Purification to Homogeneity and Initial Physical Characterization of Secondary Amine Monoxygenase*

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Secondary amine monoxygenase from *Pseudomonas aminovorans* grown on trimethylamine has been purified 265-fold to apparent homogeneity. The purified enzyme exhibits a specific activity of 14.7 μmol of NADPH oxidized per min per mg of protein, a native molecular weight of 210,000, and nondisulfide-linked subunits of molecular weight 42,000, 36,000, and 24,000, each of which is required for activity. The enzyme is extremely labile during purification; rapid handling and the presence of 5% ethanol are essential to enzyme stability. Storage at 77 K in the presence of NADH (1 mM) also confers considerable stability to the purified enzyme. The heme prosthetic group in the enzyme has been identified as protoporphyrin IX. The quantification of prosthetic group components reveals the presence of 1.6 mol of flavin as FMN, 2.0 mol of heme iron, 4.0 mol of acid-soluble (nonheme) iron, and 3.6 mol of free sulfide/210,000 molecular weight enzyme. Ferric and ferrous-CO secondary amine monooxygenase exhibit electronic absorption spectra that are very similar to those of analogous myoglobin derivatives and, therefore, quite distinct from parallel forms of cytochrome P-450, the most extensively studied heme iron-containing monoxygenase. Like myoglobin and, again, in contrast to P-450, this enzyme forms a very stable dioxygen complex. In fact, it is this oxygen-bound form of the enzyme that is obtained from the purification procedure. Once again, the absorption spectrum of oxygensenated secondary amine monoxygenase is nearly identical to that of oxyhemoglobin. The spectroscopic similarities between secondary amine monoxygenase and myoglobin suggest the presence of an endogenous histidine fifth ligand to the heme iron of the enzymes.

Secondary amine monoxygenase was first identified by Eady and Large (1) in 1969 and partially purified from extracts of *Pseudomonas aminovorans* grown on trimethylamine. The enzyme was shown to catalyze the NAD(P)H- and molecular oxygen-dependent N-dealkylation of secondary amines. Dimethylamine was reported to be the most active substrate and to form methylamine and formaldehyde as products. Initial studies revealed that the enzymatic activity was extremely sensitive to inhibition by carbon monoxide (2). This implicated the presence of an active site heme iron cofactor, which was later confirmed (3). Large and co-workers also observed the presence of flavin and acid-soluble (non-heme) iron prosthetic groups in the partially purified preparation. However, since no evidence concerning the subunit structure or molecular weight of secondary amine monoxygenase was presented, it was not clear whether the three prosthetic groups were present in a single oligomeric structure or on separable proteins as in the case of cytochrome P-4501 (4), the most extensively studied heme iron-containing monoxygenase. Preliminary spectroscopic studies of partially purified secondary amine monoxygenase revealed that the UV-visible absorption spectrum of the carbon monoxide-bound ferrous form had a Soret maximum at 420 nm (2). In contrast, the same derivative of P-450 exhibits a Soret peak at 450 nm (5-7).

The heme iron coordination structure of cytochrome P-450 has been the object of intensive study in numerous laboratories. The fifth ligand to the heme iron of P-450 has been identified as cysteinate (5-7). In order to further define the structural requirements for molecular oxygen activation and subsequent oxygen atom insertion by heme iron enzymes, we have begun to investigate the secondary amine monoxygenase system. To that end, we present herein a refined large scale purification procedure which allows for the rapid and efficient isolation of relatively large amounts of secondary amine monoxygenase. This purification scheme results in more than a 3-fold improvement in the final specific activity of the isolated enzyme compared to previous studies (8). Apparent homogeneity of the oligomeric enzyme as judged by native and denaturing polyacrylamide gel electrophoresis and by gel filtration chromatography is demonstrated for the first time. We also report our initial determination of the molecular weight and subunit composition and the prosthetic group quantification. The purified enzyme exhibits spectroscopic properties which are clearly different from those of P-450, suggesting the presence of an axial ligand other than cysteinate. Because both P-450 and secondary amine monoxygenase catalyze NAD(P)H and molecular oxygen-dependent N-dealkylations and are inhibited by CO, they provide an interesting pair of enzymes with which to study the structure-activity relationships within the functional class of heme iron monoxygenase enzymes.2

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1 The term "P-450" refers to the cytochrome P-450 in general and is used as a generic term regardless of the source of the enzyme. When referring to the *Pseudomonas* bacterial P-450 monoxygenase, the enzyme is specified as P-450-CAM throughout the text.

2 Peroxidase enzymes also catalyze N-dealkylation reactions but do so in a peroxide-dependent manner that is not inhibited by CO (9).
P. aminovorans was grown on trimethylamine as described by Large and co-workers (2,10) and modified by Andersson (11). Packed cells were stored at -70 °C until use. Ultrapure ammonium sulfate was the product of Schwarz/Mann. NADPH, NADH, N,N-tetramethylthelylenediamine, polyethylentlenimine, bovine serum albumin, reative red agarose (Type 6000-CL), molecular weight standards, Sepharose 6B, and Sepharose 4B were purchased from Sigma, sodium dodecyl sulfate from United States Biochemicals, DNase and RNase from Accurate Chemical and Scientific Corp., and Bio-Gel HTP, Bio-Gel P-6DG, and acrylamide from Bio-Rad. Dimethylamine (Aldrich) was recrystallized twice from absolute ethanol before use. Ethanol (95%) was distilled prior to use. All other chemicals were of reagent grade purity or better and were used without further purification.

**Activity Assays**

Enzymatic activity was measured by monitoring the oxidation of NADPH at 340 nm of a Cary 210 spectrophotometer at 25 °C. The assay mixture contained 158 μM NADPH, 80 μM dimethylamine in 50 mM potassium phosphate buffer, pH 7.0, and the reaction was initiated by the addition of the enzyme. One unit of activity is defined here as the oxidation of 1 μmol of NADPH/min at 25 °C in the assay buffer. The formation of formaldehyde was followed using the method described by Werringloer (12) for the Nash reaction (13).

**Concentration**

Purified enzyme was concentrated by using either a Centricon concentrator (Amicon) with a YM-30 membrane or a dialyzing concentrator (Pro-DiCon) with a PA-30 membrane. Concentration devices that involved stirring of the protein solution were found to substantially inactivate the enzyme in the time required for concentration.

**Protein Determination**

Protein concentration was determined by the method of Lowry et al. (14) when possible, using bovine serum albumin as a standard. For samples containing NADH, a modified Lowry method was employed in which the protein was precipitated with trichloroacetic acid prior to the assay (15). For affinity column fractions, protein was determined by the dye binding assay of Sedmak and Grossberg (16), initiated by the addition of the enzyme. One unit of activity is defined here as the oxidation of 1 pmol of NADPH/min at 25 °C in the assay (15). For affinity column fractions, protein was determined by the dye binding assay of Sedmak and Grossberg (16) when possible, using bovine serum albumin as a standard. For samples containing NADH, a modified Lowry method was employed in which the protein was precipitated with trichloroacetic acid prior to the assay (15). For affinity column fractions, protein was determined by the dye binding assay of Sedmak and Grossberg (16), initiated by the addition of the enzyme. One unit of activity is defined here as the oxidation of 1 pmol of NADPH/min at 25 °C in the assay (15).

**Spectrophotometric Measurements**

Spectrophotometric measurements were made at 4 °C unless otherwise noted on a Cary 210 spectrophotometer as interfaced to an IBM-PC via a digital interface port (Varian). A custom data manipulation program was used to normalize and plot the data. The oxygen-bound ferrous enzyme was isolated as a result of the purification procedure. Conversion of this form to the deoxyferrous ferrous enzyme was accomplished by exchanging the atmosphere of the cuvette with nitrogen, followed by gentle mixing of the sample via repeated inversion of the sealed cuvette. Bubbling of the sample at this stage was avoided since the stability of the enzyme complex was extremely sensitive to agitation. Complete conversion to the deoxyferrous enzyme was accomplished by the addition of a small excess of solid sodium dithionite. The CO-bound form of the ferrous enzyme was generated by very gentle bubbling of carbon monoxide through the solution of the deoxyferrous protein. Ferric secondary amine monoxygenase was prepared by addition of solid ferricyanide to the enzyme carrier protein and subsequent desalting on a Bio-Gel P-4 column to remove the excess oxidant.

**Prosthetic Group Determinations**

The heme prosthetic group was identified as protoporphyrin IX, and its concentration was determined by the alkaline pyridine hemochromogen method using ε_max = 34.4 mm−1 cm−1 for the pyridine hemochromogen (19, 20). The value was determined to be 0.075 μmol of 1 mL of 15 mM pyridine. Quantitation of acid-soluble (nonheme) iron, acid-insoluble (heme) iron (2), flavin (21), and free sulfide (22) was performed according to literature procedures. The results of determinations from two independently purified enzyme samples were in very good agreement; the average values are reported herein.

**Purification of Secondary Amine Monoxygenase**

**Step 1, Sonication—**P. aminovorans cells (~90 g) were thawed at 4 °C for 8 h and then suspended in 2 volumes (180 ml) of 50 mM potassium phosphate buffer, pH 7.5, by stirring for 20 min. Cells were disrupted using a Heat Systems/Ultrasonics, Inc. W-220F sonicator at full power in 3-min bursts with cooling to 5 °C between bursts in a dry ice-acetone bath for a total sonication time of 18 min. The maximum temperature of the mixture remained less than 15 °C during this procedure. Sonicated cells were centrifuged at 4 °C in a Sorvall GSA rotor at 12,000 rpm for 30 min. All steps following the sonication were carried out at 4 °C unless otherwise indicated.

**Step 2, Nucleic Acid Removal—**The supernatant from above was made 40 mM in MgSO4 by slow addition of a 400 mM MgSO4 stock solution in 50 mM potassium phosphate buffer, pH 7.5. DNase and RNase were added to a final concentration of 0.4 μg/ml each. This mixture was then allowed to stir for 30 min. Precipitation of nucleic acids was brought about by the dropwise addition of 6.0 ml of 5% trichloroacetic acid for each 100 ml of crude solution. The resulting suspension was allowed to stir an additional 15 min and then centrifuged as above for 30 min.

**Step 3, Ammonium Sulfate Precipitation—**The supernatant was made 5% in ethanol by the dropwise addition of 95% ethanol and allowed to stir for 5 min. The protein solution was then subjected to a 40–52% ammonium sulfate fractionation. The final pellet was resuspended in 40 mM potassium phosphate buffer containing 5% ethanol, pH 6.8. Fractions containing greater than 0.2 unit/ml activity were pooled, diluted 1:1 with Column Buffer, and centrifuged at 12,000 rpm in a Sorvall SA-600 rotor for 10 min.

**Step 4, Hydroxyapatite Column Chromatography—**The supernatant from above was loaded at 90 ml/h onto a 4 x 30 cm hydroxyapatite column which had been pre-equilibrated with Column Buffer. The loaded column was flushed with Column Buffer until the eluate was free of protein as determined by absorbance at 280 nm. Elution of the enzyme was effected with 100 mM potassium phosphate buffer containing 5% ethanol, pH 6.8. Fractions containing greater than 0.2 unit/ml activity were pooled. The hydroxyapatite column material could be reused after the elution of extraneous proteins from the column with 1 M potassium phosphate buffer, pH 6.8, containing 5% ethanol followed by extensive re-equilibration with Column Buffer.

**Step 5, Affinity Column Chromatography—**The hydroxyapatite pool was loaded at 30 ml/h onto a 3 x 30 cm reactive red agarose column which had been pre-equilibrated in Column Buffer. The affinity column was flushed at 60 ml/h with Column Buffer until the eluate was free of protein as determined by the absorbance at 280 nm. The active enzyme was eluted with 30 ml of Column Buffer containing 7 mg/ml NADH, followed by more Column Buffer. Active fractions were pooled and either placed into the dialyzing concentrator and dialyzed against Column Buffer for 10–12 h at concentrated using the Centricon system. Concentration yields approximately 1 ml of homogeneous enzyme (6-8 mg). Excess NADH was removed by desalting on a Bio-Gel P-4 column.

**Subunit Separation**

Subunits were separated under denaturing conditions in the presence of 2-mercaptoethanol by loading 0.75 mg of purified secondary
amino monooxygenase onto each of two 15% acrylamide slab gels followed by electrophoresis according to the method of Laemmli (17). Individual bands were visualized by immersing the gels into an aqueous 1 M potassium chloride solution. Sodium dodecyl sulfate was precipitated rapidly in the presence of a high concentration of potassium, yielding a gel which has clear bands corresponding to regions containing protein and a cloudy background. The bands corresponding to the appropriate subunit were removed from the gel using a razor blade.

Recovery of the individual polypeptides was by electroelution of the bands using a tube gel apparatus and dialysis tubing in a manner similar to that described for the electroelution of dye from polyacrylamide gels (23). Eluted protein was determined by the dye binding method of Sedmak and Grossberg (24) in order to eliminate interference from extraneous materials such as SDS.

**Induction of Monospecific Polyclonal Antibodies**

Antibody induction generally followed the procedure of Palmiter et al. (25) with the exception that such smaller amounts of protein were used to induce maximal specificity. Primary injections of 100 μg of protein in 0.5 ml of electrophoresion buffer and an equal volume of Freund's complete adjuvant were given to each of the three rabbits subcutaneously. The primary injection was followed 2 weeks later with a secondary injection of 50 μg of each subunit in 0.5 ml of electrophoresion buffer plus an equal volume of Freund's incomplete adjuvant. Blood was collected at approximately 3-day intervals beginning 2 weeks after the secondary injection and continued for 4 weeks.

**Antibody Isolation**

Blood from each rabbit was processed essentially as described by Palmiter et al. (25). Aliquots were placed in Eppendorf tubes and stored either frozen at -70 °C or refrigerated at 4°C. Azide was not added to the antibody pools to avoid any possible interference in enzymatic assays due to the presence of azide.

**Antibody Assays**

The presence of active antibody was assayed by Western blots of the various pools. SDS-polyacrylamide gels (1.5 mm) of purified enzyme were run with a 5% stacking gel and no well comb. Each gel has approximately 40 μg of purified enzyme loaded and was run as described above. The protein was then transferred to nitrocellulose using the Bio-Rad electrophoretic transfer cell, following the directions for the use of that apparatus, with a transfer time of 3-4 h at 70 V. Visualization of antibody was performed by using goat-anti-rabbit IgG horseradish peroxidase conjugates from Bio-Rad, again following the manufacturer's instructions. Samples which exhibited antigenic activity specific to the desired (injected) subunit was determined by specific band development were pooled. These pools were then re-examined by the same method to determine a useful amount (titer) of antibody to be used in later studies.

**Antibody Inhibition**

Enzyme samples were prepared at 25 °C with enzyme plus monospecific antibody toward subunit I, II, or III, or enzyme plus preimmune control serum in Eppendorf tubes. Aliquots of the incubation mixture were removed as a function of time to determine the remaining activity.

**RESULTS AND DISCUSSION**

**Purification of Secondary Amine Monooxygenase**—The procedure described above results in the rapid and efficient purification of secondary amine monooxygenase to apparent homogeneity as judged by native gel electrophoresis and gel filtration (vide infra). Yields of 4-10 mg of homogeneous enzyme can be obtained in a total of 36 h. The rapid performance of this procedure is essential to the recovery of large amounts of highly active enzyme. Table I outlines a typical purification of the enzyme. The overall activity, yield, and fold purification values are determined in a manner different from that previously reported (8). The earlier work reported dimethylamine-dependent NADPH oxidation for all purification steps. However, the very low amount of dimethylamine-dependent activity in crude extracts (approximately 10% of total activity) cannot account for the amount of activity following the ammonium sulfate precipitation and subsequent purification steps. This anomaly is likely caused from the presence of dimethylamine and trimethylamine in the crude cell extracts which is removed during the salt fractionation procedure. Nevertheless, the present procedure represents a considerable improvement over the previous work (8) not only in an increase in specific activity by greater than 3-fold but also by the demonstration of apparent homogeneity by native gel electrophoresis, gel filtration, and SDS-PAGE. The availability of homogeneous enzyme opens the way to additional physical and mechanistic characterization of the secondary amine monooxygenase system.

Enzyme concentration is an important factor in retaining enzymatic activity throughout the procedure. An illustration of this phenomenon is provided by the hydroxyapatite chromatography step where elution of the enzyme from the column was most effectively brought about by using a step gradient in phosphate concentration. This led to elution of the enzyme in sufficiently high protein concentrations to maintain activity. Attempts to utilize a gradient elution failed, due at least in part to the broad range of phosphate concentrations that would elute the enzyme, resulting in greater dilution and lower stability.

The elution profile for the reactive red agarose column is shown in Fig. 1. This column acts as an affinity column; the nucleotide-binding site of the enzyme apparently binds to the dye, which is covalently linked to the agarose support (26, 27). Although the elution profile differs only slightly with repeated use of the column, the overall yield of enzyme is quite dependent upon the age of the column material. In general, the affinity column material can be used up to 19 times before it must be replaced. Fresh column material usually gave a lower yield than the same material in subsequent uses. This is most likely due to the binding of enzyme to nonspecific binding sites on the column material, from which elution with NADH would be ineffective. Attempts at pre-equilibrating the column with bovine serum albumin resulted in good yields of total enzymatic activity from the column; however, some of the bound albumin was found to elute with the enzyme, yielding very low specific activity values for the final pool.

| Table 1 Purification of Secondary Amines monooxygenase |
|-----------------|---------------|-------------|--------------|---------|---------|
| Purification step | Volume | Total units | Total protein | Specific activity | Recovery | Purification |
| Sonication | 250 | 450 | 8550 | 0.056 | 100 | 1.0 |
| Nucleic acid removal | 250 | 450 | 656 | 0.078 | 98 | 1.4 |
| Ammonium sulfate fractionation | 55 | 362 | 1960 | 0.183 | 80 | 3.3 |
| Hydroxyapatite pool | 105 | 250 | 261 | 0.785 | 46 | 14.0 |
| Affinity chromatography pool | 0.8 | 107 | 7.4 | 14.9 | 24 | 267 |
Stability—Optimal enzyme stability was observed in the presence of a final concentration of 1 mM NADH. In the presence of 5% ethanol but without NADH, the purified enzyme retains about 80% of its activity over 3 months when stored at 77 K. In the presence of 1 mM NADH and 5% ethanol, 90–95% of the original activity is retained following storage at 77 K for 3 months. The loss of 5–10% of the activity can be attributed to the freeze-thaw cycle for the storage of the enzyme and cannot be avoided at the present time. Thus, freshly purified enzyme was used whenever possible; freeze-thaw cycles were avoided unless absolutely necessary.

Verification of Enzyme Identity—The identity of the enzyme purified in this work as the secondary amine monooxygenase previously described by Eady and Large (1) was verified in three ways. First, the NADPH- and molecular oxygen-dependent N-dealkylation of dimethylamine producing formaldehyde. As originally reported by Eady et al. (2), this activity was also observed to be inhibited by small amounts of CO (data not shown). Second, the Michaelis constants for NADH and NADPH measured in this work were found to be 6.1 and 15.0 μM, respectively, in good agreement with the published values of 6.5 μM for NADH and 13.2 μM NADPH (2). Dimethylamine had a much lower K_m (24 μM) than that reported earlier (58 μM) (28). The reason for this discrepancy is not clear at the present time but may be a result of the greater purity of the enzyme preparation. Finally, the electronic absorption maxima of the deoxygenated and carbon monoxide-bound forms of the ferrous enzyme are similar to those reported earlier, although the spectral features reported herein are much better defined (2, 3).

Molecular Weight Characterization—Secondary amine monooxygenase, purified by the procedure described herein, exhibited a single band upon examination by native polyacrylamide gel electrophoresis and three bands in SDS-polyacrylamide gels in the presence of 2-mercaptoethanol (Fig. 2). These three polypeptides have estimated molecular weights of 42,000, 36,000, and 24,000 for subunits I, II, and III, respectively. Electrophoretic examination of the enzyme under denaturing conditions in the absence of 2-mercaptoethanol did not reveal any evidence for intersubunit disulfide bonds. However, intrachain disulfide linkages may be present since the bands traveled with less resolution and altered mobility in the absence of 2-mercaptoethanol (data not shown).

Immunological Characterization—The results of Western blot analysis of secondary amine monooxygenase are shown in Fig. 4. The presence of noncross-reacting bands in the Western blot indicate that the three subunits are individual polypeptides and not a result of proteolytic degradation. The antibody inhibition results displayed in Fig. 5 demonstrate that all three subunits are required for activity and that none are artifacts of the purification procedure. Thus, secondary amine monooxygenase can be properly called a multisubunit enzyme according to classical standards.

Prosthetic Group Identification and Quantification—Heme concentration was measured by the pyridine hemochromogen method (19, 20). This procedure was also used to identify the heme prosthetic group as protoporphyrin IX. The reduced pyridine hemochrome complex of the enzyme (Fig. 6) exhibited absorption maxima at 556, 524, and 418 nm, in good agreement with those reported for protoporphyrin IX (29). Determination of heme type is essential when evaluating the spectroscopic characteristics of a given system (see below).
Secondary Amine Monooxygenase

*FIG. 3.* Sepharose 6B elution profile of secondary amine monooxygenase. A column of Sepharose 6B (1.2 × 56.5 cm) was equilibrated with 40 mM potassium phosphate buffer containing 5% ethanol, pH 6.8. Secondary amine monooxygenase (20 units) was loaded onto the column at a rate of 10 ml/h, and 0.5-ml fractions were collected. Activity (O) and absorbance at 280 nm (□) were monitored. Proteins used to calibrate the column included thyroglobulin (669,000), amylase (200,000), alcohol dehydrogenase (150,000), and bovine serum albumin (66,000). Fractions were analyzed by SDS-PAGE as described under “Experimental Procedures.” SDS-PAGE analysis of fractions 55, 62, and 68 are shown in the inset.

*FIG. 4.* Western blot analysis of monospecific antibodies. Antibodies induced independently from each SDS-PAGE-purified subunit were used to probe the proteins transferred to nitrocellulose following SDS-PAGE. Reactive areas were visualized using goat-anti-rabbit horseradish peroxidase-conjugated IgG. Lanes 1, 2, and 3 show antibodies directed toward subunit I, II, or III, respectively, and lane 4 shows control serum.

*FIG. 5.* Antibody inhibition of secondary amine monooxygenase with time. In a final volume of 350 μl, 50 μl of antibody directed to subunit I, 50 μl to anti-subunit II, 50 μl to anti-subunit III, or the corresponding amount of control serum was added to 33 μg of enzyme in 46 mM potassium phosphate, 5% ethanol, pH 6.8 buffer. NADPH oxidation activity was assayed after incubation at 25 °C for 0, 1, 2, and 4 h.

*FIG. 6.* UV-visible absorption spectra of the pyridine hemochrome derivative of secondary amine monooxygenase. Purified enzyme was concentrated and converted to the pyridine hemochrome as described under “Experimental Procedures.” The heme content of the concentrated sample was 41.3 μM which corresponds to a protein concentration of 5.6 mg/ml. In addition, the possibility that an unusual heme group is responsible for the reactivity of secondary amine monooxygenase is ruled out.

Quantification and verification of the enzyme prosthetic groups have also been carried out. These results are as follows: 2.0 nmol of acid-insoluble (heme) iron, 4.0 nmol of acid-labile (nonheme) iron, 1.6 nmol of flavin/nmol of Mₙ = 210,000 enzyme oligomer. We have also determined that 3.6 mol of free sulfide are present per Mₙ = 210,000 oligomer, strongly indicating that one or more ferredoxin-type iron-sulfur clusters is bound to the enzyme. Given the native molecular weight of 210,000 and subunit molecular weights of 42,000, 36,000, and 24,000, it is reasonable to suggest that the enzyme may exist in an oligomeric form where each polypeptide identified on SDS-PAGE is represented twice. If the subunits were identical, this would imply that two Fe₃S₄ clusters are present in the enzyme. However, it should be noted that the stoichiometry of subunits in oligomeric enzymes does not always follow directly from a simple interpretation of apparent molecular weights; further studies addressing this point are in progress.
Secondary Amine Monoxygenase

Preliminary Spectroscopic Investigations—As a heme iron-containing monoxygenase, secondary amine monoxygenase might be expected to mimic the spectral properties of the best known of this class of enzymes, cytochrome P-450, particularly since the N-dealkylation reaction is catalyzed by both enzyme systems. The preliminary investigations here, however, argue against any such spectroscopic similarity. The establishment of iron protoporphyrin IX as the heme prosthetic group in secondary amine monoxygenase (vide supra) makes direct spectroscopic comparisons between the three proteins possible in order to establish similarities and differences in the heme iron coordination structures of these proteins. The spectral differences observed between P-450 and secondary amine monoxygenase most likely reflect the presence of different axial ligands in the two enzymes. The sharp spectral features seen for this enzyme suggest that both hemes have the same axial ligation and geometry, indicating the presence of two identical active-site heme iron prosthetic groups.

The dimethylamine-free form of ferric secondary amine monoxygenase exhibits a Soret absorption maximum at 406 nm as well as a peak at 530 nm, with additional shoulders at 530 and 574 nm (Fig. 7). These absorbance maxima are compared to those of high spin ferric P-450 and aquo-metmyoglobin in Table II. Note the Soret maximum for secondary amine monoxygenase occurs at a wavelength that is very close to that of aquo-metmyoglobin. In addition, both proteins have a peak at approximately 630 nm. Since the extinction coefficients for flavins and iron sulfurs are much less than those for ferric heme iron, the contribution of these groups to the observed absorption spectrum should be relatively small. Since flavins absorb only weakly above 550 nm, it is safe to assume that the band at 630 nm originates from the heme iron. In ferric heme iron systems, the presence of a peak in the vicinity of 630 nm is often indicative of a high spin heme iron (29).

The ferrous carbon monoxide complex of heme proteins has long been used as a benchmark for quantitation as well as identification. Cytochrome P-450 is unusual in having a very red-shifted Soret maximum in the ferrous-CO case, presumably caused by the axial thiolate ligand trans to the carbon monoxide (6, 7). The UV-visible absorption spectrum of the ferrous-CO derivative of secondary amine monoxygenase is shown in Fig. 7, and the absorption maxima are given in Table II. The Soret maximum is at 423 nm, as opposed to 446 nm for P-450CAM, suggesting the presence of a fifth ligand other than thiolate. The overall spectral characteristics of this form of secondary amine monoxygenase closely match those of the analogous myoglobin species. In contrast to the case of ferrous-CO P-450 where only a single band is observed in the visible region of the spectrum, clearly defined α and β peaks

![Fig. 7. UV-visible absorption spectra of secondary amine monoxygenase. Spectra of the ferricyanide-oxidized (—) and ferrous-carbon monoxide (−−−) forms of the enzyme were obtained at 4 °C in 40 mM potassium phosphate buffer containing 5% ethanol, pH 6.8.](image)

![Fig. 8. Reversible conversion of oxygenated secondary amine monoxygenase to the deoxygenated form. The UV-visible absorption spectrum of the oxygenated enzyme at 4 °C in the presence of 1 mM NADH was recorded (—). The atmosphere above the cuvette was exchanged with a constant flow of nitrogen over the sample. Spectra were recorded after 5, 10, and 20 min (see arrows). No further changes were observed in subsequent scans. The atmosphere in the cuvette was then exchanged with oxygen for 10 min, and the final oxygen-bound form was scanned (−−−). The enzyme concentration was 8.3 μM in 40 mM potassium phosphate + 5% ethanol, pH 6.8.](image)

<table>
<thead>
<tr>
<th>Species Secondary Amine Monoxygenase</th>
<th>Myoglobin</th>
<th>P-450CAM</th>
<th>Cytochrome b5</th>
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<tr>
<td>Ferric</td>
<td>408 (185)</td>
<td>409.5 (157)</td>
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<td>355*</td>
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<td>596 (28)</td>
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*a The λ and fλ values are expressed in nm and nm⁻¹ cm⁻¹, respectively, normalized per heme.
*b Values determined at pH 7.0 and 20 °C from Antonini and Brunori (29).
*c Values determined at pH 7.4 and 4 °C from Ref. 6.
*d Values determined at pH 7.9 and 4 °C from Ref. 30.
*e Due to the intense absorption of NADH at 340 nm, the extinction coefficient for the δ band (which can be seen in the absence of NADH) is not reported here.
are observed for CO-bound myoglobin and secondary amine monoxygenase. This striking similarity is most consistent with the presence of a nitrogenous ligand in the fifth coordination position of secondary amine monoxygenase.

Perhaps the most interesting feature of secondary amine monoxygenase is its ability to form a very stable oxygen-bound form in the ferrous state. Following purification of the enzyme in the absence of substrate (dimethylamine) but in the presence of reducing equivalents (NADH) and atmospheric oxygen, the enzyme is found to exist predominantly in the oxygen-bound form (see below). Complete conversion to the oxyferrous species can be easily accomplished by addition of a small amount of NADH and pure oxygen to the sample cuvette followed by gentle inversion. Evidence that this form is indeed the molecular oxygen complex is presented in Fig. 8, where the oxygenated form is shown to convert to the deoxygenated state upon the exchange of the atmosphere over the cuvette with nitrogen. This conversion is nearly complete after 1 h at 4 °C as judged by the lack of substantial additional change in the spectrum upon the addition of solid dithionite (data not shown). The deoxygenated enzyme binds oxygen very rapidly in the presence of a small amount of NADH (1 mM), with complete generation of the oxy species after 10 min. In both the oxy to deoxy and deoxy to oxy conversions, the spectra are very sharp and exhibit clear isosbestic points at 390, 425, 453, 506, 528, 549, and 568 nm.

The oxygen-bound derivative spontaneously forms the ferrous-CO species when the atmosphere in the cuvette is exchanged with carbon monoxide (data not shown). This conversion is reversible upon the addition of molecular oxygen to the ferrous-CO enzyme over a period of 6–8 h at 4 °C in the presence of NADH. The increased time required for regeneration of the oxygenated enzyme is consistent with the presence of exogenous CO strongly bound to the heme iron. The electronic absorption maxima for the fully formed oxygen-bound form of secondary amine monoxygenase, listed in Table II, are very close to the corresponding maxima for oxymyoglobin and appreciably different from those of oxy-P-450am.

The UV-visible absorption properties of the ferrous deoxygenated state of the enzyme (Table II) compare less favorably with those of deoxymyoglobin than has been observed for any of the other enzyme derivatives examined here. In addition, the spectral properties of this enzyme state are distinct from those of deoxyferrous P-450. These spectral differences make it difficult to reach a firm conclusion about the heme iron coordination structure of deoxyferrous secondary amine monoxygenase. The presence of a nitrogenous base as a sixth ligand in deoxygerated secondary amine monoxygenase could explain the ~10-nm blue shift of the Soret maximum relative to deoxymyoglobin. Ferrous cytochrome b6, which contains a bis-histidine-ligated iron protoporphyrin IX (30), exhibits a similar Soret maximum (423 nm). The visible region (500–700 nm) absorption maxima of deoxyferrous secondary amine monoxygenase (530 and 557 nm) and ferrous cytochrome b6 (526 and 556 nm) are also very similar, as are the general appearance of the two spectra (see, (30)). The very facile binding of molecular oxygen or CO to ferrous secondary amine monoxygenase indicates that any endogenous sixth ligand is not bound very tightly to the heme iron (in contrast to the cytochrome b6 case). The presence of a shoulder in the 430–440 nm region of the spectrum of the deoxyferrous enzyme is also consistent with a complex having a rather loosely bound sixth ligand since this is the region where a 5-coordinated deoxymyoglobin-like species would absorb (Table II).

In summary, we have described the purification to apparent homogeneity of a M, = 210,000 oligomeric enzyme from P. amniosorum, Ed F. Phares and Mary V. Long of Oak Ridge National Laboratories for their helpful advice and assistance concerning bacterial growth procedures, and Drs. Edmund W. Svasits and Massanori Sono for many helpful discussions.

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