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Reactions of bovine serum amine oxidase with *NN*-diethyldithiocarbamate

Selective removal of one copper ion

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NN-Diethyldithiocarbamate (DDC) was able to bind, at 1.0 mM concentration, only about 50% the Cu(II) ions of bovine plasma amine oxidase. Under reducing conditions, this Cu(II) was removed with inactivation of the enzyme. Up to 90% activity could be recovered by treatment with excess Cu(II). The organic cofactor, sensitive to carbonyl reagents, was reduced in the half-Cu-depleted protein and no longer bound phenylhydrazine. The fully reacted protein, in the presence of 10 mM-DDC, lost 50% Cu(II) upon storage at −20 °C, but in this case the residual Cu(II) was in the DDC-bound form and the cofactor was in the oxidized state, as it could still bind phenylhydrazine. In the presence of DDC, the rate of reaction with phenylhydrazine was always low, even at 50% DDC saturation, and all derivatives showed identical modifications of the optical and e.p.r. spectra with respect to the phenylhydrazone of the native protein. It is concluded that the two Cu(II) ions are not equivalent, that removal of a single Cu(II) is sufficient to inhibit the re-oxidation of the organic cofactor, and that both Cu(II) ions are in some way involved in the reaction with phenylhydrazine. After reaction with DDC, the optical and e.p.r. spectra of ^{63}Cu(II)-amine oxidase and of ^{63}Cu(II)-carbonic anhydrase [Morpurgo, Desideri, Rigo, Viglino & Rotilio (1983) Biochim. Biophys. Acta **746**, 168–175] are very similar and show distorted equatorial co-ordination to Cu(II) of two sulphur atoms and two magnetically equivalent nitrogen atoms.

INTRODUCTION

Amine oxidase from bovine serum (BSAO) catalyses the oxidation of aliphatic and aromatic primary amines by O_2. The corresponding aldehyde is formed in the reaction, together with NH_3 and H_2O_2. The enzyme is made up of two subunits of identical M_r (approx. 90000), and contains two e.p.r.-detectable Cu(II) ions. A single further prosthetic group can be quantitatively titrated by carbonyl reagents (phenylhydrazine, hydroxylamine etc.) with inactivation of the enzymic activity (Pettersson, 1985). This organic cofactor, after much debate as to whether or not it was pyridoxal phosphate, has now been identified as pyrroloquinoline quinone or a closely similar derivative (Lobenstein-Verbeek *et al.*, 1984; Ameyama *et al.*, 1984; Mogg *et al.*, 1986; Knowles *et al.*, 1986).

The e.p.r. spectrum at 35 GHz has indicated a structural heterogeneity of the two Cu(II) ions in the analogous enzyme from pig serum, which disappears on reaction with inhibitors such as cyanide and azide (Barker *et al.*, 1979). Similar evidence is not available for BSAO, but it was reported (Mondovì *et al.*, 1984) that only one of its two Cu(II) ions could be removed by treatment with *NN*-diethyldithiocarbamate (DDC), without loss of enzymic activity. This result contrasts with that of Suzuki *et al.* (1982), who reported that DDC reacted with BSAO to form an inactive adduct with characteristic optical and e.p.r. spectra, diagnostic of sulphur (from DDC) and nitrogen (from the protein) co-ordination to copper, with only 20% decrease of the copper content. An inactive derivative of the protein, containing a single Cu(II) ion in the molecule, has been obtained (Suzuki *et al.*, 1986) by treating with stoichiometric Cu(II) the apoenzyme prepared by treatment with cyanide and dithionite (Suzuki *et al.*, 1983). We therefore re-examined the reactions of BSAO with DDC in an attempt to understand the reasons for the discrepancy. The interactions between the metal sites and the organic cofactor were also investigated.

MATERIALS AND METHODS

The amine oxidase was purified from bovine serum by a previously reported procedure (Turini *et al.*, 1982) with minor modifications. Incubation of the crude enzyme with $(NH_4)_2SO_4$ was limited to 1 h and a Whatman CM-23 CM-cellulose column in 0.01 M-potassium phosphate buffer, pH 5.8, was added before the third step involving chromatography on aminohexyl- (AH-)Sepharose 4B. This treatment significantly decreased the contamination by haem impurities absorbing at 410 nm.

The sodium salt of DDC from Merck was recrystallized from ethanol. ^{63}CuO (> 98%) was obtained from Oak Ridge National Laboratories and dissolved in HCl. Optical spectra were recorded on a Beckman UV 5230 spectrophotometer. E.p.r. spectra were recorded on a Varian E-9 spectrometer.

Half-Cu-depleted BSAO was prepared by dialysing a 20 μM sample against a solution containing 1.0 mM-DDC and 1.0 mM-Na_2S or 1.0 mM-$Na_2S_2O_4$ in 0.1 M-potassium

Abbreviations used: BSAO, bovine serum amine oxidase; DDC, *NN*-diethyldithiocarbamate; PHy, phenylhydrazine.

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Table 1. Cu(II) content, percentage activity and reactivity with PHy of native, half-Cu-depleted and reconstituted BSAO

BSAO concentration was calculated from the 280 nm absorption, and that of PHy-reacted BSAO from the 445 nm absorption.

Sample	[BSAO] (μM)	[Cu(II)]/ [BSAO]	Activity (%)	[PHy]/ [BSAO]
Native BSAO	32	1.9	100	0.95
DDC + sulphide-treated BSAO	27	0.94	< 2	0.02
Reconstituted BSAO	15	1.8	80	0.75
Native BSAO	26	2.0	100	0.94
DDC + dithionite-treated BSAO	22	0.92	16	0.25
Reconstituted BSAO	17	1.9	95	0.63

phosphate buffer, pH 7.2, for 48 h at 4 °C. The solution was previously de-aerated by a stream of bubbling N_2, which was kept at a minimum flow during the dialysis. A brown precipitate of Cu(II)–DDC was removed by centrifugation (at 50000 *g*). Excess DDC, sulphide and dithionite were removed by dialysis against three changes of buffer. Reconstitution of the samples was achieved by 48 h dialysis against 2.0 mM-$CuSO_4$ in 0.1 M-Tris/HCl buffer, pH 7.2. Excess Cu(II) ions were removed by two changes of 1.0 mM-EDTA in the same Tris buffer, followed by extensive dialysis against 0.1 M-potassium phosphate buffer, pH 7.2.

Copper content was determined by the biquinolyl method (Brumby & Massey, 1967) and by double integration of the e.p.r. spectra, with Cu(II)–EDTA as a standard solution (Vänngård, 1972). The presence of DDC did not interfere in the biquinolyl determinations. Controls by atomic absorption spectrophotometry, with a Perkin–Elmer 3030 apparatus equipped with an HGA-400 graphite furnace, gave identical results. The protein concentration was determined from the 280 nm absorbance, by using $a = 1.74$ litre·mg^{-1}·cm^{-1} (Suzuki *et al.*, 1983). The enzyme activity was assayed with 2.5 mM-benzylamine in 0.1 M-potassium phosphate buffer, pH 7.2, at 25 °C. Samples with activity lower than 0.2 μmol of benzaldehyde formed/min per mg of BSAO were discarded. Benzaldehyde was monitored at 250 nm, by using $\epsilon = 12500$ M^{-1}·cm^{-1} (Suva & Abeles, 1978). The absorption band at 445 nm ($\Delta\epsilon = 38000$ M^{-1}·cm^{-1}) formed with phenylhydrazine (PHy) in slight excess over the stoichiometric amount (Yamada & Yasunobu, 1963) was employed as an additional control of the protein purity.

RESULTS

Reactions with DDC and preparation of half-Cu-depleted BSAO

The dialysis of BSAO against DDC in anaerobic reducing conditions, as described in the Materials and methods section, caused the removal of about half the copper content from the protein (Table 1). The enzyme activity was nearly completely abolished by the treatment with Na_2S as a reducing agent and decreased to only 10–20 % by the treatment with $Na_2S_2O_4$. The intensity of the visible-region absorption band at 480 nm was also decreased in both cases. Except for a small new peak at $g = 2.004$ (Suzuki *et al.*, 1986), the shape of the e.p.r. spectrum was unchanged (Fig. 1) and its integrated

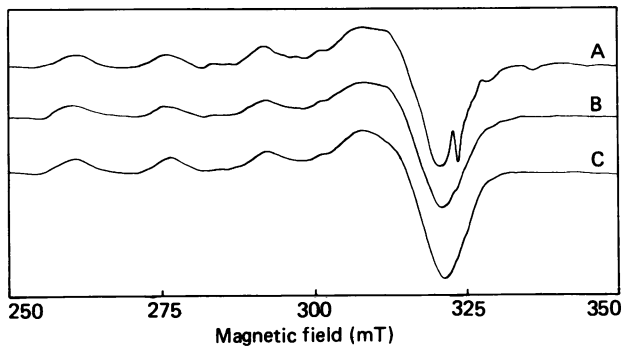

Fig. 1. E.p.r. spectra of half-Cu-depleted BSAO (spectrum A), of the reconstituted protein (spectrum B) and of the native protein (spectrum C)

Solutions of 58 μM protein in 0.1 M-phosphate buffer, pH 7.2, were used. Setting conditions were: 9.15 microwave frequency; 20 mW microwave power; 1.0 mT modulation amplitude; 100 K temperature; spectrum A was recorded at double gain.

intensity accounted for only about one Cu(II) ion per dimeric molecule. Spectral properties and activity could be restored to a large extent by Cu(II) re-incorporation as described in the Materials and methods section. Sulphide is reported to inactivate BSAO (Dooley & Coté, 1984), but in the present procedure it only behaved as a reductant, as effective as dithionite in the Cu(II) abstraction. In agreement with the report by Suzuki *et al.* (1982), only 10–15 % of the Cu(II) was removed when the protein was dialysed against solutions of recrystallized DDC in the absence of reductant.

At 1.0 mM-DDC, the concentration used for Cu(II) removal, only about 50 % of the Cu(II) was able to react with BSAO, as shown by the decrease of the first hyperfine line at low field in the e.p.r. spectrum (Fig. 2, spectrum B). To obtain total conversion into the enzyme–Cu(II)–DDC species, i.e. disappearance of the hyperfine line at low field (Fig. 2, spectrum C) and full formation of the absorption band at 380 nm (Fig. 3, spectrum B), 24 h incubation with 10 mM-DDC was necessary. The reaction with DDC could be reversed to a very limited extent by dialysis against phosphate buffer, pH 7.2, even after 3 days with repeated buffer changes. Storage in the frozen state at −20 °C of the samples incubated with 10 mM-DDC caused the loss of one Cu(II) ion, while the other remained in the DDC-bound form, after centrifugation of the free Cu(II)–DDC

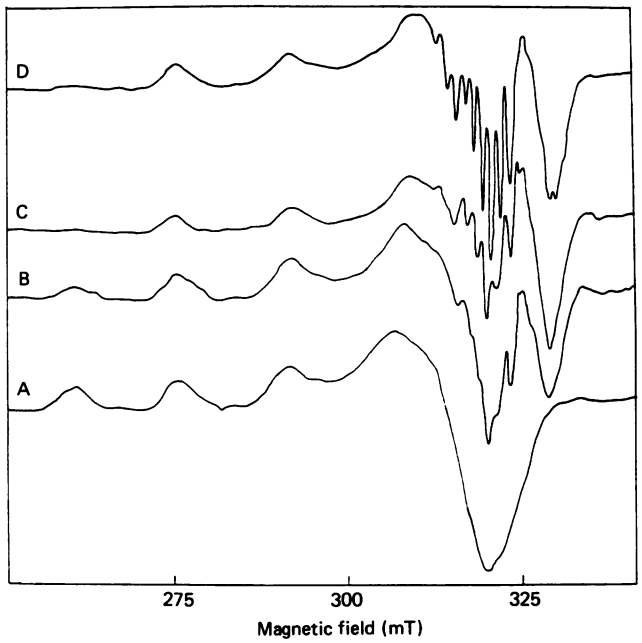

Fig. 2. E.p.r. spectra of DDC-reacted BSAO: 40 μM native protein in 0.1 M-phosphate buffer, pH 7.2 (spectrum A), after 24 h incubation with 1.0 mM-DDC (spectrum B), after 24 h incubation with 10 mM-DDC (spectrum C) and after further 24 h incubation of solution C with stoichiometric PHy (spectrum D)

Setting conditions were as given in Fig. 1 legend.

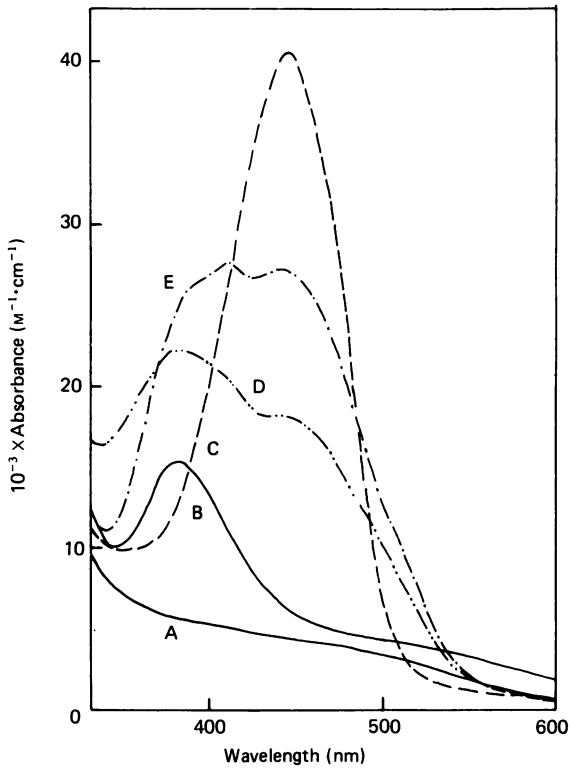

Fig. 3. Optical spectra of BSAO that had reacted with DDC and PHy: native protein in 0.1 M-phosphate buffer, pH 7.2 (spectrum A), after 24 h incubation with 10 mM-DDC (spectrum B), after addition of stoichiometric PHy to the native protein (spectrum C), solution B after 24 h incubation with stoichiometric PHy (spectrum D) and solution B after 24 h incubation with 2.5 mol of PHy/mol of protein or solution C after 24 h incubation with 10 mM-DDC (spectrum E)

complex and removal by dialysis of uncomplexed DDC. This is shown by the shape of the e.p.r. spectrum, identical with that of spectrum C in Fig. 2, and by the intensity decrease to about one-half the original value. The intensity of the 380 nm band was proportional to residual Cu(II) and the 480 nm band was slightly increased. Attempts to remove DDC and to re-activate this derivative with Cu(II) were unsuccessful. An inactive derivative with similar properties was obtained on incubation of BSAO in aerobic conditions, at room temperature, with non-recrystallized DDC. Crude DDC, unlike the purified material, decreased the e.p.r. signal intensity of the protein, suggesting the presence of reducing impurities. No attempts were made at their identification.

Reactions with PHy

Native BSAO reacts within the mixing time with stoichiometric PHy giving an intense absorption band at 445 nm (Yamada & Yasunobu, 1963), and the line shape of the e.p.r. spectrum acquires a more rhombic character with decreased hyperfine splitting in the parallel region (Mondovì *et al.*, 1986; Rinaldi *et al.*, 1983). This reaction was strongly affected by the presence of DDC. A less intense and broader absorption band was slowly formed over the Cu(II)–DDC absorption band at 380 nm, with two new peaks at about 410 and 450 nm and a weak shoulder on the low-energy side at about 510 nm (Fig. 3, spectrum D). The e.p.r. spectrum of the DDC-treated protein (Fig. 2, spectrum C) was not modified by PHy (Fig. 2, spectrum D) in the $g_{\parallel}$ region, showing that DDC was not displaced from Cu(II) co-ordination. However, PHy produced some modification in the $g_{\perp}$ region, resulting in a better resolution of the superhyperfine structure. It is worth noting that at the point when only 50% of the Cu(II) had reacted the rate of reaction with PHy and the shape of the absorption band were modified as in the fully reacted protein. Conversely, addition of DDC to PHy-treated samples caused a substantial decrease and broadening of the phenylhydrazone absorption band at 445 nm in spectrum C in Fig. 3. After 24 h incubation with 10 mM-DDC the spectrum became very similar to spectrum E in Fig. 3. Attempts to reverse these reactions were unsuccessful. Only excess DDC was removed by dialysis against buffer, whereas dialysis against Cu(II) salts resulted in loss of bound DDC without recovery of the initial phenylhydrazone optical spectrum.

The half-Cu-depleted sample, obtained by storage at −20°C, reacted with PHy in the same way as did the DDC-treated holoprotein, giving a very similar optical spectrum. The e.p.r. spectrum had identical line shape and approx. 50% intensity. The half-Cu-depleted protein prepared with DDC and sulphide or dithionite produced with PHy a band at 445 nm less intense than that of native BSAO. Since it was roughly proportional to the residual activity of the sample (Table 1), and was unaffected by excess PHy and prolonged incubation time, it is probably to be related to unmodified molecules.

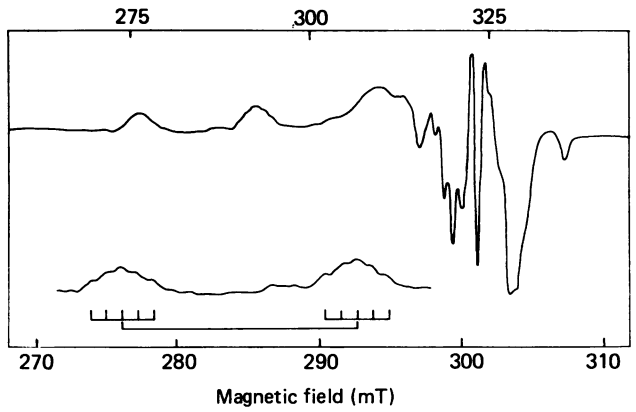

Fig. 4. E.p.r. spectrum of ^{63}Cu-reconstituted DDC-reacted BSAO

A solution of 0.1 mM protein (1.8 of Cu/mol) in 0.1 M-Tris/HCl buffer, pH 7.2, was used after 24 h incubation with 10 mM-DDC. Setting conditions were as given in Fig. 1 legend, except that the modulation amplitude of the lower curve was 0.05 mT.

Reaction with DDC of the ^{63}Cu-reconstituted apo-BSAO

Apo-BSAO, prepared by treatment with cyanide and dithionite by the procedure of Suzuki *et al.* (1983), was 90% re-activated by 24 h incubation with excess Cu(II) (3 mol/mol of enzyme), followed by extensive dialysis against EDTA and then buffer. A 50%-active sample was obtained by using stoichiometric Cu(II), one per dimer. Suzuki *et al.* (1986) reported the preparation of an inactive derivative with stoichiometric Cu(II) and full re-activation only with a large excess of Cu(II). The different results may be related to the use of apo-BSAO as prepared, with the organic cofactor in the reduced state, or to the use of previously re-oxidized apo-BSAO, respectively. This was achieved by Suzuki *et al.* (1986) with a cycle of Ni(II) reconstitution and subsequent depletion. The accessibility to Cu(II) of the oxidized and reduced derivative is apparently different.

Fig. 4 shows the e.p.r. spectrum of a BSAO sample that was reconstituted with ^{63}Cu and then incubated with 10 mM-DDC for 24 h at 5 °C. A five-line superhyperfine pattern with 1.1 mT splitting is detectable on the first and second hyperfine lines in the $g_{\parallel}$ region (the lower trace shows an enlarged portion of the spectrum), besides the nitrogen superhyperfine pattern in the $g_{\perp}$ region (upper trace) also observable in the derivative with natural copper abundance (Fig. 2). The relevant parameters are reported in Table 2, together with those of Cu(II)-substituted bovine carbonic anhydrase, which gave with DDC a derivative with similar spectral properties (Morpurgo *et al.*, 1983).

DISCUSSION

Reactivity with DDC and co-ordination of the Cu(II) sites of BSAO

The present re-investigation of the reactions of BSAO with DDC confirms previous reports (Suzuki *et al.*, 1983) that this reagent is unable, in the pure state, to remove more than 10–15% of the Cu(II) from the protein. About 50% of the Cu(II) could, however, be reversibly removed from a partially DDC-reacted protein in the presence of reducing agents, with loss of the enzymic activity (Table 1). Removal of 50% of the Cu(II) and enzyme inactivation were also obtained by storage at −20 °C of the fully DDC-reacted protein, or by the aerobic use of non-recrystallized DDC. The two similar derivatives obtained with the latter procedures were not re-activated by Cu(II). Freezing or the particular reducing conditions with the use of impure DDC seem to cause an irreversible conformational change, leading to labilization of a single DDC-bound Cu(II). The oxidized state of the organic cofactor in both derivatives and the presence of bound DDC are additional unfavourable factors for Cu(II) re-incorporation.

Previous experiments by Mondovì *et al.* (1984), leading to a fully active half-Cu-depleted enzyme, could not be reproduced.

The reaction of BSAO with DDC was slow and required a large DDC excess for completeness. At the DDC concentration used for Cu(II) removal only about 50% of the Cu(II) reacted. Bound DDC was removed by dialysis to a very limited extent. Removal in the form of a Cu(II)–DDC complex, in the presence of reductants or on storage in the frozen state, was also slow. This behaviour is significantly different from that of Cu(II)-carbonic anhydrase, which reacted with DDC within the mixing time and started to form a precipitate of the Cu(II)–DDC complex as soon as DDC was in excess of 1:1 ratio to Cu(II) (Morpurgo *et al.*, 1983). Nonetheless the DDC adducts of the two proteins appear to be very similar (Table 2). They display an intense absorption band, at 380 nm in BSAO and at 390 nm in carbonic anhydrase, that is assigned to a S→Cu(II) charge transfer (Suzuki *et al.*, 1982; Morpurgo *et al.*, 1983). The corresponding e.p.r. spectra are also similar, and $g_{\parallel}$

Table 2. Spectral properties of DDC-reacted BSAO and Cu(II)-carbonic anhydrase

Δε values refer to the absorbance increase per Cu(II). The data for the experiments with carbonic anhydrase are taken from Morpurgo *et al.* (1983).

Sample	$g_{\parallel}$	$A_{\parallel}$ (mT)	A_N (mT)	λ (nm)	Δε ($M^{-1} \cdot cm^{-1}$)
BSAO	2.30	15.5			
DDC-reacted BSAO	2.17	16.3	1.1	380	5000
Carbonic anhydrase	2.29	14.3			
DDC-reacted carbonic anhydrase	2.20	14.7	1.1	390	4300

values imply sulphur co-ordination from a bidentate DDC molecule in the Cu(II) equatorial plane (Blumberg & Peisach, 1968; Sakaguchi & Addison, 1979; Morpurgo *et al.*, 1983). In addition, the e.p.r. spectra of the ^{63}Cu-substituted proteins display (Table 2 and Fig. 4) identical five-line superhyperfine patterns with 1.1 mT splitting constant in the $g_{\parallel}$ region, diagnostic of two magnetically equivalent ^{14}N nuclei, in a distorted equatorial co-ordination (Guzy *et al.*, 1969; Buluggiu *et al.*, 1972). For the DDC derivative of BSAO N_2S_2 co-ordination was also suggested by Suzuki *et al.* (1982) by analogy with the spectra of a model copper complex Cu(II)–DDC–bipyridyl. In carbonic anhydrase the two nitrogen atoms were supplied by two histidine residues, and a third histidine nitrogen atom and a loosely bound water molecule were believed to occupy the axial positions (Morpurgo *et al.*, 1983).

Since the products of the reaction with DDC of BSAO and of Cu(II)-carbonic anhydrase are similar, the different rates of reaction may be due to some difference in the unmodified site. This is tetra-co-ordinated to three histidine nitrogen atoms and a water molecule in native carbonic anhydrase and in the inactive Cu(II) derivative (Lindskog *et al.*, 1971). The latter readily binds bidentate ligands, yielding penta- or hexa-co-ordinate adducts (Morpurgo *et al.*, 1983). The Cu(II) sites of BSAO display a more tetragonal geometry, which is believed to arise from three nitrogen atoms and one water molecule binding in the equatorial plane, plus one water molecule and possibly one more ligand in the axial positions, as suggested for the similar pig plasma protein (Baker *et al.*, 1986). To accommodate a bidentate ligand, a site with this structure may require displacement of protein ligands, with a more extensive conformational re-arrangement. The difference of reactivity observed between the two sites of BSAO may be related to different accessibility rather than different co-ordination, since the e.p.r. spectra of native and half-Cu-depleted BSAO look identical, even after reaction with DDC or with DDC and PHy.

Oxidation state and interactions with Cu(II) of the organic cofactor

The decreased optical absorbance at 480 nm and the failure to react with PHy show that the organic cofactor was reduced in the half-Cu-depleted BSAO, as demonstrated for the apoprotein prepared by treatment with cyanide and dithionite (Suzuki *et al.*, 1983). The reduced state of the organic cofactor apparently facilitates the removal of Cu(II) from the protein and also its recovery, as pointed out in a previous section above.

The presence of Cu(II) is, on the other hand, essential in the re-oxidation process of the carbonyl cofactor, which is prevented by the loss of even a single Cu(II). The presence of intact Cu(II) sites is also important in other reactions of the carbonyl cofactor, such as PHy binding. The rate of this reaction was substantially decreased in the apo-BSAO and single-Cu-containing BSAO (Suzuki *et al.*, 1986) and in the DDC-treated protein, either at 100% or 50% saturation. The interaction of the organic cofactor with the metal sites is further documented by the modifications of the phenylhydrazone optical spectrum induced by DDC binding to Cu(II) and of the Cu(II) e.p.r. spectrum induced by PHy binding to the carbonyl. Both Cu(II) ions seem to be involved, since identical effects were observed in either case with 50%- or 100%-DDC-saturated BSAO and with the half-Cu-depleted BSAO DDC derivative obtained by storage of the latter sample in the frozen state. Which is the mechanism operating in such interactions is not yet clear, but conformational effects seem to be involved, since the possibility of simultaneous binding of PHy and DDC to the protein excludes a close proximity of the metal ions to the carbonyl, in agreement with n.m.r. studies by Williams & Falk (1986).

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