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Amine Oxidase. XII. The Association and Dissociation, and Number of Subunits of Beef Plasma Amine Oxidase*

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ABSTRACT: Previous studies on the sedimentation of the beef plasma amine oxidase indicated that it might be an associating–dissociating system. This was confirmed by the studies reported here. A reinvestigation of the molecular weight of the native enzyme by the methods of gel filtration and sedimentation equilibrium gave a (minimum) molecular weight of 166,700 and 170,000, respectively, compared with the average value obtained previously of 260,000. In addition, the sedimentation velocity pattern of the enzyme was symmetrical at pH 7.0, but was sharpened considerably in acidic or alkaline solutions. Whether this is due to a pH-dependent association–dissociation process or charge effect was not ascertained definitively. The sedimentation velocity patterns of the enzyme were also dependent upon time of storage and upon concentration of the enzyme solution. After 8 weeks at 4°, multiple boundaries were observed in the ultracentrifuge pattern which exhibited $s_{20,w}$ values corresponding to monomer, dimer, and trimers. This may be due to the fact that with time and at high protein concentrations, the association of the monomer becomes slowly irreversible. The enzyme in its monomeric form of mol wt 170,000 was not easily dissociated into subunits. Acid, base, 8 M urea, and 6 M guanidine hydrochloride failed in this regard, indicating the presence of covalent bonds. Guanidine hydrochloride (6 M) in combination with 0.1 M mercaptoethanol was found to be efficient for the dissociation process. A molecular weight of 87,000 was obtained from the studies of the reduced enzyme by combined sedimentation and diffusion measurements and by the sedimentation equilibrium studies, suggesting the monomer is composed of two polypeptide chains of equivalent size.

The *S*-sulfo derivative was hydrolyzed by trypsin and the fingerprint pattern disclosed that there were about 38 peptides. From the known lysine and arginine content of the enzyme, it is concluded tha the enzyme consists of two identical subunits.

In a previous paper (Yamada *et al.*, 1964), the molecular weight of the amine oxidase from beef plasma was reported to be 261,000. However, there was an increase in the sedimentation coefficient with increasing protein concentration, suggesting the presence of reversible association of a monomeric unit to polymers. The molecular weight of the native enzyme has been reinvestigated by the methods of sedimentation equilibrium and Sephadex gel filtration, and a value of the molecular weight of the monomeric unit has been obtained. Results of some dissociation and molecular weight studies indicate that the monomer consists of two polypeptide chains which are covalently linked by disulfide groups. Other preliminary studies on the association of plasma amine oxidase are also presented.

Materials and Method

Materials. Reagent grade guanidine hydrochloride and 2-mercaptoethanol were purchased from Eastman Organic Chemicals. The guanidine hydrochloride was recrystallized from methanol as described by Reithel and Sakura (1963). Bovine serum albumin and *p*-mercuribenzoate (sodium salt) were purchased from the Nutritional Biochemical Corp. Bovine γ-globulin was obtained from Pentex, Inc. Acetic acid was a product of Mann Research Laboratories. Sephadex G-200, G-15, and Blue Dextran 2000 were obtained from Pharmacia Fine Chemicals, Inc. Cytochrome *c* was prepared as described earlier (Matsubara and Yasunobu, 1961). Cellulose for thin-layer chromatography (Sigmacell, type 19) was purchased from Sigma Chemical Co. Trypsin was obtained from Worthington Biochemical Corp.

Crystalline beef plasma amine oxidase was prepared

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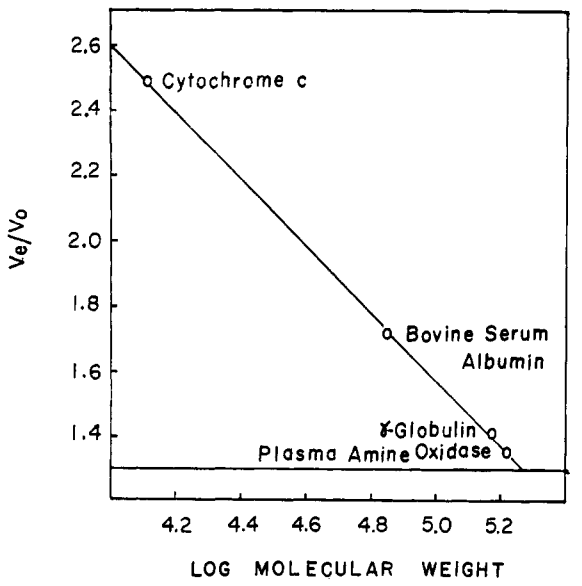

FIGURE 1: Determination of the molecular weight of plasma amine oxidase by gel filtration on Sephadex G-200. Experimental details are given in the text. The V_e/V_0 values obtained for the standard proteins used were: bovine γ-globulin, 1.41; bovine serum albumin, 1.70; and beef cytochrome *c*, 2.49.

as described previously (Yamada and Yasunobu, 1962). The enzyme was recrystallized repeatedly from ammonium sulfate until homogeneous, as judged by sedimentation velocity experiments in 0.06 M phosphate buffer (pH 7.0). For the study of the effect of pH upon the sedimentation rates, plasma amine oxidase solutions were dialyzed against the various buffers of 0.1 ionic strength at 4°. Dialysis was carried out against two changes of 500 ml of solvent for 24 hr. A control sample was dialyzed against 0.06 M phosphate buffer (pH 7.0), at the same time. For the study of the effect of guanidine hydrochloride on the sedimentation of plasma amine oxidase, solid recrystallized reagent was added directly to 1–2-ml samples of the enzyme in 0.06 M phosphate buffer (pH 7.0) to give approximately the desired concentration of guanidine hydrochloride. The treated samples were then dialyzed against 500 ml of a guanidine hydrochloride solution of the desired concentration for 24 hr in a closed container. Reduction of disulfide bonds was accomplished by dialyzing the enzyme against a guanidine hydrochloride solution containing 0.1 M mercaptoethanol. For sedimentation equilibrium measurements of the molecular weight of the reduced enzyme, solutions were dialyzed against a 6 M guanidine hydrochloride solution containing 0.1 M mercaptoethanol at pH 7.0. Appropriate dilutions of the protein were made with the same solvent.

Methods. MOLECULAR WEIGHT BY GEL FILTRATION. Molecular weight of "native" plasma amide oxidase was estimated by gel filtration following the suggestions of Andrews (1964) and of Whitaker (1963). Sephadex G-200 was hydrated in 0.06 M phosphate buffer (pH 7.0) for 5 days before packing in a 2 × 50 cm column. Protein samples (1–5 mg in 0.5 ml) were layered beneath the eluent and fractions of 1 ml were collected at a rate of 10 ml/hr. The elution volume, V_e, of each substance was defined as the effluent volume corresponding to maximum concentration of the solute as determined by the optical density at 280 mμ. The void volume, V_0, was determined with Blue Dextran 2000, a polysaccharide with an average molecular weight of 2×10^6. The column was calibrated with crystalline bovine serum albumin, bovine serum γ-globulin, and beef cytochrome *c*.

ULTRACENTRIFUGE STUDIES. All measurements were made in the Beckman–Spinco Model E analytical ultracentrifuge. Rotor temperature measurements were made by means of the RTIC unit. For sedimentation velocity studies with solute concentrations of 2 mg/ml or more, 12-mm single- or double-sector cells and schlieren optics were used. For lower concentrations, 12-mm double-sector cells and absorption optics were used in a machine equipped with the electronic speed control and with the photoelectric scanner (Hanlon *et al.*, 1962; Schachman *et al.*, 1962) and multiplex attachment. The minimum molecular weight of the enzyme was determined by the technique of miniscus depletion sedimentation equilibrium (Yphantis, 1964), and both absorption optics with photoelectric scanning at 280 mμ and interference optics (Richards and Schachman, 1959) were used. Point-average molecular weight calculations were made using data from interference experiments. The molecular weights of the denatured and reduced enzymes were determined using the sedimentation equilibrium technique of combined optics (Chervenka, 1966), for which no synthetic boundary determination of initial concentration is required. In all equilibrium runs, a sample column height of 3.0–3.5 mm over a transparent base of 0.5–1.0 mm of FC-43 fluorocarbon oil was used. The sample was considered to be at sedimentation equilibrium when no further measurable change in the total fringe found across the cell image (Richards and Schachman, 1959) occurred in a time period of one-fifth or more of the previous elapsed run time.

For sedimentation equilibrium experiments for which a synthetic boundary determination of initial concentration was required (finite meniscus concentration and interference optics), a special technique was employed (Boeye, 1965). A 12-mm double-sector, capillary synthetic boundary cell was used for the equilibrium run. The cell was filled in the normal manner with a sample fluid height of 3–3.5 mm (which can be done easily so that the fluid in both sectors is below the capillary). After the usual equilibrium run, the cell was carefully removed from the rotor, the solute was redistributed by gently tilting the cell (but so that the capillary was not wetted), the solvent sector was opened and filled with solvent, and the cell was replaced in the rotor and rerun in the normal fashion for the synthetic boundary determination.

A value of 0.76 ml/g was used for the partial specific volume of native plasma amine oxidase (Yamada *et al.*, 1964).

DIFFUSION. Diffusion measurements were also made in the Model E ultracentrifuge, utilizing the technique of boundary spreading at low speeds. The double-sector capillary synthetic boundary cell was used. The speed was 8225 rpm. The diffusion coefficient at 22.5° was cal-

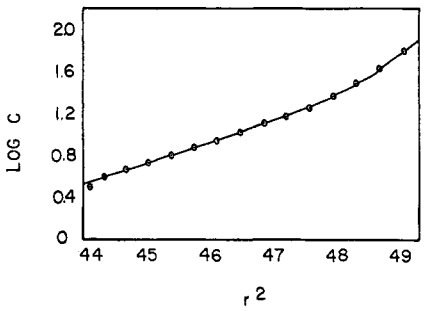

FIGURE 2: Molecular weight of plasma amine oxidase by sedimentation equilibrium. Native enzyme at 0.20 mg/ml in 0.06 M phosphate buffer (pH 7.0). The rotor was run at 7160 rpm for 36 hr at 14.5°. Concentrations were determined from absorbance values at 280 mμ.

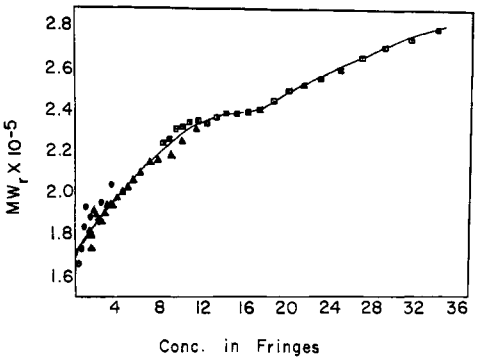

FIGURE 3: Point-average molecular weight of plasma amine oxidase as a function of concentration at 14° in 0.06 M phosphate buffer (pH 7.0). (—O—); Initial concentration (1.3 mg/ml), run at 14,000 rpm for 24 hr, interference fringe calibration based on the depletion of solute in the upper portion of the cell (25). (—△—) Initial concentration (1.3 mg/ml), run at 7200 for 27 hr, interference fringe calibration based on conservation of mass (Richards and Schachman, 1959). (—□—) Initial concentration (4.8 mg/ml), run at 5200 rpm for 46 hr, interference fringe calibration based on conservation of mass.

culated according to Ehrenberg (1957). The measured values were converted into standard conditions, $D_{20,w}$, by means of the relationship given by Schachman (1957).

ENZYME ASSAY AND PROTEIN DETERMINATION. The enzyme assay and protein determinations were carried out spectrophotometrically as previously described (Yamada and Yasunobu, 1962). One unit of activity is defined as the amount of enzyme catalyzing an absorbance change of 0.001/min under standard conditions.

PREPARATION AND DIGESTION OF *S*-SULFOPLASMA AMINE OXIDASE. Cleavage of disulfide bonds by sulfite was performed essentially as described by Pechere *et al.* (1958). Preliminary experiments indicated that urea was not required for complete sulfitolysis and it was therefore not used.

Crystalline plasma amine oxidase (25 mg) was dialyzed against 1% pyridine. To the final dialysate (15 ml) was added 200 mg of $NaSO_3 \cdot 7H_2O$ and 0.1 ml of 2 M $Cu(NO_3)_2$. The pH of the solution was raised to 9.3 by the addition of 5 M NH_4OH. The reaction mixture was allowed to stand for 4 hr at 55°. Copper ions were removed by six dialyses of 12 hr each against 250 ml of the following buffers: (1) 1 M NH_4CO_3–0.02 M EDTA, (2 and 3) 1 M NH_4CO_3–0.01 M EDTA, (4) 1 M NH_4CO_3–0.05 M EDTA, and (5 and 6) 0.2 M NH_4CO_3.

To the *S*-sulfoplasma amine oxidase in 15 ml of 0.2 M NH_4CO_3 was added 0.5 mg of TPCK-trypsin. After 6 hr at 37°, another 0.5 mg of TPCK-trypsin was added (final enzyme: substrate ratio of 1:25) and the digestion was allowed to continue for 10 hr. Acetic acid was added to lower the pH 5.5. The digest was desalted on a 1.5 × 25 cm column of Sephadex G-15, using 1% acetic acid for elution.

PEPTIDE MAPPING. Glass plates (8 in.^{2}) were coated with a 250-μ layer of cellulose. Digest (0.3 mg) was applied as a 2-cm band, 7.5 cm from the side, near the bottom of the plate. Electrophoresis was performed for 70 min in pH 6.5 buffer (pyridine–acetic acid–water, 50:2:448), at 400 V, in a Desaga–Brinkmann migration chamber. The plate was dried by fanning at room temperature. The partially separated peptides were now assumed to be present as a 2-cm wide band across the bottom of the plate. These were concentrated by placing the plate in 1% acetic acid, allowing the solvent to rise to a height of 3 cm, at right angles to the direction of electrophoresis. Chromatography in butanol–pyridine–acetic acid–water (15:10:3:12) was then carried out for 6.5 hr.

The completed peptide map was thoroughly dried and sprayed with 0.2% ninhydrin in acetone, containing 1% collidine. Colors appeared after a few hours at room temperature.

Results

Molecular Weight of Native Plasma Amine Oxidase. ESTIMATION OF MOLECULAR WEIGHT BY SEPHADEX GEL FILTRATION. According to Whitaker (1963), the ratio of the elution volume, V_e, of a protein to the void volume of the column, V_0, is proportional to the log of the molecular weight of the protein. By means of this relationship, a standard curve (Figure 1) was established with proteins of known molecular weight from which the estimated molecular weight of the plasma amine oxidase was determined. An experimental value of 1.35 for V_e/V_0 for the enzyme was obtained which corresponded to a molecular weight of 167,000.

MOLECULAR WEIGHT BY SEDIMENTATION EQUILIBRIUM. The minimum molecular weight was determined by sedimentation equilibrium measurements at low initial concentrations, using absorption optics (Hanlon *et al.*, 1962). Figure 2 shows a plot of log c *vs.* r^2 for a typical experiment at pH 7. The plot is linear below a concentration of 0.2 mg/ml, indicating that only the enzyme monomer is present in the corresponding region of the cell. The slope for this portion of the curve yields a value for the molecular weight of 170,000.

A preliminary study of the concentration dependence of the molecular weight of plasma amine oxidase was carried out using interference optics (Richards *et al.*, 1959; Yphantis, 1964). Some results are given in Figure 3. Each point on this plot was computed as a least-squares slope of five adjacent measurements of fringe

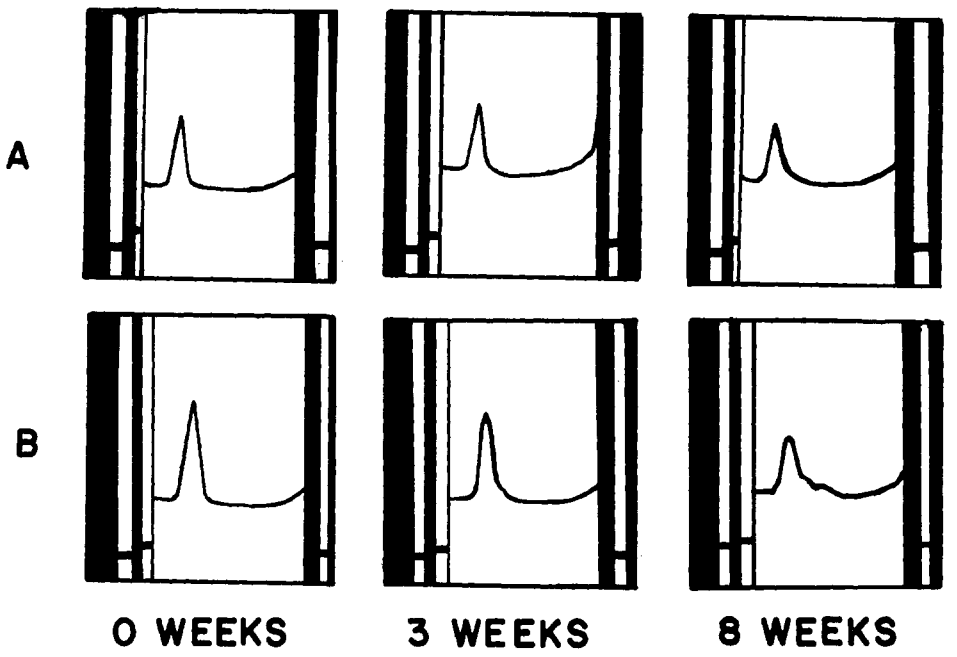

FIGURE 4: Effect of concentration and storage time on the sedimentation of the enzyme. 5-mg/ml (A) and 10-mg/ml (B) solutions of plasma amine oxidase in 0.06 M phosphate buffer (pH 7.0) were allowed to stand at 4°. All pictures were taken 16 min after reaching a speed of 59,780 rpm at bar angles of 45° (A) and 55° (B).

position (Yphantis, 1964), without any preliminary data smoothing. The increase in molecular weight with increasing concentration and the continuous nature of the data (Squire and Benson, 1967) suggest that plasma amine oxidase is indeed another example of a reversibly associating enzyme system (Reithel, 1963). While the values for molecular weight at low concentrations show the scatter typical of point average values, a plot of the data from the same experiment on the usual log c *vs.* r^2 graph is linear below a concentration of about 0.25 mg/ml (1 fringe), with a slope corresponding to a molecular weight of 175,000.

Irreversible Association Studies. Some evidence for the chemical interaction of the plasma amine oxidase in neutral solutions was obtained in a study of the effect of concentration and storage time on the plasma amine oxidase. Two solutions of fresh enzyme of approximately 10- and 5-mg/ml concentrations were made up after final recrystallization in 0.06 M phosphate buffer (pH 7.0) and allowed to stand at 4° over a period of 8 weeks. Sedimentation velocity analyses were carried out over this period with the results shown in Figure 4. Initially, the sedimentation patterns of the two solutions consisted of a single peak, but after a period of 3 weeks the 10-mg/ml solution showed a slight shoulder indicating the presence of a higher molecular weight fraction. The 5-mg/ml solution remained virtually unchanged. After 8 weeks both samples showed multiple peaks with the relative concentrations of the heavier components being greater in the case of the 10-mg/ml solution.

Approximate sedimentation coefficients were calculated for the observed peaks after 8 weeks and are given in Table I. The values for the faster moving peaks in the 5-mg/ml solution could only be regarded as estimates

TABLE I: Sedimentation Coefficients of Components Observed in Plasma Amine Oxidase Solutions Allowed to Stand for 8 Weeks at 4°.

Sample	$s_{20,w}$ (S)		
1% enzyme solution	9.67	14.3	19.2
0.5% enzyme solution	9.30	11.6	20.4

TABLE II: Sedimentation Rates of Plasma Amine Oxidase.

pH	Buffer System[a]	Protein Concn (mg/ml)	$s_{20,w}$ (S)
7.1	Tris-HCl	0.59	8.38
2	HCl-KCl	0.22	8.06
		0.69	8.11
		6.0	7.80
4	Acetate	0.27	8.02
		1.0	8.06
		6.1	8.12
11	Carbonate	0.17	8.10
		0.69	8.14
		5.2	8.21

[a] Ionic strength (0.1) buffers made up from tables given in Long (1961). Dialysis was carried out for 24 hr at 4°.

TABLE III: Effect of Guanidine Hydrochloride on Sedimentation of Plasma Amine Oxidase.

Guanidine Hydrochloride Concentration (M)	Possible No. of Components	$s_{20,w}$ (S)				
2	2	7.80	*a*			
3	5	6.48	9.60	12.4	14.5	*a*
4	3	5.80	*a*	*a*		
6	1	4.88				
6 + 0.1 mercaptoethanol	1	2.53				

[a] Sedimentation coefficients which could not be evaluated.

because they were diffuse and never completely resolved during an experiment.

Reversible Association–Dissociation Studies. SEDIMENTATION VELOCITY OF PLASMA AMINE OXIDASE. In light of the apparent self-association of the enzyme, a reevaluation of sedimentation coefficients were carried out at low concentrations, using absorption optics and photoelectric scanning. Figure 5 shows the concentration dependence of the sedimentation rate of plasma amine oxidase in pH 7 phosphate buffer. Table II lists some sedimentation data for the enzyme in other buffers and no concentration dependence of the sedimentation coefficient was observed. It was observed also that schlieren patterns at pH 7 were distinctly more diffuse than those at pH 4 or 11. A preliminary conclusion fom these data is that the self-association occurs only around pH 7.

THE EFFECT OF GUANIDINE HYDROCHLORIDE. The results of sedimentation velocity studies in guanidine hydrochloride solutions are given in Table III. In the absence of the denaturing agent a single component is observed. Similarly, in 6 M guanidine hydrochloride the sedimentation pattern is that of a single, sharp boundary with a sedimentation coefficient of 4.9 S. At intermediate guanidine hydrochloride concentrations the patterns are complex, showing two to five peaks, with sedimentation coefficients indicating possible sequential aggregation.

The molecular weight of the enzyme in 6 M guanidine hydrochloride was determined by sedimentation equilibrium at pH 7, using the technique of combined optics (Chervenka, 1966), which does not require the synthetic boundary determination of initial concentration. A solution of approximately 2.1 mg/ml was run at 8000 rpm and 12.5° for 46 hr. A plot of the data as $1/r\ dc/dr$ *vs.* c was linear over the entire sample column; that is, over a concentration range of 0.3–2.7 mg/ml. The slope of the plot yielded a value of $M_{app}(1 - \bar{v}) = 2.84 \times 10^4$. If the partial specific volume of the enzyme is the same in guanidine hydrochloride as in dilute buffers, then this value corresponds to a molecular weight of 184,000. Hade and Tanford (1967) found that in general the partial specific volumes of proteins were 0.01–0.02 ml/g less in 6 M guanidine hydrochloride than in dilute buffers. Thus this estimate of the molecular weight of the denatured protein is probably 5–10% too high. In either case, the value obtained is sufficiently close to the molecular weight of the monomer to indicate that the enzyme does not associate in 6 M guanidine hydrochloride.

The Reduced, Denatured Enzyme. As shown in Table III, the addition of the 0.1 M mercaptoethanol to the solution of the enzyme in 6 M guanidine hydrochloride results in a further, marked reduction in the sedimentation coefficient. This further dissociation, which occurs in the presence of a reducing agent, indicates that disulfide bonds may be involved in the binding of subunit polypeptide chains of plasma amine oxidase.

SEDIMENTATION AND DIFFUSION STUDIES. From the preliminary experiments on the dissociation of the plasma amine oxidase molecule, it appeared that ultimate dissociation occurred in 6 M guanidine hydrochloride upon reduction of the disulfide bonds. Consequently, sedimentation and diffusion measurements were made in 6 M guanidine hydrochloride plus 0.1 M mercaptoethanol (pH 7.0) in order to evaluate the molecular size of the subunit polypeptide chains.

The concentration dependence of the diffusion and sedimentation coefficients of the denatured, reduced enzyme exhibited the negative trend typical of noninteracting, nonideal proteins. The sedimentation coefficient of reduced plasma amine oxidase in 6 M guanidine hydrochloride was found to vary with concentration in a manner which is best described by the relation, $s_{20,w} = 3.32(1 - 0.028c)$, where c is in milligrams per milliliter. The results for the diffusion measurements were found to fit the relationship, $D_{20,w} = 3.83(1 - 0.016c)$. A pre-

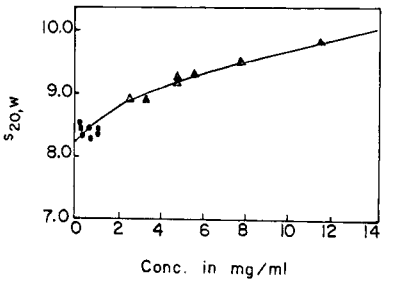

FIGURE 5: Sedimentation rate of plasma amine oxidase as a function of concentration in 0.06 M phosphate buffer (pH 7.0). (—○—) Values determined using absorption optics. (—△—) Values determined using schlieren optics.

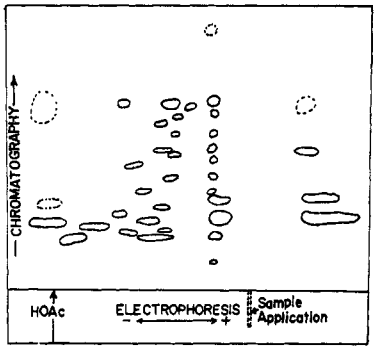

FIGURE 6: Peptide map of a tryptic digest of *S*-sulfoplasma amine oxidase.

liminary molecular weight was calculated from these results by the Svedberg equation (Svedberg and Pedersen, 1940) and found to be 87,000.

Sedimentation Equilibrium. The molecular weight of plasma amine oxidase in 6 M guanidine hydrochloride containing 0.1 M mercaptoethanol was determined by sedimentation equilibrium, again using the technique of combined optics. A solution of approximately 3.9 mg/ml was run at 12,000 rpm and 12.7° for 50 hr. A plot of $1/r\ dc/dr$ *vs. c* was essentially linear over the entire sample column; that is, over a concentration range of 0.8–4.2 mg/ml. The slope of the plot yielded a value of $M_{app}(1 - v) = 1.09 \times 10^4$. If the partial specific volume of the enzyme is the same in this solvent as in dilute buffers, then this value corresponds to a molecular weight of 80,500.

Peptide Mapping. The peptide map of the tryptic digest of *S*-sulfoplasma amine oxidase is shown in Figure 6. A total of 38 ninhydrin-positive spots is present, 4 of them relatively faint. Amino acid analysis of plasma amine oxidase shows the presence of approximately 36 moles of lysine and 63 moles of arginine per mol wt 170,000. Therefore, one would expect a total of approximately 99 peptides from a tryptic digest of plasma amine oxidase. The number obtained is less than half of this. This strongly suggests that the molecule with a molecular weight of 170,000 is composed of two identical subunits.

Discussion

The number of proteins which are known to form stable configurations by the assembly of discrete subunits is now very large as witnessed by the recent review article of Reithel (1963), and it is felt that those proteins whose preparative molecular weights are over 50,000–100,000 may be expected to reveal such subunit structure (Kendrew, 1959). Under the usual isolation conditions, dissociation does not occur and the study of the association and dissociation of a protein generally involves some moderate environmental changes. In most cases, the bonding is that of the noncovalent linkages, although some proteins have been found to be made up of several polypeptide chains covalently linked by disulfide bridges. In addition to these "static" proteins, there are also those proteins which undergo a reversible interaction. An equilibrium is established under certain conditions between "monomers" and higher aggregates of the monomeric unit and what may be regarded as a continual association and dissociation takes place in solution.

The studies on the beef plasma amide oxidase have given some evidence to support the hypothesis that the native enzyme is a reversible interacting system at neutral pH. Yamada and Yasunobu (1962) reported a molecular weight of 261,000 and an $s_{20,w}$ of 9.23 S, but the *s vs. c* curve, described by the relation $s_{20,w} = 9.23(1 + 0.005c)$, had a positive concentration dependency. According to Gilbert (1963) one can assume the presence of a reversible aggregation if the sedimentation velocity of a constituent increases with concentration. A reinvestigation of the minimum molecular weight of plasma amine oxidase yielded a value of 170,000 for the native enzyme. The discrepancy between the present results and the previously reported value can be explained on the basis of an associating–dissociating system. Plots of *log c vs.* r^2 in sedimentation equilibrium runs of plasma amine oxidase in pH 7.0 buffer showed an upward curvature which is characteristic of such a system or a polydisperse system of noninteracting components (Schachman and Edelstein, 1966). The positive slope of the *s vs. c* curve together with the fact that the enzyme was a recrystallized preparation with high amine oxidase activity led us to reject the latter possibility. Other studies also indicated a reversible association–dissociation and it seemed likely that the earlier value was an average of the molecular weight of the monomer and higher polymers.

The effect of concentration and of storage time on the plasma amine oxidase solutions suggested that monomers, dimers, and trimers could be formed. Whether this is a different reaction from the rapid reversible association–dissociation at neutral pH or whether the higher aggregates were unable to dissociate to monomer with time requires further investigations. Nonetheless, higher aggregates appeared upon standing in the sedimentation patterns of both the 5 and 10 mg per ml, with the relative proportions of the components being larger in the more concentrated sample and increasing with time. The sedimentation coefficients of the components were in the approximate ratios of 1:1.47:1.99 for the 10-mg/ml solution. If the enzyme "monomer" ($s_{20,w} = 8.3$ S) is considered to be spherical and the dimer and trimer also assumed to be spheres, then it can be shown that the theoretical sedimentation coefficients for such a case would lie in the ratio 1:1.59:2.08, derived from the relationships of the molar frictional constant (Svedberg and Pedersen, 1940). For a spherical monomer and cylindrical dimer and trimer with axial ratios of 2 and 3, respectively, the ratios would be 1:1.52:1.87. These values are similar to those obtained experimentally within the limits of the experiment.

In the preparation of the plasma amine oxidase, after repeated crystallizations, a single peak which approximates gaussian form emerges in the ultracentrifuge pattern. This fact in conjunction with an interacting system would also seem to suggest the presence of monomers, dimers, and trimers in equilibrium since theory predicts one boundary for mixtures of monomers and

dimers or monomers, dimers, and trimers (Rao and Kegeles, 1958).

In the pH studies, it was noted that the boundaries of the acid- or base-treated enzyme were sharper than the control (pH 7) sample. In addition, the pH dependence of the sedimentation coefficient was observed only near neutrality. If we consider plasma amine oxidase at pH 7 to contain appreciable quantities of monomers and higher polymers, then this polydispersity is reflected in the greater boundary spreading, due to the fact that the boundary is a site of continually readjusting equilibria in cases of interacting systems. The reversible polymerization of plasma amine oxidase appears to be pH dependent with the interactions occurring more predominantly around neutrality as in the case of lysozyme, which undergoes a reversible association between pH 5 and 9 (Sophianopoulos and Van Holde, 1964). If only a single species were present at acid and alkaline pH, then this would explain the sharper peaks and the lower sedimentation coefficients might reflect the decrease in $s_{20,w}$ with concentration that is typical of most proteins suffering hydrodynamic effects. The polymerization around pH 7 is not due to the specific effect of phosphate, since the same sedimentation coefficients were obtained in Tris buffer at pH 7. However, charge effects must be ruled out clearly before the pH dependence of the association–dissociation can be considered conclusive.

The dissociation of reduced plasma amine oxidase shows clearly the dissociation of the molecule into subunits. However, the enzyme could not be readily dissociated by moderate means as evident from the studies in acidic or basic solutions or with low concentrations of guanidine hydrochloride, indicating either a very strong interaction between the subunits or covalent linkages. Sedimentation and diffusion coefficients of the reduced enzyme is 6 M guanidine hydrochloride yielded a molecular weight value of 87,000. This value was confirmed by sedimentation equilibrium studies which gave a result of 80,500. Thus, on the basis of the molecular weight studies, it was concluded that the beef plasma amine oxidase is composed of two subunit polypeptide chains which are covalently bonded (–S–S–), and very probably of the same size.

Finally, from the fingerprint pattern of the tryptic digest of the *S*-sulfo derivative and from the known content of lysine and arginine in the enzyme, one can conclude that the two subunits present in the enzyme molecule are probably identical. Thus, it appears that the enzyme has two active centers per molecule of enzyme. It has recently been shown that the enzyme has two unessential sulfhydryl groups (Wang *et al.*, 1968) and thus, each subunit has one sulfhydryl group.

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