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Stoichiometry of Phenylhydrazine Inactivation of Pig Plasma Amine Oxidase†

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ABSTRACT: Pig plasma amine oxidase is irreversibly inactivated by phenylhydrazine. The stoichiometry of this inactivation was determined by monitoring the loss of catalytic activity, the formation of a new visible spectral band, changes in the circular dichroic spectrum and by equilibrium binding studies. In all cases, only 1 mol of phenylhydrazine reacted with the dimeric pig plasma amine oxidase; further additions of phenylhydrazine had no effect. Pretreatment of the enzyme with phenylhydrazine inhibited the binding of amine substrate. The phenylhydrazine–enzyme complex was found to be stable under various experimental conditions for at least 72 h. Circular dichroic spectra revealed the conformation of the phenylhydrazine-treated enzyme to be altered in the region around prosthetic groups and indicated some changes about the aromatic amino acids. No major conformational changes were detected by this technique. Isoelectric focusing experiments exposed no differences in the band pattern or isoelectric point between the untreated and phenylhydrazine-treated enzymes.

Plasma amine oxidase (PAO)[1] from pig[2] is one of a family of soluble amine oxidases containing Cu^{2+} ions and an organic prosthetic group capable of interacting strongly with carbonyl reagents (Buffoni & Blaschko, 1964; Blaschko & Buffoni, 1965; Buffoni, 1968). The molecular weight of pig PAO has been reported between 186 000 and 196 000 (Buffoni & Blaschko, 1964; Boden et al., 1973; Barker et al., 1979). In the presence of denaturing reagents, it behaves as a single species of molecular weight 95 000–97 000 (Boden et al., 1973; Barker et al., 1979). Barker et al. (1979) suggest the subunits have identical primary structure on the basis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis and isoelectric focusing under denaturing conditions. The closely related bovine PAO has been shown to be composed of two identical subunits by tryptic peptide mapping (Achee et al., 1968).

There is suggestive evidence that the "active-carbonyl" cofactor is pyridoxal phosphate or a modified form of pyridoxal phosphate (Malmstrom et al., 1975). Reaction of the active-carbonyl cofactor with hydrazine and other carbonyl reagents inactivates the enzyme competitively with amine substrates (Lindstrom et al., 1974). Lindstrom & Pettersson (1973, 1978) have demonstrated that hydrazine reagents irreversibly inactivate pig PAO with a stoichiometric ratio of 1 mol of hydrazine per 196 000 g of PAO. Anaerobic titration of pig PAO with substrates yields the same ratio (Lindstrom & Pettersson, 1978; Massey & Churchich, 1977). This contradicts the ratio of 3 mol of phenylhydrazine per 195 000 g of PAO of Buffoni & Ignesti (1975), the 3 mol of [^{14}C]histamine bound per mol of enzyme in the form of a Schiff base (Buffoni, 1968), and the 3.8 mol of phosphate bound per dimer

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[1] Abbreviations: PAO, plasma amine oxidase (from pig); Temed, *N,N,N'N'*-tetramethylethylenediamine; Bis, *N,N'*-methylenebis(acrylamide); CD, circular dichroism; DEAE, diethylaminoethyl; EPR, electron paramagnetic resonance.

[2] Amine:oxygen oxidoreductase (deaminating) (EC 1.4.3.6).

(assuming the cofactor to be pyridoxal phosphate) (Blaschko & Buffoni, 1965).

The possibility that PAO is composed of two identical subunits yet contains only one active-carbonyl raises some intriguing questions as to the location of the cofactor, the relationship of the active-carbonyl cofactor to the bound metal, and the possibility of half of the sites reactivity.

The present studies were undertaken to resolve the controversy surrounding the stoichiometry of the active-carbonyl cofactor involved in the active sites. These studies also investigated the possibility that additional active-carbonyl cofactor is present on the enzyme but remains undetected by any of the means utilized so far. Finally, the properties of the inactivated enzyme–phenylhydrazine complex were studied, yielding information about the conformation of the enzyme.

Experimental Procedures

Materials

Pig blood was obtained from the slaughterhouse. Benzylamine was purchased from Aldrich Chemical Co, converted to the hydrochloride in aqueous ethanol, and twice recrystallized from ethanol. Phenylhydrazine hydrochloride was purchased from Aldrich Chemical Co. and twice recrystallized from ethanol. Histamine dihydrochloride was purchased from Calbiochem-Behring and was recrystallized from aqueous ethanol. [*ring*-^{14}C(U)]Phenylhydrazine hydrochloride was purchased from New England Nuclear; [7-^{14}C]benzylamine hydrochloride and [*ring*-2-^{14}C]histamine dihydrochloride were purchased from Amersham-Searle, Inc. Electrophoresis reagents were purchased from Bio-Rad Laboratories, except Pharmalytes, which were from Pharmacia Fine Chemicals, Inc. Serva Blue G was purchased from Serva Fine Biochemical, Inc. All other reagents were of analytical grade.

Methods

Enzyme Purification and Assay. Pig plasma amine oxidase was prepared by the method of Buffoni & Blaschko (1964, 1971) as modified by Falk et al. (1983). The enzyme was homogeneous by both gradient gel and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The purified enzyme had a specific activity of 0.097 unit/mg of protein; 1 unit is defined as the amount of enzyme required to catalyze the production of 1 μmol of benzaldehyde per min at 25 °C under the assay conditions described in Falk et al. (1983). Protein concentration was determined by the 260/280 method of Warburg & Christian (1941). This method had been reported to overestimate protein concentration by a factor of 1.3 (Lindstrom & Pettersson, 1973) and was corrected accordingly. Spectral measurements were made on a Gilford Model 250 spectrophotometer.

Phenylhydrazine Titrations. Aqueous solutions of phenylhydrazine hydrochloride were found to be unstable. The instability was monitored by the loss in ability to react with pyridoxal. A 10 mM stock solution was prepared fresh daily by dissolving phenylhydrazine hydrochloride in deionized water and storing it at 5 °C in the dark. Under these conditions, the solution remained stable for several hours. In experiments involving reaction of phenylhydrazine with the enzyme, the stock solution was diluted with deionized water just before use. For the titrations monitored by the increase in OD_{430}, the enzyme solution was treated with phenylhydrazine and allowed to incubate for 15 min before a reading was taken. For the titrations monitored by the loss of catalytic activity, separate aliquots of enzyme solution were treated with increasing amounts of phenylhydrazine, allowed to incubate for 30 min at 25 °C, and then assayed in duplicate under standard conditions. The amounts and concentrations of phenylhydrazine solutions were adjusted so that 6–10 additions were required to reach the equivalence point. The enzyme concentration, 2 mg/mL, was chosen to allow complete reaction in approximately 10 min as reported by Lindstrom & Pettersson (1978). This was confirmed by monitoring changes in OD_{430} with time after treatment with phenylhydrazine.

CD Spectra. CD spectra were obtained on a JASCO J-500A spectropolarimeter equipped with a DP-500 data processor and corrected for base line. All spectra were the average of at least four scans. Water-jacketed, cylindrical quartz cells were used for all experiments, and the temperature was maintained at 90 °C with a Lauda K4R circulating bath. The ordinate for the UV CD spectrum is expressed in terms of mean residue ellipticity; the mean residue molecular weight is 111 (M. C. Falk and T. J. Williams, unpublished results).

Isoelectric Focusing Experiments. Isoelectric focusing was performed on 1 mm × 115 mm × 230 mm polyacrylamide gels on a Pharmacia FBE 3000 flat-bed electrophoresis apparatus. The temperature during focusing was maintained at 9 °C by using a Lauda K2R circulating bath. The gels were composed of 3.1% (v/v) pH 2.5–5 Pharmalyte, 3.1% (v/v) pH 4–6.5 Pharmalyte, 13% (v/v) glycerol, and 5% monomeric/3% cross-linking polyacrylamide:Bis. Polymerization was catalyzed by 2.2% (v/v) Temed and 1.5% (w/v) ammonium persulfate. The catholyte was 0.1 M H_2SO_4, and the anolyte was 0.1 M NaOH. The gels were prefocused at 5 W for 30 min. The samples were applied to the gel on filter paper wicks, and electrofocusing was continued at 15 W for 45 min. The filter wicks were removed, and the focusing was continued for 60 min at 20 W. The gels were stained for protein by using the method of Reisner et al. (1975). The gels were stained and fixed in a solution of 0.05% (w/v) Serva Blue G in 4.1% perchloric acid for 1 h, destained in 4.1% (v/v) perchloric acid (three changes, 5 min each), and preserved in 7:20:25:48 (v/v/v/v) acetic acid:glycerol:methanol:water. The gels were stained for catalytic activity by first immersing the gel in a solution of 5 mM benzylamine–50 mM sodium phosphate, pH 7.2, for 30 min (two changes). Then 0.5 mg of nitro blue tetrazolium and 0.25 mg of phenazine methosulfate are added per mL of sodium phosphate buffer. The gel was gently agitated at room temperature in the dark for 2–4 h. The staining solution was removed and the gel rinsed with water and destained overnight in acetic acid–glycerol–methanol–water as above. During all these procedures, the gel was protected from the light as much as possible. Before the gel was destained, the catalytically active proteins appear as a purple band surrounded by a colorless halo on a faint yellow background. Protein species that are catalytically inactive under these conditions appear as a clear patch. After being destained, active protein bands remain purple on a clear background; inactive proteins are not visible.

Kinetic Dialysis Binding Experiments. Kinetic dialysis binding experiments were conducted by using the method of Womack & Colowick (1973). The cell, constructed out of Plexiglass, had an upper chamber 1.1 cm deep × 1.4 cm in diameter and a lower chamber 0.4 cm deep × 1.4 cm in diameter. Experiments were conducted in 50 mM sodium phosphate–150 mM sodium chloride, pH 7.2. The flow rate of buffer through the lower chamber was 1.8 mL/min, and 1-mL fractions were collected with a Gilson FC-80 fraction collector. Binding studies were begun by adding 1×10^{-4} M PAO in the phosphate–sodium chloride buffer to the upper chamber and then adding 2×10^{-5} M radioactive ligand (either [^{14}C]histamine or [^{14}C]benzylamine) in a 2.5-μL aliquot.

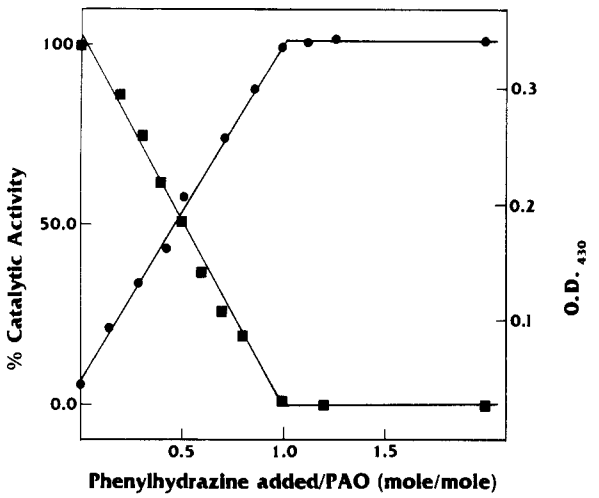

FIGURE 1: Titration of PAO with phenylhydrazine. (■) Loss of catalytic activity; (●) change in OD_{430}.

Table I: Titration of Pig PAO with Phenylhydrazine

chromatographic fraction	mol of phenylhydrazine added/195 000 g of PAO[a]	
	OD_{430}	activity
A1	0.98	1.01
A2	1.04	1.04
B1	0.90	0.91
B2	0.93	0.98

[a] The values reflect the extrapolated inflection point where addition of phenylhydrazine induced no further change in the measured parameter.

After 10 fractions were collected, a 5-μL addition of 2 mM nonradioactive ligand (either histamine or benzylamine) was made to the upper chamber. The addition of nonradioactive ligand was repeated 6 more times, each time collecting 10 fractions. Finally, a 5-μL aliquot of 100 mM ligand was added, and 15 fractions were collected. The control experiment was performed identically except no PAO was added. The experiments were conducted at room temperature.

Results

Pig PAO exhibits heterogeneity on both DEAE-cellulose and hydroxyapatite column chromatography (Buffoni & Blaschko, 1964; Falk et al., 1983). Falk et al. (1983) have separated the various fractions resolved by DEAE-cellulose chromatography and purified each separately. Each of these fractions (labeled A, B, and C) was further resolved into two forms by hydxroxyapatite column chromatography (labeled A1, A2, B1, B2, etc.). The titration of each of these forms with phenylhydrazine was monitored both by the increase in absorbance at 430 nm and by the loss of catalytic activity (Figure 1 and Table I). All the chromatographic fractions of PAO were fully titrated and catalytically inactivated by 1 mol of phenylhydrazine per 195 000 g of PAO in agreement with the results of Lindstrom & Pettersson (1973, 1978). In subsequent experiments, these fractions were used interchangeably.

The interaction of PAO and phenylhydrazine was monitored by circular dichroism to examine the effects on the structure of the enzyme and to detect any possible additional interactions not seen in the visible absorption spectrum. The addition of phenylhydrazine to pig PAO induces many changes throughout the near-ultraviolet and visible regions of the circular dichroic (CD) spectrum. The CD spectrum of the native enzyme (Figure 2) has a weak broad band above 600 nm, a broad

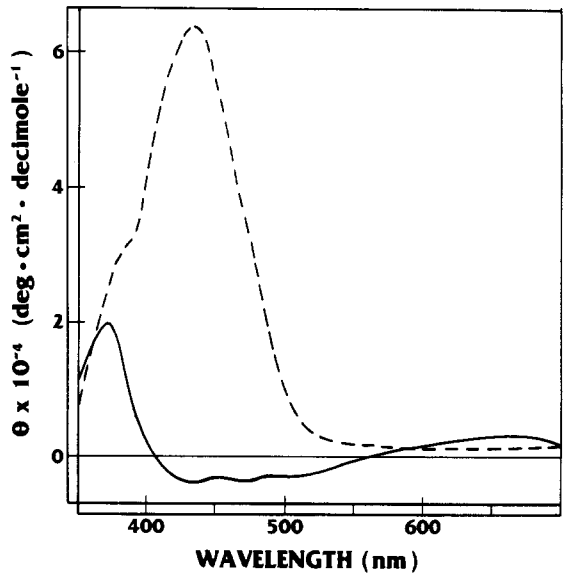

FIGURE 2: Visible CD spectra of PAO. Solid curve, native enzyme; dashed curve, native enzyme + phenylhydrazine. All spectra were the average of at least four scans and are corrected for base line. Enzyme concentration was 2 mg/mL, the path length was 1 cm, and the buffer was 50 mM sodium phosphate, pH 7.2.

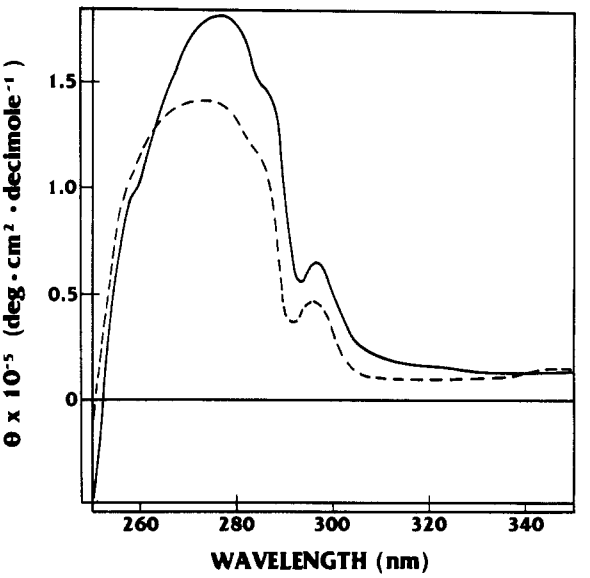

FIGURE 3: Near-UV CD spectra of PAO. Solid curve, native enzyme; dashed curve, native enzyme + phenylhydrazine. The enzyme concentration was 2 mg/mL, the path length was 0.5 cm, and the buffer was 50 mM sodium phosphate, pH 7.2.

negative band with a λ_{max} at 435 nm and shoulders at 470 and 500 nm, and a positive band with a λ_{max} at 372 nm, all presumably associated with the active-carbonyl and metal cofactors. This spectrum is insensitive to changes in temperature between 9 and 25 °C. The most striking change in the CD spectrum of PAO after the addition of phenylhydrazine is the appearance of a new positve band with a λ_{max} at 435 nm. The band above 600 nm is diminished, and the band at 372 nm is shifted bathochromically. These changes indicate substantial changes in the environment surrounding all the cofactors upon treatment with phenylhydrazine.

In the region of the spectrum most commonly associated with the aromatic amino acids, there is a positive band at 278 nm, a shoulder at 286 nm, and a band at 297 nm for the native enzyme (Figure 3). All of these bands are diminished in intensity, and the band at 278 nm is broadened by the addition of phenylhydrazine. The ultraviolet CD spectrum of the native

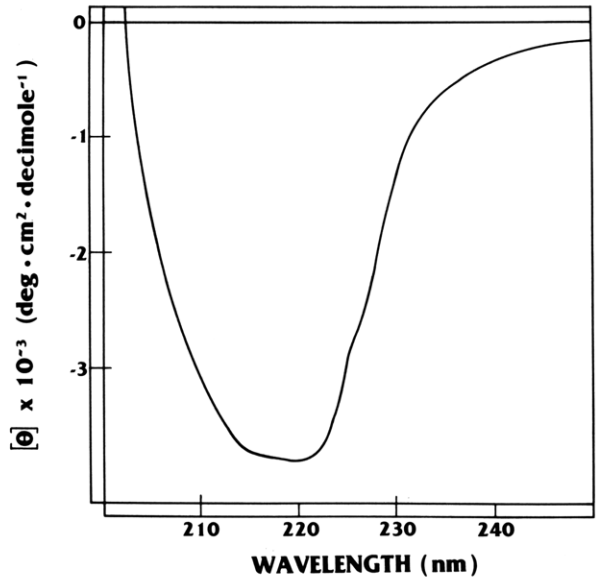

FIGURE 4: Far-UV CD spectrum of PAO. Solid curve, native enzyme or native enzyme + phenylhydrazine. The enzyme concentration was 0.2 mg/mL, the path length was 0.1 cm, and the buffer was 50 mM sodium phosphate, pH 7.2.

Table II: Equilibrium Binding of Phenylhydrazine with Pig PAO[a]

mol of [^{14}C]phenylhydrazine/mol of PAO		
	bound	
added	native[b]	inactivated[c]
1 (2)[d]	1.09	0.008
2 (1)	1.11	0.27
5 (2)	0.7	0.029
10 (2)	1.34	0.014
(7)[e]	1.05 ± 0.10[e]	0.05 ± 0.04[e]

[a] The enzyme concentration was 0.75 mg/mL in 50 mM sodium phosphate–150 mM sodium chloride, pH 7.2. Dialysis was carried out at 5 °C for 22 h. [b] The enzyme was allowed to react with [^{14}C]phenylhydrazine for 3 h at 5 °C and then loaded into the equilibrium dialysis chamber. [c] The enzyme was first treated with 1 equiv of nonradioactive phenylhydrazine for 1 h at 5 °C, then allowed to equilibrate with [^{14}C]phenylhydrazine for 2 h, and finally loaded into the chamber. [d] Number of replicates in parentheses. [e] Mean values ± standard error of mean.

enzyme had a negative extremum at 220 nm with a shoulder at 210 nm and was unaffected by the addition of phenylhydrazine (Figure 4).

Throughout the entire range of the CD spectrum, the changes induced by the addition of phenylhydrazine were fully realized by the addition of 1 mol of phenylhydrazine per 195 000 g of enzyme. Additional phenylhydrazine had no effect on the spectrum. The CD spectrum of phenylhydrazine by itself is undetectable at the concentrations employed in these experiments.

Although no additional interactions of phenylhydrazine with PAO could be detected by CD, direct binding studies were attempted to further investigate this possibility. Only 1.05 ± 0.05 mol of [^{14}C]phenylhydrazine bound per 195 000 g of enzyme in equilibrium dialysis binding experiments (Table II). Moreover, when the enzyme was first inactivated by 1 mol of nonradioactive phenylhydrazine per mol of enzyme, the ability of the enzyme to bind additional phenylhydrazine was inhibited greater than 95%. The possibility remained that the active-carbonyl/PAO ratio was higher than the value of 1, but the phenylhydrazine–PAO complex was unstable. For this hypothesis to be valid, all the various experimental techniques would have to have been fortuitously determined at the same

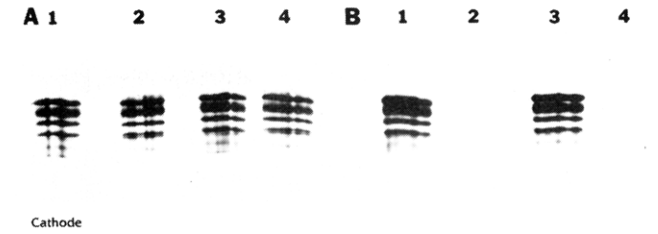

FIGURE 5: Isoelectric focusing of native and phenylhydrazine-inactivated PAO. Phenylhydrazine (1×10^{-4} M) was added to 1×10^{-4} M PAO and allowed to incubate 30 min at 5 °C before being focused. (A) Protein stain: positions 1 and 3, native PAO; positions 2 and 4, phenylhydrazine-inactivated PAO. (B) Activity stain: positions 1 and 3, native PAO; positions 2 and 4, phenylhydrazine-inactivated PAO.

point in the decomposition. Nevertheless, the hypothesis was investigated.

The stability of the phenylhydrazine–PAO complex was tested by treating the enzyme (1.5 mg/mL) with an excess of [^{14}C]phenylhydrazine for 1 h and gel filtering the mixture on Bio-Gel P2 to remove any unbound [^{14}C]phenylhydrazine (0.9 mol of phenylhydrazine remained bound per 195 000 g of enzyme). The complex was incubated at room temperature. At various time intervals, aliquots were removed and gel filtered on smaller Bio-Gel P2 columns. Column fractions were assayed for radioactivity. Those fractions eluting in the protein peak (exclusion volume) were combined, and OD_{280}, OD_{430}, radioactivity, and catalytic activity were measured. Over a 72-h period, there was no change in the OD_{430}/OD_{280} or cpm/OD_{280} ratios, total cpm eluted in the protein peak, or catalytic activity. By the above criteria, the phenylhydrazine–PAO complex was stable at room temperature. Subsequently, only the ultraviolet–visible absorption spectrum was used to monitor the stability of this complex. It was found to be stable at pH 7 in the presence and absence of 150 mM sodium chloride, both at room temperature and at 5 °C.

Isoelectric focusing was applied to test if any of the forms of PAO reacted differently with phenylhydrazine and to examine conformational differences between the native and inactivated forms. Each of the chromatographically separable fractions of PAO has been shown to contain multiple forms by isoelectric focusing (Falk et al., 1983). In the following experiments, one of these forms was treated with 1 equiv of phenylhydrazine, incubated for 30 min, and compared to native PAO on isoelectric focusing. The native and phenylhydrazine-inactivated PAO's were compared by staining for protein (Figure 5A). There was no difference in either the band pattern or the isoelectric points. When the isoelectric focusing gel of the native PAO sample was stained for catalytic activity (Figure 5B), all the bands formerly revealed by protein staining were catalytically active. However, none of the bands of the phenylhydrazine-inactivated PAO were catalytically active.

Although it was conclusively demonstrated that phenylhydrazine completely inhibits the oxidation of amine substrates by PAO, it was still possible that amines could bind to the inactivated enzyme. Therefore, the binding of benzylamine and histamine to phenylhydrazine-inactivated PAO was investigated by using the kinetic dialysis binding technique (Womack & Colowick, 1973). The phenylhydrazine-inactivated PAO concentration was 1×10^{-4} M, and the substrate (histamine or benzylamine) concentration varied between 2×10^{-5} and 1.6×10^{-4} M. No binding could be detected for either substrate. Under these conditions, this technique could easily detect binding of ligand to enzyme with a dissociation constant of 10 times the enzyme concentration

(i.e., $K_d \geq 1 \times 10^{-3}$ M). The dissociation constants for the binding of benzylamine or histamine to PAO have not been reported in the literature. The K_m for benzylamine is 9×10^{-5} M (Falk et al., 1983) and for histamine is 1.1×10^{-3} M (Buffoni & Blaschko, 1964). It would appear from these data that phenylhydrazine partially, if not completely, inhibits the binding of amine substrate to PAO.

Discussion

The kinetics of the reaction of substrates and inhibitors with pig PAO are fairly well understood. Yet the chemistry of these reactions, indeed the nature of the reactants and intermediates, remains elusory. It seems to be commonly agreed that the primary interaction of amine substrate with PAO involves a reactive group on the enzyme and this same group is inactivated by carbonyl reagents such as hydrazine derivatives (Malmstrom et al., 1975). This active-carbonyl cofactor is thought to be pyridoxal phosphate, or at least an analogue of pyridoxal (Malmstrom et al., 1975). The exact identity of the active-carbonyl cofactor has not been conclusively elucidated. Not only is the chemical structure of the active-carbonyl cofactor in doubt but also the stoichiometric ratio of active-carbonyl cofactor to the dimeric enzyme has been questioned.

The earliest reports on the stoichiometry of the cofactor of pig PAO yielded a ratio of 3.8 on the basis of phosphate analysis, assuming the cofactor to be pyridoxal phosphate (Blaschko & Buffoni, 1965). This ratio was supported by experiments in which the enzyme was reduced with borohydride in the presence of [^{14}C]histamine, resulting in a ratio of 3 (Buffoni, 1968). Both of these experimental approaches have shortcomings. The former approach assumes the identity of the cofactor, which is still in question many years after the report was published. Moreover, the enzyme is purified in the presence of phosphate buffers, and both adventitious binding of phosphate and contamination of glassware are serious sources of possible error. The latter approach could yield high ratios if any of the [^{14}C]histamine substrate was converted to the aldehyde and subsequently reacted with available amino groups on the enzyme. In fact, N^{ϵ}-benzyllysine was the product of a similar experiment with bovine PAO using [^{14}C]-benzylamine as substrate (Inamasu et al., 1974).

On the basis of titrations with hydrazine derivatives, Lindstrom & Pettersson (1973) found a ratio of 1 cofactor per dimer. Buffoni & Ignesti (1975), repeating these titration experiments, found a ratio of 3 and suggested the discrepancies were due to differences in the specific activity of the enzymes. Finally, Lindstrom & Pettersson (1978) repeated the phenylhydrazine titrations, included anaerobic titrations of the enzyme with substrate, and again found a ratio of 1. In the same paper, they suggested the discrepancy between their titration results and those of Buffoni & Ignesti (1975) was because the latter authors used a much lower concentration of enzyme. The titration reaction had not been allowed to reach equilibrium at the lower concentration, resulting in curvature of the titration curve. If this curvature is neglected, it could lead to an overestimate for the observed stoichiometry. Massey & Churchich (1977) found a stoichiometric ratio of 1 by anaerobic titrations with substrate.

The question of the identity of the subunits further complicates the above discrepancies. None of the experimentally derived ratios are equal to the number of subunits, yet there is some evidence that the enzyme is composed of subunits of identical primary structure (Barker et al., 1979). Although these two facts are not mutually exclusive, their simultaneous validity would raise many interesting possibilities, which are described later in this discussion. Therefore, the stoichiometry ratio of active-carbonyl cofactor per dimer enzyme is important to the basic understanding of the structure and how it relates to the function of this enzyme.

In the present study, the titrations of pig PAO with phenylhydrazine are in agreement with the results of Lindstrom & Pettersson (1973, 1978) demonstrating a ratio of 1 active-carbonyl cofactor per dimer of enzyme. This ratio was determined by monitoring spectral changes and by loss of catalytic activity. Identical results were obtained by using various different chromatographic fractions. Further, all the bands seen on isoelectrophoretic focusing were catalytically active and were fully inactivated by the addition of 1 mol of phenylhydrazine per mol of dimer of enzyme. This would seem to discredit earlier suggestions that the diffferent ratios were due to different enzyme purity (Buffoni & Ignesti, 1975).

While these results clearly demonstrated that 1 mol of phenylhydrazine inactivates pig PAO, it was still conceivable that additional sites capable of reacting with phenylhydrazine were present on the enzyme but not yet detected. Hence, the reaction of phenylhydrazine with pig PAO was followed by circular dichroism and resulted in the same ratio of 1. Finally, direct binding studies showed only 1 mol of phenylhydrazine tightly bound per dimer. If another site on pig PAO binds phenylhydrazine, it must do so with a dissociation constant of greater with 10^{-3} M, which was the limit of detection in the binding studies. These results conclusively demonstrate that the ratio of active-carbonyl cofactor per dimer of enzyme is 1.

It has been reported that the reaction of bovine PAO and various hydrazine derivatives was unstable, decomposing to active PAO and a product incapable of inhibiting PAO (Hucko-Haas & Reed, 1970). In this investigation, the phenylhydrazine–pig PAO complex was found to be stable for days under various conditions. This eliminates the possibility that decomposition of the complex has any effect on the determination of the stoichiometric ratio. Other experimental factors that might influence the ratio are the stability of the phenylhydrazine titrant and the degree of completion of the reaction during titration (which is affected by the concentration of reactants). Both of these factors were carefully controlled in these experiments.

Implicit in all of these experiments and those of previous authors was the assumption that the same group on the enzyme is inactivated by hydrazine and other derivatives and interacts with amine substrates during catalysis. This had been justified by the fact that catalysis was inhibited by hydrazine derivatives in a kinetically competitive manner with the reaction of amine substrate and enzyme (Lindstrom et al., 1974). That assumption was put on much firmer ground by the demonstration that phenylhydrazine inhibited, if not abolished, the binding of amine substrates by direct binding studies.

The results presented herein provide additional information about the nature of the phenylhydrazine–enzyme complex and by inference the substrate–enzyme complex. Reaction of the enzyme with phenylhydrazine induced several changes in the CD spectra of the enzyme. Several CD bands in the visible region were altered. These bands had been shown to involve both the active-carbonyl and metal cofactors in the bovine PAO (Ishizaki & Yasunobu, 1975). Since they were all altered by the binding of phenylhydrazine, all must be in the vicinity of the bound phenylhydrazine, or their environment must have been altered by a conformational change induced by phenylhydrazine. Phenylhydrazine-induced changes in the CD spectrum throughout the near-ultraviolet support a conformational change reflecting differences in the environment

about the aromatic amino acids and/or disulfide bonds. There were no detectable differences in the far-ultraviolet CD spectra, which limits the extent of the conformational change. Similarly, reaction with phenylhydrazine induced no changes in the number of bands, relative amount, or isoelectric point of pig PAO as demonstrated by isoelectric focusing.

Several explanations could describe how the dimeric enzyme binds only 1 equiv of phenylhydrazine, assuming the two subunits of pig PAO have the same primary structure. One possibility is that the enzyme in its native state contains two active-carbonyl cofactors, one of which is inactivated during purification. Another possibility is that the enzyme exhibits negative cooperativity and only one active site is functional at a time. Lindstrom & Pettersson (1973) titrated the urea-denatured enzyme with 1 mol of phenylhydrazine. Any effects of the conformation of the enzyme on the reactivity of the active-carbonyl cofactor would be abolished under these conditions, and this explanation would be unlikely. Pig PAO binds two Cu^{2+} ions, both of which are fully EPR detectable and structurally distinct, which minimally suggests the enzyme is not symmetrical (Boden et al., 1973; Grant et al., 1978; Barker et al., 1979). However, treatment with azide or cyanide makes the two Cu^{2+} sites identical (Barker et al., 1979). The enzyme could operate through a flip-flop mechanism in which only one cofactor could be active at any one time, e.g., one pyridoxal-like cofactor in the aldehyde form and the other in the amine form. Alternatively, the cofactor could be in a single symmetrical site shared between the two subunits. Neither of these last two possibilities has any experimental evidence to recommend or reject them. However, the latter explanation has no known precedent. The evidence for the identity of the two subunits in pig PAO is not overwhelming and is the subject of ongoing investigations. If the subunits are chemically distinct, then their respective functions and the interplay between them forecast an interesting future for the study of the enzyme.

Registry No. EC 1.4.3.6, 9001-53-0; phenylhydrazine, 100-63-0.

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