Quaternary Structure of δ-Aminolevulinate Dehydratase from *Rhodopseudomonas spheroides**

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ABSTRACT: δ-Aminolevulinate dehydratase (EC 4.2.1.24) from *Rhodopseudomonas spheroides* was examined by polyacrylamide gel electrophoresis, gel filtration, and analytical ultracentrifugation. Although electrophoresis of the purified enzyme showed it to be apparently not homogeneous in the native state, electrophoresis in the presence of sodium dodecyl sulfate showed essentially only one species of mol wt 40,000 ± 10%. This species was found to be ^{14}C labeled after reduction of the native enzyme with borohydride in the presence of [^{14}C]δ-aminolevulinate, a treatment which is known to bind substrate to the enzyme. Gel filtration chromatography showed one species of molecular weight about 240,000 (oligomer) and some material of molecular weight several million. In the presence of potassium ions dioligomers and possibly trioligomers besides very high molecular weight material were found. Ultracentrifugation showed a concentration-dependent state of aggregation with average molecular weights both higher and lower than that of the oligomer. At protein concentrations below 0.1 mg/ml most of the oligomers were apparently dissociated. The conclusions reached are that the enzyme exists as a hexamer of molecular weight about 240,000 which can apparently dissociate into monomeric units and can aggregate to material having higher molecular weights.

δ-Aminolevulinate dehydratase (EC 4.2.1.24) is one of the early enzymes in the biosynthesis of tetrapyrroles. It catalyzes the condensation of two molecules of δ-aminolevulinic acid (ALA)[1] to one molecule of porphobilinogen (Gibson *et al.*, 1955; Schmid and Shemin, 1955; Granick and Mauzerall, 1958; Burnham and Lascelles, 1963). A mechanism of this reaction has been proposed by Nandi and Shemin (1968b). They (Nandi *et al.*, 1968; Nandi and Shemin 1968a) have also published details of their work on a rather pure preparation of the enzyme, which show it to be a high molecular weight protein, having complex association properties and kinetics, both of which vary with the metal ions in solution. A plot of the reaction velocities against substrate concentration of the enzyme assay solution containing no metal ions or only sodium ions was sigmoidal, whereas in the presence of either potassium or ammonium or rubidium ions the curve was hyperbolic. Furthermore, under the former conditions, the enzyme was shown to sediment in a sucrose gradient in one single band whose molecular weight was calculated to be about 250,000, whereas sedimentation in a sucrose gradient of a similar sample in the presence of potassium ions showed an equilibrium mixture of this species with others of higher molecular weight, calculated as about 500,000 and 750,000. The 250,000 molecular weight species was shown to be dissociated by 1 M urea or after dilution into a small species of about half the molecular weight. This smaller species can be reassociated into the equilibrium mixture by potassium ions or into the 250,000 molecular weight species by ALA.

This paper presents further work on the state of association and dissociation of the enzyme in solution, and also evidence for its subunit and quaternary structure.

Materials and Methods

δ-Aminolevulinic acid hydrochloride, sodium dodecyl sulfate (SDS), and Tris (trizma base) were purchased from Sigma. [4-^{14}C]δ-Aminolevulinic acid hydrochloride (32.0 mCi/mmole, 100 μCi/ml) was from Schwarz BioResearch. Coomassie Brilliant Blue R-250 and ammonium sulfate (special enzyme grade) were from Mann, and Amido Schwarz and the polyacrylamide gel reagents were from Canalco. Myoglobin (sperm whale), chymotrypsinogen (bovine), serum albumin (bovine), γ-globulin (human), and apoferritin (horse) were from Mann; catalase (bovine), pyruvate kinase (rabbit), and cytochrome *c* (horse) from Sigma; and glyceraldehyde-3-phosphate dehydrogenase (rabbit) from Calbiochem.

Enzyme activity was assayed as previously described (Nandi *et al.*, 1968) by the method of Mauzerall and Granick (1956) except that 1.0-ml reaction volume was used and the quantities of reagents were reduced proportionately. The definition of a unit of activity was also as given in Nandi *et al.* (1968). Protein concentrations were estimated by the method of Lowry *et al.* (1951).

Polyacrylamide gel electrophoresis in the presence of SDS was carried out essentially as described by Weber and Osborn (1969). Purified enzyme was dialyzed against water and freeze dried. It was then dissolved (0.5 mg/ml) in 0.01 M sodium phosphate buffer (pH 7.0) containing 10 g/l. of SDS and 1% (v/v) 2-mercaptoethanol and incubated at 37° for 2 hr. It was dialyzed overnight against the same buffer with one-tenth the concentration of SDS and 2-mercaptoethanol. About 0.02 mg was then applied to a 10% acrylamide gel (containing SDS), subjected to electrophoreses for 3 hr at 8 mA (about 60 V), stained for 2 hr with coomassie brilliant blue, and destained electrophoretically.

The identification and localization of the subunit of ALA dehydratase were also achieved by labeling the enzyme with

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[1] Abbreviations used are: ALA, δ-aminolevulinate; SDS, sodium dodecyl sulfate.

radioactive ALA as found by Nandi and Shemin (1968b). A sample of enzyme (specific activity 20 units/mg) was dialyzed against 0.1 M Tris buffer (pH 8.5)–0.05 M KCl. A 0.5-ml sample containing 5 mM [^{12}C]ALA and 25 μl of the [^{14}C]ALA solution was incubated for 30 min at 0°, and treated with sodium borohydride and acetic acid. Control samples were treated with isotopic ALA or with borohydride and acetic acid. After incubation, the enzyme samples were precipitated by adding solid ammonium sulfate to 80% of saturation and dissolved in the Tris buffer for assay. The enzyme treated with both ALA and borohydride was about 70% inactivated compared to the controls.

Purification of the Enzyme

Unless otherwise stated, all buffers used in the enzyme purification contained 2-mercaptoethanol (5 mM) and the operations were conducted between 0° and 5°.

Growth of Bacteria. The enzyme was purified from *Rhodopseudomonas spheroides* (ATCC 11167) by a modification of the method previously described (Nandi *et al.*, 1968). The bacteria were grown photosynthetically in the medium S of Lascelles (1956) supplemented with 1 g/l. of yeast extract (Difco). They were grown in flat 1-l. Roux culture bottles arranged upright and lengthwise in rows between rows of showcase lamps. The bottles and lamps were immersed in a water bath maintained at 30°. This arrangement was designed to ensure that the bacteria were evenly illuminated throughout the suspension.

The intensity of the light had a very critical effect on the ultimate yield of enzyme. At high intensity (about 500 ft-candles), the bacteria reached stationary phase after about 30-hr growth, but yielded only about 25 units of activity/g dry weight. At 40 ft-candles, growth required 4 days but the yield was 75 units/g. This was the light intensity normally used. The final yield of bacteria was 0.8–0.9 g dry weight/l. of growth medium whatever the light intensity.

After growth, the bacteria were harvested by centrifugation on a Sharples high-speed centrifuge, washed once with NaCl solution (9 g/l.) and twice with water, and then freeze-dried and stored at −20° until used.

Cell-Free Extract. The freeze-dried bacteria (usually about 100 g) were suspended in 0.1 M Tris buffer (pH 7.6; 15 ml/g of dried bacteria) containing 0.5 M KCl and sonicated for three periods each of 5 min. The temperature was never allowed to rise about 30° and the suspension was cooled to 5° between each sonication.

The broken cell suspension was centrifuged for 1 hr at 13,000*g* and the thick dark supernatant was heated to 55° in about 10 min and maintained at that temperature for a further 5 min. It was then rapidly cooled in ice and centrifuged for 3 hr at 105,000*g*. The residue from the low-speed centrifugation was resuspended in buffer and sonicated, heat treated, and centrifuged as before. The pale red-brown supernatants were pooled.

First Ammonium Sulfate Fractionation. Solid ammonium sulfate was slowly added to this solution (about 5 mg of protein/ml) up to 40% saturation and the solution gently stirred for 30 min. It was centrifuged and the precipitate taken up in enough 0.001 M sodium phosphate (pH 6.8) solution containing 0.05 M KCl to give a protein concentration of about 3 mg/ml. This solution was centrifuged to remove insoluble material when necessary.

Hydroxylapatite Column. The solution was poured onto a column (5 cm diameter × 10 cm high) of hydroxylapatite (prepared according to Levin (1962)) which had been previously equilibrated in the same 0.001 M phosphate buffer. The column was washed with this buffer at a flow rate of about 2 ml/min until the eluate was protein free (as judged by its absorption at 280 nm). The buffer was then changed to 0.03 M sodium phosphate (pH 6.8)–0.05 M KCl and the column again washed until protein free. This buffer sometimes eluted a little active enzyme but only about 2% of the total. The bulk of the enzyme was eluted with 0.09 M sodium phosphate (pH 6.8)–0.05 M KCl.

DEAE-cellulose Column. The active eluate was adjusted to pH 7.2 with NaOH and applied to a column (2.5 cm diameter × 5 cm high) of diethylaminoethylcellulose (Whatman De-52) which had been preequilibrated with 0.09 M sodium phosphate buffer (pH 7.2)–0.05 M KCl, and from which the fines had been removed by decantation. The column was washed with phosphate buffer until the eluate was protein free and then developed with a linear gradient of 0.05–0.20 M KCl in the same phosphate buffer (200 ml of each) using a three-channel peristaltic pump (Ayad *et al.*, 1968) at a flow rate of 1.5 ml/min. Elution was completed with a further 100 ml of the more concentrated buffer. The enzyme emerged during the gradient elution and the active fractions were pooled and concentrated down to about 30 ml with an Amicon Model 200 ultrafiltration cell, using a UM-20E membrane.

Second Ammonium Sulfate Fractionation. The concentrated solution (about 1.5 mg of protein/ml at this stage) was taken to 35% saturation with solid ammonium sulfate, adjusted to pH 6.0 with 2 M acetic acid, readjusted to 35% saturation, and stirred gently for 30 min. The precipitate was collected by centrifugation, dissolved in 2 ml of 0.05 M potassium phosphate buffer (pH 6.8), and clarified by centrifugation.

Gel Filtration. The solution was applied to a column (2.5 cm diameter × 95 cm high) of Bio-Gel A 1.5m (100–200 mesh, Bio-Rad Laboratories, Inc.) equilibrated and developed in 0.05 M potassium phosphate buffer (pH 6.8) which was pumped up the column, against gravity, at 20 ml/hr. The eluted fractions with the highest specific activity were pooled and concentrated by ultrafiltration as before to about 30 ml and then further concentrated to about 3 ml with a model 12 cell. The state of the enzyme eluting from this column is discussed below.

Results

The purification of the enzyme through the second ammonium sulfate fractionation is shown in Table I. The enzyme was stored at this degree of purification; samples were applied to the Bio-Gel column for further purification when required. A typical gel filtration column profile is shown in Figure 1A and the material eluting between 0.45 and 0.57 column volumes (which had the highest specific activity) was normally used. The remainder was concentrated and repurified. This most active fraction was generally about 60% of the total activity eluted from the column and had a specific activity of 130–140 units/mg. Polyacrylamide gel electrophoretograms of this material are shown in Figure 2. A is a run of the native material and it appears to be far from homogeneous. However electrophoresis in the presence of SDS (see below) shows only one main band with traces of contamination (Figure 2B). This, together with the ultracentrifugal data also discussed below, suggests that the material is almost pure ALA dehydratase but that it has undergone extensive disaggregation and aggregation before and during the elec-

TABLE I: Purification of ALA-dehydratase from *Rhodopseudomonas spheroides*.

Stage	Vol (ml)	Act. Units/ml)	Protein (mg/ml)	Total Protein (mg)	Sp Act. (Units/mg)	Total Act. (Units)
Crude, low-speed supernatant	810	4.5	19.5	15,800	0.2	3600
Heat-treated high speed supernatant	690	3.5	5.8	4,000	0.6	2400
First ammonium sulfate precipitate	400	6.2	3.8	1.523	1.6	2450
Hydroxylapatite eluate	230	6.7	0.85	196	7.8	1540
DEAE-cellulose eluate	255	4.3	0.23	57	18.9	1090
Second ammonium sulfate precipitate	2.2	433	6.32	13.9	70.1	955

trophoresis. Enzyme of this purity was used in most of the experiments described below. It lost more than half its activity on storage for a few days.

Dissociation into Subunits. ALA dehydratase activity was found to be completely inhibited by SDS (1 g/l.) in 0.05 M sodium phosphate buffer (pH 6.8). No activity was recovered after passing the enzyme through a column of Sephadex G-25 or after exhaustive dialysis against the phosphate buffer containing potassium chloride. Urea (8 M) and guanidine hydrochloride (6 M) also bring about complete inactivation which thus far cannot be reversed by dialysis against a phosphate buffer. These results suggest that SDS, urea, and guanidine hydrochloride bring about irreversible conformation changes in the enzyme and we decided to investigate this further by polyacrylamide gel electrophoresis in the presence of SDS (Weber and Osborn, 1969; Dunker and Rueckert, 1969; Shapiro *et al.*, 1967). The results are shown in Figure 2B. There is essentially one strong band of stained protein. To determine the molecular weight of the protein in this band, we ran a series of marker proteins treated with SDS in exactly the same way and measured their mobility relative to bromophenol blue. A semilogarithmic plot of molecular weight against mobility is shown in Figure 3. It is a straight line within the range shown. We found that the mobilities of the marker proteins were reproducible to within less than 5% from run to run so that is was not necessary to run all the markers at the same time as the experimental sample. Most of the commercial proteins used for the calibration showed several minor bands after electrophoresis, but provided that only one protein sample was run on each gel, there was no problem in selecting the major band. From these data, the mobility of the band from ALA dehydratase was found to be 0.40 which fits best a molecular weight of 39,500 ± 2000.

The identification of this band as coming from ALA dehydratase was confirmed by labeling an impure sample of the enzyme with radioactive ALA, as described in Materials

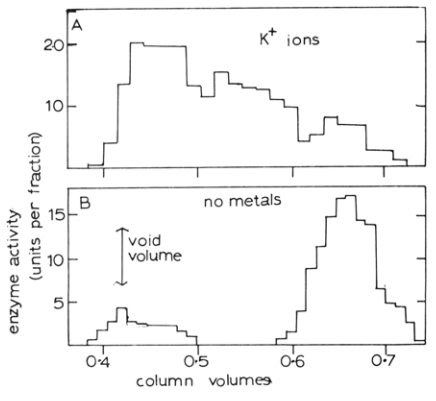

FIGURE 1: Chromatography of samples (about 500 units) of ALA-dehydratase on a column of Bio-Gel A 1.5m developed at a flow rate of 20 ml/hr. (A) Chromatography in 0.05 M potassium phosphate buffer (pH 6.8); (B) chromatography in 0.05 M Tris buffer (pH 7.1).

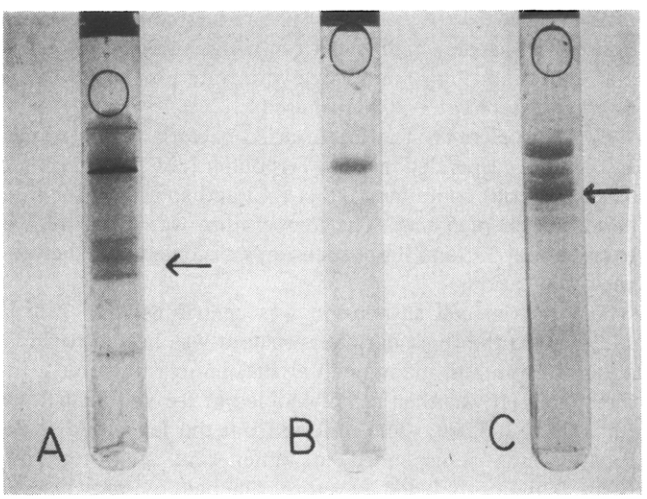

FIGURE 2: Polyacrylamide gel electrophoretograms of ALA-dehydratase. (A) Purified native preparation (50 μg) stained with Amido Schwarz for 2 hr. The arrow indicates the approximate position of enzyme activity which was carried out as follows. A similar gel transferred to a close fitting test tube was incubated in assay medium (Nandi *et al.*, 1968) for 10 min at 37° and, after pouring off medium, Ehrlich's solution (Mauzerall and Granick, 1956) was added. A rather diffuse pink band appeared, presumably at the position of active enzyme. (B and C) Samples of enzyme (25 μg) treated with SDS were subjected to electrophoreses and stained with coomassie brilliant blue. (B) Specific activity, 130 units/mg; (C) specific activity, 20 units/mg. The latter sample (C) was covalently linked to radioactive ALA prior to the SDS treatment. The arrow indicates the radioactive area.

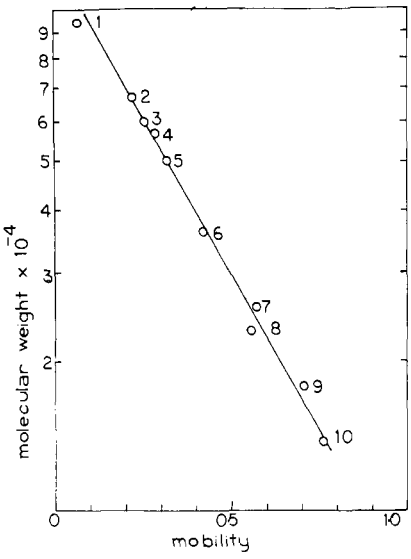

FIGURE 3: A semilogarithmic plot of molecular weight of various proteins against their mobility, compared to that of bromophenol blue, in polyacrylamide gels containing SDS. Key: 1, phosphorylase *a;* 2, serum albumin; 3, catalase; 4, pyruvate kinase; 5, γ-globulin, H chain; 6, glyceraldehyde-3-phosphate dehydrogenase; 7, chymotrpsinogen; 8, γ-globulin, L chain; 9, myoglobin; 10, ribonuclease. About 10 μg of each protein was applied to the gels.

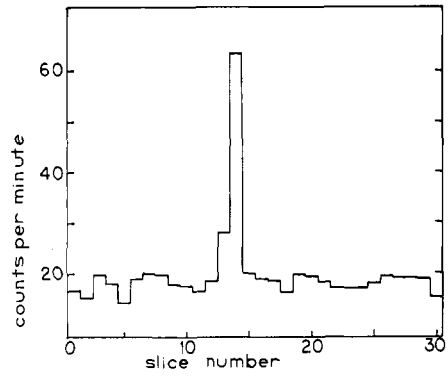

FIGURE 4: Radioactivity of slices prepared from a polyacrylamide gel in which the ALA-dehydratase, covalently labeled with radioactive ALA (Nandi and Shemin, 1968b), was subjected to electrophoreses after treatment with SDS.

and Methods above. After this treatment the enzyme solutions were dialyzed for 5 hr at 30° against 0.01 M sodium phosphate buffer (pH 7.0) containing 10 g/l. of SDS and 1% (v/v) 2-mercaptoethanol, and then overnight against the same buffer with one-tenth the concentrations of SDS and 2-mercaptoethanol. Samples (50 μl) were then electrophoresed on SDS-containing gels and stained with coomassie brilliant blue as described above. The result is shown in Figure 2C. There are three main bands and several minor bands. The mobility of each band was measured and the gels were then cooled to 3° and sliced into 33 slices each about 1.6 mm thick with Canalco lateral gel slicer. The radioactivity of each slice was then measured by the method of Tishler and Epstein (1968). Figure 4 shows a plot of counts against slice number for the enzyme sample which had been reduced with $NaBH_4$ in the presence of radioactive ALA; there was no detectable radioactivity in the control samples. It shows that almost all the radioactivity was concentrated in one slice and could be attributed to one stained band alone (as indicated by an arrow in Figure 2C). The mobility of this band was 0.39, which is very close to that found with the purified enzyme.

Gel Filtration. Nandi and Shemin (1968a) have reported evidence from ultracentrifugation in sucrose gradients that ALA dehydratase associates into an equilibrium mixture of oligomer, dioligomer, and trioligomer (Monod *et. al.*, 1965) in the presence of potassium, rubidium, or ammonium ions, whereas, in the absence of these ions, only the oligomer can be found. Gel filtration chromatography is a useful alternative way of looking at molecular weight distributions, and we looked at the chromatography of the enzyme on Bio-Gel A 1.5m (8% agarose gel, 100–200 mesh) which has an exclusion limit of about 1.5 million. A column (2.5 cm diameter × 95 cm high) was calibrated with certain proteins of known molecular weight as shown in Figure 5. The result compares quite closely with the data provided by the makers of the gel (Bio-Rad Laboratories, Inc.) in their own publications. Figure 1A shows chromatography of an enzyme sample (specific activity 80 units/ml) in 0.05 M potassium phosphate buffer (pH 6.8). Figure 1B shows chromatography of a similar sample in 0.05 M Tris buffer (pH 7.1). There is a marked difference in the two profiles. Both show some material coming through at the void volume (determined with Blue Dextran (Pharmacia)) and some more retarded material. The most retarded material in both cases emerges at about 0.65 column volume which corresponds to a molecular weight of about 220,000. The material coming through at the void volume should have a molecular weight of about 1.5 million or more. Chromatography (in the absence of metal ions) on Bio-Gel A 5m which has an exclusion limit of 5 million also shows some active material coming through at the void volume. The major difference betweeen the two chromatograms (Figure 1A,-B) is that in the presence of potassium there is some material of intermediate molecular weight (corresponding to a molecular weight of about 500,000) which is absent when there are no metal ions.

Some of the aggregation to very high molecular weight material must be reversible for when material coming through at a void volume is collected, concentrated, and rechromatographed in the same system (both in the presence and absence of potassium) it gives the same profile as the original material.

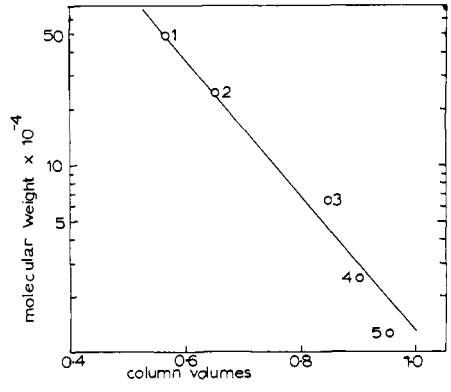

FIGURE 5: Calibration of a column of Bio-Gel A 1.5m with proteins of known molecular weight. Each protein (2 mg) in 2 ml of 0.05 M potassium phosphate buffer (pH 6.8) was applied and the column (2.5 cm diameter × 91 cm) was developed in that buffer at a rate of 35 ml/hr. Key: 1, apoferritin; 2, catalase; 3, serum albumin; 4, chymotrypsinogen; 5, cytochrome *c*.

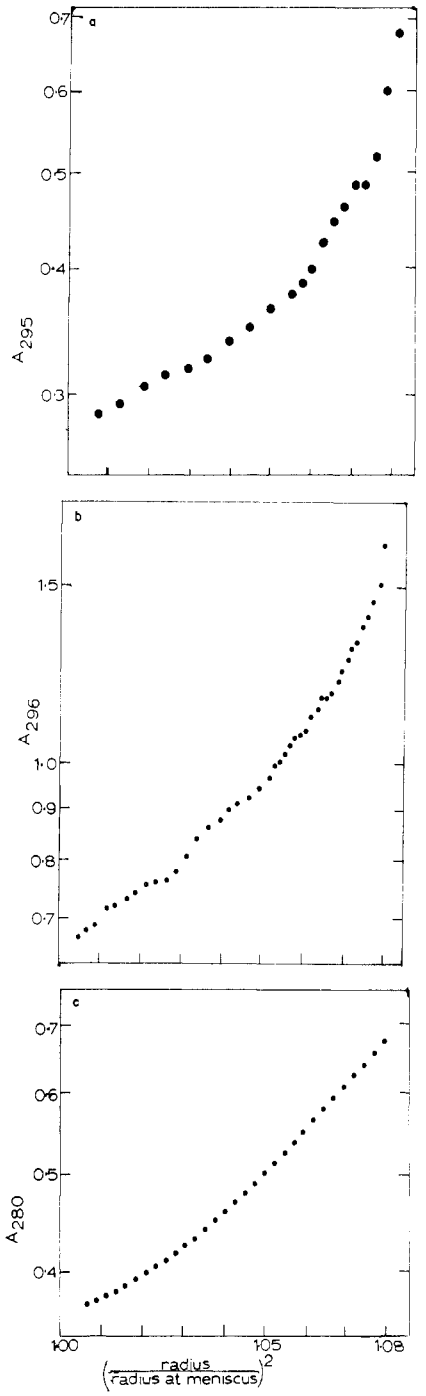

FIGURE 6: Sedimentation equilibrium plots of ALA-dehydratase at different concentrations. (a) 0.5 mg/ml of protein. The rotor was run for 3.5 hr at 8000 rpm and then for 48 hr at 5200 rpm. (b) 0.24 mg/ml of protein. The rotor was run for 1.8 hr at 8000 rpm and then for 80 hr at 5200 rpm. (c) 0.12 mg/ml of protein. The other conditions were the same as in part (b). All runs were at 5°. The ordinate is the absorbancy of the solution at the givenwave length (in nanometers). The abscissa is given in terms of the square of the ratio of the radius at a point to the radius at the meniscus, so that the three plots may be more easily compared.

Ultracentrifuge Experiments. We decided to investigate the situation further using the analytical ultracentrifuge (Beckman, Model E, fitted with a photoelectric scanning system). Sedimentation velocity experiments (see Chervenka, 1969) at 60,000 rpm showed considerable differences in the presence or absence of K^+ ions. In 0.05 M Tris (pH 7.2), there was one boundary moving with a sedimentation coefficient of 13 S. There was also some material which sedimented very fast right down to the bottom of the cell. In 0.05 M potassium phosphate buffer (pH 6.8) the situation was more complex. There was still some material sedimenting very fast, but this time there were two other boundaries sedimenting at 13 and 19 S (a molecular weight ratio of 1.8 using the approximation of Martin and Ames (1961)). In both cases, the optical density profile at the top of the cell was not completely flat, suggesting the presence of some material of much lower molecular weight. The protein concentration in these experiments was about 1 mg/ml.

Several sedimentation equilibrium runs were performed in 0.05 M Tris buffer (pH 7.2). A three-sector Yphantis cell (12-mm path length) was used and one sample was run at 1.0, 0.5, and 0.3 mg per ml. After centrifugation for about 1 hr at 8000 rpm all three sectors had about the same optical density profile in spite of the very different original protein concentrations. This curious effect was explained by the fact that a large amount of protein was spun rapidly to the bottom of the cell and remained in equilibrium with the lower molecular weight species in solution. The optical density gradient at the bottom of the cell was very steep compared to the rest. From the scanner record found after equilibrium was reached for the cell loaded with the protein solution at 0.5 mg/ml the plot of optical density against $(\text{radius/meniscus radius})^2$ gives the curve in Figure 6a (Chervenka, 1969). The slope of this curve at any point is proportional to the molecular weight at that point. However, the plot is a curve rather than a straight line and shows that the enzyme solution is a mixture of proteins of various molecular weights, some of which are both higher and lower than the 240,000 found for the oligomer by gel filtration and by sucrose density sedimentation studies (Nandi and Shemin, 1968a).

Since these runs show great heterogeneity partly because of the extensive aggregation, we decided to try more dilute solutions in the hope that equilibria in such solutions would be more in favor of lower molecular weight species. To increase the optical density we used a three-section cell with 30-mm path length filled with protein at 0.24, 0.12, and 0.04 mg per ml. Figure 6b shows the result of the run with 0.24 mg/ml. This again is a continuous curve of the sort that would be expected for a heterogeneous solution. However, at the top of the cell the apparent slope suggests the existence of protein whose molecular weight is below 240,000. Figure 6c shows the result at 0.12 mg/ml. In this case, the direct scanner trace was a rather irregular curve (presumably because of electronic noise and other spurious effects at this low concentration) and we therefore used a computer to construct a smooth curve from it by second-order least-squares approximation. At this concentration the curve at the bottom of the cell (1.05–1.08 of the abscissa) has a slope which corresponds to proteins having an apparent or average molecular weight of 130,000, whereas the slope at the top of the cell corresponds to a mixture of species of even lower molecular weight. The results at 0.04 mg/ml were very similar.

When this experiment was repeated at the same protein and buffer concentrations, but in the presence of 0.05 M KCl, the results were very similar except that there were more aggregates and also low molecular weight material.

Discussion

These experiments show that the quaternary structure of ALA dehydratase is a complex one; solutions of the native

enzyme are highly heterogeneous since almost any species in solution rapidly aggregates and dissociates into other species.

The polyacrylamide gel experiments show that ALA dehydratase dissociates in the presence of SDS into one band of molecular weight about 40,000. The system is capable of, resolving proteins differing only about 10% or less in molecular weight (Weber and Osborn, 1969) so that it is unlikely (but possible) that there is in fact more than one product from the dissociation, but that they are not resolved on the gel. The reproducibility of the method and the accuracy of the calibration (Figure 3) also suggest an accuracy of better than 10% in the figure of 40,000 for the molecular weight of the subunit. It is ture that Dunker and Rueckert (1969) have reported some "anomalous" proteins, *e.g.*, ribonuclease, that do not have the mobility that would be expected from their molecular weight. However, in our case, ribonuclease ran in accordance with expectations and in general it seems most unlikely that the subunit of ALA dehydratase has a molecular weight greatly different from the one found by this method. The fact that protein labeled with radioactive substrate can be clearly identified with this product further confirms its identification as a subunit of ALA-dehydratase.

Before electrophoresis, the enzyme had been incubated in a strong solution of 2-mecaptoethanol as well as in SDS. This should have been sufficient to cleave any disulfide bonds present in the native enzyme and the lower concentration of thiol present during the electrophoresis should have kept them reduced. This is certainly the case for the human γ-globulin used as a marker, which did split into the heavy (H) and light (L) chains known to be linked by disulfide bonds in the native molecule (Fleischman *et al.*, 1963). Since ALA-dehydratase gives only one product under these conditions, it is reasonable to conclude that the subunits of the enzyme as isolated by this procedure are single polypeptide chains and presumably identical.

Altogether, then, the experiments with SDS show that ALA-dehydratase is composed of single-chain monomer whose molecular weight is about 40,000.

It is worth remarking at this stage that the experiment on electrophoresis of radioactively labeled enzyme suggests a general method for determining subunits of impure proteins which can be specifically labeled in a similar manner. Methods using gel filtration (Andrews, 1964) have been used extensively for impure, native proteins which can be specifically assayed (*e.g.*, for enzymic activity). However, this method is generally impossible for subunits because the treatment which causes dissociation is usually irreversible and inactivates the protein. Our method evaded this problem by making use of specific binding by the enzyme before it was inactivated.

The gel filtration experiments confirm the result of Nandi and Shemin (1968a) that this enzyme is more associated in the presence of potassium ions than in their absence. The column (Figure 1A) run in the presence of potassium has not resolved the dioligomers and trioligomers they found very clearly, but it does show material having a molecular weight around 500,000 and which is absent in Tris buffer alone. There is also an indication of some material larger than that, but nevertheless not coming through at the void volume. The calibration of the column (Figure 5) is not very accurate partly because it is difficult to find commercial markers whose molecular weight is in this high range and accurately known. However, quantitative results should certainly be as accurate as the sucrose gradient data (Nandi and Shemin, 1968a) and suggest a minimum molecular weight of about 220,000.

Both columns show some material of much higher molecular weight than a trioligomer of 240,000. There was no sign of this in the earlier work either in the presence or in the absence of potassium ions. The fact that active material comes through at the void volume of Bio-Gel A 5m suggests that it must be made of aggregates of molecular weight several million or more.

Before considering possible interpretations of the data from the experiments with the ultracentrifuge, it is important to remember that the protein used in these experiments was all purified by gel filtration as described above. Since proteins elute from gel filtration columns at a rate roughly proportional to their molecular weight and calibration with known markers showed the molecular weight of the eluted enzyme to be in the range between 200,000 and one million, anything outside this range found when the eluted enzyme was ultracentrifuged must have come from association or dissociation; it cannot be impurity for that would have been removed in the gel filtration. It is possible, though unlikely, that some very low molecular weight material might have arisen from digestion by proteolytic enzymes not removed during the purification.

The sedimentation velocity experiments confirm that the protein is in different states in the presence and absence of potassium ions. They also showed that there was some rapidly sedimenting and therefore high molecular weight material.

The sedimentation equilibrium studies were carried out in general in the absence of potassium ions so that the dioligomers and trioligomers found in sucrose gradients and suggested by the gel filtration experiments should have been absent. Nevertheless, the experiments at 0.3–1.0-mg/ml protein concentration showed the presence of high molecular weight aggregates, as well as some proteins whose apparent average molecular weights were somewhat lower than 240,000. At lower concentrations, the amount of aggregates becomes smaller or negligible and smaller species begin to predominate. Thus, at 0.12 and 0.04 mg per ml, the lower part of the cell contains protein whose apparent molecular weight is about 130,000 whereas the upper part of the cell contains material whose average molecular weight is considerably lower. Assuming that the previously found 250,000 species is the oligomer and that the 120,000 species (trimer) found by Nandi and Shemin (1968a), on treatment with 1 M urea, exists, these experiments appear to show that an equilibrium exists among the monomeric units, oligomer, and aggregates and that it is dependent on the protein concentration. Nandi and Shemin (1968a) further showed that association of the trimer to oligomer was aided by the presence of 0.05 M ALA. It would have been interesting to have done an experiment with the analytical ultracentrifuge in the presence of ALA, perhaps making it possible to get a nearly homogeneous solution and thence an accurate value for the molecular weight. However, in 0.05 M ALA, we found that the extinction of the solution was so great that we could not use the scanner at any wavelength where that of the protein was significant.

The electrophoresis, chromatography, ultracentrifugation, and the previous data obtained from sedimentation studies in a sucrose gradient (Nandi and Shemin, 1968a) suggest the following set of concentration-dependent states of aggregation (D = dehydratase monomer). Potassium ions cause an apparent stabilization of the intermediates between D_6 and $(D_6)_n$, where n is greater than 3 and probably variable. The

$$D \overset{?}{\rightleftharpoons} (D_3) \rightleftharpoons D_6 \rightleftharpoons (D_6)_2 \rightleftharpoons \cdots\cdots \rightleftharpoons (D_6)_n$$

inclusion of a possible trimer is exclusively conjectured from the previous findings of Nandi and Shemin (1968a).

It is very difficult to determine which of these species is enzymically active since they are all in equilibrium with other forms under the assay conditions. D_6 is presumably active, for this species is predominantly found when the enzyme is activated by Mg^{2+} ions (Nandi *et al.*, 1968). It is not clear whether the associated species $(D_6)_2$.....$(D_6)_n$ are active as such but due to dissociation of the oligomer D_6. In regard to the possible D_3 a decision concerning its enzymic activity cannot be made for it is converted to the D_6 oligomer in the presence of the substrate or by K^+ ions. The purified enzyme loses activity on storage even in the presence of ammonium sulfate (30% of saturation); this might be due to formation of inactive aggregates or subunits.

If the active oligomer (D_6) is a hexamer as these experiments and previous experiments suggest, its quaternary structure may have cyclic or dihedral symmetry (Klotz *et al.*, 1970). The dihedral symmetry is somewhat favored for the enzyme gives rise to a species of about 120,000 in the presence of 1 M urea (Nandi and Shemin, 1968a). This dihedral symmetry is similar to the quaternary structure proposed for the hexamer, glutamic dehydrogenase, by Eisenberg (1970) and Reisler and Eisenberg (1970).

It is worth noting that the ALA dehydratase of bovine liver also has a molecular weight of 235,000 and is composed of six subunits having a molecular weight as determined by gel electrophoresis of 42,000, although its enzymic properties in regard to cation activation, EDTA inhibition, and association properties are vastly different than those of the enzyme of *R. spheroides* (B. S. Schearer and D. Shemin, unpublished data).

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References

Andrews, P. (1964), *Biochem. J. 91*, 222.
Ayad, S. R., Bonsall, R. W., and Hunt, S. (1968), *Anal. Biochem. 22*, 533.
Burnham, B. F., and Lascelles, J. (1963), *Biochem. J., 87*, 462.
Chervenka, C. H. (1969), A Manual of Methods for the Analytical Ultracentrifuge, Palo Alto, Calif., Beckman Instruments Inc.
Dunker, A. K., and Rueckert, R. R. (1969), *J. Biol. Chem. 244* 5074.
Eisenberg, H. (1970), *in* Pyridine Nucleotide Dependent Dehydrogenases, Sund, H., Ed., New York, N. Y., Academic Press, p 293.
Fleischman, J. B., Porter, R. R., and Press, E. M. (1963), *Biochem. J. 88*, 220.
Gibson, K. D., Neuberger, A., and Scott, J. J. (1955), *Biochem. J. 61*, 618.
Granick, S., and Mauzerall, D. (1958), *J. Biol. Chem. 232*, 1119.
Klotz, I. M., Langerman, N. R., and Darnall, D. W. (1970), *Annu. Rev. Biochem. 39*, 25.
Lascelles, J. (1956), *Biochem. J. 62*, 78.
Levin, O. (1962), *Methods Enzymol. 5*, 27.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Martin R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.
Mauzerall, D., and Granick, S. (1956), *J. Biol. Chem. 219*, 435.
Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Nandi, D. L., Baker-Cohen, K. F., and Shemin, D. (1968), *J. Biol. Chem. 243*, 1224.
Nandi, D. L., and Shemin, D. (1968a), *J. Biol. Chem. 243*, 1231.
Nandi, D. L., and Shemin, D. (1968b), *J. Biol. Chem. 243*, 1236.
Reisler, E., and Eisenberg, H. (1970), *Biopolymers, 9*, 877.
Schmid, R., and Shemin, D. (1955), *J. Amer. Chem. Soc. 77*, 506.
Shapiro, A. L., Vinuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Tishler, P. V., and Epstein, C. J. (1968), *Anal. Biochem. 22*, 89.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.