed in terms of possible oxidative mechanisms. The binding site for LSD is also reported.

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Introduction

Ceruloplasmin (CP), a copper-containing glycoprotein with a molecular weight of 132 kDa, is found mainly in the plasma. It belongs to a family of multinuclear "blue" oxidases [1] together with ascorbate oxidase and laccase and is composed of a single chain of 1046 amino acids with a carbohydrate content of 7–8%. The X-ray structure of human ceruloplasmin (hCP) [2] reveals that the molecule is composed of six cupredoxin-type domains with large loop insertions. There are six integral copper atoms. Three of these form a trinuclear cluster sited at the interface of domains 1 and 6 and comprise one type II and a pair of type III copper atoms [3]. The remaining coppers occupy mononuclear sites in domains 2, 4 and 6. The copper in domain 2 is probably in the reduced form [4], whereas domains 4 and 6 carry typical type I blue copper atoms. The trinuclear centre and the mononuclear copper in domain 6 form a cluster essentially the same as that found in the structure of ascorbate oxidase, strongly suggesting an oxidase role for hCP in the plasma.

Since the discovery of CP in 1944 by Holmberg [5], an overwhelming number of papers have been published dealing with the different facets of CP in terms of its multi-functionality. In spite of these extensive studies, the precise functions of CP have not yet been defined, but it has been associated with ferroxidase activity, amine oxidase activity, pro- and anti-oxidant activities, inhibition of myeloperoxidase activity [6] and copper transport. The physico-chemical properties, the biological roles and oxidase activity of CP have been the subjects of several comprehensive reviews [7–10].

CP is unique in being able to oxidise both organic and inorganic substrates. The value of the apparent Michaelis constant for Fe(II) is low (values of 0.6 and 5.6 μM have been reported), whereas K_m for organic

substrates is some three orders of magnitude higher. However, this wide difference in K_m is not reflected in the values of V_m, which appear to vary by only an order of magnitude [10]. Such findings have prompted the suggestion that the limiting step in catalysis is a function of the rate of reduction of the blue copper centres and not the affinity of the substrate for the binding site of the protein. Indeed, transient kinetics have shown that for Fe(II) the rate of reduction of the blue copper centres is faster than steady state turnover [11]. Frieden and Hsieh [8] have proposed three major groups of substrates to describe the oxidase action of CP:

1. Fe(II), the substrate with the highest V_m and the lowest K_m.
2. An extensive group of bi-functional aromatic amines and phenols which do not depend on traces of iron for their activity. This group includes two classes of biogenic amines, the epinephrine and 5-hydroxyindole series, and the phenothiazine series.
3. A third group of pseudo-substrates comprising numerous reducing agents which can rapidly reduce Fe(III) or partially oxidised intermediates of class (2) substrates.

It has been suggested that CP catalysed oxidation of biogenic amines, such as epinephrine (also known as adrenalin), norepinephrine (noradrenalin) and serotonin, may be of importance in regulating the level of these stress hormones in the bloodstream and eventually in those areas of the brain where they act as neurotransmitters. Thus hCP by its effect on the lifetime of biogenic amines in the bloodstream could play an important role in the regulation of brain chemistry necessary for mental function. Enhanced oxidation of dopamine by hCP has been invoked to explain the lower dopamine levels found in Parkinson's disease. The effects of drugs used in the treatment of mental illness, e.g. tranquilizers and anti-depressants, on the oxidation of biogenic amines by hCP have also been studied [12]. The mode of reaction of (+)-lysergic acid diethylamide (LSD) with hCP is of particular interest since LSD inhibits the oxidation of serotonin and enhances the oxidation of epinephrine and norepinephrine by hCP [7].

Curson and Cummings [13] have identified seven categories of inhibitors of CP: inorganic anions, carboxylate anions, -SH compounds, chelating agents, hydrazines, 5-hydroxyindoles and a miscellaneous group including divalent and trivalent metal cations. The inorganic anions include two of the strongest inhibitors of oxidase activity, cyanide and azide, both with an inhibitory constant of $K_i \approx 2 \times 10^6$ M. Azide has been frequently used in attempts to distinguish between CP catalysis of Fe(II) oxidation and other ferroxidase activity in the plasma. The binding of azide (and fluoride) to the resting enzyme appears to change the EPR signal from the type II copper [14–15]. These results together with binding and kinetic studies [16–17] indicate that one anion binds at low concentration, but two at high concentration. Recent X-ray studies of the azide complex with ascorbate oxidase [18] clearly indicate that the two azide anions bind to one of the two type III copper atoms. One interpretation of these results could be that during catalysis the trinuclear centre acts as a unit and there is little distinction between the type II and III coppers.

In addition to the X-ray structure determination of the native enzyme, X-ray studies have also been undertaken with the cations Co(II), Fe(II), Fe(III) and Cu(II) [19] bound to hCP. These structural results in conjunction with studies associating mutations in the hCP gene with systemic haemosiderosis (aceruloplasmenia [20–21]) appear to substantiate a ferroxidase role for the enzyme in the plasma. In this context, and with the conventional wisdom, hCP may serve to assist the release of iron from cells prior to uptake of the metal by transferrin. However, it has been recently proposed that CP as a ferroxidase facilitates the uptake of iron by cells [22].

This article will address further X-ray studies of hCP involving complexes with the azide inhibitor, aromatic diamines such as *p*-phenylenediamine, several substrates from the series of biogenic amines (epinephrine, norepinephrine, serotonin and dopa) and LSD. The results will be discussed in terms of the molecular structure and possible oxidation mechanisms will be proposed.

Materials and methods

Crystals of hCP were obtained as previously described [2]. Complexes with inhibitor and substrates were prepared by soaking experiments. In each case, binding was accompanied by a change in colour of the blue native crystals to pale green (azide), dark violet (*p*-phenylenediamine), green (serotonin), grey (dopa), yellow (epinephrine and norepinephrine) or dark grey (LSD). The conditions for which diffraction data were usefully collected are listed in Table 1. Diffraction data were collected in each case at 276 K on station 9.5 at the SRS, CCLRC Daresbury Laboratory, using the oscillation method and a wavelength in the range 0.97–1.05 Å. The detector was a 30 cm MAR-Research image plate system. Prior to synchrotron data collection, diffraction patterns of crystals under a range of soaking conditions were tested on an in-house rotating anode generator equipped with a Rigaku R-axis II image plate detector system. The conditions shown in the table are not exhaustive, but significant variations from them led either to very poor quality diffraction patterns or to no significant changes in intensities from the native pattern. All the images were processed using the MOSFLM [23] program suite; the final scaling and data reduction were achieved with ROTAVATA and AGROVATA (CCP4 suite of programs [24]). The principal statistics of the data collection are also given in Table 1.

For each soaking experiment, the data were scaled against the data for the native protein using SCALEIT [24] and a difference Fourier synthesis was calculated using amplitudes $|F_{\text{native+inhibitor}} - F_{\text{native}}|$ or $|F_{\text{native+substrate}} - F_{\text{native}}|$ and phase information resulting from a combination of the original MLPHARE [24] phases modified by density modification using the program DM [25]; see [2] for further details. The features in the difference electron density maps were then interpreted in terms of the binding sites. No attempts have been made to refine the binding sites owing to the limitations in resolution of the diffraction data sets.

Table 1 Crystal soaking conditions and data collection

	Azide	*p*PD	Epinethrine	Serotonin	Dopa	Norepinethrine	LSD
Soaking condition							
Concentration	10 mM	1 mM	1 mM	1 mM	1 mM	1 mM	3 mM
Time	5 days	22 h	6 days	28 h	6 days	4 days	2.5 days
Data collection							
Number of crystals	2	2	1	1	1	1	2
Total number of measurements	234,035	155,897	108,399	82,059	87,345	113,303	130,058
Number of unique *hkl*	30,272	30,063	35,506	27,897	24,446	30,707	28,472
Resolution (Å)	3.3	3.3	3.2	3.1	3.4	3.3	3.3
Overall R_{merge} [a]	0.083	0.081	0.078	0.082	0.095	0.071	0.086
R_{merge} for highest resolution shell	0.283	0.289	0.304	0.266	0.229	0.272	0.284
Overall % of completeness	88.9	89.3	95.9	70.1	80.0	90.6	83.5
Multiplicity	3.8	2.8	2.0	1.8	1.4	2.7	2.9
Overall % of *hkl*, $I > 3\sigma(I)$	81.1	80.1	69.9	76.6	85.5	76.4	80.3
Overall I/s(I)	6.3	7.9	7.8	6.9	4.7	5.6	7.3
% Completeness in highest resolution shell	90.6	92.9	95.1	70.9	81.0	91.4	84.2
% of *hkl* with $I > 43\sigma(I)$ in highest resolution shell	53.3	50.6	26.2	40.4	62.1	48.6	51.4
$I/\sigma(I)$ for highest resolution shell	2.6	2.5	2.4	2.7	2.6	2.2	2.5

[a] $R_{merge} = \Sigma\Sigma|I(h)_i - <I(h)>|/\Sigma<I(h)>$, where $I(h)_i$ is the observed intensity of the ith measurement of a reflection *(h)* and $<I(h)>$ is the mean intensity of reflection *(h)* over the i measurements

Results and discussion

In the following sections the limitations in the resolution of the diffraction data sets, 3.1 Å at their best, should be borne in mind. Various attempts have been made to improve the resolution of the diffraction patterns, including cryo-cooling methods and the use of the high brilliance synchrotron source at the ESRF, Grenoble, France. The solvent content of the crystals is high, between 65 and 70%, and attempts to increase the percentage of cryo-protectant in the solution followed by flash freezing have so far proved problematic. At the ESRF, using beam line ID2, only some 3–4° of data per crystal could be collected at ambient temperatures before radiation damage became significant. The resolution is very anisotropic, varying from 2.6 Å to 3.0 Å in accordance with the crystal packing. This limit in the resolution clearly means that no detailed information on the binding sites is available, but it is possible to describe their location in the hCP molecule and to postulate possible mechanisms. Figure 1 is an overall view of the hCP molecule viewed almost perpendicular to the pseudo three-fold axis and shows at A the binding site for the biogenic amines, at B the binding site for aromatic diamines and at C the binding site for LSD. The X-ray co-ordinates of the native hCP enzyme are available in the Protein Data Base [26] (formerly at the Brookhaven National Laboratory, USA, and now at The Research Collaboratory for Structural Bioinformatics with the www address: http://www.rcsb.org/pdb/), under the deposition code [1kcw]. The intensity data and phase information used in these studies will be made available on request by the authors.

The azide inhibitor

A single peak with a height of 7.5 rms was found in the difference map close to one of the type III copper atoms in the trinuclear copper cluster and by its elongated nature can clearly accommodate an azide anion, as shown in Fig. 2. A comparison with the structure of the azide/ascorbate oxidase (AO) complex [18] shows that this binding site in hCP occupies a homologous position to one of the two possible azide molecules in the AO structure. Thus, the azide anion is bound to one of the type III copper atoms at a distance of around 2.2 Å in the vicinity of H1020, one of the two histidines implicated in electron transfer between the type I copper centre in domain 6 and the trinuclear cluster; for AO the corresponding distance is 2.19 Å and the histidine residue is H506. Under the crystal soaking conditions used for data collection (relatively high concentration of 10 mM and a long soaking time of 5 days), there was

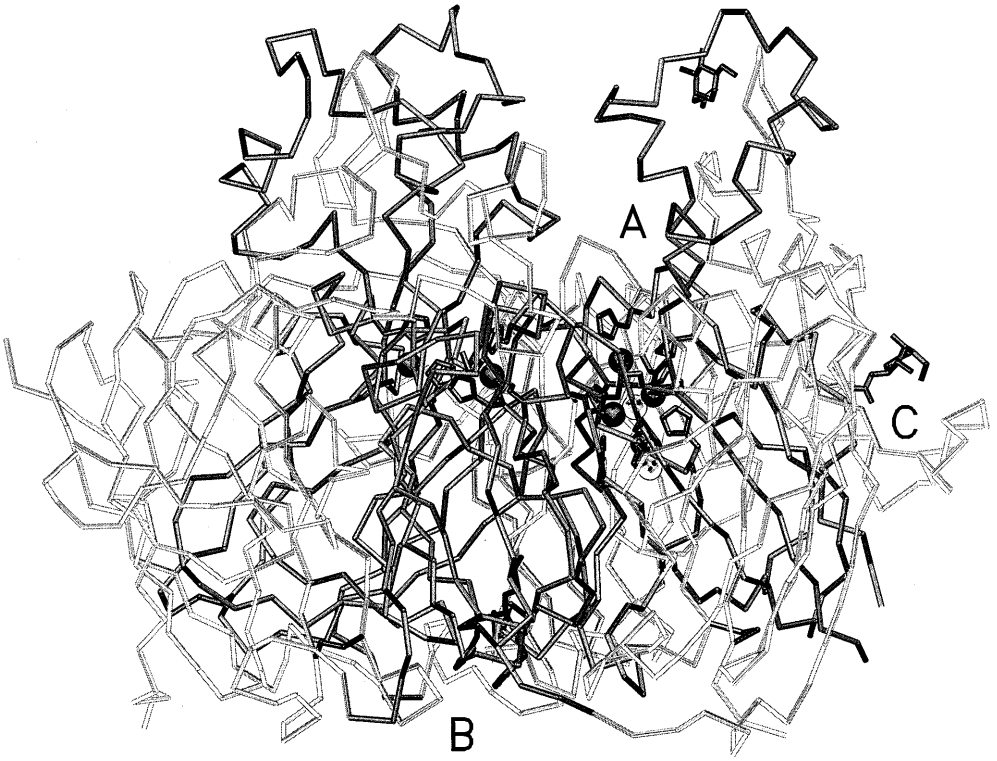

Fig. 1 An overall view of the human ceruloplasmin molecule viewed almost perpendicular to the pseudo three-fold axis. The letter *A* marks the binding site for biogenic amines close to the oxidation site for metal cations in domain 6, *B* marks the binding site for aromatic diamines at the bottom of domain 4, and *C* marks the binding site for (+)-lysergic acid diethylamide close to N119, the attachment site for one of the glycan chains

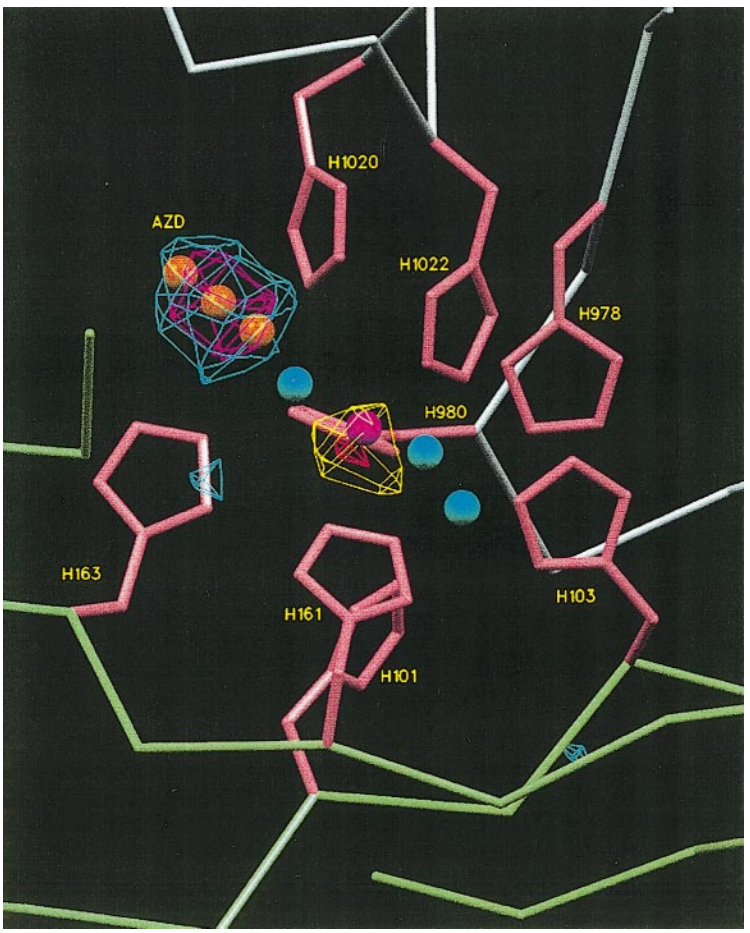

Fig. 2 A portion of the Fourier difference map calculated with data collected from native hCP crystals and one soaked with the inhibitor, azide (see Table 1). Positive density can be seen corresponding to an azide anion attached to one of the type III copper atoms in the trinuclear cluster. This position is almost identical to one of the two positions occupied by azide in the case of ascorbate oxidase (AO) [18]. In addition, a negative peak can be seen between the two type III copper atoms which can be interpreted as loss of the bridging oxygen (OH^-) atom and an increasing separation of the type III coppers as observed for AO

no indication of additional azide anions bound either to the type III copper (as found in AO) or elsewhere in the cluster as predicted by some solution spectroscopic measurements. Whether access to the trinuclear copper cluster is restricted in the crystal owing, for example, to the presence of PEG 20,000 in the crystallisation medium, is a matter of conjecture.

A further interesting feature of the difference map for the azide/hCP complex is the presence of a strong negative peak (–11 rms) between the two type III copper atoms of the trinuclear site (see Fig. 2). For the native hCP structure the resolution did not permit the unequivocal assignment of an oxygen atom (OH^-) between the two type III coppers, although the electron density could accommodate it and its presence has been well established for AO [18]. It has also been shown for AO that, when azide binds, this oxygen is removed and the distance between the type III coppers increases from 3.5 Å to 5.1 Å. The negative peak in the azide/hCP difference map can also be interpreted as loss of the bridging oxygen and increased separation of the type III copper atoms, reinforcing the homology between AO and hCP. Thus, in both cases it appears that the inhibitory effect of azide can be explained in terms of a charge redistribution around the trinuclear copper site, followed by release of the bridging oxygen and an increase in the distance between type III coppers in the trinuclear site. These geometrical distortions then make the binding of the molecular oxygen unfavourable so that electrons can no longer be accepted from substrates, with concomitant loss of oxidation activity by the enzymes.

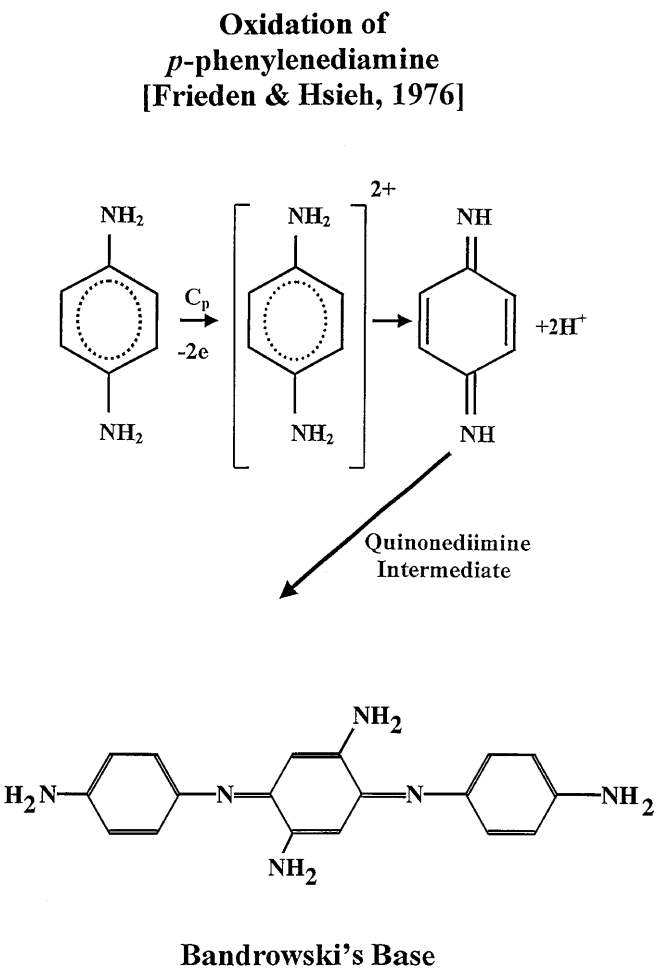

Fig. 3 The oxidation of *p*-phenylenediamine to give a Bandrowski base *via* a quinonediimine intermediate [8]

p-Phenylenediamine

p-Phenylenediamine (*p*PD) is known as a classic assay for the determination of the oxidase activity by CP. It has been shown that the principal product of *p*PD oxidation by CP is a Bandrowski's base [8]; the reaction proceeds *via* formation of the quinonediimine intermediate [27] as shown in Fig. 3. A peak of about 10 Å in length with a maximum height of 11 rms has been identified on the *p*PD/hCP difference map in close proximity (~2.8 Å) to the side chain of W669 in domain 4 (Fig. 4). The shape and size of the peak is sufficient to accommodate the quinonediimine intermediate, but not the complete Bandrowski's base. A second soaking experiment using the chlorine derivative of *p*PD produced an almost identical result. This binding site is intriguing since W669 and H667 are stacked almost parallel to one another so that the histidine ring overlaps the six-membered ring of the tryptophan residue at a distance of around 3.5 Å. This combination of amino acid residues is unique to domain 4. In between these two residues, M668 is oriented inside the domain so that the sulfur atom points upwards towards the type I mononuclear copper site. It is therefore plausible that oxidation of the substrate could take place through its interaction with the π orbitals of W660 and H667 and electron transfer *via* M668 and the cupredoxin domain to the mononuclear type I copper. Further electron transfer to the trinuclear copper centre with concomitant reduction of oxygen can then take place, as previously suggested [19]. The identification of electron density corresponding to the quinonediimine intermediate on the difference maps, as opposed to the substrate or the final product, may be a result of the crystal soaking procedure so that a time-averaged result of the oxidation of *p*PD by hCP is observed.

Biogenic amines: norepinephrine, epinephrine, serotonin and dopa

Figure 5 shows the oxidation reactions involving the biogenic amines norepinephrine, epinephrine, dopa and serotonin. All four substrates bind at the same

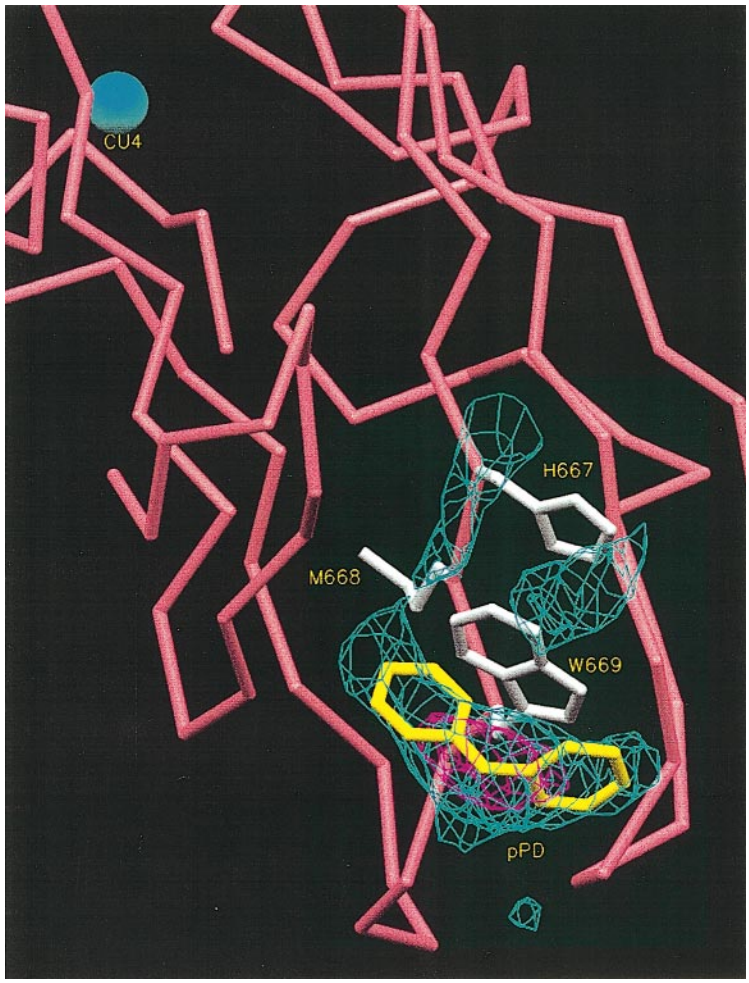

Fig. 4 A portion of the Fourier difference map calculated with data collected from native hCP crystals and one soaked with *p*-phenylenediamine (see Table 1). The elongated density in the region of W669 and H667 could readily accommodate the quinonediimine intermediate

place in the hCP molecule (Fig. 6). In the difference maps a single peak is observed very close to D1025 in domain 6, and some 5.7 Å distant from the labile copper site [19]. The distance from the mononuclear copper site is 9.7 Å. In addition, a negative peak is observed corresponding to loss of the labile copper. Again, the resolution of the data does not permit an unequivocal location of the substrate nor, indeed, whether the peak corresponds to the substrate alone, its oxidation product or a mixture of the two. D1025 is one of the ligands which bind the labile copper together with H940, E935 and E272. According to our previous results on Fe binding [19], the labile copper sites act as a primary electron acceptor in the oxidation of Fe(II) to Fe(III). Unlike the case of Fe(II), there is no evidence, under the soaking conditions used, of binding to the equivalent site in domain 4. The binding of the biogenic amines close to the domain 6 labile site does not seem to be a random choice of nature and the oxidation of biogenic amines may proceed by a similar mechanism to that proposed for the oxidation of Fe(II), i.e. binding of the substrate with displacement of any labile copper, transfer of an electron to the domain 6 copper causing oxidation of the substrate, and eventual reduction of oxygen at the trinuclear copper site. Although the reactions with epinephrine, norepinephrine, serotonin and dopa were observed in crystals, the identical location of all four binding sites suggests an identical mechanism of oxidation of these substances *in vivo*.

(+)-Lysergic acid diethylamide (LSD)

The binding site for LSD (Fig. 7) is completely different to that of either the aromatic diamines or the biogenic amines, as shown in Fig. 8. A difference map reveals a substantial peak close to the glycan moiety at N119 in domain 1, which can readily accommodate a molecule of LSD. The binding site has a closest approach of some 20 Å to the trinuclear copper centre, but how such binding could modulate the oxidation of biogenic amines bound close to the domain 6 mononuclear copper is a matter of conjecture. Quite clearly if LSD has a physiological role in controlling the oxidation of biogenic amines such as serotonin and dopa, it does not do this by blocking the binding sites for biogenic amines in CP.

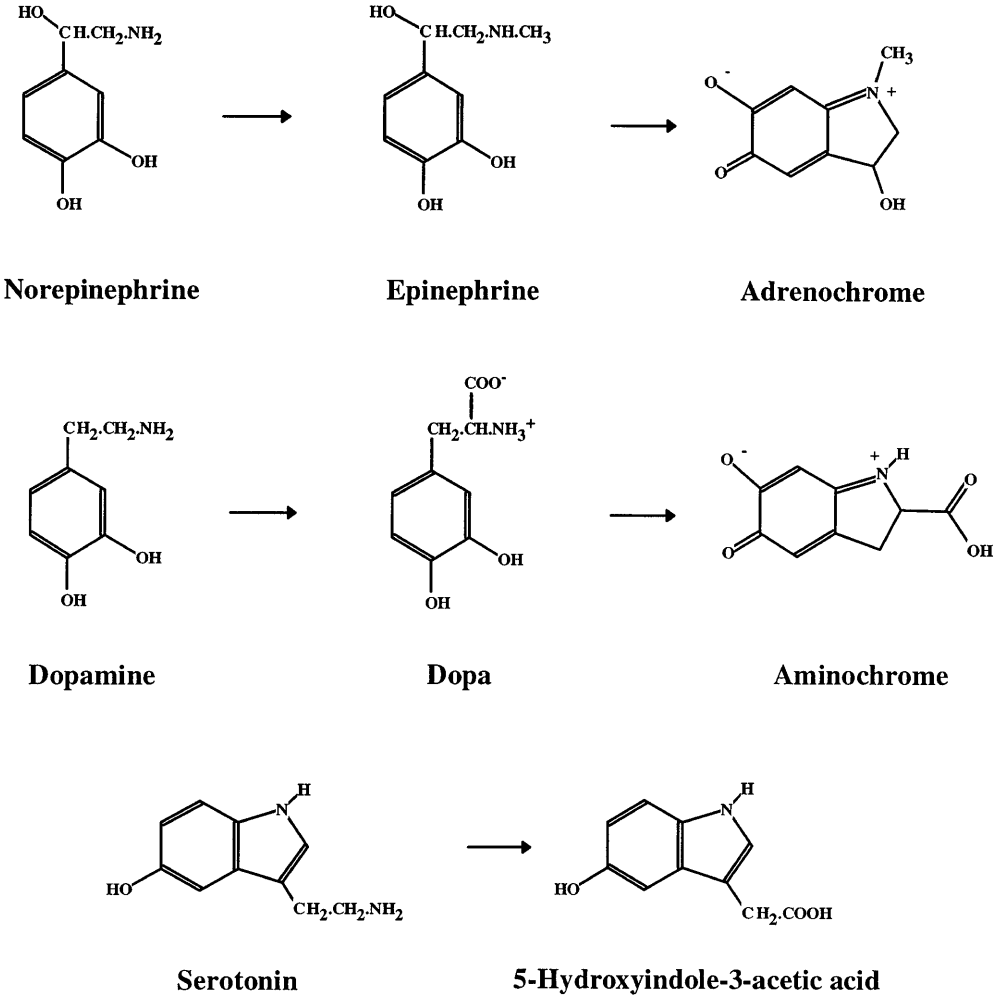

Fig. 5 Oxidation reactions and products of the biogenic amines norepinephrine, epinephrine, dopamine, dopa and serotonin

Conclusions

The soaking and X-ray experiments described in this paper, albeit at a resolution insufficient to show detailed interactions, clearly indicate that the inhibition of hCP by azide is analogous to that of AO. The azide binds to one of the type III coppers in the trinuclear centre, resulting in loss of the bridging oxygen (OH^-) and increase in separation of the type III coppers. These changes in local charge and molecular geometry may then mitigate against oxygen binding, thus removing the electron acceptor required for oxidation of substrates. In addition, there are two distinct binding sites for aromatic amines. The binding site for the aromatic diamines is in the vicinity of W669 in domain 4 and oxidation may take place by electron transfer through the type I copper atom in that domain. This binding site on the external flat bottom side of the molecule is perhaps not too surprising considering the steric requirements of both the intermediate and the final product. On the other hand, the binding site for biogenic amines appears to be associated with the labile copper site in domain 6 and oxidation may take place in a similar manner to that previously proposed for Fe(II). It is interesting to note that in no case does the mononuclear copper in domain 2 appear to be directly involved in any electron transfer processes. This is consistent with the findings of Machonkin et al. [4] that this copper, which lacks the methionine ligand, is in the reduced form. The results on the binding of LSD clearly indicate that further biochemical and physiological studies are required before the precise roles of CP in the plasma can be fully characterised. However, the experiments reported herein seem to give additional credence to the view that the role of CP in the plasma may be multi-functional. In an acute-phase condition (infection, tissue damage, etc.), when the normal physiology of the body is disrupted, the requirement for such multi-functionality may be very important.

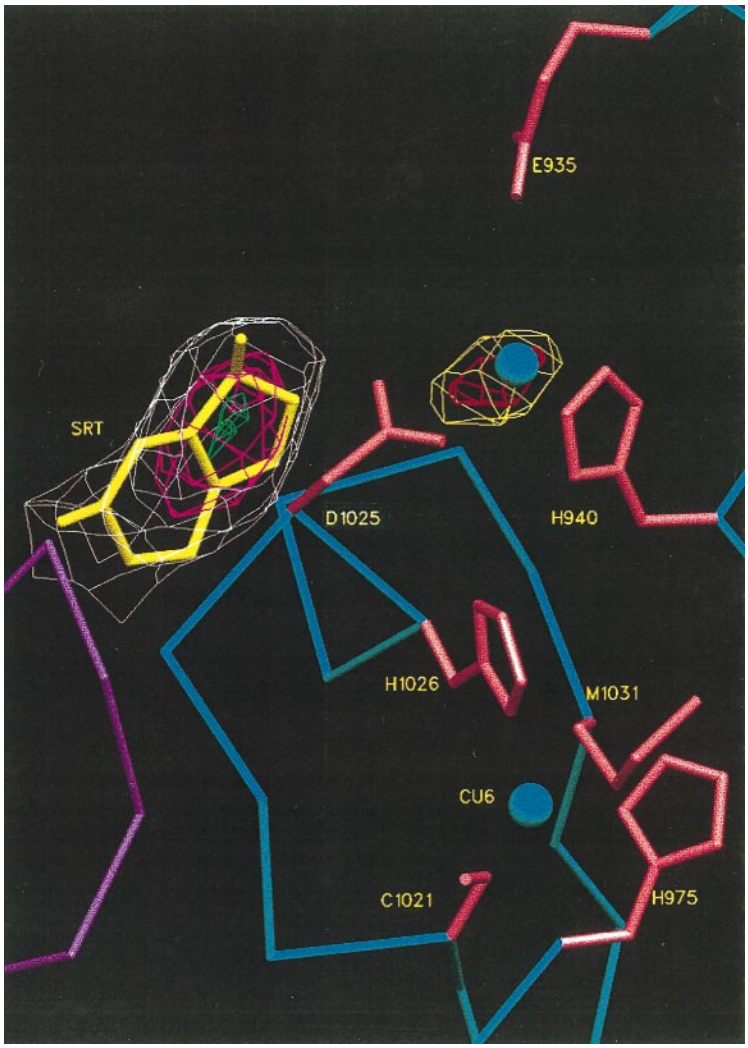

Fig. 6 A portion of the Fourier difference map calculated with data collected from native hCP crystals and one soaked with serotonin. The binding site is close to the labile copper site in domain 6. Oxidation of the substrate can take place in a similar manner to that proposed for the oxidation of Fe(II) [19]

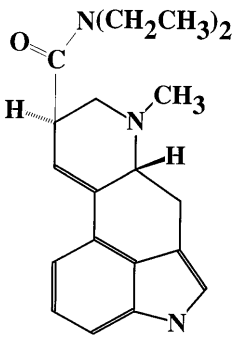

Fig. 7 (+)-Lysergic acid diethylamide (LSD)

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Fig. 8 A portion of the Fourier difference map calculated with data collected from native crystals and one soaked with LSD. The binding site for LSD appears to be in close proximity to the glycan moiety attached to N119 in domain 1. It is some 20 Å distant from the domain 1 mononuclear copper site and totally different to the binding site for the biogenic amines

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