Heterogeneity of Pig Plasma Amine Oxidase: Molecular and Catalytic Properties of Chromatographically Isolated Forms†

Michael C. Falk,* Annie J. Staton, and Taffy J. Williams

ABSTRACT: Pig plasma amine oxidase was resolved into several fractions by ion-exchange and hydroxyapatite chromatography. These fractions were separately purified, and each fraction was analyzed for catalytic and structural properties. The relative amount of these fractions varied between preparations. Each fraction was composed of a unique set of bands on isoelectric focusing, as revealed by activity and protein staining. All the fractions contained 2 mol of Cu^{2+} and one "active-carbonyl" cofactor per 195 000 g of protein. There was no detectable difference in the amino acid contents of the fractions. The fractions all had similar catalytic properties using benzylamine as the substrate. The chromatographically resolved fractions had differing carbohydrate contents as revealed by gas chromatographic analysis and interaction with lectins. Further, some of the isoelectric focusing bands interacted with lectins of differing affinities. The results suggest that the heterogeneity may be due to variable carbohydrate content. Further, the practice of pooling the various chromatographic fractions may yield misleading results under certain circumstances.

Plasma amine oxidase (PAO)[1] from pig[2] is a member of a class of extramitochondrial, copper-dependent enzymes that catalyze the oxidative deamination of biogenic amines. Benzylamine is the most rapidly oxidized of a series of primary amines that act as substrates for pig PAO, though the physiological substrate is unknown (Buffoni & Blaschko, 1964).

Pig PAO has been purified and crystallized to homogeneity as detected by analytical ultracentrifugation and various types of electrophoresis, and many of its properties have been reported (Buffoni & Blaschko, 1964; Barker et al., 1979; Lindstrom & Pettersson, 1973). Both ion-exchange and hydroxyapatite chromatographies are employed in the enzyme purification, and PAO elutes in multiple peaks during both types of chromatography (Buffoni & Blaschko, 1964). These chromatographically separated fractions were only partially characterized prior to conducting the remaining studies on the pooled, chromatographically heterogeneous enzyme (Buffoni & Blaschko, 1964). Surprisingly, the reasons for the heterogeneity of these fractions have been ignored.

The present study was aimed at determining the structural and functional differences between the chromatographically separable fractions. Such a characterization would help determine when the use of pooled enzyme would be misleading. This study also examined the structural basis for the observed chromatographic heterogeneity. To do this, the various fractions were purified separately, and each fraction was analyzed for catalytic and structural properties. No differences were found in catalytic properties between the fractions. Many similarities were found, as well as some significant compositional differences between the fractions.

Experimental Procedures

Materials

Pig blood was obtained from a slaughterhouse. Benzylamine was purchased from Aldrich Chemical Co. and histamine dihydrochloride from Calbiochem-Behring. Both were purified as described in Falk (1983). Electrophoresis reagents, Bio-Gel A1.5M and hydroxyapatite, were obtained from Bio-Rad Laboratories. Pharmalytes were purchased from Pharmacia Fine Chemicals, Con A–agarose was from Sigma Chemical Co., and DEAE-cellulose (DE-52) was from Whatman Co. Lectins were purchased from Vector Laboratories, Inc. All other reagents were of analytical grade.

Methods

Assay of enzyme activity was carried out by the method of Tabor et al. (1954) at 25 °C in 50 mM sodium phosphate buffer, pH 7.2, containing 1 unit of catalase (Beers & Sizer, 1952) and 3.33 mM benzylamine. The production of benzaldehyde was monitored spectrophotometrically at 250 nm (11.3 mM^{-1} cm^{-1} is the difference between the optical density at 250 nm of 1 mM of benzaldehyde and 1 mM benzylamine under these conditions). A unit of activity is defined as the amount of enzyme required to catalyze the production of 1 μmol of benzaldehyde per min under assay conditions (Tabor et al., 1954). Protein concentration was determined by the method of Warburg & Christian (1941). This method has been reported to overestimate protein concentration by a factor of 1.3 (Lindstrom & Petterson, 1973), and the results were corrected accordingly. Spectral measurements were made on a Gilford Model 250 spectrophotometer equipped with a Lauda K2R circulating fluid temperature controller.

Enzyme Purification. Enzyme was purified by a modification of the procedure of Buffoni & Blaschko (1971). Only those steps that have been changed will be described fully (Table I).

(*A*) *Step 1.* Fresh pig blood (30 L) was citrated, cooled to 4 °C, and centrifuged at 25000*g* for 10 min at 4 °C, and the plasma was collected. All subsequent steps were carried out at 4 °C.

(*B*) *Step 2.* The plasma was treated with solid ammonium sulfate to 35% saturation (209 g/L). After centrifugation, the supernatant was brought to 55% saturation (129 g/L) and centrifuged again, and the fraction precipitating between 35%

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[1] Abbreviations: PAO, plasma amine oxidase (from pig); $NaDodSO_4$, sodium dodecyl sulfate; Tes, 2-[[tris(hydroxymethyl)methyl]amino]-ethanesulfonic acid; Bistris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; Con A, concanavalin A.

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

Table I: Purification of Pig PAO

	peak	total units	total protein[a]	sp act.[b]	x-fold purification	yield (%)
plasma		149.1	3612	4.8×10^{-5}	1	100
ammonium sulfate		108.8	339	3.2×10^{-4}	6.7	72.9
DEAE	A	42	23.1	1.8×10^{-4}	37.5	28.2
	B	24.8	19	1.3×10^{-3}	27.1	16.6
Con A	A	38.6	16.1	2.4×10^{-3}	50.1	25.9
	B	21.0	1.1	1.9×10^{-2}	396	14.1
gel filtration (A1.5M)	A	12.4	1.52	7.9×10^{-3}	164	8.3
	B	24.8	0.460	5.4×10^{-2}	1130	16.7
hydroxyapatite	A1	5.6	0.051	1.1×10^{-1}	2292	3.7
	A2	3.8	0.030	1.0×10^{-1}	2083	2.5
	B1	11.8	0.12	1.0×10^{-1}	2083	7.8
	B2	8.1	0.077	1.05×10^{-1}	2188	5.3

[a] Protein expressed in grams. [b] Units per milligram of protein.

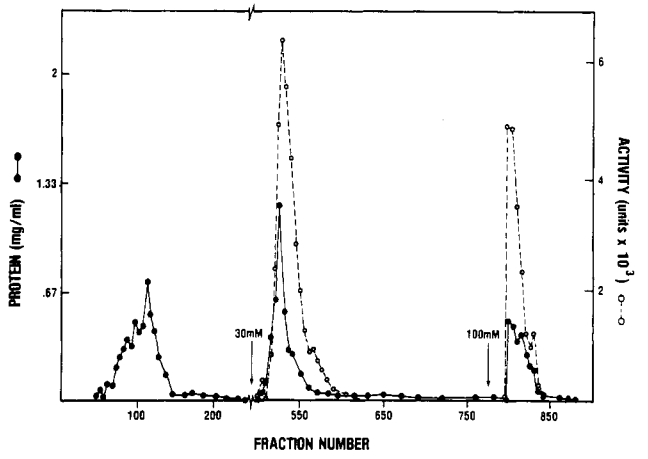

FIGURE 1: DEAE-cellulose chromatography of pig PAO. (●) Protein concentration; (○) amine oxidase activity. Buffer changes are depicted by arrows. Twenty-two-milliliter fractions were collected.

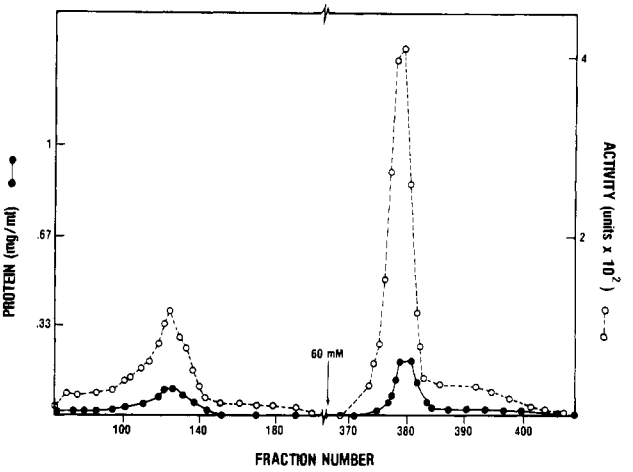

FIGURE 2: Hydroxyapatite chromatography of pig PAO. (●) Protein concentration; (○) amine oxidase activity. Buffer changes are depicted by arrows. Five-milliliter fractions were collected.

and 55% saturation was dissolved in a minimum amount of 10 mM sodium phosphate buffer, pH 7.0. The solution was dialyzed against six changes of 20 L of 10 mM sodium phosphate buffer at pH 7.0 for 76 h.

(*C*) *Step 3.* The protein solution was applied to a column (5 cm × 100 cm) of DEAE-cellulose and washed with 10 mM sodium phosphate buffer, pH 7.0. Then it was eluted with 6 L of 30 mM sodium phosphate buffer, pH 7.0, followed by 100 mM sodium phosphate buffer, pH 7.0. Assay for enzyme activity showed the presence of two peaks of enzyme activity, one eluted by the 30 mM buffer and the other eluted by the 100 mM buffer (Figure 1). The most active areas about each peak were pooled and labeled fractions A and B, respectively. The remaining steps of the purification were carried out in tandem on these two fractions. Sometimes a third peak of enzyme activity was detected as the column was being washed with the 10 mM buffer. This weakly bound peak, labeled peak C, eluted at 1.3 times the total column volume. Peak C, when found, was purified as a third fraction.

(*D*) *Step 4.* Each fraction from step 3 was applied without further treatment to a 2.5 cm × 75 cm column of Con A–agarose equilibrated with a buffer containing 10 mM sodium phosphate, 150 mM sodium chloride, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 7.0. The protein solution was applied and eluted at a flow rate of 12 cm/h. The column was washed with the same buffer until the protein concentration dropped below 0.1 mg/mL. Enzyme was eluted by 100 mM methyl α-mannopyranoside dissolved in the same buffer.

(*E*) *Step 5.* The most active fractions of step 4 were pooled, concentrated in an Amicon concentrator by using a PM-30 membrane, and dialyzed against 10 mM sodium phosphate, pH 7.0. This was loaded on a 5 cm × 100 cm Bio-Gel A1.5m column and eluted with the same buffer at 5 cm/h.

(*F*) *Step 6.* The most active fractions of step 5 were pooled, concentrated as above, dialyzed in 6 mM sodium phosphate, pH 7.0, and loaded on a 2.5 cm × 100 cm hydroxyapatite column. The column was eluted with 600 mL of 6 mM phosphate buffer and the 600 mL of 60 mM phosphate buffer. Two peaks of enzyme activity were identified (peaks 1 and 2) (Figure 2), eluting in 6 and 60 mM phosphate buffer, reepectively. The pooled material of each peak was separately concentrated and then dialyzed against 50 mM sodium phosphate, pH 7.0. Thus, the A fraction of step 3 was now separated into A1 and A2, B into B1 and B2, and C into C1 and C2.

Enzyme Kinetics. Michaelis constants were obtained at 0.31 μM enzyme concentration by varying benzylamine concentration between 0.01 and 10 mM in air-saturated 50 mM sodium phosphate buffer, pH 7.0 and 25 °C. All samples were run in triplicate, and the reaction velocities were determined spectrophotometrically from the initial rates of benzaldehyde formation. The kinetic parameters were estimated by fitting the results to the Michaelis–Menten equation by means of a nonlinear regression analysis (Horwitz & Homer, 1970).

Electrophoretic Experiments. Isoelectric focusing was performed as in Falk (1983). $NaDodSO_4$–polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970) using a Bio-Rad Model 220 vertical slab gel electrophoresis cell equipped with 1.5-mm spacers. The gels were fixed and stained by heating to 40 °C in 0.25% (w/v) Coo-

massie Brilliant Blue R-250 dissolved in 40:10:50 (v/v/v) methanol:acetic acid:water. Destaining was accomplished by several changes of the same solvent with slow agitation. Gels were stained for carbohydrate by utilizing the periodic acid stain (Fairbanks et al., 1971). Gradient polyacrylamide gel electrophoresis was performed on Pharmacia PAA 4/30 gels (80 mm × 80 mm × 2.7 mm) by using a Pharmacia GE-2/4 electrophoresis apparatus and an electrode buffer comprised of 40 mM Tes–40 mM Bistris, pH 7.1, cooled to 5 °C. Gels were first prerun 50 min at 125 V, and then the samples were loaded. Preelectrophoresis (20 min at 70 V) was followed by separation for 15 h at 125 V. The gels were stained for protein as described above for $NaDodSO_4$–polyacrylamide gel electrophoresis and destained electrophoretically in the same solvent; activity staining is described in Falk (1983).

Carbohydrate Analysis. (A) Neutral and Amino Sugars. Carbohydrate determinations were performed by gas chromatography (Porter, 1975) with both arabinose and *myo*-inositol used as internal standards. Samples were prepared as in Porter (1975); thermal decomposition of amino sugars was prevented by avoiding any heating after the hydrolysis step. A Varian 3700 gas chromatograph equipped with CDS-111 integrator/microprocessor and a 3% SP-2330 on 100/120 Supelcoport glass column (6 ft × 2 mm) were used for the analysis. Temperature was programmed from 180 to 210 °C over 16 min.

(B) Sialic Acid. Sialic acid was released from the protein by hydrolysis in 0.1 N HCl at 80 °C for 80 min and assayed by the thiobarbituric acid method of Kuwahara (1980) using *N*-acetylneuraminic acid (0–400 ng) as a standard. Fluorescence was monitored on an SLM Model 4800 spectrofluorometer (SLM Instrument Inc., Champaign-Urbana, IL). The efficiency of release of sialic acid by acid hydrolysis was confirmed by hydrolysis with neuraminidase.

Precipitin and Precipitin-Inhibition Reactions. Precipitin reactions were carried out by a modification of the method of Williams et al. (1979). Various known quantities of PAO (or glycogen), dissolved in buffer (10 mM phosphate, 500 mM NaCl, 0.1 mM $MnCl_2$, and 0.1 mM $CaCl_2$, pH 7.2), were added to tubes containing 17 μg of Con A and brought to a total volume of 0.5 mL with buffer. The tubes were incubated at 25 °C for 48 h, centrifuged, and washed with buffer. The precipitates were dissolved in 0.05 M NaOH, and protein was determined by the Lowry procedure (1951). Precipitin-inhibition reactions were performed as above, except each tube contained, in addition to Con A, 70 μg of either PAO or glycogen and known quantities of methyl α-mannopyranoside.

Amino Acid Analysis. Protein samples were hydrolyzed with 6 M HCl in sealed tubes for 22 h 110 °C and analyzed by using a Beckman 119 automatic amino acid analyzer. Analyses were performed on a single column by using a sodium citrate buffer system (M. L. Tanzer, personal communication). Loss of labile amino acids and degree of release were determined by hydrolyzing samples for 22, 48, and 72 h. Cysteine and cystine were determined as cysteic acid after oxidation by performic acid (Hirs, 1967). Tryptophan was estimated by utilizing the *N*-bromosuccinimide technique (Spande & Witkop, 1967).

Results

Chromatographic Separation. The various fractions resolved on DEAE-cellulose chromatography (Figure 1) were separated and purified each in tandem. Subsequently, each of these fractions (labeled A, B, and C) was resolved into two forms by hydroxyapatite chromatography (Figure 2) and labeled A1, A2, B1, B2, C1, and C2.

Table II: Relative Amounts of Material in Peaks Eluting from DEAE-cellulose Chromatography

prepn	peak A	peak B	peak C
1	25.8	74.2	0
2	12.6	87.4	0
3	10.1	85.2	4.7
4	43.5	39.1	17.4
5	40.3	33.2	28.5
6	59.8	40.2	0
7	14.2	85.8	0

Table III: Cofactor Content and Michaelis-Menten Parameters

fraction	Cu^{2+} [a]	active carbonyl [a]	k_{cat} [b]	K_m [c]
A1	2.0	1.01	11.3	9.5×10^{-5}
A2	2.1	1.04	11.6	9.3
B1	1.9	0.91	11.9	7.5
B2	1.7	0.98	11.9	8.5
	1.9 [d]	0.985 [d]	11.7 [d]	8.7 [d]

[a] Moles per 195 000 g of PAO. [b] Micromoles of benzaldehyde per minute per 195 000 g of PAO. Kinetic analyses were carried out at 0.31 μM PAO; the standard deviations for these determinations varied from 0.51 to 0.93. [c] In molar concentrations of benzylamine. The standard deviations for these parameters varied from 0.35 to 0.55. [d] Mean values.

Although every preparation eluted in at least two, and sometimes three, distinct peaks on DEAE-cellulose chromatography (Figure 1), relative amounts of material in these peaks differed from batch to batch (Table II). These relative amounts were established by integrating the areas under the activity profiles of the DEAE-cellulose columns.

Fresh preparations of all the fractions obtained after hydroxyapatite chromatography migrated as a single band on gradient gel electrophoresis with a molecular weight of 195000, as visualized by protein, activity, and periodic acid staining. On $NaDodSO_4$ gel electrophoresis, all the fractions migrated as a single, somewhat broad band, as seen by protein and periodic acid staining, with a molecular weight of 95 000.

Activity Measurements and Copper Content. For activity, PAO requires both Cu^{2+} and an active-carbonyl cofactor (thought to be a pyridoxal-based coenzyme) (Malmstrom et al., 1975). The Cu^{2+} content of the various fractions was determined by atomic absorption spectrophotometry, and the active-carbonyl cofactor content was determined by titration with phenylhydrazine (Falk, 1983) (Table III). There were no significant differences in the content of either cofactor among the fractions. The Michaelis–Menten constants of the fractions, utilizing benzylamine as the substrate, were not significantly different (Table III). On long storage, the k_{cat} of highly concentrated solutions sometimes decreased as a visible precipitate formed. This also correlated with the appearance of higher molecular weight forms as seen on gradient gel electrophoresis.

Amino Acid and Carbohydrate Composition. The amino acid content of the fractions was determined as the mean of at least two separate hydrolyses and at least four analyses for each fraction (A1, A2, etc.). No convincing evidence of differences was found as determined by analysis of variance. This does not rule out the existence of differences but does place bounds on the likely magnitude of such differences, as shown in Table IV. The amino acid content in Table IV represents the mean of 40 analyses, combining the results of all the fractions and the means of other representative fractions. All the chromatographic fractions of PAO contained

Table IV: Amino Acid Content of Pig PAO

amino acid	av of all fractions[a]	fraction A1[b]	B1[c]	B2[d]
aspartic acid	151.4[e] ± 4[f]	155.0 ± 2.7	150.9 ± 2.7	149.0 ± 3.1
threonine	81.1 ± 6.1	81.5 ± 2.9	79.5 ± 2.1	78.0 ± 4.2
serine	118.2 ± 7.6	122.6 ± 2.5	112.8 ± 3.3	114.4 ± 6.4
glutamic acid	175.1 ± 6.6	175.4 ± 3.7	171.2 ± 5.8	173.8 ± 4.5
proline	132.5 ± 10.3	140.7 ± 4.1	130.7 ± 4.9	138.5 ± 12.5
glycine	131.0 ± 5.0	131.0 ± 4.0	131.0 ± 6.5	131.0 ± 5.0
alanine	135.8 ± 4.9	136.7 ± 0.7	134.3 ± 4.4	135.3 ± 7.6
cysteic acid	22			
valine	113.6 ± 7.1	113.1 ± 7.3	120.1 ± 5.9	112.5 ± 7.1
methionine	35.7 ± 10.0	35.7 ± 10.3	36.2 ± 11.6	34.6 ± 10.0
isoleucine	52.6 ± 9.8	53.9 ± 4.5	56.2 ± 2.4	52.0 ± 4.0
leucine	152.6 ± 6.5	158.4 ± 2.5	161.5 ± 4.5	153.2 ± 6.3
tyrosine	56.6 ± 2.9	58.2 ± 2.7	58.3 ± 1.3	54.9 ± 2.1
phenylalanine	98.8 ± 3.8	102.3 ± 4.5	101.4 ± 1.5	97.2 ± 3.2
histidine	55.7 ± 8.6	54.7 ± 1.5	58.5 ± 5.1	54.4 ± 4.8
lysine	36.0 ± 7.6	31.1 ± 1.4	35.4 ± 12.5	33.4 ± 3.0
arginine	86.2 ± 3.6	88.1 ± 3.4	89.8 ± 2.4	85.6 ± 2.6
tryptophan	25			

[a] $n = 40$. [b] $n = 4$. [c] $n = 6$. [d] $n = 18$. [e] Moles of amino acid per 195 000 g of PAO adjusted for carbohydrate content. [f] Standard deviation.

Table V: Carbohydrate Content of Pig PAO

carbohydrate	chromatographic fraction A1	A2	B1	B2
fucose	3.3[a] ± 0.17	6.8 ± 0.98	5.6 ± 0.12	5.4 ± 0.27
glucosamine	19.4 ± 2.5	20.5 ± 0.6	13.1 ± 0.11	11.1 ± 0.79
mannose	20.9 ± 2.5	27.3 ± 0.6	20.8 ± 0.39	16.8 ± 0.31
galactose	6.7 ± 0.96	9.0 ± 1.1	9.0 ± 1.1	11.2 ± 1.3
sialic acid	<1	<1	<1	<1

[a] Residues of carbohydrate per 195 000 g of PAO.

the same types of carbohydrate residues, i.e., fucose, glucosamine, mannose, and galactose (Table V). However, there were significant differences in the amounts of these carbohydrates among the chromatographic fractions. Moreover, the carbohydrate content of the combined fractions varied from preparation to preparation.

Two other lines of evidence suggest that a variable carbohydrate content is at least partly responsible for the heterogeneity of the enzyme. One line of evidence stems from the pattern of elution from the Con A–agarose column. PAO was eluted from the Con A–agarose column as a large peak followed by a long tail. This protein was eluted only by methyl α-mannopyranoside or other carbohydrates that bind to Con A, but not by high concentrations of salt, suggesting the enzyme is solely bound via the carbohydrate residues. The relative amounts of material in the peak and tail could be varied by altering the concentration of methyl α-mannopyranoside, but a clean separation could not be accomplished.

A second line of evidence for the heterogeneity of carbohydrate content comes from precipitin interactions of PAO and Con A. The addition of increasing amounts of PAO to Con A solution yielded a precipitin curve similar in shape to that obtained with glycogen and Con A (not shown). The amount of precipitin increased with increasing PAO (at constant Con A concentration) up to a plateau region where no further precipitin was observed. Upon the addition of much greater amounts of PAO (or glycogen), the amount of precipitin eventually decreased. A value of PAO (or glycogen) within the plateau region was chosen for the precipitin inhibition experiments. In these experiments, the concentrations of Con A and either PAO or glycogen were held constant, while a variable amount of methyl α-mannopyranoside was added to inhibit precipitin formation (Figure 3). The glycogen–Con A precipitin inhibition curve was a simple, S-shaped curve, while the PAO–Con A curve was more complex. This suggests a heterogeneous population of PAO carbohydrate residues binding with different affinities to Con A. These experiments were all carried out in the presence of Mn^{2+} and Ca^{2+}, which rules out heterogeneity effects arising from the loss of the metal. It is also possible that attachment of Con A to agarose may affect the binding capability of a fraction of the population of Con A and thus give rise to the long-tailed elution pattern for PAO from the Con A–agarose column. However, the precipitin curves also reflect the heterogeneity of the PAO fractions, although the Con A in these experiments was not modified in any way.

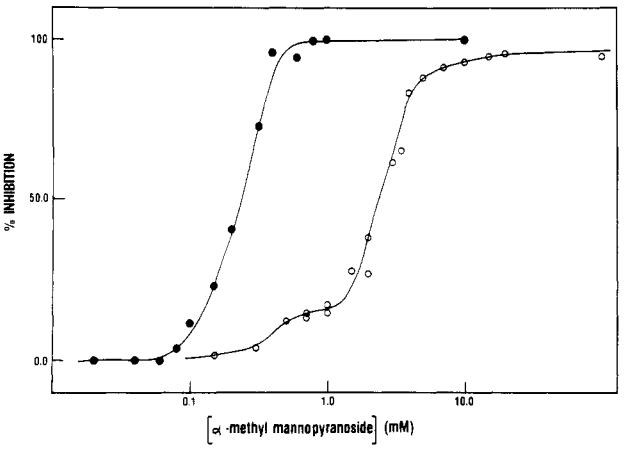

FIGURE 3: Inhibition of precipitin reaction. Samples contain 17 mg of Con A. (●) 70 μg of glycogen; (○) 70 μg of PAO. All samples contain varying amounts of methyl α-mannopyranoside as inhibitor.

Isoelectric Focusing. The chromatographically resolved fractions all yielded several bands when analyzed by isoelectric focusing (Figure 4). The p*I* of isoelectric focusing bands ranged from pH 4.5 to 5.0, with each fraction exhibiting a distinct and stable pattern. No two chromatographic fractions from different preparations or within a preparation exhibited the same isoelectric focusing band patterns. All the bands revealed by protein staining were shown to possess the same catalytic activity by activity staining (Falk, 1983). This multiple-band pattern does not seem to be due to a variable content of sialic acid, since pretreatment with 50 units of neuraminidase, 22 h, 37 °C, pH 7, had no affect on the

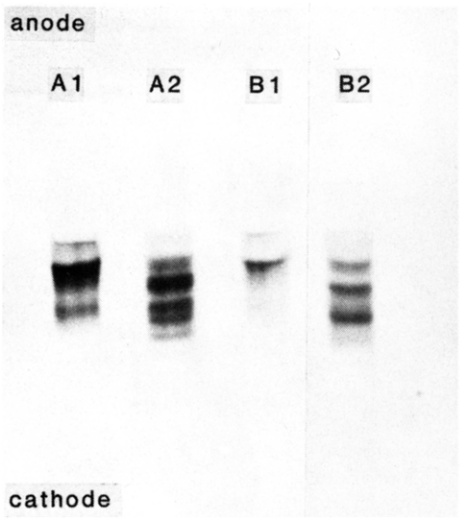

FIGURE 4: Isoelectric focusing of chromatographically resolved fractions of pig PAO. The gels were stained with Coomassie Blue as described under Methods. Forty micrograms of protein was loaded on each gel position.

isoelectric focusing band pattern.

To test the possibility that the multiple isoelectric focusing bands were due to the interaction of amino groups in the ampholines with the enzyme, ampholines from several manufacturers (Pharmacia, LKB, and Serva) were tested and found to have no affect on the band pattern. Further, inactivation of the enzyme with phenylhydrazine, followed by isoelectric focusing, had no affect on the band pattern revealed by protein staining, while no bands could be detected by activity staining (Falk, 1983). Inactivation by phenylhydrazine has been shown to inhibit amine binding to PAO (Falk, 1983). Moreover, the fact that the various fractions exhibit different band patterns argues that isoelectric focusing must be revealing structural or functional differences among them.

Other interaction of PAO with lectins provides further information about the carbohydrate content and possible modes of heterogeneity. In these experiments, PAO (6 mg/mL in 50 mM sodium phosphate, pH 7.2) was incubated with lectin (10 mg/mL in 50 mM sodium phosphate, pH 7.2) for 30 min at room temperature and then analyzed by isoelectric focusing. A precipitin reaction resulted in diminution of the normal PAO band pattern and formation of a visible precipitate at the site of application. Such was the result when Con A and *Lens culinaris* agglutinin were tested. Both of these lectins precipitated all of the PAO under these conditions. Wheat germ agglutinin and *Ricinus communis* agglutinin I also precipitated PAO, although not quite as efficiently as Con A. Pretreatment of the PAO with neuraminidase did not alter its interaction with wheat germ agglutinin. Soybean agglutinin, peanut agglutinin, *Dolichos biflorus* agglutinin, and *Ulex europaeus* agglutinin I did not precipitate PAO by this procedure. The qualitative characterization of the PAO carbohydrate content, as determined by these binding reactions and the known specificity of the lectins (Goldstein & Hayes, 1978), was in good agreement with the chemical carbohydrate analysis. The sole exception was the failure of the *Ulex europoaeus* agglutinin to bind PAO even though chemical analysis revealed that fucose was present in the enzyme. This result suggests that the linkages by which fucose is bound to the enzyme may preclude lectin binding.

No substantial differences among the various chromatographic fractions could be demonstrated in regard to their interaction with any of the lectins. However, some of the

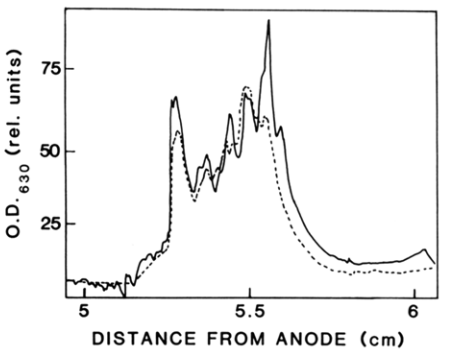

FIGURE 5: Isoelectric focusing of pig PAO and ricin. Solid line, 0.18 nmol of PAO (fraction B2); dashed line, 0.18 nmol of PAO + 0.33 nmol of ricin.

isoelectric focusing bands did precipitate preferentially with *Ricinus communis* agglutinin (Figure 5). The isoelectric focusing band pattern of pig PAO fraction B2 contained dominant bands at both the cathodic and anodic ends, as seen in Figure 5. The product of the reaction of *Ricinus communis* agglutinin I with PAO fraction B2 can be seen in Figure 5. The relative intensities of several of the bands are altered, suggesting differences in the ability to complex with the *Ricinus* lectin. For example, the prominent band nearest the cathode appeared to be selectively eliminated from the isoelectric focusing band pattern, indicating a more favorable complex formation. In the corresponding experiment, in which the lectin was Con A, a strong precipitin interaction was observed, but there were no alterations in the relative intensities of the individual bands.

Discussion

Although the chromatographic heterogeneity of PAO has been recognized for some time, neither the structural basis for this heterogeneity nor its functional consequences have been investigated (Buffoni & Blaschko, 1964). Elucidation of these questions is important on several levels. Uncovering functional differences for the chromatographically resolved forms could help clarify the biochemical role of this enzyme. The chromatographic heterogeneity of the enzyme provides a convenient aspect to study why it is a dimer of structurally similar, if not identical, subunits (Barker et al., 1979) yet seems to contain only one active site and two dissimilar Cu^{2+} binding sites (Barker et al., 1979; Lindstrom & Pettersson, 1973; Falk, 1983). Finally, an understanding of the causes of the heterogeneity would provide the requisite foundation of knowledge around which other experiments may be more rationally designed.

The present investigation was designed to determine the structural and functional differences among the chromatographically resolved forms of PAO. All the chromatographic forms were equally purified, and each migrated as a single band on both gradient gel polyacrylamide electrophoresis (M_r 196 000 for dimer) and NaDodSO$_4$–polyacrylamide electrophoresis (M_r = 95 000 for monomer). Each fraction contained one active-carbonyl cofactor and two Cu^{2+} per dimer and exhibited the same k_{cat} and K_m values with benzylamine as substrate. The only differences observed were in the carbohydrate content of each fraction and in the isoelectric focusing band pattern.

The complex isoelectric focusing patterns reveal that all the various chromatographically resolved fractions show further heterogeneity but do not provide any information about its cause. One explanation for the differences in isoelectric focusing patterns is that it is the result of an artifact of puri-

fication or of the isoelectric focusing conditions. However, all of the isoelectric focusing bands had the same catalytic activity, and the banding pattern as revealed by protein staining was unaffected by enzyme inactivation or by ampholines from different manufacturers.

On the other hand, ricin binding data suggest that some of the heterogeneity revealed by isoelectric focusing is associated with variable carbohydrate content of the active enzyme. Although there is clear evidence that the various chromatographic and isoelectric focusing variants of PAO have differing carbohydrate contents, a causal relationship has not been proven, and there is no reason to believe that the causes of the heterogeneities revealed by chromatography and isoelectric focusing are the same.

Indeed, it is not easy to rationalize how changes in the carbohydrate content could cause variations in the isoelectric point or chromatographic properties. Perhaps the simplest explanation would be that the carbohydrate residues themselves carry a charge at physiological pH. Chemical analysis reveals only trace amounts of sialic acid present on PAO, not enough to account for the isoelectric focusing or chromatographic heterogeneity. Glucosamine is positively charged at physiological pH, but it is almost always neutralized by acetylation when attached to glycoproteins. Our methods of carbohydrate analysis could not have distinguished the extent of acetylation of the glucosamine residues on PAO. The other carbohydrate residues known to be contained on PAO are uncharged. Further, attachment of the carbohydrate residue on glycoproteins by the two most common modes, *N*-aspartylglycosylamine and *O*-serylglycosyl, results in no net alteration in the charge of the aminoacyl residue.

Conceivably, the attachment of carbohydrate residues results in conformational changes and consequent alterations in the isoelectric point or chromatographic behavior. These conformations must be manifold and relatively stable in order to explain the large number of discrete variants revealed by isoelectric focusing and chromatography. Yet, the various conformations must not be extensive enough to affect the enzyme's catalytic activity.

It is feasible that the heterogeneity revealed by chromatography and isoelectric focusing is due to some as yet undetected factor other than carbohydrate content. Other possibilities that could bring about such heterogeneity are partial proteolysis or, as has been suggested for serum albumin, an altered disulfide pairing system (Yamamoto et al., 1981).

However, it is clear from all of the above that the use of a pooled enzyme source is fully acceptable for many types of experiments including studies on the cofactors, enzyme kinetics, and mechanism of action. Furthermore, the present data show that it would not be useful in any circumstance to employ enzymes that have been resolved chromatographically, since these species are themselves not homogeneous.

The effect of selective removal of the carbohydrate residues on the isoelectric focusing patterns should help determine if the variation in carbohydrate content is the cause of the multiplicity of forms. It seems likely that more definitive conclusions can be drawn about the structural and functional differences, carbohydrate content, and isoelectric focusing properties if future experiments were conducted on individual species as resolved by isoelectric focusing.

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