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Studies on L-Amino Acid Oxidase. II. Dissociation and Characterization of Its Subunits*

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ABSTRACT: The quaternary structure of L-amino acid oxidase has been investigated by ultracentrifugation both in the presence of and in the absence of denaturing agent and by gel electrophoresis. Three variants of this enzyme (Wellner, D., and Meister, A. (1960), *J. Biol. Chem. 235*, 2013) are evident in starch gel electrophoresis experiments. While removal of the sialic acid residues of this glycoprotein results in an altered electrophoretic mobility, three bands are still observed. The native enzyme has been shown to be a noncovalent dimer, consisting of two subunits of molecular weight near 70,000. Two types of polypeptide chain occur in this enzyme system but in unequal amounts (a ratio of near 2.5:1). The three isozymes appear to be the result of the various combinations of these differing subunits to give native dimer. Peptide mapping studies are consistent with a high degree of homology between these two kinds of polypeptide chain. The chains differ, however, in both their electrophoretic mobility and amino-terminal residues.

Carboxymethylation studies indicate that the native enzyme contains both free sulfhydryl groupings and intrachain disulfide bridges.

L-Amino acid oxidase (EC 1.4.3.2) has been purified from *Crotalus adamanteus* venom by Wellner and Meister (1960). Upon crystallization, this enzyme was shown to be a glycoprotein containing approximately 5% carbohydrate and 2 moles of FAD/mole (130,000 g). While the enzyme appeared pure by several physical criteria, it could be resolved by both electrophoresis and ion-exchange chromatography into three components of equal specific activity.

As in the case of other flavoproteins, such as glucose oxidase (EC 1.1.3.4), lipoamide dehydrogenase (EC 1.6.4.3), and D-amino acid oxidase (EC 1.4.3.3) where two flavins per molecule of enzyme have also been found, the possibility of interaction between the two FAD moieties of L-amino acid oxidase during catalysis has been discussed (Wellner and Meister, 1961; Meister and Wellner, 1966; Massey and Curti, 1967; deKok and Veeger, 1967). While some spectral evidence has appeared which might suggest the possible role of an FAD–$FADH_2$ interaction during enzymatic catalysis, recent studies by Massey and Curti (1967) and by deKok and Veeger (1967) have attributed this spectral intermediate to a semiquinoid species of FAD and suggest independence of the FAD moieties. From a catalytic point o[view, the enzyme may therefore be considered to consist of two identical and independent active sties.

Because two similar FAD-containing enzymes, D-amino acid oxidase and lipoamide dehydrogenase, have been reported to consist of a pair of homologous or identical polypeptide chains (Massey *et al.*, 1962; Kotaki *et al.*, 1967), this has been suggested by Kotaki *et al.* as a characteristic of this group of flavoproteins. Because in these cases, an apparent dissociation to subunits was observed when the apoproteins were prepared, these authors suggest that FAD is the cementing force between the subunits. The existence of interchain disulfide bridges suggested by Massey *et al.* (1962) for lipoamide dehydrogenase has recently been questioned (Visser and Veeger, 1968). In the present paper we have studied L-amino acid oxidase under dissociating conditions both with and without prior reduction and alkylation. Information concerning the degree of homology between the peptide chains of

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this enzyme has been obtained from peptide mapping of a tryptic digest of reduced and *S*-carboxymethylated L-amino acid oxidase and from amino-terminal studies. A quantitative analysis of the amino acids and the carbohydrates in this glycoprotein and a study of the state of oxidation of the half-cystine residues have also been carried out. The nature of the three electrophoretic bands observed in the native enzyme has been clarified. Some differences between the two kinds of polypeptide chains of L-amino acid oxidase have been demonstrated.

Experimental Procedure

Materials and Methods

Dried *Crotalus adamanteus* venom was obtained from Sigma Chemical Co. Guanidine hydrochloride was obtained from J. T. Baker Co. L-(1-Tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin[1] was obtained from Worthington Biochemical Corp. Neuraminidase (purified from *Vibrio cholerae*) was obtained from General Biochemicals. Iodoacetic acid (sodium salt) was obtained from Eastman Organic Chemicals. Glass-distilled water was used throughout.

Enzyme Preparation. L-Amino acid oxidase was prepared from dried venom of *Crotalus adamanteus* according to the method of Wellner and Meister (1960). The once-recrystallized enzyme was stored either as a concentrated solution in 0.2 M KCl at 0°, as a precipitate under ammonium sulfate, or as freeze-dried material. Isozyme A was obtained in pure form by means of preparative disc electrophoresis at pH 8.6 on a commercial apparatus (Shandon Scientific Co., London). The gel column consisted of a 12-cm lower segment of 5% gel and a 2-cm upper section of 3% gel, both in 0.05 M glycine (pH 8.6). The gel column was prerun for 1 hr to eliminate polymerization catalysts. The sample was applied in 0.1 M KCl and the gel was cooled to 4° during the run.

Starch gel electrophoresis was carried out in a horizontal apparatus as described by Carsten and Pierce (1960). Runs were in either Tris-HCl (pH 8.0) or in sodium glycinate (pH 9.4) for periods of from 8 to 18 hr and at a potential of 11 V/cm. The gels were stained for 3 min with Amido Black (Schwartz Biochemicals, Orangeburg, N. Y.), destained by washing with a methanol–acetic acid–water (5:1:5, v/v).

Disc Electrophoresis. Polyacrylamide disc electrophoresis was performed in 0.03 M glycine buffer (pH 9.0) both in the presence of and in the absence of 8 M urea and 0.5% β-mercaptoethanol. The gels were stained with Amido Black, destained electrically, and scanned in a densitometer (Photovolt Corp., New York, N. Y., modified to a slit width of 0.05 mm). The densitometer patterns were used to estimate the relative amounts of each component present.

Reduction and Alkylation. Small amounts of L-amino acid oxidase (10–40 mg) were reduced and alkylated with iodoacetic acid as described by Crestfield *et al.* (1963), and dialyzed against distilled water for 12 hr followed by dialysis against two changes of 2 l. of 0.5% ammonium bicarbonate. Material for physical studies was then recovered by lyophilization while that for peptide mapping was maintained in the ammonium bicarbonate solution and the tryptic degradation carried out immediately.

Tryptic Digestion and Peptide Mapping. Tryptic digestion of the reduced and alkylated protein was as previously described (Rawitch *et al.*, 1968) in 1% ammonium bicarbonate with 2 mg of L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin/100 mg of protein added initially and an equal quantity was added after 2 hr. The digestion was terminated after 4 hr by freezing and the material was recovered by lyophilization. A small amount of the freeze-dried peptide mixture (3–4 mg) was dissolved in 0.05 ml of 50% acetic acid and spotted on a sheet of Whatman No. 3MM chromatography paper (46 × 57 cm) and descending chromatography initiated immediately with butanol–pyridine–acetic acid–water (15:10:3:12, v/v). The chromatography was allowed to proceed until the solvent front reached the bottom of the paper, approximately 16 hr, and the paper was air dried for several hours. High-voltage electrophoresis was carried out in the second dimension at pH 6.4 in pyridine–acetic acid–water (10:0.4:90, v/v) for 55 min at 40 V/cm. Several replicate peptide maps were prepared in order to facilitate the detection of peptides containing specific amino acids. Peptides were revealed by dipping the maps in either cadmium–ninhydrin reagent (Atfield and Morris, 1961) or collidine–ninhydrin reagent (Canfield and Anfinsen, 1963). Peptides containing arginine were identified by spraying with a modified Sakaguchi reagent, those containing tryptophan were revealed by the Ehrlich reaction. Tyrosine- and histidine-containing peptides were identified by the Pauly reaction (Easley, 1965).

Carbohydrate Analyses. Neutral sugars were determined by hydrolysis and gas chromatography of alditol acetate derivatives as described by Kim *et al.* (1967). Amino sugars were determined after hydrolysis in 6 N HCl for 4 hr at 100°, by ion-exchange chromatography on the 50-cm column of a Spinco Model 120C amino acid analyzer. Sialic acid was determined by the method of Warren (1959). Sialic acid was removed from the intact protein with neuraminadase as described by Spiro (1962). Analysis of the enzyme after neuraminidase treatment indicated 90% removal of sialic acid.

End-Group Analysis. Amino-terminal studies were carried out according to the method of Stark and Smyth (1963) including a step to eliminate pyrrolidonecarboxylic acid. The carbamylation was carried out in 6 M guanidine hydrochloride. A protein control was subjected to identical treatment but with no cyanate addition. Quantitation was by amino acid analysis on a Spinco Model 120C amino acid analyzer.

Amino Acid Analysis. Hydrolysis of protein samples was carried out in the following manner. Hydrolysis vials were prepared from thick-walled Pyrex glass

[1] The trypsin was treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone as described by Koska and Carpenter (1964) in order to minimize cleavage due to chymotrypsin.

TABLE I: The Amino Acid and Carbohydrate Composition of L-Amino Acid Oxidase.

	Isozymes A–C		Isozyme A	
	g/100 g of Protein[b]	Residues/Mole[d]	g/100 g of Protein[e]	Residues/Mole[d]
Lysine	7.5	75.7	7.2	73.1
Histidine	2.5	27.1	2.5	27.1
NH_3	1.0	81.0	n.d.[f]	n.d.
Arginine	7.9	65.4	7.4	61.1
Aspartic acid	8.8	100.0	8.8	100.0
Threonine	5.2	67.2	4.3	55.2
Serine	4.0	59.3	3.9	58.4
Glutamic acid	10.0	100.5	10.0	100.8
Proline	3.3	44.3	3.3	44.8
Glycine	3.2	72.2	3.0	67.2
Alanine	4.1	75.6	4.1	75.2
Half-cystine[g]	1.3	16.5	1.1	14.4
Valine	4.1	53.7	3.8	48.8
Methionine[g]	2.0	15.9	2.0	16.0
Isoleucine	5.6	64.8	5.1	58.4
Leucine	6.1	69.8	6.3	72.0
Tyrosine	6.9	55.4	6.5	51.2
Phenylalanine	6.8	59.8	5.7	49.6
Tryptophan[a]	2.0	14.2	n.d.	n.d.
N-Acetylglucosamine[a]	1.7	11.1	n.d.	n.d.
Fucose[a]	0.4	3.3	n.d.	n.d.
Mannose[a]	1.6	12.7	n.d.	n.d.
Galactose[a]	1.2	9.5	n.d.	n.d.
Sialic acid[a]	0.4	2.0	n.d.	n.d.
FAD[c]	1.2	2.0	n.d.	n.d.
Weight recovery	99.2			

[a] Determined separately as described in the text. [b] Average or extrapolated values of the data obtained from three hydrolysates with hydrolysis times of 22, 48, and 72 hr. Values were corrected for moisture content of the sample. [c] Taken from Meister and Wellner (1966). [d] The molecular weight assumed was 135,000. [e] Average or extrapolated values of the data obtained from two hydrolysates with hydrolysis times of 22 and 72 hr; 15% moisture was assumed in the calculations. [f] Not determined. [g] Determined as oxidized derivatives after performic acid oxidation in the case of the mixture of isozymes. Determined as half-cystine in the case of isozyme A.

tubing and washed with concentrated nitric acid followed by multiple washings in deionized and finally glass-distilled water. Protein samples of 1–2 mg were weighed into the dried vials and 2 ml of 6 N HCl (prepared by diluting a fresh bottle of reagent grade concentrated HCl 1:1 with glass-distilled water) was added to each vial. The vials were frozen in an ethanol–Dry-Ice bath, carefully evacuated, and sealed under high vacuum as suggested by Crestfield *et al.* (1963). Hydrolysis was carried out at 110° for periods of 22, 48, or 72 hr. Cysteine plus cystine and methionine were determined after performic acid oxidation as described by Hirs (1956). The HCl was removed on a rotary evaporator, a small amount of water was added, and the samples were redried. The hydrolysates were then redissolved in citrate buffer (pH 2.2) and stored frozen until the amino acid analyses could be carried out. Tryptophan was determined by the method of Spies and Chambers (1949).

Sedimentation Analyses. Sedimentation equilibrium experiments were carried out on a Spinco Model E analytical ultracentrifuge equipped with interference optics and an electronic speed control. Measurements of the molecular weight of both the native and dissociated enzyme were obtained by the high-speed equilibrium method of Yphantis (1963). A rotor speed of 12,590 rpm was used for the native enzyme and a speed of 22,000 rpm was used for the dissociated form of the enzyme. The protein concentrations employed were in the range of 0.25–1.0 mg/ml. Interference plates were analyzed by reading half-fringes on a Gaertner microcomparitor (Gaertner Scientific Corp., Chicago, Ill.). The partial specific volume used for calculations of experiments both in the presence and absence of guanidine hydrochloride was that given by Wellner and Meister (1966), 0.733 cm^3/g. Apparent weight-average molecular weights were calculated from the slope of a plot of log c *vs.* $(\text{radius})^2$ as suggested by Yphantis (1963), where the concentration, c, was expressed in fringe increments and the radius was in centimeters from the axis of rotation. No detectable change in the fringe distribution after an additional 24-hr centrifuga-

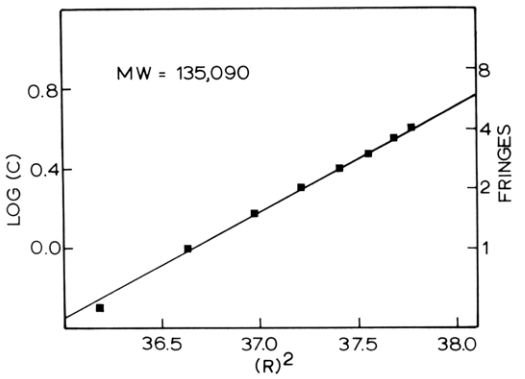

FIGURE 1: A representative plot of sedimentation equilibrium data obtained on the native enzyme. The solvent system was 0.1 M sodium phosphate plus 0.1 M potassium chloride (pH 7.0). The experiment was carried out at 20° and a rotor speed of 12,590 rpm. These data were obtained 28 hr after reaching the equilibrium speed and at a protein concentration of 0.35 mg/ml.

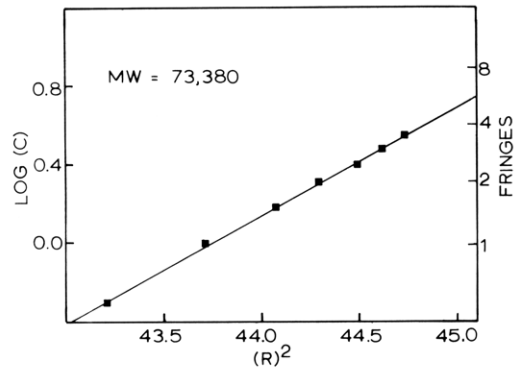

FIGURE 2: A representative plot of sedimentation equilibrium data obtained on reduced and *S*-carboxymethylated L-amino acid oxidase. The solvent was 6 M guanidine hydrochloride–0.1 M sodium phosphate (pH 7.0). This experiment was carried out at 20° and a rotor speed of 22,000 rpm. These data were obtained 48 hr after reaching the equilibrium speed. The protein concentration was 0.5 mg/ml.

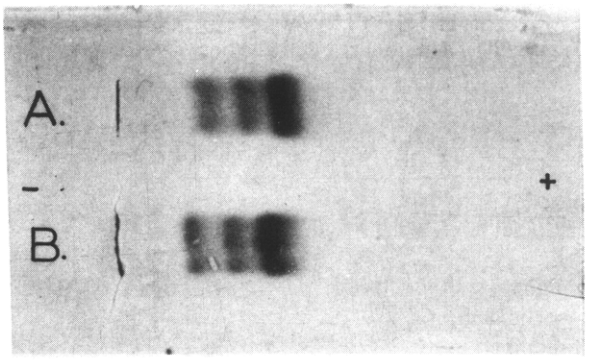

FIGURE 3: Starch gel electrophoresis of L-amino acid oxidase at pH 9.4 in 0.03 M sodium glycinate. Details are given in the text. Electrophoresis was for 18 hr at 4°. (A) Native enzyme. (B) L-Amino acid oxidase which had been treated with neuraminidase in order to remove its two residues of sialic acid.

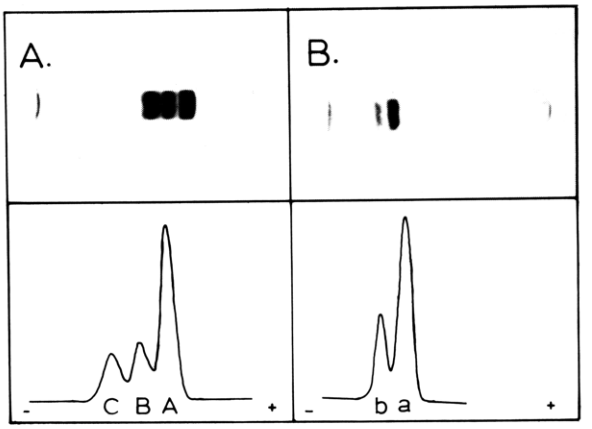

FIGURE 4: Disc gel electrophoresis of L-amino acid oxidase. (A) In 0.03 M glycine (pH 9.0). The gels contained 5% acrylamide and the run was for 90 min at 3 mA/tube. (B) In that which had been preincubated for 20 hr in 8 M urea and for 2 hr in 0.5% β-mercaptoethanol. The gels contained 6% acrylamide, 0.5% β-mercaptoethanol, 8 M urea, and 0.03 M glycine (pH 9.0). Run conditions were as in part A. The gels were scanned as described in the text.

tion was used as a criterion for the attainment of equilibrium.

Results

Amino Acid and Carbohydrate Analyses. Amino acid composition data obtained on our enzyme preparation and on pure isozyme A are shown in Table I. The data obtained for the enzyme containing all three isozymes are in reasonable agreement with the values reported earlier by Wellner and Meister (1966), but with a slightly lower recovery in the weight of amino acid residues per milligram of protein and lower quantities of half-cystine. We have also determined that this enzyme contains 12–14 residues of tryptophan/mole. Carbohydrate analyses revealed 3–4 residues of fucose, 13 residues of mannose, 9–10 residues of galactose, 2 residues of sialic acid, and 11 residues of glucosamine (assumed to be present as *N*-acetylglucosamine) (Wellner and Meister, 1966). Alkylation of the enzyme in 8 M urea without reduction revealed 4 residues of *S*-carboxymethylcysteine after acid hydrolysis. It may thus be concluded that of the half-cystine residues shown in amino acid analysis, 4 are found as cysteine in the enzyme and the remaining 12 or 13 are either involved in intrachain disulfide bridges or are not accessible to iodoacetic acid under these conditions. While the amino acid composition of isozyme A differs slightly from that of the mixture of isozymes with respect to a few amino acids, the over-all analyses were quite similar (Table I). The large difference in arginine content between isozyme A and the mixture of isozymes A, B, and C reported recently by Wellner and Hayes (1968) was not observed.

Physical Properties. Sedimentation equilibrium studies on the native L-amino acid oxidase indicated a molecular weight of 130,000–140,000 (Figure 1). In the presence of 6 M guanidine hydrochloride at pH 7.0, dissociation of reduced and *S*-carboxymethylated L-amino acid oxidase to subunits of near mol wt 70,000 was

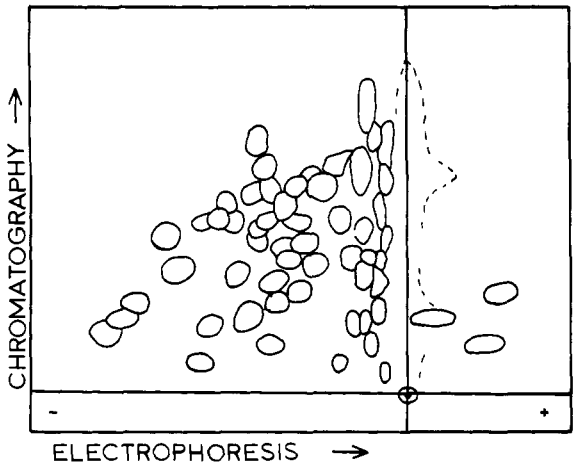

FIGURE 5: A tracing of a typical tryptic peptide map of reduced and *S*-carboxymethylated L-amino acid oxidase. The spots were revealed with collidine–ninhydrin dip. Details of the mapping procedure are given in the text.

TABLE II: A Summary of the Tryptic Maps of L-Amino Acid Oxidase.

Amino Acid	Residues/ Mole[e]	No. of Peptides Found
Lysine plus arginine	141	60–70[a]
Arginine	65	30–35[b]
Tyrosine plus histidine	82	19[c]
Tryptophan	14	6[d]

[a] Ninhydrin-positive peptides. From the known specificity of trypsin we should expect 142 peptides in a tryptic digest if there were no repeating sequences in the primary structure of the enzyme. [b] Sakaguchi-positive peptides. [c] Pauly-positive peptides. [d] Ehrlich-positive peptides. [e] The molecular weight was assumed to be 135,000.

observed (Figure 2). Virtually identical results were obtained when the native enzyme was studied by sedimentation equilibrium in 6 M guanidine hydrochloride at pH 7.0. In all cases the plots of log *c vs.* (radius)^2 were linear throughout the cell and indicated no major heterogeneity in the molecular size of either the native enzyme or its subunits (Yphantis, 1963). No significant concentration dependence of the molecular weight of the native enzyme or its subunits was observed over the range of 0.25–1.0 mg/ml.

The results of a typical starch gel electrophoresis experiment on the native enzyme at pH 9.0 are shown in Figure 3A. Three components were observed. The fastest migrating component appeared to represent approximately 50% of the protein. Removal of the two sialic acid residues in the enzyme by digestion with neuraminidase produced a very similar three-banded pattern (Figute 3B), but with a modified electrophoretic mobility indicating less electronegative character. Disc electrophoresis at pH 9 showed patterns similar to the starch gel experiments (Figure 4A). Scanning of these polyacrylamide gels on a densitometer indicated that the three components, A, B, and C, were present in relative amounts of 0.58, 0.22, and 0.20 respectively. When the enzyme was subjected to disc electrophoresis in the presence of 0.5% β-mercaptoethanol and 8 M urea at pH 9.0, two components, a and b, were observed (Figure 4B). The relative amounts of these two components were 0.69 and 0.31, respectively. Also shown in Figure 4 are densitometer tracings of the gel patterns, indicating relative amounts of each electrophoretic component. We have assumed equal staining properties for the various electrophoretic components. When isozyme A was subjected to electrophoresis as described above, a single band was observed both with and without 8 M urea and β-mercaptoethanol.

Peptide Mapping. Peptide maps of the tryptic digest of reduced and *S*-carboxymethylated L-amino acid oxidase revealed between 60 and 70 ninhydrin-positive spots (see Figure 5) with no significant ninhydrin-positive material at the origin. As may be seen in Table I, the sum of the number of lysine plus arginine residues in L-amino acid oxidase was found to be 141. The Sakaguchi reagent revealed 30–35 arginine-containing peptides while the Erlich reagent indicated 6 tryptophan-containing peptides. The Pauly reagent showed 19 peptides containing tyrosine or histidine. Three peptides showed visible fluorescence when illuminated with a short-wavelength ultraviolet lamp. These data are summarized in Table II.

Amino-Terminal Analysis. Analysis of amino-terminal residues by the cyanate method showed 1.33 residues/mole of alanine, 0.39 residue/mole of tyrosine, and small quantities (less than 0.1 residue/mole) of leucine. Significant amounts of glutamic acid were found both in the control and carbamylated samples even though the additional step recommended to eliminate pyrrolidonecarboxylic acid (Stark and Smyth, 1963) was carried out. Since the amount of glutamic acid found in the control sample was equal to that in the carbamylated sample, this amino acid was not regarded as amino terminal.

Discussion

Our determination of the molecular weight of native L-amino acid oxidase by high-speed sedimentation equilibrium is in good agreement with that reported by Wellner and Meister (1960), *i.e.*, between 130,000 with 140,000. Combining this information with a sedimentation coefficient of 6.70 S (Meister and Wellner, 1966), an apparent diffusion coefficient, $D_{20,w}$, of 4.52×10^{-7}cm^2/sec may be calculated by the Svedberg equation. These data may be combined as suggested by Svedberg and Pedersen (1940) to give an apparent f/f_0 value of 1.39. This would be consistent with a prolate ellipsoid of revolution with an axial ratio near 7. From these parameters it can be stated that the hydrodynamic properties of L-amino acid oxidase differ significantly from those of an anhydrous sphere. We have shown that this enzyme is composed of two polypeptide chains of equal size and near 70,000

molecular weight. Since the dissociation of the enzyme may be achieved in either 8 M urea or 6 M guanidine hydrochloride without prior reduction of disulfide bridges, it may be concluded that the enzyme contains no covalent lingages between its two polypeptide chains and thus the 12 or 13 half-cystine residues which were not alkylated by iodoacetic acid are involved in intrachain disulfide bridges. The enzyme contains 4 residues of cysteine as judged from alkylation by iodoacetic acid in 8 M urea.

Gel electrophoresis studies in both polyacrylamide and starch gels indicate three electrophoretic components in this enzyme, while under dissociating conditions only two components in a ratio of about 2.5 to 1 were observed. Removal of the sialic acid residues results in the retention of a three-banded pattern with modified electrophoretic mobility and would seem to eliminate these residues as a source of the electrophoretic heterogeneity in the enzyme. These patterns are consistent, however, with a system in which two types of subunits, a and b, may combine to form three differing dimeric species aa, ab, and bb. This model is further supported by the fact that isozyme A shows a single electrophoretic band before and after dissociation with urea and β-mercaptoethanol. Our data show that the two kinds of polypeptide chain which make up L-amino acid oxidase are found in unequal amounts in this system. This may be due to an unequal synthesis or degradation of the two chains in individual reptiles or since we have used pooled snake venom to prepare our enzyme, it may reflect a heterogeneous genetic pool. In this connection, the observation of Wellner and Meister (1966) that upon electrophoresis of L-amino acid oxidase from a single snake, only two of the electrophoretic components were observed, is rather puzzling.

The distribution of material among the three components of the native enzyme from pooled snake venom differs significantly from a normal distribution[2] and the homodimeric species, *aa* and *bb*, seem to be favored over the heterodimeric species *ab* (assumed to be the component of intermediate electrophoretic mobility.) Isolation of the individual chains for hybridization experiments is currently in progress and further experiments with regard to this point may clarify this observation.

The results of amino-terminal analysis fully support a two-chain model for this enzyme and further confirm the unequal amounts of the two kinds of chain found in gel electrophoresis. The predominant polypeptide chain has an amino-terminal alanine residue while the chain found in lesser amounts has an amino-terminal tyrosine.

Peptide mapping studies on the tryptic digest of reduced and alkylated L-amino acid oxidase show near half the number of ninhydrin-positive spots one would expect if the two chains of L-amino acid oxidase were completely different with respect to their amino acid sequence. This observation would be consistent with two polypeptide chains showing a high degree of homology in their primary structure. This conclusion is further supported by the number of peptides found to contain specific amino acids.

In summary, we have demonstrated through ultracentrifugal studies and chemical end-group analysis that L-amino acid oxidase is composed of two polypeptide chains of similar amino acid sequence but differing at least in their amino-terminal residues and electrophoretic mobilities. These two subunits occur in unequal amounts in the L-amino acid oxidase system and are combined through noncovalent forces to form the dimeric native enzyme. Gel electrophoresis experiments in the presence and absence of 8 M urea indicate that the three electrophoretic bands in the native enzyme are due to the various dimers formed between the two types of subunit and that the formation of dimers between like subunits is favored over that between unlike subunits. It will be of interest to determine if, in fact, this enzyme contains two active sites. The recent observation of Massey *et al.* (1968) on the borohydride reduction of this enzyme in the presence of substrate may allow the active site or sites of L-amino acid oxidase to be labeled and further studied. Additional studies on the isolated chains of L-amino acid oxidase and on the individual isozymes will certainly be required in order to clarify our knowledge of the structure and catalytic mechanism of the enzyme.

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[2] A normal distribution of two species which can form a dimer is taken to mean that the three possible dimers formed, AA, AB and BB, will be found in relative amounts corresponding to the terms, a^2, $2ab$, and b^2 of the binomial expansion $(a + b)^2$ if a and b are the relative fractions of each monomer and we assume equal stability and probability of formation for each type of dimer. In this case, we would expect relative amounts of 0.475, 0.428, and 0.096 for a normal distribution of the species AA, AB, and BB, respectively. The relative amounts found, however, 0.58, 0.22, and 0.20, show significant deviations from the values calculated above and suggest that the homodimeric species are favored in some way.

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Binding of Adenosine 5′-Monophosphate and Substrate by Rabbit Liver Fructose 1,6-Diphosphatase*

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ABSTRACT: The binding of adenosine 5′-monophosphate and fructose 1,6-diphosphate by rabbit liver fructose 1,6-diphosphatase has been studied by the technique of gel filtration. Four binding sites for each ligand per enzyme molecule were estimated from Scatchard plots. In the presence of substrate and absence of divalent cation, the tightness of adenosine 5′-monophosphate binding to the enzyme was greatly increased and positive cooperative interaction was observed among the adenosine 5′-monophosphate binding sites. No cooperative interaction was detectable among the substrate binding sites.

At pH 7.3 and 3°, the respective primary association constants were estimated to be 3.2×10^5 M^{-1} for adenosine 5′-monophosphate and 1.6×10^5 M^{-1} for fructose 1,6-diphosphate.

Numerous kinetic studies have shown that adenosine 5′-monophosphate is a specific inhibitor for fructose 1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) from a variety of sources (Taketa and Pogell, 1963; Newsholme, 1963; Scala *et al.*, 1968). The selective removal of AMP inhibition by various treatments of the enzyme from liver, yeast, and kidney (Taketa and Pogell, 1965; Rosen and Rosen, 1966; Marcus and Hubert, 1968) supports the view that AMP is bound at allosteric sites. In the case of the rat liver enzyme, the inhibition is reversible and noncompetitive. The sigmoid nature of the inhibition curve has been explained from theoretical considerations by suggesting cooperative interactions of three or four AMP molecules per molecule of enzyme (Taketa and Pogell, 1965). In general, rabbit liver fructose 1,6-diphosphatase has kinetic properties, including inhibition by AMP, which are very similar to those of the rat liver enzyme (Pontremoli *et al.*, 1965; this paper).

Our preliminary experiments on the direct binding of AMP by rabbit liver fructose 1,6-diphosphatase showed that AMP is bound much more tightly in the presence of the substrate, fructose 1,6-diphosphate (Watanabe *et al.*, 1968). These results also suggested that the enzyme formed intermediate binary and ternary complexes with substrate and inhibitor in the absence of divalent cation. Either Mg^{2+} or Mn^{2+} is needed for over-all catalysis of substrate hydrolysis. Direct evidence for the existence of an enzyme–substrate complex was recently reported by Pontremoli *et al.* (1968a).

Detailed studies by gel filtration of the binding of AMP and substrate to homogeneous preparations of rabbit liver fructose 1,6-diphosphatase are reported in the present communication. These results are consistent with our kinetic observations.

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