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An Investigation of Bovine Serum Amine Oxidase Active Site Stoichiometry: Evidence for an Aminotransferase Mechanism Involving Two Carbonyl Cofactors per Enzyme Dimer†

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ABSTRACT: Recent evidence has shown that the active site cofactor in bovine serum amine oxidase (BSAO) is 2,4,5-trihydroxyphenylalanine or 6-hydroxydopa [Janes et al. (1990) *Science 248*, 981]. However, much ambiguity remains regarding the mechanism of the enzymatic reaction. Conflicting data exist for both the number of functional active sites in the dimeric enzyme and for the oxygen dependence of product release. To resolve these questions, a new method has been developed for the purification of BSAO which leads to the isolation of specific activity ≥0.4 unit/mg of enzyme in 2–3 weeks. This highly active enzyme has been used to quantitate both aldehyde and ammonia release in the reductive half-reaction. Anaerobic incubation of enzyme and substrate resulted in the production of 2 mol of aldehyde/mol of enzyme, indicating the presence of a cofactor at each enzyme subunit. As anticipated for an aminotransferase reaction, no ammonia release was detected under comparable conditions. Active site titration of enzyme samples of varying specific activity with phenylhydrazine extrapolates to 1 mol of inhibitor/mol of enzyme subunit for BSAO of specific activity = 0.48 unit/mg. These findings contrast with numerous, previous reports of only one functional cofactor per enzyme dimer in copper amine oxidases.

Bovine serum amine oxidase (BSAO)[1] catalyzes the oxidative deamination of primary amines using molecular oxygen as a terminal $2e^-$ acceptor:

$$RCH_2NH_3^+ + H_2O + O_2 \rightarrow RCHO + NH_4^+ + H_2O_2 \quad (1)$$

The enzyme is a dimer of 170 kDa, comprised of identical subunits joined by disulfide bonds (Achee et al., 1968). Pure enzyme is peach colored and exhibits a broad absorption band centered around 480 nm. This band is lost when the enzyme is reduced, either with substrate or by inhibitors. Carbonyl reagents such as semicarbazide, phenylhydrazine, and 2,4-dinitrophenylhydrazine are potent inactivators of BSAO (Buffoni, 1966; Yasunobu et al., 1976; Moog et al., 1986). Reductive trapping experiments have shown that the active site prosthetic group is capable of forming a Schiff base with the amine of substrate during the course of catalysis (Hartmann & Klinman, 1987). The enzyme has also been reported to contain two tightly bound coppers per dimeric unit (Yasunobu et al., 1976). Experiments with copper-depleted enzyme indicate that the metal center is necessary for enzyme activity (Suzuki et al., 1986); however, no significant Cu(I) species is formed during catalysis, suggesting that enzyme-bound Cu(II) acts as an electron shuttle in the oxidation of reduced cofactor by molecular oxygen.

Although BSAO has been studied extensively since the 1950s, identification of the carbonyl cofactor has been hindered by its inherent reactivity and the fact that it is covalently linked to enzyme. The inability to isolate and characterize the cofactor has resulted in conflicting hypotheses regarding cofactor structure, which was originally attributed to pyridoxal phosphate (Buffoni, 1966; Buffoni & Cambi, 1990) and, later, to pyrroloquinoline quinone (Lobenstein-Verbeek et al., 1984; Ameyama et al., 1984). Recently, this ambiguity has been resolved with the identification of 6-hydroxydopa (topa) at the active site of BSAO (Janes et al., 1990).

The net reaction catalyzed by serum amine oxidases can be formalized as a series of partial reactions involving, first, proton abstraction and concomitant enzyme reduction, second, hy-

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[1] Abbreviations: BSAO, bovine serum amine oxidase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; FPLC, fast protein liquid chromatography; PH, phenylhydrazine; Con-A, concanavalin A; topa, 2,4,5-trihydroxyphenylalanine; NADH, α-nicotinamide adenine dinucleotide, reduced form; ADP, adenosine 5′-diphosphate.

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drolysis of a product imine intermediate, and third, reoxidation of reduced cofactor by dioxygen to produce hydrogen peroxide:

$$E_{ox} + RCH_2NH_3^+ \rightarrow E_{red}—NH^+=CHR \quad (2)$$

$$E_{red}—NH^+=CHR + H_2O \rightarrow E_{red}—NH_2 + RCHO \quad (3)$$

$$E_{red}—NH_2 + O_2 \rightarrow E_{ox} + H_2O_2 + NH_4^+ \quad (4)$$

According to this mechanism, reduced enzyme can hydrolyze in the absence of O_2 to release product aldehyde, eq 3, whereas ammonia release requires cofactor reoxidation, eq 4. The latter point is somewhat controversial, due to conflicting reports of the position of ammonia release during catalysis. In particular, Berg and Abeles (1980) have reported a stoichiometric release of ammonia following reaction of BSAO with benzylamine under anaerobic conditions, while Oi et al. (1970) found evidence for only benzaldehyde under anaerobic conditions. A more recent study using kinetic techniques has demonstrated that ammonia release is oxygen dependent for porcine serum amine oxidase (Rius et al., 1984). In order to resolve this issue for BSAO, a radiometric assay for anaerobic ammonia production has been developed: in support of eqs 2–4, no ammonia release could be detected during the anaerobic portion of the catalytic cycle.

Although serum amine oxidases are dimeric enzymes (Knowles & Yadav, 1984), the number of functional active sites has not been well understood. Experiments with phenylhydrazine and BSAO suggest that there is only one reactive carbonyl per enzyme dimer (Rinaldi et al., 1983; Suzuki et al., 1986), while earlier studies of the binding of mechanism-based inhibitors or quantitation of product release had indicated the presence of two active sites per enzyme dimer (Neumann et al., 1975; Berg & Abeles, 1980). A similar enzyme, porcine serum amine oxidase, has been studied extensively using inhibitors and anaerobic reaction conditions. Active site titration with phenylhydrazine indicates derivatization of only one carbonyl per enzyme dimer (Lindstrom & Pettersson, 1978a; Falk, 1983). More recently, Collison et al. (1989) have shown that 2-hydrazinopyridine can react with two carbonyls per dimer, albeit at different rates; however, the titration of one cofactor was sufficient to eliminate enzyme activity. In a similar manner, quantitation of anaerobic product release has indicated the presence of only one active site per enzyme dimer (Lindstrom & Pettersson, 1978b). Overall, the majority of investigators have concluded that dimeric serum amine oxidases contain a single active site. We have reinvestigated this question with BSAO using highly active preparations of enzyme. As shown herein, the monitoring of either anaerobic product formation or enzyme inhibition by phenylhydrazine indicates that BSAO contains two active sites per enzyme dimer.

Experimental Procedures

Materials

Methyl α-D-mannopyranoside, β-D-glucose, and HEPES were purchased from Calbiochem. Catalase, glucose oxidase, glutamate dehydrogenase, α-ketoglutarate, NADH, and ADP were purchased from Boehringer Mannheim Biochemicals. [7-^{14}C]Benzylamine and α-[1-^{14}C]ketoglutarate were purchased from Amersham Corp.; [^{14}C]phenylhydrazine hydrochloride was from ICN. CrystalFluor and Surfactol 100 were purchased from E & K Scientific Products. Phenylhydrazine hydrochloride and phenylhydrazine (>99% pure GC grade) were from Fluka, and potassium phosphate dibasic trihydrate was purchased from Sigma. Con-A–Sepharose, Ultrogel AcA34, and Q-Sepharose fast flow were purchased from Pharmacia/LKB. Ultrogel AcA 34 is now purchased from IBF Biotechniques. All other chemicals were of reagent grade unless otherwise noted.

Methods

Enzyme Purification and Assay. Bovine serum amine oxidase with a specific activity of ca. 0.4 unit/mg was prepared by a modification of existing procedures (Ishizaki & Yasunobu, 1980). The dialyzed pellet obtained after ammonium sulfate fractionation of 20 L of beef blood was chromatographed on 700 mL of Q-Sepharose fast flow using a gradient of 50–350 mM NaCl in 10 mM phosphate, pH 7.5. The BSAO activity was then applied to a 300-mL Con-A–Sepharose column, washed with 100 mM phosphate, pH 7.2, and eluted with 0.5 M methyl α-D-mannopyranoside in 100 mM phosphate, pH 7.2. The active fractions were collected, pooled, and concentrated by using an Amicon cell with a PM-30 membrane. The protein was then chromatographed on 1 L of Ultrogel AcA34 (10 mM KP_i, pH 7.2). We have found that large amounts of protein can be fractionated efficiently on a 1-L column with a diameter to length ratio of 1:25 rather than the 1:50 ratio traditionally recommended for sizing columns. This modification was necessary for the column to fit into our cold room. The most active fractions from the sizing column were subjected to back to back chromatography on 70-mL, pH 8.0 and 6.5, Q-Sepharose columns, using a stepwise gradient of 150, 180, 210, and 300 mM NaCl in 20 mM phosphate at pH 8.0, or a gradient of 50–150 mM NaCl in 20 mM phosphate at pH 6.5. The last two anion-exchange chromatography steps can also be done on an FPLC system using a Mono-Q Sepharose column. The purified enzyme was concentrated by using an Amicon cell and frozen. Protein concentrations were determined spectrophotometrically on either a Cary 118B or a Varian DMS 200 spectrophotometer at 280 nm using $E^{1\%}_{1\,cm}$ = 20.8 (Zeidan et al., 1980) and a molecular weight of 170 000 (Yasunobu et al., 1976). Although there has been some discrepency regarding the extinction coefficient and molecular weight of BSAO (Yamada & Yasunobu, 1962; Turini et al., 1982; Suzuki et al., 1983; Ishizaki & Yasunobu, 1980; Achee et al., 1968; Yasunobu et al., 1976), we find that (i) protein concentrations determined by Bio-Rad assay are consistent with those calculated from absorbance at 280 nm and (ii) the subunit molecular weight is clearly 85 000 on SDS gel electrophoresis. Enzyme activity was assayed according to the method of Neumann et al. (1975) and expressed in terms of international units (μmol/min·mg).

Anaerobic Reaction Conditions. Anaerobic conditions were obtained by using 99.999% pure argon from Matheson Gas Products, house vacuum, two gas bubblers, a firestone valve, a manifold capable of holding five stoppered reaction vessels, and a glucose oxidase/catalase O_2 scrubbing system. All liquids were dispensed with Hamilton gas-tight syringes. A solution of BSAO (100–400 μM) in 100 mM β-D-glucose and 10 mM potassium phosphate, pH 6.5, was introduced into a reaction vessel; a solution of benzylamine (1.0–1.5 mM) in the same glucose-containing buffer was put into a second reaction vessel, and three 1-mL test tubes were placed in the third reaction vessel. All reaction vessels were sealed with serum caps. Solutions were frozen in liquid nitrogen, and the enclosed system was subjected to repeated flush/purge cycles using the firestone valve and house vacuum to achieve anaerobic conditions. After the solutions were thawed, aliquots of a stock mixture of glucose oxidase and catalase were added to each solution to achieve a final concentration of 125 units/mL of each enzyme. Aliquots (50–150 μL) of the benzylamine solution were added to the three test tubes, and all

solutions were left at room temperature for 20 min to allow the glucose oxidase and catalase to remove remaining oxygen from the system.

Anaerobic Ammonia Production and Assay. Ammonia production under anaerobic conditions was determined according to the above procedure with the addition of a fourth vessel containing buffer and a fifth vessel containing the ammonia assay solution (3.5 mM NADH, 225 mM HEPES, pH 7.5, 8 mM ADP, 1.0 mM α-ketoglutarate, and 100 units/mL glutamate dehydrogenase). BSAO (50–250 μL) was added to the benzylamine aliquots in the test tubes, incubated for 3 min, and quenched with 50 μL of 1.0 N HCl. At the end of the reaction, the remaining enzyme solution and the buffer were removed from their reaction vessels and added to a set of ammonia standards (in scintillation minivials, containing 0–20 nmol of ammonium sulfate solutions and 50 μL of 1.0 N HCl). In this way a set of standard curves was generated both in the presence and in the absence of high enzyme concentrations. All samples were then neutralized by the addition of 1.0 M Na–HEPES base. α-[1-^{14}C]Ketoglutarate was added to the ammonia assay solution (final activity = 1.0 μCi/mL), and 100 μL of the radioactive assay was added to each sample. These were incubated for 2.5 h, the assay was quenched by the addition of 250 μL of acetone, and the samples were then heated at 80 °C for 20 min. The remaining α-[1-^{14}C]ketoglutarate in the samples was decarboxylated by treatment with 200 μL of fresh 4 M H_2O_2 for 20 min at 80 °C, followed by reaction with 100 μL of 1 N HCl for 20 min at 80 °C. Radioactivity remaining in the samples was determined by using a cocktail containing 3.6 g/L CrystalFluor in a mixture of water, toluene, and Surfactol 100 (10:60:30 v/v) on a Beckman LS 8000 liquid scintillation system. The amount of ammonia produced by the reaction of BSAO with benzylamine was determined by comparing the radioactivity remaining in the samples to those of the standard addition curve.

Anaerobic Benzaldehyde Production. Benzaldehyde production under anaerobic conditions was determined by using the aforementioned anaerobic reaction conditions with radiolabeled benzylamine (0.5–1.0 μCi/mmol). When the oxygen scrubbing was complete, 100–450 μL of the BSAO solution was added to benzylamine aliquots in test tubes, allowed to react for 3–5 min, and quenched with a 55–60% volume of spectral-grade acetone. The reaction mixtures were then removed from the apparatus, cooled to −20 °C, and spun in a microcentrifuge for 10 min. The supernatants were removed and the pelleting process was repeated until no further pellet was obtained. Samples were stored at −20 °C until injection on the HPLC. Benzylamine and benzaldehyde were separated at 34 °C on an Altex C-18 Ultrasphere ODS column using 12.5% methanol and 12.5% acetonitrile in 0.5% acetic acid at pH 4.0, while monitoring at 254 nm. Benzylamine eluted at 5.0 min and benzaldehyde eluted at 28.0 min using a flow rate of 0.8 mL/min. Fractions were collected and counted to determine radioactivity, and turnover was calculated on the basis of percent conversion of the benzylamine added to the reaction mixtures. HPLC was performed on Shimadzu or Beckman isocratic liquid chromatography systems.

Phenylhydrazine Titrations. Phenylhydrazine solutions were freshly made before each titration with 100 mM potassium phosphate, pH 7.2, and were protected from light. For a typical titration, 1 mL of BSAO (2.5 mg/mL) was titrated with 1.0–2.0-μL aliquots of 2.5 mM phenylhydrazine and allowed to react for 5 min between additions. The course of the titration was monitored by measuring the absorbance of the solution at 450 nm. The phenylhydrazine solutions were judged to be stable over the hour needed to complete the titrations. The activity of the phenylhydrazine-treated enzyme was determined with benzylamine as substrate (Neumann et al., 1975). Incorporation of phenylhydrazine into inactivated enzyme was also determined by using radiolabeled inhibitor. Aliquots of BSAO pools (0.7–1.2 mg/mL) with assorted specific activity were reacted with 21.5 nmol of [^{14}C]-phenylhydrazine (2.1 × 10^4 dpm/nmol) for 20 min. Unbound radioactivity was separated from the inhibited enzyme complex by chromatography on Bio-Rad 10-DP columns using 10 mM ammonium bicarbonate, pH 8, as elutant. Radioactivity in each fraction was determined by scintillation counting using Ecolite(+) cocktail.

Table I: Improved Purification Scheme for BSAO[a]

step	protein	enzyme units	specific activity (unit/mg)	yield of units (%)
serum	304 g	336	1.1×10^{-3}	100
supernatant	178 g	403	2.4×10^{-3}	100
dialyzed pellet	73 g	358	4.8×10^{-3}	100
Q-Sepharose	7.6 g	294	3.9×10^{-2}	82
Con-A–Sepharose	1.46 g	297	0.20	82
Ultrogel	816 mg	234	0.29	65
Q-Sepharose, pH 8.0	628 mg	197	0.31	55
Q-Sepharose, pH 6.0	336 mg	138	0.41	39

[a] See Methods for experimental details.

Results and Discussion

Enzyme Purification. Numerous purification schemes for BSAO have been presented in the literature (Tabor et al., 1954; Yasunobu et al., 1976; Summers et al., 1979; Turini et al., 1982). In general, the crude enzyme, obtained after ammonium sulfate fractionation of bovine serum, is purified through a combination of anion exchange, sizing, and affinity chromatography steps. The crucial step in these methods is the initial anion-exchange purification of the crude enzyme on either DEAE-Sephadex (Hartmann, 1988; Oda et al., 1981), DEAE-cellulose (Yasunobu et al., 1976), DE-52 (Summers et al., 1979), or AH-Sepharose 4B (Turini et al., 1982; Mondovi et al., 1983) Loading of enzyme onto these materials is generally fraught with problems, being characterized by protein breaking through the column or extreme swelling or shrinking of the column material. Our best preparation of enzyme, obtained with DEAE-Sephadex, was 30 mg of BSAO, specific activity = 0.36 unit/mg.

Although the latter specific activity is fairly high, the yield of enzyme was insufficient for the desired characterization of BSAO; this necessitated improving the enzyme preparation to the point where hundreds of milligrams of high activity enzyme could be readily obtained. Accordingly, we have modified the BSAO preparation to take advantage of a new class of preswollen column materials that can withstand higher flow rates and more rigorous conditions than Sephadex-type precursors (Table I). The use of Q-Sepharose fast flow in the initial anion-exchange column eliminates any difficulty associated with the loading of crude enzyme, decreasing the time required for this step from a week to a day. We believe this decrease in time is an essential feature in obtaining high specific activity enzyme. Con-A–Sepharose has been used instead of hydroxyapatite, due to high protein recovery and the ability to remove ceruloplasmin (a common contaminant of the enzyme after the initial anion-exchange step). Subsequent size fractionation of the enzyme was done on a single large column of Ultrogel AcA34 (see Methods), rather than multiple 200–300-mL capacity columns. Two additional anion-exchange columns were used at the end of the preparation

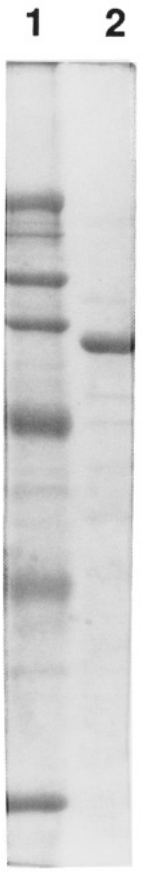


FIGURE 1: Gel electrophoresis profile of purified bovine serum amine oxidase. SDS gel electrophoresis run according to Laemmli (1970), using 10% acrylamide. Lane 1: Standards contain myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa). Lane 2: 1 μg of BSAO, prepared according to Table I.

Table II: Comparison of Enzyme Preparations

preparation	specific activity (unit/mg)	yield (uints)	yield[a] (%)	duration of preparation (weeks)
standard[b]	0.18–0.27	363	70	4–6
improved[c]	0.34–0.38	115	50	2–3
improved[d]	0.41	138	39	3

[a] Percent yield calculated from the units of enzyme in the dialyzed pellet that was loaded on the first column. [b] Preparation used in Hartmann (1988). [c] As in Table I, but Ultrogel column ran poorly. [d] Table I.

to perform a rough isoelectric focusing purification of the enzyme. With these changes it is now possible to obtain 300–400 mg of highly active BSAO in 2–3 weeks. The most active fractions from the final column were pooled and concentrated such that 0.41 unit/mg represents an average activity; the highest enzyme activity observed during the course of enzyme preparation has been 0.44 unit/mg. Pooled enzyme is a single band on gel electrophoresis and is judged to be >95% pure (Figure 1).

In Table II, yields and specific activities have been compared for several enzyme preparations. In all cases, protein preparations appear equivalent on gel electrophoresis, suggesting that changes in enzyme specific activity are due to inactivation, rather than incomplete separation of BSAO from contaminating proteins. As will be shown below, these enzyme preparations indicate different stoichiometries with regard to their carbonyl content.

Quantitative Measurement of Anaerobic Production of NH_3. The kinetic technique used by Rius et al. (1984) for

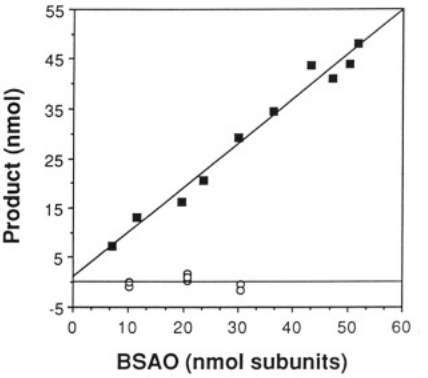


FIGURE 2: Anaerobic product release. Aldehyde production data (■). Ammonia production data (○). The slope of the benzaldehyde line is 0.89 nmol of benzaldehyde/nmol of BSAO subunits.

NH_3 release from porcine serum amine oxidase could not be applied to the bovine enzyme, due to differences in rate-limiting steps (Palcic & Klinman, 1983). In order to assess the oxygen dependence of ammonia release from BSAO, it was necessary to measure the amount of ammonia produced from the enzyme after a single anaerobic turnover. This technique required accurate measurement of nanomole quantities of ammonia and strict maintenance of anaerobic conditions. Although BSAO has a high affinity for oxygen [$K_m(O_2)$ = 14 μM] (Oi et al., 1970), it can be kept anaerobic if enzyme concentrations are greater than 10 μM and a glucose oxidase/catalase oxygen scrubbing system is present. By use of a modification of a glutamate dehydrogenase ammonia assay from Levitzki (1970) and a radiometric assay procedure from Kalb et al. (1978), a suitable assay was developed. In order to eliminate possible BSAO turnover during the lengthy NH_3 assay, it was necessary to inactivate BSAO with HCl, followed by reneutralization to pH 7.5 with buffer (cf. Methods). The ammonia content of anaerobic reaction mixtures was determined against a standard curve containing a mock reaction mixture without benzylamine, but including BSAO. Although this procedure is very enzyme intensive, it was necessary because the addition of BSAO to standards generally suppresses the slope and magnitude of standard addition curves.

As shown in Figure 2, we have been unable to detect any NH_3 production during anaerobic reaction of BSAO and benzylamine. Control experiments established that NH_3 could be measured if O_2 were introduced into the anaerobic system. In an effort to account for an earlier report of anaerobic ammonia production with BSAO (Berg & Abeles, 1980), we examined whether the previously used procedure of ammonium sulfate precipitation would lead to anaerobic ammonia release. Accordingly, purified enzyme was concentrated by precipitation with ammonium sulfate, followed by extensive dialysis to remove noncovalently bound NH_3. When this enzyme was reacted with benzylamine under anaerobic conditions, 21% of the enzyme active sites released ammonia (Table III). We note that the measured NH_3 release cannot be the result of simple NH_3 contamination in enzyme samples, since BSAO is present in standard curves. It appears likely that high concentrations of ammonium sulfate result in addition of ammonia to one of the cofactor carbonyls. In the presence of substrate, a transimination reaction would be expected, releasing ammonia:

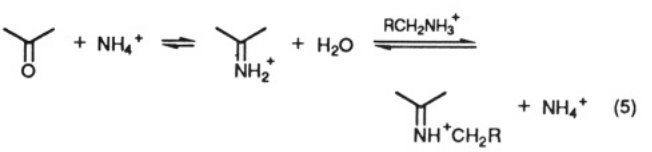
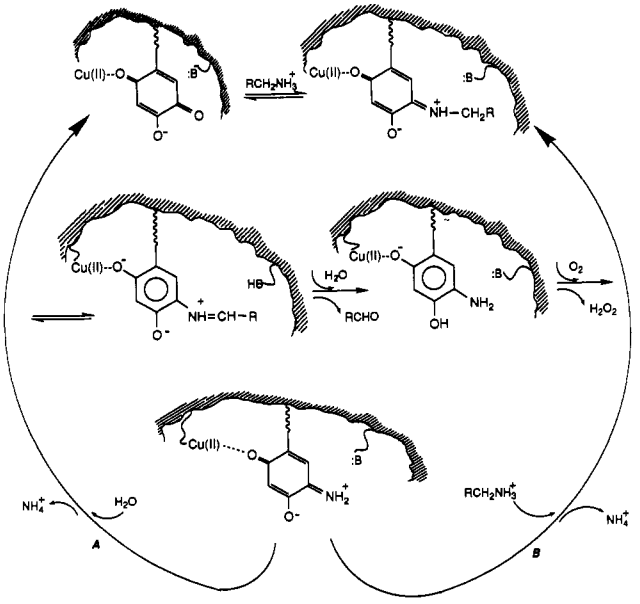


Table III: Anaerobic NH_3 Release from Bovine Serum Amine Oxidase Precipitated with Ammonium Sulfate[a]

BSAO[b]			
NH_3 release (nmol of active sites)	unit/mg	specific activity, ^{14}C cpm	nmol
6.2	0.26	6.41×10^4	1.4
6.2	0.26	6.37×10^4	1.2

[a] A BSAO stock solution was brought to 75% staturation with ammonium sulfate, incubated at 5 °C for 30 min, and centrifuged at 12000 rpm for 30 min to obtain a protein pellet. The pellet was dissolved in 0.5 mL of 10 mM phosphate buffer, pH 7.2, and dialyzed for 24 h against a total of 8 L of 10 mM phosphate buffer. This dialyzed enzyme was used in anaerobic ammonia determination (see Methods). [b] Concentration of enzyme sites, corrected for inactive subunits (cf. Results and Discussion, under Phenylhydrazine Titration as a Measure of Active Site Stoichiometry, and Figure 4).

Scheme I: Possible Steady-State Mechanisms for BSAO[a]



[a] As illustrated, interaction of enzyme with substrate produces the amino form of reduced cofactor during the first half-reaction via a base-catalyzed proton abstraction reaction [cf. Hartmann and Klinman (following paper in this issue)]. Subsequent interaction with oxygen leads to an imino form of oxidized cofactor, which can release NH_3 to solvent through attack of either water (pathway A) or substrate (pathway B). Analogous to the mechanism of Hartmann and Klinman (following paper), a possible role for copper has been incorporated into the mechanism.

We therefore propose that an earlier report of anaerobic ammonia release from BSAO (Berg & Abeles, 1980) is a result, in part, of covalent binding of NH_3 to BSAO during the final stages of enzyme preparation. The present result, which establishes an aminotransferase mechanism for BSAO, is consistent with that of Rius et al. (1984) on porcine serum amine oxidase.

The demonstration of an amino form of reduced cofactor under anaerobic conditions raises questions regarding the position of ammonia release in the presence of oxygen. As illustrated in Scheme I, steady-state incubation of enzyme with substrate is expected to produce an amino form of reduced cofactor via a base-catalyzed proton abstraction mechanism [cf. Hartmann and Klinman (following paper in this issue)]. Subsequent binding and reaction of dioxygen are expected to produce an imine species, which can recycle by two possible pathways: According to pathway A, ammonia is rapidly hydrolyzed from cofactor by active site water, such that subsequent attack of substrate occurs at a carbonyl of cofactor. For

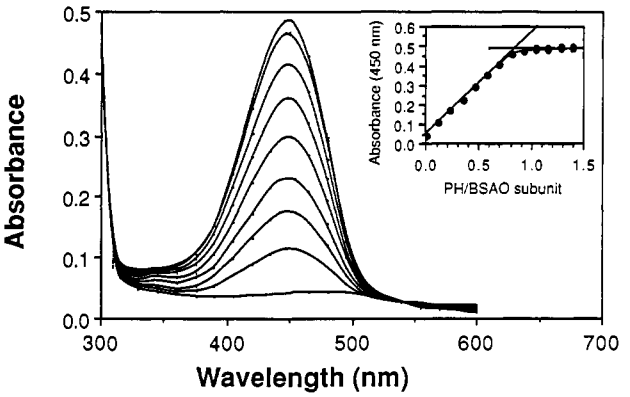


FIGURE 3: Phenylhydrazine titration. The spectra were taken during the titration of 1.46 mg/mL BSAO, specific activity = 0.36 unit/mg, with 1.00 mM phenylhydrazine in a 1-mL cuvette. The relationship between absorbance at 450 nm and the phenylhydrazine (PH) to enzyme ratio is shown in the inset.

the second mechanism, pathway B, cofactor imine undergoes direct attack by substrate. Although an imino group of oxidized cofactor is expected to be more reactive than a free carbonyl toward substrate, water is readily accessible to the active site (aldehyde is rapidly released from product imine during anaerobic turnover). The distinction between pathways A and B is clearly a kinetic one and may depend on the concentration of substrate, such that in the limit of saturating substrate pathway B predominates.

Stoichiometry of Anaerobic Benzaldehyde Production. Benzaldehyde production was measured over a large range of enzyme concentrations, with an upper limit for BSAO of 15–20 mg/mL; at this level of protein, fractional turnover from leakage of O_2 into the anaerobic system is insignificant. As shown in Figure 2, the amount of benzaldehyde produced by enzyme of specific activity 0.38 unit/mg was 0.89 ± 0.05 nmol of product/nmol of BSAO subunit. Although the stoichiometry is slightly reduced from a maximum value of 1 mol/mol of subunit, the data show that each subunit of BSAO is capable of oxidizing substrate. Although this does not demonstrate that each subunit is catalytically competent under steady-state conditions, it is proof of the presence of a functional cofactor at each subunit of the enzyme.

Phenylhydrazine Titration as a Measure of Active Site Stoichiometry. Phenylhydrazine is a potent, irreversible inhibitor of BSAO. The enzyme can be cleanly titrated with phenylhydrazine by monitoring either loss of catalytic activity or increase in absorbance at 450 nm (Figure 3). We have observed that the end point of the titration of ammonium sulfate precipitated enzyme is considerably less sharp than that shown in the insert of Figure 3, which may account for reports of a marked decrease in the ability to perform end point titrations using low levels (1–2 mg/mL) of ammonium sulfate precipitated BSAO (Rinaldi et al., 1983). Using enzyme of specific activity = 0.38 unit/mg, we find 0.82 nmol of phenylhydrazine/nmol of enzyme subunit, in good agreement with the value obtained from the anaerobic benzaldehyde titration (Figure 2). When this titration was repeated with different enzyme preparations, the end point was found to be independent of enzyme concentration but dependent on the specific activity of enzyme. Independent of enzyme source, an extinction coefficient of 32.4 ± 1.7 mM^{-1} cm^{-1} (at 450 nm) was estimated for the complex formed between enzyme and phenylhydrazine.

The apparent variation of phenylhydrazine incorporation with enzyme specific activity was investigated further by measuring the incorporation of radiolabeled phenylhydrazine

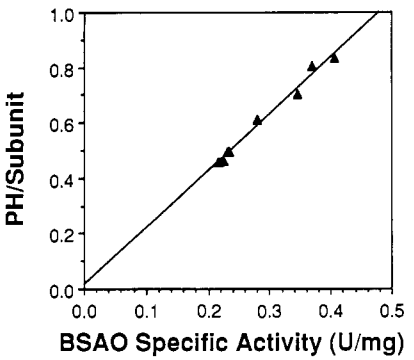
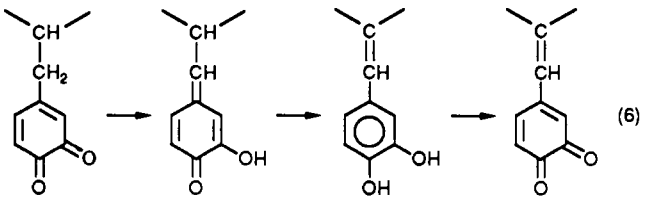


FIGURE 4: Dependence of [^{14}C]phenylhydrazine (PH) incorporation on enzyme specific activity.

into BSAO preparations characterized by specific activities 0.21–0.41 unit/mg (Figure 4). These data demonstrate a linear correlation between incorporation of radiolabel and enzyme activity, with extrapolation to 1 mol of phenylhydrazine/mol of BSAO subunit for enzyme of specific activity 0.48 ± 0.03 unit/mg. It can be seen that our best enzyme preparation of 0.41 unit/mg is still only ca. 85% active.[2]

The Myth of Half-Site Reactivity. Numerous reports exist in the literature proposing that serum oxidases are half-site reactive and/or contain only one cofactor per enzyme dimer (Knowles & Yadav, 1984; Collison et al., 1989, Falk, 1983; Suzuki et al., 1986; Rinaldi et al., 1982). However, our experiments show clearly that bovine serum amine oxidase contains two active sites per enzyme dimer. Previous reports of one cofactor per dimer with this enzyme may be due to the difficulty of obtaining enzyme with a specific activity greater than 0.3 unit/mg. As can be seen from Figure 4, use of enzyme with specific activity in the range of 0.2–0.3 unit/mg would give apparent half-site reactivity for the enzyme. As noted earlier (under Enzyme Purification), low specific activity preparations of BSAO frequently appear homogeneous by SDS gel electrophoresis, suggesting that reduced activity is due to inactive subunits, rather than the presence of protein contaminants. This raises the question of the apparent ease with which BSAO undergoes inactivation, leading to reduced titers for the active site carbonyl which parallel lowered specific activities (Figure 4). Possible insight into the inactivation process comes from recent studies of Waite and co-workers (personal communication), who have shown formation of methide species from dopa quinone:



(6)

Although analogous chemistry has not yet been demonstrated for 6-hydroxydopa (topa), it is known that the active site of BSAO contains a basic residue that catalyzes proton abstraction from both the α and β carbons of phenethylamine substrates (Farnum et al., 1986). Clearly, an acid/base-catalyzed isomerization of active site topa to either a methide structure or an α,β-unsaturated quinone would lead to significant changes in the spatial orientation of cofactor, which would be expected to preclude complex formation with substrate and subsequent catalysis.

[2] The highest specific activity previously reported for BSAO is 0.36 unit/mg (Berg & Abeles, 1980), which can be estimated as 75% active. Using this preparation, Abeles and Maycock (1976) reported two active sites per dimer, in contrast to subsequent reports of only half-site reactivity (Suzuki et al., 1986).

Added in Proof

Recent copper analyses of specific activity = 0.43 unit/mg indicate a copper content of 0.9 copper per subunit, for a ratio of copper atoms to phenylhydrazine reactive carbonyls of 1:1.

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Structure–Function Studies of Substrate Oxidation by Bovine Serum Amine Oxidase: Relationship to Cofactor Structure and Mechanism†

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ABSTRACT: The chemical mechanism of substrate oxidation, catalyzed by bovine serum amine oxidase, has been explored by a detailed investigation of structure–reactivity correlations. Past mechanistic studies, involving the reductive trapping of substrate to cofactor [Hartmann, C., & Klinman, J. P. (1987) *J. Biol. Chem. 262*, 962], implied the intermediacy of a substrate imine complex in the catalytic redox mechanism. These studies led to the proposal of a transamination mechanism for substrate oxidation, analogous to pyridoxal phosphate dependent enzymes. In pyridoxal phosphate catalyzed reactions, the transamination process involves the transient formation of a resonance-stabilized carbanion intermediate. Although evidence has been presented describing the participation of an active site base in bovine serum amine oxidase catalysis [Farnum, M. F., Palcic, M. M., & Klinman, J. P. (1986) *Biochemistry 25*, 1898], the nature of the intermediate derived from C–H bond cleavage has not been directly addressed. To examine this question, a structure–reactivity study was performed using a series of para-substituted benzylamines. Having prior knowledge of the intrinsic isotope effect for an enzymatic reaction permits calculation of microscopic rate constants from steady-state data [Palcic, M. M., & Klinman, J. P. (1983) *Biochemistry 22*, 5957]. Deuterium isotope effects on k_{cat} and k_{cat}/K_m parameters were determined for all substrates, allowing for the calculation of rate constants for C–H bond cleavage (k_3) and substrate dissociation constants (K_d). Pre-steady-state constants obtained for *p*-acetylbenzylamine, *p*-(trifluoromethyl)benzylamine, and unsubstituted benzylamine exhibited excellent agreement with values calculated from steady-state isotope effects. Multiple regression analysis yielded an electronic effect of $\rho = 1.47 \pm 0.27$ for the bond cleavage step, supporting the intermediacy of a carbanion species. An additional effect, determined from regression analysis, indicated inhibition of catalysis by hydrophobic substituents ($\pi = -0.71 \pm 0.21$). These results lead to a reaction mechanism for amine oxidation by the covalently bound cofactor in bovine serum amine oxidase, 6-hydroxydopa [Janes, S. M., Mu, D., Wemmer, D., Smith, A. J., Kaur, S., Maltby, D., Burlingame, A. L., & Klinman, J. P. (1990) *Science 248*, 981].

Bovine serum amine oxidase (BSAO)[1] belongs to the class of proteins designated copper amine oxidases. These proteins catalyze a spectrum of activities, which include the cross-linking of collagen and elastin (Siegel, 1979) and the regulation of intracellular polyamines (Bachrach, 1985). Although the precise function of the plasma amine oxidases is unknown, their broad specificity suggests a role in the regulation of blood plasma biogenic amines (Buffoni, 1966).

In addition to active site copper, the copper amine oxidases contain a covalently bound carbonyl cofactor capable of forming chromophoric derivatives with phenylhydrazines. The pursuit of detailed mechanistic studies of these proteins has been hampered by the absence of a confirmed structure for the active site cofactor. In fairly recent studies, Ameyama et al. (1984) and Lobenstein-Verbeek et al. (1984) proposed that the active site cofactor in BSAO was a pyrroloquinoline quinone (PQQ), a tricyclic quinone previously demonstrated to exist in a number of prokaryotes (Duine et al., 1987). However, new studies by Janes et al. (1990) show very clearly that the active site cofactor is a dihydroxy derivative of tyrosine, referred to as 6-hydroxydopa or topa.

Model studies of topa indicate that the reduced form [Figure 1 (**1**)] is quite unstable (Janes et al., 1990), undergoing rapid

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[1] Abbreviations: BSAO, bovine serum amine oxidase; UCB, University of California, Berkeley; NMR, nuclear magnetic resonance; PQQ, pyrroloquinoline quinone; THF, tetrahydrofuran; DME, ethylene glycol dimethyl ether.

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