Reactions of the oxidized organic cofactor in copper-depleted bovine serum amine oxidase

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A novel copper-depleted bovine serum amine oxidase (BSAO), in which about half the molecules contained the organic cofactor in the oxidized form, was prepared by adding a reductant in anaerobic conditions to the cyanide-reacted protein. The Cu^{I}-semiquinone formed in these conditions reoxidizes after the removal of copper. The inactive derivative was reduced by benzylamine at approx. 1/1000 the rate of BSAO. The pseudo-first-order reaction was preceded by the formation of a protein–benzylamine complex with dissociation constant, K_d, of 4.9 ± 0.5 mM, similar to the K_m of BSAO (2.2 mM). Also the reactions with phenylhydrazine and benzohydrazide were considerably slower than in holo-BSAO, whereas the reactions with *p*-pyridine-2-ylphenylacetohydrazide, containing a longer aromatic tail, and semicarbazide, lacking an aromatic moiety, were less severely affected. Removal of copper had no effect on the optical spectra of BSAO and of most adducts, containing the cofactor in quinol form, showing that copper is bound to neither the oxidized nor the reduced cofactor. Benzylhydrazine did not produce optical effects but was tightly bound, as inferred from its inhibitory effect on reaction with other molecules. Substrate and inhibitors might bind a hydrophobic pocket at some distance from the quinone, probably near the protein surface, with their affinity depending on the hydrophobic character and pK_a. The binding, which is not greatly influenced by copper removal, probably induces a copper-dependent change of conformation, 'opening' a pathway to the active site buried in the protein interior.

INTRODUCTION

Copper-containing amine oxidases (EC 1.4.3.6) are found in a variety of organisms, from mammals to plants, bacteria and yeasts [1,2]. They are mostly homodimers, with subunits of 70–95 kDa, and contain two Cu^{II} ions and one or two molecules of the cofactor topa quinone (TPQ), derived from the tyrosine in the consensus sequence Asn-Tyr-Asp/Glu [3,4]. The catalytic reaction occurs by a Ping Pong mechanism. In the first half-reaction the amine forms a molecular complex with the enzyme, and then a Schiff base, which isomerizes to quinol-imine and eventually hydrolyses with release of aldehyde. In the second half-reaction the enzyme is reoxidized by oxygen with the release of ammonia and hydrogen peroxide [5,6]. In spite of many similarities, enzymes from various sources differ in specificities for substrates and inhibitors. The reasons are not yet understood and even enzyme properties are not always well defined. A particularly debated issue is the role of copper. It catalyses the post-translational oxidation of tyrosine to TPQ [7,8], but its precise role in the catalysis of amine oxidation has not yet been established. Direct involvement is suggested by the formation of a Cu^{I}-semiquinone by many amine oxidases, on reaction with substrates under anaerobic conditions [9], and a structural function can be inferred from the persisting enzymic activity in bovine serum amine oxidase (BSAO) after replacement of copper by Co^{II} [10–12]. A related, still unsettled, issue is the distance between copper and quinone, direct binding having been excluded by model studies [13] and spectroscopic properties [14], whereas close proximity is suggested by the extremely fast rate of electron transfer between Cu^{II} and reduced quinone to form a Cu^{I}-semiquinone radical [15]. The X-ray structures of two amine oxidases from *Escherichia coli* (ECAO) [16] and pea seedling (PSAO) [17] show that copper is not bound to TPQ. This supports the absence of a direct Cu–TPQ bond in resting BSAO, but cannot exclude a closer interaction under different conditions, because copper and TPQ are bound in an inactive ECAO form [16].

Limited information on the role of copper has been provided so far by the study of copper-depleted BSAO, because copper was removed under conditions that left the cofactor reduced and inert towards any reagent. A procedure for cofactor reoxidation is rather complicated [10]. The present paper reports a simple method for the preparation of a novel copper-depleted BSAO, containing approx. 50 % oxidized cofactor, and the effect of copper removal on the reactions with the substrate benzylamine and with inhibitors of the hydrazine family.

EXPERIMENTAL

BSAO was purified from bovine blood as previously described [18]. The purified protein moved as a single band under SDS/PAGE. The concentration was measured spectrophotometrically at 280 nm, using $a = 1.74$ litre·g^{-1}·cm^{-1} [10]. Benzylamine oxidase activity was assayed spectrophotometrically at 25 °C by monitoring the formation of benzaldehyde at 250 nm ($\epsilon = 12\,500$ M^{-1}·cm^{-1}) [19]. All samples had a minimum specific activity of 0.3 units/mg (μmol of substrate oxidized/min) and were inactivated by 1.00 ± 0.05 molecule of phenylhydrazine per dimer ($\epsilon = 41\,500$ M^{-1}·cm^{-1} at 447 nm) [20].

Abbreviations used: BSAO, bovine serum amine oxidase; ECAO, *Escherichia coli* amine oxidase; PSAO, pea seedling amine oxidase; LSAO, lentil seedling amine oxidase; TPQ, topa quinone.

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To achieve copper removal, a 10 μM protein solution was dialysed at 5 °C against carefully de-aerated 0.1 M potassium phosphate buffer, pH 7.2. The solution was then made 10 mM in KCN, added as the solid salt. After approx. 1 h, solid sodium dithionite (or benzylamine) was added to a concentration of 1.0 mM and the dialysis was continued for 3–24 h, followed by final dialyses against three buffer changes. Reconstitution of copper-depleted BSAO was performed in 0.1 M Tris/HCl, pH 7.2, by incubation of the sample for 48 h with variable copper amounts up to two Cu ions per dimer, inclusive of residual copper, followed by extensive dialysis against buffer.

Copper concentration was measured as previously described [20] by using a spectroscopic method (with biquinolyl), atomic absorption and EPR spectra. Optical spectra were recorded on a Lambda 9 Perkin-Elmer spectrophotometer equipped with Peltier thermostat, EPR spectra on a Varian-E9 spectrometer, and Cu atomic absorption measurements on a Perkin-Elmer 3030 spectrometer equipped with a HGA-400 graphite furnace. Kinetic data were processed with Microcal Origin 3.5 software.

p-Pyridine-2-ylphenylacetohydrazide was kindly supplied by Professor M. Artico [21]. Phenylhydrazine was recrystallized from ethanol. Other chemicals were commercial reagents of analytical grade and were used without further purification.

RESULTS

Preparation and characterization of copper-depleted BSAO

Copper-depleted BSAO was previously prepared by adding KCN anaerobically to the dithionite-reduced protein [10,12,22]. TPQ was reduced in this derivative, which therefore lacked the 480 nm band and the ability to bind phenylhydrazine [10,12]. The derivative obtained by first adding KCN and then dithionite to the anaerobic solution, as described in the Experimental section, lost up to 90 % copper and 98 % catalytic activity (Table 1) but retained a substantial portion of absorbance at 480 nm (Figure 1), consistent with the presence of oxidized cofactor. This was confirmed by reaction with phenylhydrazine, which slowly formed a band at 434 nm. After incubation overnight, the concentration of reactive TPQ was calculated by means of the molar absorption coefficient $\epsilon_{434} = 32000\ M^{-1} \cdot cm^{-1}$, previously

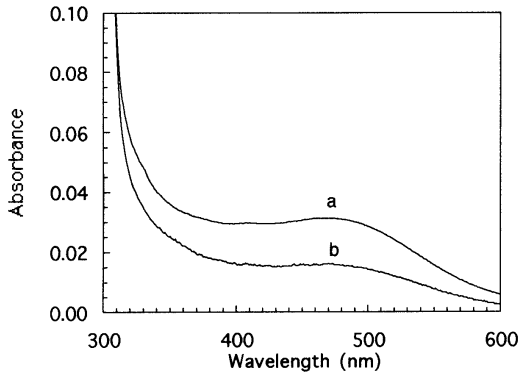

Figure 1 Absorption spectra of native and copper-depleted BSAO

Native protein (10 μM) in 0.1 M potassium phosphate, pH 7.2 (trace a); copper-depleted protein (trace b).

obtained by removing copper from the holo-BSAO adduct with one molecule of phenylhydrazine per dimer, using *N*,*N*-diethyldithiocarbamate in 5 M urea [23]. The amount of residual copper and oxidized TPQ decreased with the time of anaerobic incubation with BSAO of cyanide and dithionite, reaching a constant value after 9.0 h dialysis (Table 1). The recovery of holoenzyme properties was a linear function of copper reincorporation in accordance with the procedure described in the Experimental section (Table 1). With the substrate benzylamine as a reductant in place of dithionite, a smaller amount of copper was removed, even after a long incubation time, irrespective of the order of addition of the reagents. Residual activity and oxidized TPQ were comparable to those of corresponding dithionite preparations (Table 2).

Reaction of copper-depleted BSAO with benzylamine

The addition of benzylamine to copper-depleted BSAO in air decreased the residual TPQ band at 480 nm by a further 20–25 % of the native intensity. The process was not reversed by dialysis. At benzylamine concentrations lower than 7.5 mM the reaction

Table 1 Properties of native, copper-depleted and copper-reconstituted BSAO

BSAO form		Copper/dimer		Oxidized TPQ/dimer	Activity (%)
		Atomic absorption	Biquinolyl		
Holo		2.00	2.00	1.00*	100
	Incubation time (h)				
Cu-depleted	3	0.52	0.42	1.00†	13
	6	0.50	0.38	0.75†	5.0
	9	0.31	0.18	0.60†	3.5
	24	0.25	0.17	0.58†	2.2
	Cu/dimer added				
Cu-reconstituted	0.5	0.53		0.65†	22
	1.0	0.77		0.70*	51
	1.5	1.30		0.90*	72
	2.0	1.70		1.00*	88

* Measured from a band of phenylhydrazine adduct at 447 nm, $\epsilon = 41500\ M^{-1} \cdot cm^{-1}$.
† Measured from a band of phenylhydrazine adduct at 434 nm, $\epsilon = 32000\ M^{-1} \cdot cm^{-1}$.

Table 2 Copper depletion of BSAO with benzylamine as the reducing agent

Stage of addition of benzylamine	[Benzylamine] (mM)	Dialysis time (h)	Cu/dimer	Oxidized TPQ/dimer	Activity (%)
After KCN	0	0	1.95	1.00*	100
	1.0	6	1.30	0.40†	15
	2.0	16	0.86	0.38†	5.2
	5.0	18	0.88	0.44†	7.0
Before KCN	5.0	18	0.87	0.2†	1.8

* Measured from a band of phenylhydrazine adduct at 447 nm, $\epsilon = 41\,500\ M^{-1}\cdot cm^{-1}$.
† Measured from a band of phenylhydrazine adduct at 434 nm, $\epsilon = 32\,000\ M^{-1}\cdot cm^{-1}$.

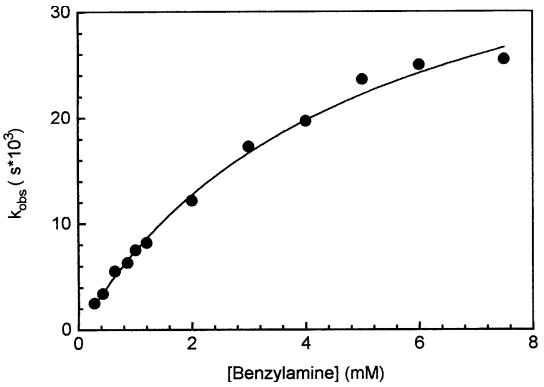

Figure 2 Copper-depleted BSAO reduction by benzylamine: plot of apparent first-order rate constants against benzylamine concentration

The reaction of 11 μM copper-depleted BSAO with benzylamine, in 0.1 M potassium phosphate, pH 7.2, at 25 °C was monitored by the absorbance decrease at 480 nm.

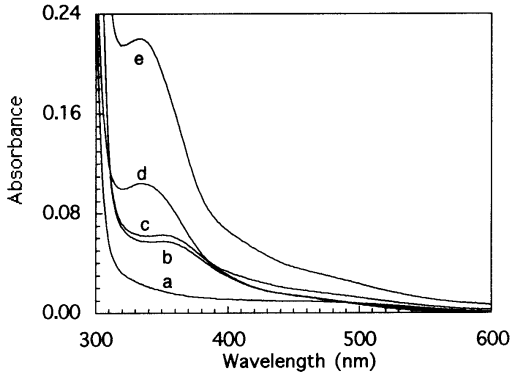

Figure 3 Absorption spectra of *p*-pyridine-2-ylphenylacetohydrazide adducts of copper-depleted BSAO

Copper-depleted BSAO (6 μM) in 0.1 M potassium phosphate buffer, pH 7.2 (trace a); plus 0.3 mM *p*-pyridine-2-ylphenylacetohydrazide (spectrum recorded after 3 min at 25 °C) (trace b); plus 0.3 mM *p*-pyridine-2-ylphenylacetohydrazide, after 24 h (trace c); incubated for 12 min at 60 °C after excess inhibitor had been dialysed away (trace d); incubated for 2 h at 60 °C in the presence of excess inhibitor (trace e).

could be monitored by conventional spectroscopic techniques. The decrease in the absorbance at 480 nm followed pseudo-first-order kinetics; k_{obs} values, in the range 10^2 to $10^3\ s^{-1}$, showed saturation behaviour at increasing benzylamine concentration (Figure 2). This suggests [24] that the reduction is preceded by formation of a protein–benzylamine complex ($E_{ox}S$) in accordance with the equation:

$$E_{ox} + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (E_{ox}S) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (E_{red}P) \quad (1)$$

The complex dissociation constant, K_d, was calculated from Eqn. (2) by assuming that $k_{-2} \ll k_{obs}$:

$$k_{obs} - k_{-2} = k_2[S]/(K_d + [S]) \quad (2)$$

At $[S] \gg K_d$, k_{obs} reaches the maximum value $k_{obs} = k_2$. The nonlinear regression analysis of data from Figure 2 gave $K_d = 4.9 \pm 0.5$ mM and $k_2 = (44 \pm 2) \times 10^{-3}\ s^{-1}$. The complex ($E_{red}P$) probably hydrolyses to benzaldehyde and E_{red}-NH_2 as in substrate-treated copper-depleted lentil seedling amine oxidase (LSAO) [25], but the presence of residual native BSAO did not permit the confirmation of benzaldehyde formation as it was not possible to discriminate between holoenzyme or copper-depleted BSAO reactions.

Reaction of copper-depleted BSAO with inhibitors

Besides phenylhydrazine, copper-depleted BSAO was assayed for reactivity with benzylhydrazine and a series of hydrazides of general formula NHNHCO-R (with R = NH_2, semicarbazide; C_6H_5, benzohydrazide; CH_2-C_6H_4-C_5H_4N, *p*-pyridine-2-yl-phenylacetohydrazide). With the last of these compounds it formed, within a few minutes, a band at 354 nm (Figure 3, trace b), as found for holo-BSAO [20]. The band intensity accounted for approx. 0.7 molecule per dimer by using the molar absorption coefficient, $\epsilon = 14\,700\ M^{-1}\cdot cm^{-1}$, of the holo-BSAO adduct containing one molecule of hydrazide per dimer [23]. Further incubation at 25 °C had only a minor effect on the absorbance, whereas at higher temperature the band was blue-shifted and the absorbance increased. At 60 °C it occurred at 335 nm as in the derivative obtained by removing copper from the holo-BSAO adduct in 5 M urea [23]. By using $\epsilon_{335} = 34\,900\ M^{-1}\cdot cm^{-1}$ for the latter species, the band intensity accounted for 0.6 or 1.2 molecules of inhibitor per dimer, depending on whether the temperature was increased after dialysis (Figure 3, trace d) or in the presence of excess hydrazide (Figure 3, trace e) respectively. This means that under the latter conditions TPQ was reoxidized up to about the native protein level of 1.3 molecules per dimer. The removal of copper did not affect the spectrum and therefore the structure of the adduct at low temperature, but greatly decreased its stability to heat [23].

Semicarbazide formed a band at 360 nm with copper-depleted BSAO as with the holoenzyme (Figure 4, trace b), whereas the reaction $t_{\frac{1}{2}}$ increased from 20 to 140 s. The band was unaffected by incubation at 25–40 °C but was irreversibly shifted to 335 nm at 60 °C with a small increase in absorbance (Figure 4, trace c).

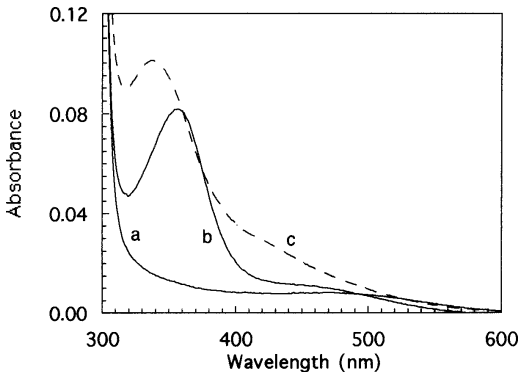

Figure 4 Absorption spectra of semicarbazide adducts of copper-depleted BSAO

Copper-depleted BSAO (6 μM) in 0.1 M potassium phosphate buffer, pH 7.2 (trace a); plus 0.3 mM semicarbazide after 15 min at 25 °C (trace b); plus 0.3 mM semicarbazide after 20 min at 60 °C (trace c).

The amount of bound inhibitor was calculated to be 0.6 molecule per dimer either by means of the 360 nm band and the $\epsilon = 24000$ $M^{-1} \cdot cm^{-1}$ of the 1:1 semicarbazide adduct of holo-BSAO dimer, or the 335 nm band and the $\epsilon = 26000$ $M^{-1} \cdot cm^{-1}$ obtained by removing copper in 5 M urea from the holoprotein adduct [23]. None of the spectral shifts occurring in semicarbazide-treated holo-BSAO with time or at high temperature [23] were detected in the absence of copper.

Thiosemicarbazide formed, with both the holoenzyme and copper-depleted BSAO, a band at 400 nm (results not shown), but the effect of heat on this band was strongly copper-dependent. At 60 °C the band moved to 460 nm in the holoenzyme [23] and to 380 nm in the copper-depleted adduct. A band at 380 nm was also produced on removing copper from the holo-BSAO adduct in 5 M urea.

Benzohydrazide formed a band at 355 nm with copper-depleted BSAO at a much slower rate than with the holoenzyme: 1 molecule per dimer inactivated holo-BSAO in 1–2 h [20], whereas the binding of 0.5 molecule per dimer (estimated by using the $\Delta\epsilon_{335}$ of 22800 $M^{-1} \cdot cm^{-1}$ of the former adduct) required overnight incubation with a 5-fold molar excess of benzohydrazide.

The pseudosubstrate benzylhydrazine seemed to be totally unreactive with copper-depleted BSAO, because no optical effects could be observed, except the small effects produced by residual active molecules [26]. It was found, however, that a low concentration of benzylhydrazine (1 molecule per dimer) completely inhibited, within the mixing time, the reaction with other molecules, such as semicarbazide or *p*-pyridine-2-ylphenylacetohydrazide.

DISCUSSION

Preparation and properties of copper-depleted BSAO

The influence on the final TPQ oxidation state of the sequence of reagents used to remove copper might be related to the BSAO property of forming a very small amount of Cu^{I}-semiquinone on reaction with substrate, and a higher amount when the reaction is performed in the presence of cyanide [9]. The Cu^{II}-quinol formed in the absence of cyanide is unable to undergo electron transfer to semiquinone on the addition of cyanide, so that TPQ remains reduced after the removal of copper. By first adding cyanide, the cofactor can be reduced in part to semiquinone, which reoxidizes after copper removal, forming a mixture of oxidized and reduced TPQ. Disproportionation of the semiquinone, via intra- or inter-molecular electron transfer, was proposed to occur in amine oxidases [9,27], but TPQ location inside the protein [16,17] makes this explanation questionable.

Both copper ions were reduced and removed by using dithionite, a non-specific reducing agent, whereas 55–60 % of the copper, at most, was removed by using benzylamine, with a decrease in enzymic activity of more than 90 %. This shows that the substrate reacted with one site per dimer of the native enzyme [20,23]. Copper-depleted BSAO containing oxidized TPQ reacted with approx. 0.5 molecule of phenylhydrazine per dimer (Table 2) and other inhibitors. If one molecule of TPQ per dimer reacts, as in holo-BSAO [23], half molecules were fully reduced. The substantial amount of residual copper in samples treated with benzylamine did not influence the reaction. This makes the presence of a second TPQ molecule in the protein unlikely. The 0.1–0.35 'less active' TPQ, previously detected [23], might be responsible for the greater than 50 % copper loss with benzylamine.

The wavelength of the TPQ band as well as the spectra of the adducts with all inhibitors examined, except phenylhydrazine, were not affected by copper removal. Because the adducts were shown to contain TPQ in quinol form [23], copper binds neither oxidized nor reduced TPQ [13]. The only spectroscopic difference from holo-BSAO was the lack of bathochromic shifts with time or at high temperature in the spectra of copper-depleted adducts with semicarbazide and thiosemicarbazide. This supports the previous conclusion that the shifts arise from perturbations of electronic transitions in TPQ induced by a closer interaction with copper [23]. Therefore the active site in BSAO is similar to those of ECAO [16] and PSAO [17], with copper and TPQ at relatively short distance but not directly bound, except in an inactive form of the protein [16].

Copper influence on BSAO reactivity with substrate and inhibitors

Copper removal made TPQ reduction by benzylamine slower by a factor of approx. 10^3 but had relatively little effect on the benzylamine affinity for BSAO because the complex dissociation constant (4.9 ± 0.5 mM) was of the same order of magnitude as the enzyme's K_m, 2.2 mM, under similar experimental conditions [28]. Changes in ionic strength had an opposite influence on the kinetic parameters of BSAO, affecting K_m but hardly affecting k_c [28]. These differences are quite understandable if TPQ is close to copper and deeply buried in the protein interior with no opening to solvent access [16,17]. This might account for the relative insensitivity of k_c to ionic strength and the high sensitivity to copper removal. However, the high sensitivity of substrate affinity to ionic strength and the low sensitivity to copper removal suggest that the binding site is far from copper and near the protein surface.

The presence of a non-covalent binding site apart from the active site might help to rationalize the variable effect of the removal of copper on the reactivity of the substrate and inhibitors and might also explain why the reactivity of BSAO with substituted benzylamines could be accounted for by hydrophobic properties [29], whereas the reactivity of the similar enzyme from pig plasma with substituted benzylamines and hydrazines was accounted for by nucleophilic properties [30,31]. The non-covalent binding is affected by hydrophobicity of the substrate and, because it precedes the nucleophilic attack, does not preclude the reaction from being dependent on nucleophilicity. Hydrophobic effects predominate when molecules with a similar pK_a,

such as hydrazides, are examined. Benzohydrazide reacted with copper-depleted BSAO at a slower rate than both semicarbazide, lacking a hydrophobic group, and *p*-pyridine-2-ylphenylacetohydrazide, carrying a longer hydrophobic chain. The fast reaction of substituted phenylacetohydrazides with holo-BSAO was related to the formation of a hydrophobic bond at some distance from TPQ [20,21]. This interaction might change the protein conformation, opening a pathway to the active site for the hydrazide moiety, which reacts faster than semicarbazide. The conformational change should be assisted by copper, being slower in the copper-depleted enzyme than in holo-BSAO. In a series of molecules such as benzylamine, benzylhydrazine, phenylhydrazine and benzohydrazide, with the benzene ring unsubstituted in the *para* position, but with different pK_a values (9.33, 7.13, 5.14 and 3.53 respectively), the nucleophilicity becomes an important factor. Benzylamine, with the highest pK_a, showed the fastest reaction. Benzylhydrazine was strongly bound and unable to react with the cofactor, whereas phenylhydrazine and benzohydrazide preserved some reactivity because the effect of a still lower pK_a value was counterbalanced by the decreased hydrophobic character owing to the absence of a methylene group.

Comparison of properties of BSAO and LSAO

BSAO and LSAO were previously shown to react with inhibitors in the ratio of one and two molecules per dimer respectively [23,32]. Some spectroscopic differences between the adducts were explained by a stronger tendency of copper to interact with TPQ in LSAO [23]. This conclusion is supported by the present results showing that the differences are abolished by copper depletion. This is apparent in the case of thiosemicarbazide, because the substantial differences between the spectra of holo-adducts [23,32] are reduced to 10 nm in the copper-depleted derivatives [33].

As far as primary structure, substrate specificity, turnover number and reactivity with inhibitors are concerned [2,17,34,35], LSAO shows a close analogy with PSAO. BSAO resembles instead the prokaryotic ECAO in substrate specificity, reactivity with one phenylhydrazine molecule per dimer [27], and in the presence of the N-terminal stretch [2] giving rise to the D1 domain of ECAO [16], absent from the X-ray structure of PSAO [17]. This suggests that the disordered TPQ location in ECAO structure, with 'not unequivocal ring orientation' [16], reflects a difference between the two subunits. The structure is well defined in PSAO, where the two subunits have identical reactivities.

Conclusions

This study represents the first attempt to explain kinetic data on the basis of new structural information. It is proposed that substrate access to the active site is mediated by the binding to a hydrophobic pocket, probably near the protein surface, which induces an 'open' conformation in a reaction controlled by copper. The metal probably also controls the oxygen access to the site, because the free quinol is easily oxidized in air [3,36]. In this respect copper most probably plays a structural role, as other metals can induce reoxidation [10,12] and fast reactions with phenylhydrazine and benzylhydrazine [11]. A relationship between protein conformation and oxygen access to the site is suggested by the reoxidation of TPQ in copper-depleted BSAO at high temperature in the presence of *p*-pyridine-2-ylphenylacetohydrazide, leading to the formation of the adduct, albeit in a denatured form (Figure 4).

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