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Benzylhydrazine as a pseudo-substrate of bovine serum amine oxidase

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Bovine serum amine oxidase is inhibited by benzylhydrazine (BHy), but recovers full activity after a few hours incubation [Hucko-Haas & Reed (1970) Biochem. Biophys. Res. Commun. **38**, 396–400]. The first phase of the process, requiring about 15 min, was found to consist of a mechanism-based hydrazine-transfer reaction leading to formation of the hydrazine-bound enzyme, benzaldehyde and H_2O_2. At variance with the enzymic process, the reaction with O_2 preceded the benzaldehyde release. Two reaction intermediates could be characterized by optical spectroscopy and were assigned as the azo derivative and the benzaldehyde hydrazone, the latter one probably being involved in the reaction with O_2. No reduction of Cu was detected at any stage. The hydrazine adduct could also be obtained by stoichiometric reaction of hydrazine with the native enzyme. The decay of this species occurred in about 8 h and was not studied in detail. The Cu-binding inhibitor *NN*-diethyldithiocarbamate affected the BHy reaction by stabilizing the benzaldehyde hydrazone form as against the azo derivative and the reaction with O_2. However, under these same conditions the initial spectroscopic properties of the diethyldithiocarbamate adduct were recovered if the oxidase was left overnight. The reaction with O_2 was abolished only upon removal of at least one Cu atom from the enzyme. On the basis of the failure to detect any change of Cu redox state and the enzyme behaviour in the presence of inhibitors, a reaction mechanism involving the formation of a hydroperoxy intermediate, as in the FAD-containing enzymes, is tentatively proposed.

INTRODUCTION

Bovine serum amine oxidase (BSAO; EC 1.4.3.6) catalyses the oxidative deamination of primary amines with production of aldehyde, H_2O_2 and NH_3. It is made up of two subunits of identical M_r (approx. 90000) and contains two Cu^{2+} ions and a single organic cofactor sensitive to carbonyl reagents, recently identified as pyrroloquinoline quinone (PQQ) (Ameyama *et al.*, 1984; Lobenstein-Verbeek *et al.*, 1984). The two tetragonally co-ordinated Cu^{2+} ions are indistinguishable by e.p.r. at X-band and spin-echo spectroscopy (Mondovi' *et al.*, 1987), but their reactivities are not the same: they show differing abilities to bind *NN*-diethyldithiocarbamate (DDC) and, thereby, to be removed from the protein under reducing conditions or by freezing (Morpurgo *et al.*, 1987). The copper contribution to the optical absorption is very low, and the intense absorption band at 480 nm ($\epsilon = 3800\ M^{-1}\cdot cm^{-1}$) is due to the organic cofactor (Suzuki *et al.*, 1983). The band, which disappears under reducing conditions, was suggested (Morpurgo *et al.*, 1988) to be an $n \rightarrow \pi^*$ transition peculiar to polycyclic quinones (Murrell, 1963).

The similar enzyme from pig plasma was shown to operate by a Ping Pong mechanism (Pettersson, 1985). In the first phase of the process the amine is oxidized to aldehyde and the cofactor is reduced to the imino derivative by an amine-transferase mechanism that does not require the presence of O_2 (Olsson *et al.*, 1976). In the second phase the enzyme is reoxidized by O_2 with release of H_2O_2 and NH_3 (Olsson *et al.*, 1977). Copper is believed to be involved in oxygen-binding, since copper binding inhibitors (N_3^-, CN^-) act competitively towards O_2 and uncompetitively towards the amine substrate (Olsson *et al.*, 1978; Barker *et al.*, 1979). The same inhibitors also decrease the rate of enzyme reduction, with release of the aldehyde, indicating a role for copper even in the first phase of the catalytic process. More precisely, copper appears to control both of the actual redox steps (Olsson *et al.*, 1978). In lentil (*Lens culinaris*) seedlings amine oxidase, the removal of copper abolishes the process of reoxidation by O_2, but it does not prevent reduction of the enzyme by the substrate and aldehyde release (Rinaldi *et al.*, 1984), although it does decrease the rate of some intermediate steps of the reaction (Bellelli *et al.*, 1985). Copper removal also prevents reoxidation of the organic chromophore of BSAO (Suzuki *et al.*, 1983, Morpurgo *et al.*, 1987), and the inhibitory effects of N_3^- and CN^- are comparable with those on the pig enzyme (Dooley & Coté, 1985).

The present paper reports a study of the reactions of BSAO with benzylhydrazine (BHy). Like other carbonyl reagents BHy forms a coloured adduct with the enzyme (Morpurgo *et al.*, 1988). In most cases the adduct is stable, but with some hydrazines, including BHy, it decomposes on prolonged aerobic incubation, with eventual recovery of the enzymic activity (Hucko-Haas & Reed, 1970). Hydrazines are inhibitors of a variety of redox-active proteins, among which are haem, copper and flavin containing proteins [Fitzpatrick & Villafranca

Abbreviations used: BSAO, bovine serum amine oxidase; BHy, benzylhydrazine; DDC, *NN*-diethyldithiocarbamate; PQQ, pyrroloquinoline quinone.

(1986) and references therein]. The inactivation mechanism leads, in the cases studied in detail so far, to the irreversible addition of the aryl or alkyl portion of the hydrazine to the cofactor (haem or flavin) or to a protein residue when no cofactor is present (Fitzpatrick & Villafranca, 1986). The latter statement refers to dopamine (3,4-dihydroxyphenethylamine) β-hydroxylase, which has recently been shown to contain PQQ (van der Meer *et al.*, 1988). A study of the reversible inactivation of BSAO therefore seemed worthwhile, especially in view of the fact that the reaction is slow enough to allow spectroscopic characterization of the intermediates and affords some analogies with the catalytic process described for the pig plasma enzyme. The effect on the reaction of Cu-binding inhibitors was also investigated in the hope of gaining more information on the still-debated catalytic role of copper.

MATERIALS AND METHODS

All chemicals were reagent grade. BHy oxalate was purchased from Fluka and the other reagents from Merck. They were used without further purification, except DDC, which was recrystallized from ethanol. BSAO was purified by the method of Turini *et al.* (1982). The protein concentration was measured from A_{280}, an absorption coefficient of 1.74 litre·g^{-1}·cm^{-1} (Suzuki *et al.*, 1983) being used. The concentration of the reactive carbonyl group was measured by means of the absorption band formed at 445 nm with phenylhydrazine by using $\Delta\epsilon = 38000$ M^{-1}·cm^{-1} (Suzuki *et al.*, 1983). Enzyme activity was determined at 37 °C by monitoring the oxidation of benzylamine to benzaldehyde at 250 nm ($\epsilon = 12500$ M^{-1}·cm^{-1}) (Suva & Abeles, 1978). The BSAO derivative containing a single DDC-bound Cu^{2+} was prepared as previously described (Morpurgo *et al.*, 1987). H_2O_2 was assayed by the horseradish-peroxidase-mediated oxidation of scopoletin (7-hydroxy-6-methylcoumarin), which was monitored by the decrease of the fluorescence emission at 470 nm (Root *et al.*, 1975). Benzaldehyde was assayed with a Beckman model 322 liquid chromatograph equipped with two high-pressure model 110 A pumps, a model 420 system controller, a model 210 sample injection valve and a model 165 variable-wavelength detector. The column was a Brownlee Laboratories Spheri-5 RP-18. Elution was first performed with a 10 min gradient from 7 to 32% (v/v) acetonitrile in 0.2% trifluoroacetic acid and then isocratically. The flow rate used was 1.0 ml/min and the effluent was monitored at 280 nm.

All experiments were carried out at 25 °C in 0.1 M-potassium phosphate buffer, pH 7.2, unless otherwise stated. Anaerobiosis was obtained by repeated cycles of evacuation and flushing with argon of the optical cuvette or the e.p.r. tube fitted to a Thunberg apparatus. Optical spectra were recorded on a Beckman DU-7 spectrophotometer. C.d. spectra were recorded on a Jasco 500 spectropolarimeter. E.p.r. spectra were recorded on a Varian E-9 spectrometer.

RESULTS

Reaction between BSAO and BHy: characterization of some intermediates and products

In Fig. 1 are reported the difference spectra obtained on treatment of BSAO solutions with a 5-fold excess of BHy. The spectra are characterized by a negative absorbance above 480 nm due to decrease of the absorption band of the native enzyme and by the formation of an intense absorption with maximum intensity at 405 nm that rapidly faded, producing an isosbestic point at 368 nm. This was lost when a new band began to be clearly formed at 335 nm, showing that more than two species were involved in the reaction. All of the enzyme was converted into the latter species in about 15 min. At the end of the reaction the spectrum showed a shoulder around 410 nm besides the maximum at 335 nm. The same absorption at 405 nm was formed by allowing BSAO and BHy to react under anaerobic conditions. Again the band rapidly decreased, forming an isosbestic point at 368 nm, and the single derivative produced under these conditions showed a broad absorbance with a maximum at 355 nm (Fig. 1, broken line). On admission of air the final spectrum was the same as that obtained in the 'aerobic' experiment. The 355 nm-absorbing species was therefore the first species formed in any event on decay of the 405 nm band, and only in the presence of air was it transformed into the species absorbing at 335 and 410 nm. The use of stoichiometric amounts of BHy did not modify the reaction pattern, but permitted the observation of the further slow decay of the 335 nm-absorbing derivative in parallel with recovery of native spectroscopic and catalytic properties. This process was completed in about 8 h and required a proportionally longer time with an excess of BHy. A spectrum identical with the last one, with maximum absorbance at 335 nm and a shoulder at 410 nm, could also be obtained, within

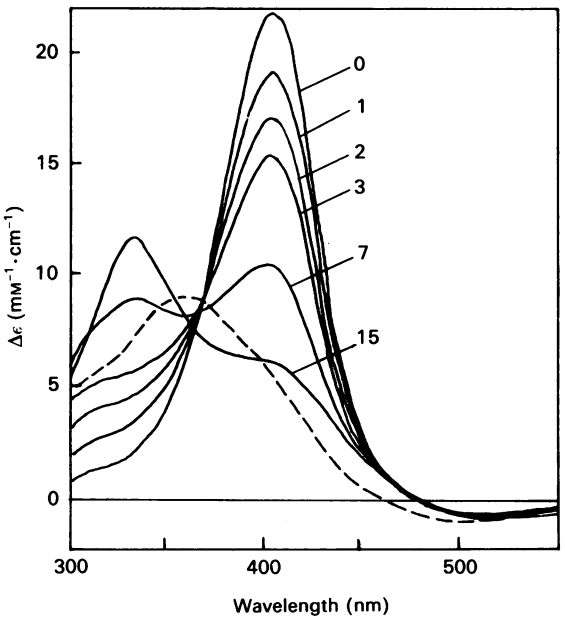


Fig. 1. Differential optical spectra of BSAO that has reacted with BHy

BSAO (20 μM) was allowed to react with 100 μm-BHy. The spectrum with highest absorbance at 405 nm was recorded immediately after BHy addition in air; the other continuous-line spectra, showing the decay of this species and the formation of the 335 nm peak, were recorded after 1, 2, 3, 7 and 15 min respectively; an identical solution gave, under anaerobic conditions, the broken-line spectrum after 10 min. BSAO that has not reacted provided the baseline.

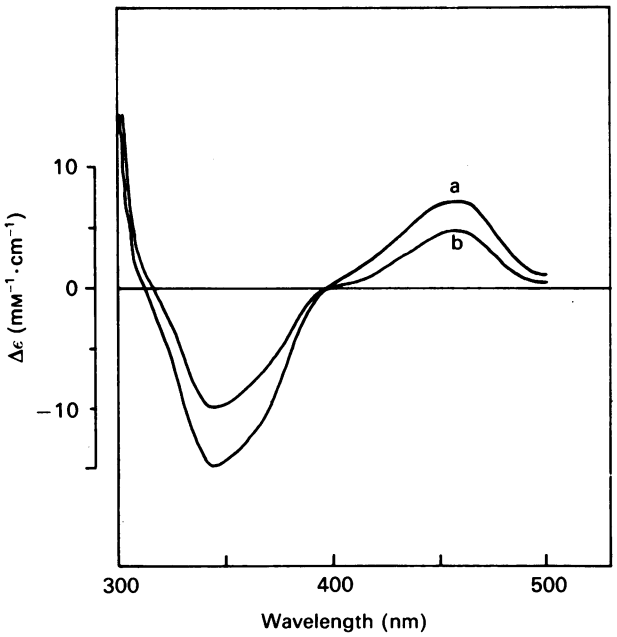


Fig. 2. C.d. spectra of hydrazine and BSAO that has reacted with BHy

BSAO (15 μM) was allowed to react with equimolar hydrazine (*a*) or equimolar BHy (b). Spectrum b was recorded after 15 min.

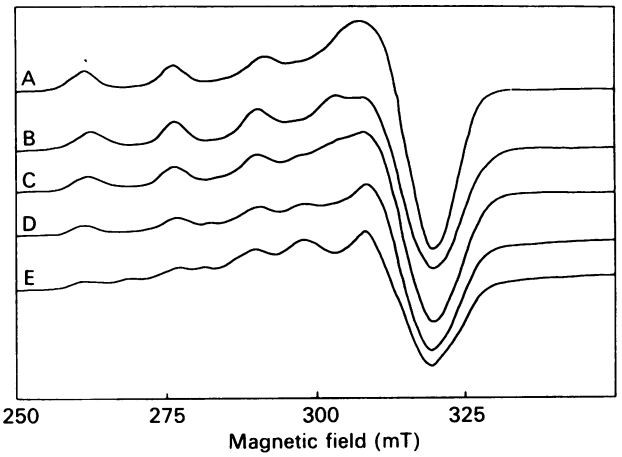


Fig. 3. E.p.r. spectra of BSAO and its BHy derivatives

A, 40 μM-native BSAO; B, frozen immediately after addition of equimolar BHy; C, thawed and frozen again after 5 min; D, thawed and frozen again after 30 min; E, native BSAO plus equimolar or excess hydrazine. Setting conditions were: microwave frequency, 9.15 GHz; microwave power, 20 mW; modulation amplitude, 1.0 mT; temperature, 100 K.

mixing time, by allowing BSAO to react with stoichiometric amounts of hydrazine. It also faded within 8 h with recovery of enzymic activity. Its shape was unaffected by up to a 100-fold excess of hydrazine. No other attempt was made to characterize this return to a protein with native properties.

When the reaction was monitored by c.d. spectroscopy, a parallel behaviour was observed. The positive Cotton effect immediately observed at 410 nm on aerobic reaction of BSAO with BHy rapidly decreased with formation of a positive band at 455 nm and a negative one at 345 nm. The latter spectrum is shown in Fig. 2 together with the identical one which was obtained on allowing BSAO to react with hydrazine. Fig. 3 shows the e.p.r. spectra of the native enzyme (curve A) and of the BHy derivative recorded immediately (curve B) and after 5 and 30 min (curves C and D respectively). The intensity of the signal did not change throughout. The last one is identical with that of the adduct of BSAO with hydrazine (curve E). It shows a large heterogeneity of the Cu^{2+} signal, unaffected by up to a 100-fold excess of hydrazine.

Optical, c.d. and e.p.r. spectra clearly show that a BSAO–hydrazine adduct was formed in the reaction. The other reaction products were found to be H_2O_2 and benzaldehyde. The formation of H_2O_2 was detected by the fluorimetric test described in the Materials and methods section; 0.4–0.5 mol of H_2O_2 were obtained per mol of enzyme as against 0.7–0.9 mol obtained when BSAO was allowed to react with stoichiometric amounts of benzylamine. The formation of benzaldehyde was determined by h.p.l.c. as also described in the Materials and methods section. The single peak obtained after 15 min incubation of BSAO with BHy had the same retention time as benzaldehyde (either the pure reagent or that produced by the enzymic reaction with stoichiometric amounts of benzylamine). This peak was well separated from that of BHy, which was not retained by the column, and from that of benzoic acid, a possible reaction product (Bellin & Mahoney, 1972), that showed a shorter retention time. The amount of benzaldehyde produced was stoichiometric with the protein. A much lower benzaldehyde yield (0.3–0.5 mol/mol of BSAO) was obtained from a sample that had been allowed to react with BHy under anaerobic conditions up to complete formation of the 355 nm-absorbing species. Some O_2 leak could have occurred during the transfer of the solution to the h.p.l.c. column, which was carried out by plunging a Hamilton syringe in the anaerobic cuvette through a rubber cap. The yield rose to 1 mol/mol of BSAO in the same sample open to air.

Effect of copper-binding inhibitors

The reaction of BSAO with BHy followed a different and much slower pattern when the protein was preincubated with DDC at 6 mM. The 405 nm-absorbing species was almost unobservable, whereas the species absorbing at 355 nm was slowly formed in air in about 30 min. Then it slowly decayed, in about 1 h, to the 335 nm-absorbing adduct; 0.3 and 0.9 mol of benzaldehyde/mol of BSAO (corresponding to the latter two intermediates) were detected by h.p.l.c. In the presence of DDC the shoulder, at 410 nm, of the 335 nm-absorbing derivative obtained from native BSAO was changed into a resolved maximum shifted to 430 nm. The same shift was observed on reaction of DDC–BSAO with hydrazine. This reaction was also extremely slow, and only 30% of the protein was converted into the adduct after 6 h incubation at room temperature with a 5-fold excess of hydrazine. At lower DDC concentrations, or at a short incubation time, the effect of the inhibition was partial. The band at 405 nm was formed, but with diminished intensity. The loss of intensity closely paralleled the loss of benzylamine oxidase activity of the samples, as shown in Fig. 4. On overnight incubation the

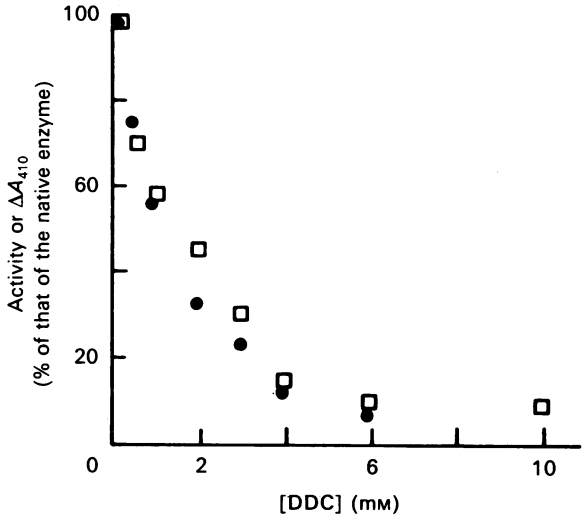


Fig. 4. Decrease of the activity (●) and of the 405 nm absorbance formed on aerobic reaction with BHy (□) of BSAO solutions preincubated 20 h with DDC

solutions treated with stoichiometric amounts of BHy recovered the original spectral properties of the BSAO–DDC adduct. However, when BHy was added to a Cu^{2+}-deprived BSAO–DDC sample (Morpurgo *et al.*, 1987), the reaction did not proceed beyond the formation of the 355 nm-absorbing species.

A somewhat similar behaviour was observed in the presence of N_3^-. The 405 nm-absorbing species was almost unobservable in the presence of 0.1 M-N_3^-, whereas the species absorbing at 355 nm was immediately formed in air. The decay to the BSAO–hydrazine adduct absorbing at 335 nm occurred in about 15 min, as with the native protein. However, the optical spectrum of the adduct was modified, i.e. the shoulder to the main band was shifted in this case to 425 nm. At 10 mM-N_3^- the effect was already very small and was not increased by preincubation, unlike the situation with DDC.

DISCUSSION

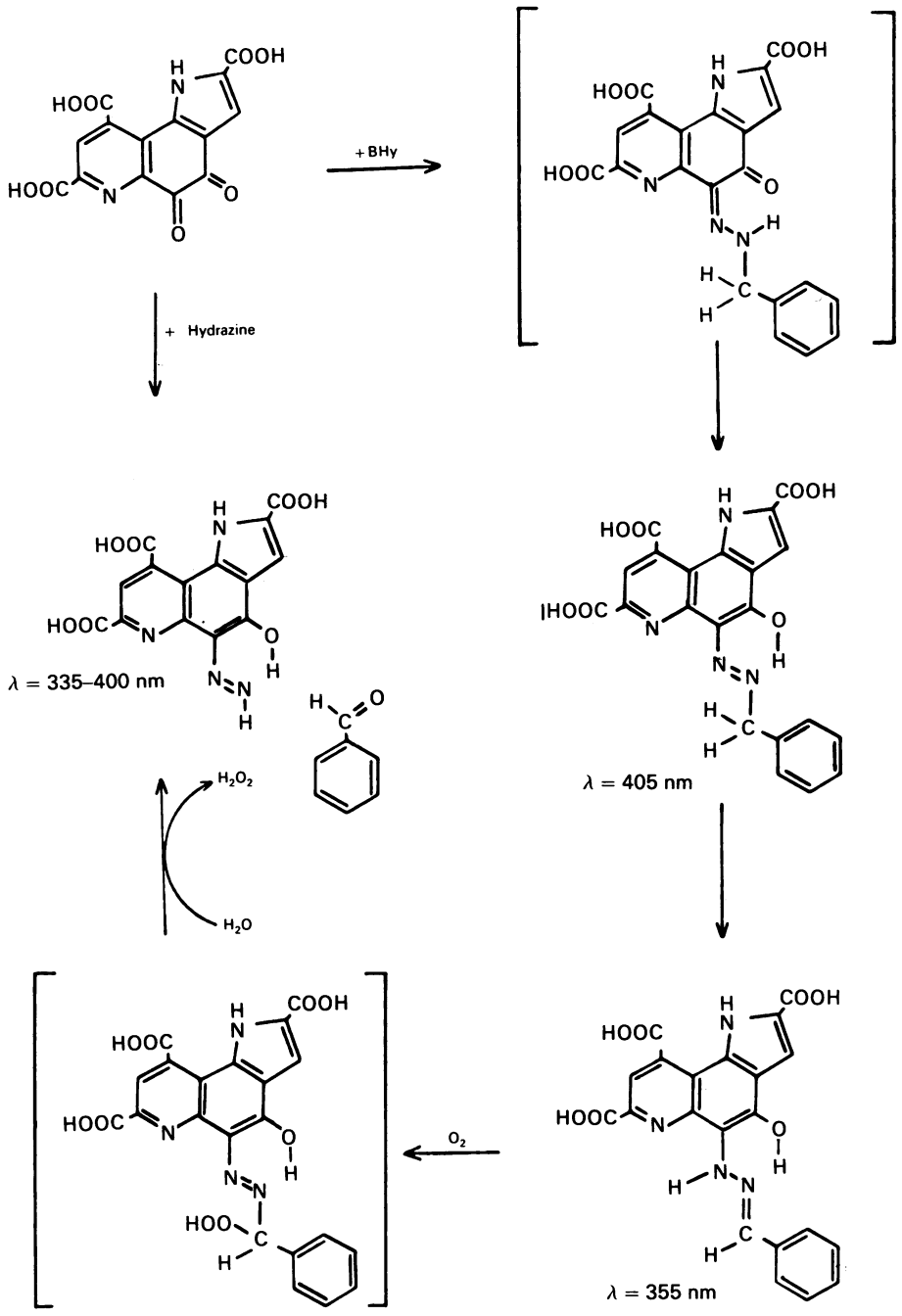
Identification of intermediates and products

Hydrazines are known to react in 1:1 stoichiometry with BSAO, forming either the hydrazone or the azo derivative (Morpurgo *et al.*, 1988). The species absorbing at 405 nm which was formed within mixing time both in air and under anaerobic conditions (Fig. 1) is assigned as the azo derivative rather than the benzylhydrazone (Scheme 1; compound in upper square brackets) because it absorbs at a relatively high wavelength (van der Meer *et al.*, 1987) and because it lacks the 480 nm band of the native protein. The disappearance of the 480 nm band was in fact related (Morpurgo *et al.*, 1988) to the formation of the azo derivative, in which the PQQ moiety is in a 'reduced' hydroquinonic conformation.

The species absorbing at about 355 nm, which was formed under anaerobic conditions on decay of the azo derivative, is assigned as the hydrazone of benzaldehyde for the low-wavelength absorbance and the persisting bleaching of the 480 nm band (Fig. 1, broken line) which makes it unlikely that it is the benzylhydrazone of PQQ. The same intermediate was also produced in the initial stage of the aerobic decay of the azo derivative, as an identical isosbestic point at 368 nm was formed. Subsequently the isosbestic point was lost, owing to the formation of a species absorbing at 335 nm with a shoulder at 410 nm. The latter derivative, which was also formed on exposure to air of the anaerobic solution, is clearly the BSAO–hydrazine adduct, because of the identity of its optical, c.d. and e.p.r. spectra with those of native BSAO that had been allowed to react with hydrazine. This is also assigned as the azo adduct (Scheme 1), because the 480 nm band of native BSAO was not restored (Fig. 1). At this stage 1 mol of benzaldehyde/mol of dimeric enzyme was detected by h.p.l.c., whereas a significantly less than stoichiometric amount (0.3–0.5 mol) was obtained from anaerobically treated solutions. Since the 355 nm band of the benzaldehyde hydrazone was stable under anaerobic conditions, it seems likely that the detected benzaldehyde results from O_2 leaks during transfer from the anaerobic cuvette to the h.p.l.c. column. H_2O_2 was also detected in the solutions after reaction with O_2.

Reaction mechanism

In the catalytic reaction of the amine oxidase from pig plasma (Olsson *et al.*, 1976) and from lentil seedlings (Bellelli *et al.*, 1985) with substrates at least two steps precede, under anaerobic conditions, the hydrolytic release of the aldehyde with formation of the reduced cofactor–amine derivative. In the scheme outlined by Petterson (1985) for the pig enzyme, the two steps are identified with formation of the Schiff base of the carbonyl group of the cofactor and isomerization to the Schiff base of the aldehyde. The reoxidation by O_2 also proceeds in two steps, with the release, in one of such steps, of H_2O_2 and NH_3 (Rius *et al.*, 1984). A very similar mechanism is proposed in Scheme 1 for the reactions of BSAO with BHy described above. Such reactions are in fact equivalent to one catalytic cycle of BSAO with benzylamine, resulting in the production of benzaldehyde and H_2O_2 and in the transfer of hydrazine from BHy to PQQ. Hydrazine, however, could not be hydrolysed away like NH_3, since the opposite reaction, i.e. formation of the adduct from BSAO and hydrazine, was favoured. Similarly the benzaldehyde hydrazone was more stable than the corresponding Schiff base and did not hydrolyse under anaerobic conditions, but only after the reaction with O_2. The oxidation was not prevented by Cu^{2+}-binding inhibitors such as N_3^- and DDC, although its rate was substantially decreased by the latter compound. The effect of the bidentate ligand was more dramatic than, but qualitatively similar to, that of N_3^-. It resulted in the stabilization of the benzaldehyde hydrazone absorbing at 355 nm with respect to the azo derivative and the O_2 reaction. N_3^- is a reversible inhibitor of amine oxidases and competitive toward O_2 (Olsson *et al.*, 1978; Barker *et al.*, 1979; Dooley & Coté, 1985), suggesting that the copper sites are implicated in O_2 binding. DDC was found instead to react slowly and irreversibly with the protein, acting as a bidentate ligand of the tetragonal Cu^{2+} (Morpurgo *et al.*, 1987). In the latter case, competitive or additional O_2 binding to Cu^{2+} seems unlikely to occur. Moreover, copper-catalysed reactions with O_2 usually involve Cu^+ intermediates (Al-Arab & Hamilton, 1986), which were never detected either in the catalytic cycle of the pig plasma amine oxidase (Grant *et al.*, 1978) or in the present study. For these reasons, in Scheme 1 the reaction with O_2 is tentatively proposed to procede



Scheme 1. Sequence of reactions of BSAO with BHy and hydrazine

The compounds are identified by their absorbance, whereas the postulated ones are enclosed in square brackets.

via a hydroperoxy intermediate. Compounds of the same type were reported to be formed in the autoxidation of phenylhydrazones (Bellamy & Guthrie, 1965) and in the oxidation of 1,3,5-triphenylformazan by singlet O_2 (Bellin & Mahoney, 1972). The mechanism of Scheme 1 does not exclude N_3^-/O_2 competition, since the inhibitor stabilizes the 355 nm species and slows down the reaction with O_2.

The stabilizing effect of DDC on particular tautomers was reported for BSAO hydrazides, and it was shown to be analogous to the effect of pH (Morpurgo *et al.*, 1988). This suggests that Cu^{2+}-bound inhibitors may affect the basicity of a group involved in the reaction, by modifying the electronic properties of the metal or its conformation. Binding of copper to the cofactor, at a site distinct from the hydrazine-binding one (Finazzi-Agro' *et al.*, 1977) provides a possible explanation for this behaviour. If such binding is to be excluded, on the basis of n.m.r. and fluorescence studies (Williams & Falk, 1986; Lamkin *et al.*, 1988), then it seems possible that copper affects the basicity of a protein residue at the catalytic site, which assists in the reaction (Farnum *et al.*, 1986). The two possibilities are not mutually exclusive, owing to the presence of two inequivalent metal ions (Morpurgo *et al.*, 1987). In the native pig plasma enzyme the two Cu^{2+} ions display Q-band e.p.r. signals slightly different

from each other (Barker *et al.*, 1979), and only one of the two Cu^{2+} ions is sensitive to the redox state of the cofactor (Grant *et al.*, 1978). In the present case the heterogeneity of the e.p.r. signal is apparent even at X-band in the BSAO–hydrazine adduct, showing again that a single Cu^{2+} ion was affected. On the other hand, both Cu^{2+} ions were modified at the BHy-binding stage, as previously found with hydrazides (Morpurgo *et al.*, 1988), probably attributable to the aromatic portion of the molecule.

When one of the Cu^{2+} ions was removed from the protein that had reacted with DDC, the first phase of the process, i.e. BHy binding with formation of the 355 nm-absorbing species, occurred at the same rate as in the holoprotein, that had reacted with DDC, but the second phase, i.e. the reaction with O_2, was abolished. The reoxidation of the chromophore by O_2 was also prevented in BSAO depleted of a single Cu^{2+} ion under reducing conditions (Morpurgo *et al.*, 1987). The crucial effect on the reactions with O_2 of the removal of even a single Cu^{2+} ion could be related either to a more dramatic structural change than that produced by DDC binding or, perhaps, to the different spin multiplicity of the system.

Conclusions

The reaction of BSAO with BHy appears to follow a pathway showing many analogies with that of the substrate. The effect of Cu^{2+}-binding inhibitors confirms a previous conclusion (Olsson *et al.*, 1978) that the catalytic role of copper is not restricted to the reaction with O_2. Whether the analogy can be extrapolated to include a hydroperoxide intermediate in the turnover reaction of BSAO like in that of flavin-containing amine oxidases (Ball & Bruice, 1980) is purely speculative. The most important difference between the enzymic and the BHy oxidation process was actually observed at this stage, since the release of the first product, the aldehyde, occurs in the absence of O_2 when the real substrate benzylamine is used (Pettersson, 1985). On the other hand, the Cu^{2+}-binding inhibitors acted in a very similar way on both the enzymic and the present reaction (Fig. 4), through the stabilization of equivalent intermediates, the benzaldehyde Schiff base and the benzaldehyde hydrazone respectively. This stabilization may cause competitive inhibition of O_2 reaction by N_3^-. An indirect involvement of copper in the reaction with O_2 was also indicated by a NMR study on pig kidney amine oxidase (Kluetz & Schmidt, 1977).

The reaction of BSAO with BHy is quite different from those reported for BHy and other hydrazines acting as inhibitors of either flavin-containing amine oxidases (Nagy *et al.*, 1979) or copper-containing enzymes such as dopamine β-hydroxylase (Fitzpatrick & Villafranca, 1986). The latter case is particularly interesting, since the enzyme was also recently shown to contain PQQ (van der Meer *et al.*, 1988). The reaction with BHy, which was described as a mechanism-based inhibitor, was different in the mechanism involving a radical and a Cu^+ intermediate and in the reaction products. Also different from the situation with BSAO (Morpurgo *et al.*, 1987) was the reaction of dopamine β-hydroxylase with phenylhydrazine (Fitzpatrick & Villafranca, 1986). The results of the present study suggest that the reactivity of the enzyme and the conformation of the adducts strongly depend on a number of factors related to the nature of the reacting molecule and to the proteinic environment. The same factors may be important in determining the particular reaction catalysed by each enzyme and the substrate specificity, in a manner very similar to that shown by the well-known pyridoxal-phosphate-dependent enzymes.

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