DIAMINE OXIDASE: MOLECULAR WEIGHT AND SUBUNIT ANALYSIS

Michael D. Kluetz[1] and Paul G. Schmidt

Department of Chemistry, University of Illinois, Urbana, IL 61801

Received March 14,1977

SUMMARY

Electrophoretically pure hog kidney diamine oxidase has been isolated by an improved procedure and subjected to molecular weight and subunit analyses. Sedimentation/diffusion and sedimentation equilibrium ultracentrifugation clearly show that the native enzyme has a molecular weight of 172,000. Acrylamide gel electrophoresis indicates that the enzyme consists of two apparently identical subunits of 91,000 daltons each. The native enzyme contains two firmly bound Cu(II) ions. The isolation procedure described provides diamine oxidase in 50-60% yield of activity and of the highest specific activity yet reported (1.2 units/mg).

For purposes of magnetic resonance studies underway in our laboratory of diamine oxidase (diamine: O_2 oxidoreductase [deaminating]; EC 1.4.3.6), it was imperative that we clarify the molecular weight and subunit structure of the enzyme. Conclusive data of this nature has not been available. It was also important that we be able to rapidly obtain large amounts of pure enzyme, which was not possible employing previously reported methods. We therefore report our ultracentrifugation and electrophoretic studies of diamine oxidase isolated by a more efficient method.

METHODS

Assays. Protein determinations were made by the biuret procedure of Goa (1) or by direct absorbance measurements of purified enzyme using an extinction coefficient (280 nm, 1% solution) of 1.7 cm^{-1}. Diamine oxidase activity was determined spectrophotometrically using p-dimethylaminomethylbenzylamine as the substrate and monitoring the change in absorbance at 250 nm.[2]

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[1]Present address: Department of Chemistry, University of Idaho, Moscow, ID 83843.

[2]This assay as originally described by Bardsley et al. (2) gives activities which are erroneously large due to an inaccurate determination of the total absorbance change for the amine oxidation. We have applied this assay to both hog kidney and pea seedling diamine oxidases and, using both enzymes, have measured $\Delta\varepsilon_{250}$ to be 16,200 M^{-1}-cm^{-1}. Using this value, one unit of activity, corresponding to the oxidation of 1 µmole of substrate per min, yields a change of 10.8 absorbance units per min (in a 1.5 ml assay volume) rather than 7.4 absorbance units per min.

ISSN 0006-291X

Gel Electrophoresis. The method of performing gel electrophoresis in the presence of SDS[3] and 2-mercaptoethanol was that of Weber and Osborn (3). Gels were made from a stock solution of 22.2 g acrylamide (Eastman) and 0.60 g Bis (Canalco) in a total volume of 100 ml. The gel buffer was 0.10 M monobasic sodium P_i (Mallinckrodt) containing 0.1% SDS (Sigma). The standard proteins used for the molecular weight determinations were as follows: aspartate transcarbamylase catalytic subunit (prepared in our laboratory); catalase, glutamate dehydrogenase and fumarase (Sigma); aldolase (Boehringer-Mannheim); bovine serum albumin and trypsin (Pentex).
Ultracentrifugation. Ultracentrifugation experiments were performed on a Beckman Model E Analytical Ultracentrifuge using the schlieren and Rayleigh interference optical systems. Kel-F double-sector centerpieces and sapphire windows were used throughout. The temperature was controlled at 20°. In sedimentation/diffusion runs, the rate of descent of the boundary was measured from the peak of the schlieren band. Schlieren peak areas were calculated gravimetrically from tracings of enlarged images of the bands.
Cu Analysis. Enzyme solutions were analyzed for their Cu content on a Perkin-Elmer 303 Atomic Absorption Spectrophotometer, monitoring at 327 nm.

RESULTS

Enzyme Preparation. After initial homogenization of the hog kidney cortex and removal of insoluble material, the solution was immersed in an 80° bath until the temperature of the material was 66° (about 5 min). This rapid heat denaturation increased the activity yield substantially. Diamine oxidase was then precipitated from the supernatant solution between 35 and 55% saturation with ammonium sulfate (Mallinckrodt).

The crude preparation obtained above was applied to a DEAE-cellulose (Sigma) column (5 x 40 cm) equilibrated with 0.015 M potassium P_i buffer, pH 7.2. At this ionic strength, diamine oxidase remains bound to the column while about 40% of the contaminating material elutes off. Upon raising the buffer concentration to 0.040 M, the activity is eluted from the column in a thin band. This step generally results in activation of the enzyme (as had been previously observed by Kapeller-Adler and McFarlane (4)) with a yield of about 200%.

Further purification of the enzyme was accomplished by elution from a second DEAE-cellulose column (2.6 x 80 cm) with a 0 to 0.050 M gradient of the substrate cadaverine (in 0.015 M potassium P_i, pH 7.2). This specific elution effected a separation which could not be obtained with a salt gradient covering the same ionic strength range.

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[3]Abbreviations used: SDS, sodium dodecyl (lauryl) sulfate; Bis, N,N'-methylenebisacrylamide.

TABLE 1

Summary of the hog kidney diamine oxidase preparation. The data listed below are the approximate averaged values from a number of enzyme isolations. The values of % yield, for example, from one step to the next were found to be quite constant in the different preparations. The data are normalized to a starting value of 100,000 mg Total Protein, which would derive from approximately 1.5 kg of wet kidney cortex.

Step	Total Protein (mg)	Volume (ml)	Specific Activity (u/mg)	Total Activity (u)	Yield (%)	Purification
Homogenization	100,000	1500	0.0005	50	100	1
Heat denaturation	19,000	1000	0.0020	38	76	4
Ammonium sulfate precipitation	6,000	200	0.006	36	72	12
DEAE-cellulose: step gradient	600	25	0.12	72	144	240
DEAE-cellulose: substrate elution	150	5	0.40	60	120	800
Low ionic strength precipitation	25	-	1.2	30	60	2400

The final purification of the enzyme was accomplished by selectively precipitating diamine oxidase at low ionic strength. After exhaustive dialysis against the 0.015 M buffer at pH 7.2 and removal of any precipitate, the diamine oxidase was precipitated by dialysis against 0.005 M potassium P_i, pH 7.0. The enzyme obtained was electrophoretically pure; the total purification was 2400-fold, the yield of activity greater than 50%, and the final specific activity approximately 1.2 u/mg. The results of the preparation are summarized in Table 1.

Cu Content.[4] The Cu content of diamine oxidase was determined on two dilute

[4]We have found that diamine oxidase as isolated generally has some amount of weakly bound or "non-specifically" bound Cu(II) associated with it which can

enzyme solutions (1 mg/ml). The average of the two determinations gave a value of 0.75 (± 0.15) μg Cu per mg enzyme, or 0.075%. This corresponds to one Cu per 85,000 daltons.

SDS-Acrylamide Gel Electrophoresis. The molecular weight of diamine oxidase was determined by comparison of its mobility on the gels with those of the standard proteins listed above. Runs were made under varying conditions of pH and percent acrylamide in the gels. Within experimental uncertainty, the results were independent of these variables. The average value of the subunit molecular weight was 91,000 (± 3,000) daltons.

Sedimentation Equilibrium Ultracentrifugation. Two meniscus depletion equilibrium runs were done, at 16,000 and at 24,000 rpm, on a 0.9 mg/ml sample. $\bar{v}_2$ for the enzyme was taken to be 0.723. The results were nearly invariant with respect to the rotor speed at equilibrium. The average value obtained was 172,000 (± 3,000) daltons.

Sedimentation/Diffusion Ultracentrifugation. A value for the standard sedimentation coefficient at zero concentration, $s^o_{20,w}$, was obtained through a series of six boundary velocity runs in which the diamine oxidase concentration was varied between 5 and 13 mg/ml. Extrapolation to zero concentration yielded a value for $s^o_{20,w}$ of 9.4 S. The diffusion coefficient, $D_{20,w}$, was calculated from the variation with time of the square of the schlieren peak area to height ratio. The runs were done at a concentration of 2 mg/ml. Large uncertainties commonly found in determinations of $D_{20,w}$ preclude the necessity of extrapolating to zero concentration. In addition, no corrections for sedimentation or radial dilution were applied. Upon calculating the molecular weight from the $s^o_{20,w}/D_{20,w}$ ratio, we obtained a value of 170,000 (± 3,000) daltons.

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be removed by dialysis against 1 mM EDTA without affecting the activity. The firmly bound Cu(II) can only be removed by dialysis against diethyldithiocarbamate followed by high-speed centrifugation. The results in this section were obtained on a sample from which this "non-specifically" bound Cu(II) had been removed and hence reflect only firmly bound active site Cu(II).

TABLE 2

Summary of molecular weights obtained for diamine oxidase by three different procedures.

Method	Molecular Weight
1. SDS-acrylamide gel electrophoresis	94,000[a]
	91,000[b]
	91,000[c]
	88,000[d]
2. Sedimentation equilibrium	175,000[e]
	169,000[f]
3. Sedimentation/ diffusion	173,000
	167,000

[a] 10% gel, pH 7.2
[b] 8.5% gel, pH 7.2
[c] 7% gel, pH 7.2
[d] 10% gel, pH 8.2
[e] Meniscus depletion at 24,000 rpm
[f] Meniscus depletion at 16,000 rpm

The results of the molecular weight determinations are summarized in Table 2. The averaged data indicates that diamine oxidase in its native form has a molecular weight of 175,000 and consists of two subunits of 91,000 daltons each which are identical by the criterion of electrophoretic mobility.

DISCUSSION

The molecular weight of diamine oxidase was initially reported to be 87,000 as determined by Cu analysis (5). However, these authors' ultracentrifugation results yielded values of 119,500 to 135,000 daltons. More recently, Yamada et al. (6) have reported 185,000 daltons as determined by sedimentation/diffusion alone, which is oftentimes less reliable than equilibrium methods. Finally, Pionetti (7) has obtained a value of 185,000

daltons in oxygenated solutions, but 725,000 daltons in deoxygenated solutions, again by sedimentation/diffusion. We therefore feel that this work provides the necessary clarification of both the native enzyme and subunit molecular weights. In addition, this is the only evidence to date that diamine oxidase is a multisubunit enzyme with the exception of the fact that previous Cu analyses yielded values which were substantially smaller than those obtained by centrifugation. The elucidation of the subunit structure is important in that our preliminary magnetic resonance results suggest the possibility of cooperative binding of substrate analogs and electronically inequivalent Cu(II) sites which are presumably on the different subunits.

Finally, the use of "ion-exchange filtration" with DEAE-cellulose and specific elution from this resin with substrate appear to be valuable techniques in allowing one to isolate relatively large amounts of diamine oxidase in high yield. This procedure is currently being applied to the pea seedling system as well.

ACKNOWLEDGEMENT

The authors wish to acknowledge the support of the National Institutes of Health (Grant GM 18038, to PGS).

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