Purification and General Properties of δ -Aminolaevulate Dehydratase from Cow Liver

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1. δ -Aminolaevulate dehydratase, the enzyme catalysing the condensation of δ -aminolaevulinic acid to porphobilinogen, has been prepared from cow liver and its properties have been studied. The enzyme has been purified 310-fold. 2. The purified preparation behaves as a single protein under gel filtration on Sephadex and Bio-Gel columns; it migrates as a single band in disk and starch-gel electrophoresis at different pH values and it sediments as a single symmetrical peak in the ultracentrifuge. 3. The pH optimum for the pure enzyme was 6.8, the K_m at pH 6.8 and 38° was $1.5 \times 10^{-4} M$, the isoelectric point was about pH 4.9 and the molecular weight was $140\,000 \pm 14\,000$ by the gel-filtration method. Maximal enzyme activity was observed at 65°. 4. The presence of thiol groups in the enzyme system, essential for its activity, was indicated and the total number of thiol groups was determined. 5. After the first steps of purification the enzyme required cysteine or reduced glutathione for activity.

The condensation of two molecules of δ -aminolaevulinic acid to form porphobilinogen by aminolaevulate dehydratase (δ -aminolaevulate hydrolyase, EC 4.2.1.24) has been firmly established (Shemin & Russell, 1953; Neuberger & Scott, 1953).

This enzyme is widely distributed in animals, plants and micro-organisms, and there have been several reports of its partial purification from ox liver (Gibson, Neuberger & Scott, 1955; Iodice, Richert & Schulman, 1958), avian erythrocytes (Granick, 1954; Schmid & Shemin, 1955; Granick & Mauzerall, 1958), pigeon and rat liver (Schulman, 1955), rabbit reticulocytes (Granick & Mauzerall, 1958), *Rhodopseudomonas spheroides* (Gajdos & Gajdos-Török, 1955, 1956; Lascelles, 1959, 1960; Burnham & Lascelles, 1963), *Corynebacterium diphtheriae* and *Bacterium cadaveris* (Gibson *et al.* 1955), propionic acid bacteria (Walerych, 1963) and soya-bean callus (H. A. Tigier, G. Locascio & A. M. del C. Batlle, unpublished work).

The enzyme is readily soluble in water. In an extensive study of its distribution in the subcellular particles of rat-liver homogenates, Gibson *et al.* (1955) showed that it was present almost exclusively in the soluble fraction of the cells.

Electrophoretically pure δ -aminolaevulate dehydratase (purified 20-fold) required GSH or cysteine for maximum activity, whereas crude extracts did not. However, the activity of crude extracts was diminished by thiol inhibitors; it behaved like a typical thiol enzyme. It was also

inhibited by EDTA and tris buffer although the need for metal ions could not be demonstrated directly.

Whether only one or both condensations involved in porphobilinogen biosynthesis are enzymically catalysed is uncertain. There is little doubt, however, that a single protein is sufficient to carry out the complete reaction (Gibson *et al.* 1955; Granick & Mauzerall, 1958).

The present paper describes further purification of the enzyme from cow liver as well as some of its properties.

MATERIALS AND METHODS

δ -Aminolaevulinic acid. This was prepared as described by Mitta, Ferramola, Sancovich & Grinstein (1965). δ -Aminolaevulinic acid (lot 64B-1900) from Sigma Chemical Co. (St Louis, Mo., U.S.A.) was also used.

Other compounds. Starch gel for electrophoresis was obtained from Connaught Medical Laboratories, Toronto, Canada. Different types of Sephadex were obtained from Pharmacia (G.B.) Ltd. (London, W. 13). Bio-Gel P was a generous gift from Bio-Rad Laboratories (Richmond, Calif., U.S.A.). Calcium phosphate gel was prepared by the method of Keilin & Hartree (1951). Colloidal Fe_2O_3 or 'dialysed iron' [approx. 5% (w/v) Fe_2O_3] was purchased from British Drug Houses Ltd. (Poole, Dorset). All other reagents were analytical-grade products obtained from several commercial sources. All solutions were made up in ion-free glass-distilled water.

Source material of enzyme. Fresh cow liver was supplied by 'Química Estrella' Laboratories, SACII, Buenos Aires,

Argentina; it was stored and transported frozen in solid CO_2 , and the frozen material was cut into thin slices and allowed to thaw before being homogenized.

Ultracentrifugal analyses. These were carried out at 59780 rev./min. (260000g) in a Spinco model E analytical centrifuge equipped with a phase-plate optical system. Photographs were taken at 8 min. intervals. In each analysis the concentration of the enzyme solution was about 6 mg./ml.

Spectrophotometric measurements were carried out in a Beckman model DU spectrophotometer.

Porphobilinogen estimation. In previous experiments porphobilinogen was estimated by the method of Mauzerall & Granick (1956), but it was found later that the assay method used by Moore & Labbe (1964) is simpler and it was therefore adopted. Results obtained with both procedures were the same. The porphobilinogen present was calculated by applying the extinction coefficient $E_{\text{max}}^{1\text{cm}}$ 25400, determined by using crystalline porphobilinogen obtained from the urine of acute porphyria cases, as described by Cookson & Rimington (1954).

Estimation of enzyme activity and incubation conditions. To estimate enzyme activity the procedure described by Gibson *et al.* (1955) was followed with slight modifications. The standard conditions were as follows: Incubations were carried out *in vacuo* in Thunberg tubes at 38°. The enzyme preparation, together with 0.067 M-phosphate buffer, pH 6.8, and 0.1 ml. of 0.025 M-cysteine, in a final fluid volume of 2.5 ml., was preincubated for 1 hr. to activate the enzyme. At the end of this time, 0.1 ml. of 0.05 M- δ -aminolaevulinic acid hydrochloride, pH 6.8, was added from the side arm of the Thunberg tube, and the incubation was continued for another hour, with mechanical shaking at about 80–90 oscillations/min. After incubation, the contents of the tubes were poured into 1 ml. of colloidal Fe_2O_3 , then 0.1 ml. of saturated aq. CuSO_4 was added, and the tube stoppered and shaken vigorously for about 15 sec. The precipitate was removed by centrifuging and the amount of porphobilinogen was determined in the supernatant as described above.

As was found by Gibson *et al.* (1955) with purified preparations, addition of colloidal Fe_2O_3 was unnecessary after the first step of purification and the reaction mixture was poured directly into a centrifuge tube containing 0.1 ml. of saturated

CuSO_4 and treated as before. Moreover, we have found that, when colloidal Fe_2O_3 is added to the reaction mixture, the amount of porphobilinogen recovered is 20% lower than that obtained when only CuSO_4 is added as protein precipitant, i.e. there is some loss of porphobilinogen when colloidal Fe_2O_3 is used as co-precipitant.

Enzyme unit. A dehydratase unit was defined as the amount of enzyme that catalyses the formation of 1 μ mole of porphobilinogen/min. under the standard conditions described above, the specific activity being the units of enzyme/mg. of protein.

Chromatography on Sephadex columns. Sephadex columns were prepared as described by Batlle, Benson & Rimington (1965).

Estimation of molecular weight. Sephadex-gel filtration was carried out according to the method of Andrews (1964, 1965) and Bio-Gel-gel filtration according to the method of Batlle (1966). Sephadex G-75, G-100 and G-200 and Bio-Gel P-200 for molecular-weight estimations were calibrated with sucrose, cytochrome c, ovalbumin, serum albumin, catalase and urease.

Fractions obtained from Sephadex or Bio-Gel columns were concentrated by pressure filtration through Visking tubing as described by Wildy, Nizet & Benson (1961).

The protein content was determined by the Folin-Ciocalteu micro-method as described by Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard.

Polyacrylamide-gel and starch-gel electrophoresis. The technique employed was based on the methods described by Bodman (1960). A preliminary survey was made to find the most suitable conditions for running the different samples.

Assay for thiol groups. The disulphide 5,5'-dithiobis-(2-nitrobenzoic acid) was used as suggested by Ellman (1958, 1959).

RESULTS

Extraction and purification of the enzyme (Table 1)

All operations were carried out in the cold room at 4°, unless otherwise stated.

(1) *Preparation of the homogenate.* Homogenate

Table 1. *Purification of δ -aminolaevulate dehydratase from cow liver*

Incubation conditions were as described in the Materials and Methods section.

Fraction	Total protein (mg.)	$10^3 \times$ Total units	Yield (%)	$10^3 \times$ Specific activity	Purification
(1) Cow-liver homogenate	23 800	443.5	100	0.300	1
(2) 11 750g supernatant	10 650	418.9	94	0.515	2.1
(3) Heat treatment	3089	299.6	68	1.633	5.25
(4) 30–55%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction	486	137.8	29	4.733	15.75
(5) $\text{Ca}_3(\text{PO}_4)_2$ -gel treatment	104.6	114.4	24.5	18.20	60.6
(6) Second 55%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction	61.1	100.1	21	27.31	90.9
(7) Combined fractions from first Sephadex G-100 column	17.7	87.0	18.2	81.96	272.7
(8) Combined fractions from second Sephadex G-100 column	14.2	79.6	16.7	93.41	310.9

(10%, w/v) of liver (20–50g.) was prepared in a Potter–Elvehjem-type homogenizer (glass tube and Teflon pestle); the homogenizing medium was 0.25M-sucrose.

(2) *Centrifugation at 11750g.* The homogenate thus obtained was centrifuged at 11750g for 10min. The sediment had very little activity and was discarded. More than 90% of the activity was in the supernatant.

(3) *Heat treatment.* Portions (about 300ml.) of the supernatant were heated with constant stirring until the temperature rose to 50°, the heat source was then removed and the system was stirred for 5min. During this time the temperature reached 56–58°, after which the supernatant was immediately cooled to 34–36° in an ice bath. The system thus treated was centrifuged at 1060g for 10min. and the sedimented protein precipitate was discarded.

(4) *First ammonium sulphate fractionation.* To the supernatant obtained from the heat treatment neutralized saturated ammonium sulphate was added to 30% saturation. After 1hr. the material was centrifuged at 11750g for 10min.; the sediment had negligible activity and was discarded. The supernatant solution was then brought to 55% saturation with ammonium sulphate and after 1hr. was centrifuged at 11750g for 10min.

(5) *Calcium phosphate-gel treatment.* The fraction precipitated between 30% and 55% ammonium sulphate saturation was dissolved in 0.134M-phosphate buffer, pH 6.8 (protein content being about 16mg./ml.). To this enzyme solution was added an equal volume of calcium phosphate gel (containing 36mg. dry wt. of gel/ml.). After being stirred for 10min. the mixture was centrifuged at 1060g for 10min. The enzyme was adsorbed on the gel, from which it was eluted successively with three portions (1–2ml.) of 0.134M-phosphate buffer, pH 6.8, 5% saturated with respect to ammonium sulphate.

(6) *Second ammonium sulphate fractionation.* The three eluates were combined and neutralized saturated ammonium sulphate was added to 55% saturation. After 1hr. the material was centrifuged at 11750g for 10min.

(7) *Gel filtration on Sephadex G-100.* The second 55%-saturated ammonium sulphate fraction was suspended in a small volume of 0.067M-phosphate buffer, pH 6.8, containing potassium chloride (0.1M) (approx. 8–10mg. of protein/ml.), and applied to a Sephadex G-100 column (1.8cm. x 30cm.). Protein was eluted with 0.067M-phosphate buffer, pH 6.8, containing potassium chloride (0.1M), at a flow rate of 16–18ml./hr. Fractions of volume 3–4ml. were collected. The protein content and specific activity were determined in each fraction eluted from the column. Those fractions containing activity were pooled. Fig. 1 shows an

Fig. 1. Chromatographic behaviour of the dehydratase preparation obtained from stage 6 on Sephadex G-100. The enzyme preparation (2ml., 14mg. of protein) was applied to Sephadex G-100 column (1.8cm. x 30cm.) in 0.067M-phosphate buffer, pH 6.8, containing KCl (0.1M), and eluted with the same buffer at a flow rate of 0.3ml./min. in the cold room. ○, Specific activity, determined under standard conditions and represented as μmoles of porphobilinogen formed/hr./mg.; ●, protein (mg./ml.).

Fig. 2. Chromatographic behaviour of the dehydratase preparation obtained from stage 7 on Sephadex G-100. Conditions are the same as those given in Fig. 1.

elution diagram. Only one main protein peak was obtained, and the dehydratase activity was associated with this.

(8) *Second gel filtration on Sephadex G-100.* The pooled fractions containing the dehydratase activity were concentrated by pressure filtration to give a protein content of about 10mg./ml. and rechromatographed on Sephadex G-100, yielding a

main band with a specific activity 310.9 times that of the crude preparation. Fig. 2 depicts an elution diagram. The fractions containing the dehydratase activity were pooled, giving a pale-yellow clear solution with an absorption band, characteristic of proteins, at $273\text{m}\mu$ and a second maximum at $406\text{m}\mu$. The yellow coloration was always associated with the enzymic activity and was not lost on dialysis. Solutions were stored at -15° .

In some preparations the homogenate was centrifuged at $105\,000g$ for 30 min. at stage 2 of the purification. The specific activity of the supernatant was slightly higher than that obtained by centrifuging at $11\,750g$ for 10 min. and this procedure was finally adopted.

Attempts were made to purify the enzyme by using hydroxyapatite instead of calcium phosphate gel, but more reproducible results were obtained with the latter.

Gel filtration on Bio-Gel P-200 after stage 6 also purified the enzyme, but it yielded a fraction with a specific activity only 2.5 times that of the previous stage; as better results were obtained with Sephadex G-100, this material was the one adopted.

Preliminary trials indicated that some purification could be achieved by dialysis under a variety of conditions, but this method was nevertheless abandoned since it caused some inactivation of the enzyme (see the Discussion section).

Properties of δ -aminolaevulate dehydratase

Studies were made with different preparations of the enzyme, but the results recorded here correspond to those obtained with the dehydratase purified 270-fold, unless otherwise indicated.

Optimum activity was observed at pH 6.8 in 0.067M -phosphate buffer; the isoelectric point was about pH 4.9. Activity was measured over the temperature range 20 – 80° and the maximal rate was observed at 65° . Heating the system above 85° or boiling it completely destroyed its activity.

Fig. 3. Sedimentation pattern of δ -aminolaevulate dehydratase from cow liver. The rotor speed was $59\,780\text{ rev./min.}$ ($260\,000g$), the temperature 14.2° , the phase-plate angle 40° and voltage 92 v. The initial enzyme concentration was 7.0 mg./ml. in phosphate buffer. The photographs were taken at 8 min. intervals.

As was reported by Gibson *et al.* (1955), after the first steps in purification there is no activity at all unless the enzyme has been previously activated with a thiol compound such as cysteine or GSH. The time-course of the activation by cysteine and the effect of its concentration was studied, and it was found that it is complete in 1 hr. at a final concentration of 1 mM.

Under the standard conditions described, the amount of porphobilinogen formed increased in direct proportion with time up to 1.5 hr. The amount was also proportional to the concentration of enzyme over a wide range.

The Michaelis constant, K_m , estimated by plotting the reciprocal of the initial velocity against the reciprocal of the concentration of δ -aminolaevulic acid under the standard conditions, was $1.5 \times 10^{-4}\text{ M.}$

Purity of the enzyme. This was tested with highly purified enzyme preparations (after stage 8). The enzyme preparation behaved as a single protein under gel filtration on Sephadex and Bio-Gel columns.

The purified enzyme sedimented as a single symmetrical peak in the ultracentrifuge (Fig. 3); with partially purified preparations two protein peaks were obtained in the ultracentrifuge.

The protein migrated as a single band in disk and starch-gel electrophoresis; in this latter analysis the homogeneity of the enzyme was tested at different pH values. Here also two protein bands were obtained with partially purified preparations of the dehydratase. The electrophoretic migration rate of the highly purified preparation was compared with that of the enzyme derived from earlier stages of purification and with δ -aminolaevulate dehydratase obtained from other sources; there was practically no difference in the migration rate of the enzyme, whether isolated from cow liver, rat liver, rat Harderian gland or soya-bean callus.

Stability. Repeated freezing and thawing, prolonged storage at -15° or storage at 4° for 7–10 days partially inactivate the enzyme, which loses 50% of its activity when stored at room temperature for 24 hr. Ammonium sulphate (55% saturation) precipitates the enzyme in a very stable form at -15° .

Cofactors. The possibility of diffusible cofactors being present was investigated. Dialysis of the supernatant obtained after stage 2 of purification against glass-distilled water for 24 and 48 hr. at 4° caused a heavy flocculent precipitate to form. The mixture was centrifuged and activity was determined in both supernatant and precipitate, the latter suspended in 0.067 M -phosphate buffer, pH 6.8 (it was impossible to get complete dissolution of the precipitate). After dialysis for 24 hr. the activity was distributed almost equally between the supernatant and the precipitate; after dialysis for 48 hr.,

more than 90% of the total activity was found in the precipitate; moreover, specific activity was slightly increased, but the yield at this step was too low to warrant use of this long treatment as a purification procedure.

On the other hand, dialysis against phosphate buffer at various pH values (between 5.4 and 7.0) at several molarities (between 6.7 mM and 0.42 M) for different time-intervals (between 24 and 48 hr.) produced interesting results; in general after dialysis for 48 hr., as the pH and molarity of the buffer increased, the degree of protein precipitation and specific activity of the precipitate decreased; attempts to reactivate the enzyme to its original level of activity were unsuccessful. The diffuse was also tested, but it had no dehydratase activity, nor did it increase the activity of the precipitate or an active enzyme preparation. Partially purified enzyme obtained after stage 2 was heated at 100° for 5 min., the protein precipitate was removed by centrifugation and the supernatant tested for enzymic activity; it had no activity, but it slightly increased the activity of the partially purified preparation (about 20%).

Molecular weight. The weight-average molecular weight determined by the gel-filtration method at various enzyme concentrations was estimated to be $140\,000 \pm 14\,000$. Estimations of molecular weight at low concentrations by means of calibrated Sephadex G-200 columns provided evidence of some dissociation of the enzyme into sub-units of molecular weight $70\,000 \pm 7\,000$; complete dissociation was not observed; it therefore seems that dilution of the enzyme would induce partial dissociation into sub-units. The association-dissociation behaviour of proteins at extremely low concentrations under gel filtration is a phenomenon that has already been studied by Andrews (1964, 1965). Attempts to obtain further evidence of dissociation by varying experimental conditions have met with no success so far, and the present finding, though suggestive, needs additional investigation.

Effect of some reagents on the dehydratase activity. Various compounds have been examined for their effect on the dehydratase activity. When partially purified preparations that do not require thiol activation were used, results obtained were essentially the same as those reported by Gibson *et al.* (1955). With pure preparations, after activation for 1 hr. with cysteine, the reagent to be tested was added from the side arm. The results obtained are summarized in Table 2, which shows the final concentration of the additive in the reaction mixture. Of the thiol inhibitors studied (Table 2, group A), all produce marked inhibition of activity, confirming the presence of thiol groups in the enzyme. Cyanide up to 1 mM, thiourea up to 10 mM and sulphite up to 1 mM (Table 2, Group B) did not inhibit the dehydra-

tase activity; 10 mM-cyanide and 10 mM-sulphite (Table 2, group B) inhibited activity by about 20% and 30% respectively, and high concentrations of cysteine (0.1 M) produced almost complete inactivation of the dehydratase. Inhibition by sulphite suggested that disulphide groups were also involved, and this would be supported by the deactivating effect of cysteine at high concentrations. The reagents designated group B in Table 2 are known to react with the disulphide bonds of certain proteins and therefore on the basis of these findings it cannot be concluded that the enzyme requires disulphide groups for its activity, but perhaps these groups are necessary to maintain a specific protein structure, or perhaps a balance between thiol and disulphide groups is needed for optimum activity.

Thiol groups of the dehydratase. The number of total thiol groups was determined by the procedure of Ellman (1958, 1959) and 11 thiol groups/enzyme molecule were found; the molecular weight of the enzyme was considered to be 140 000 for all the calculations.

Enzymic synthesis of porphobilinogen. A mixture of 2.5 ml. of the purified dehydratase (3.75 mg. of protein, 0.475 μ mole of porphobilinogen/hr./ml.) in 175 ml. of 0.067 M-phosphate buffer, pH 6.8, 0.25 ml. of 0.025 M-cysteine and 0.5 ml. of 0.05 M- δ -aminolaevulinic acid was preincubated for 2 hr., then incubated for 10 hr. *in vacuo* at 38° in a 200 ml. Thunberg-like flask. After incubation the compo-

Table 2. *Effect of some compounds on the activity of δ -aminolaevulinate dehydratase*

	Additions	Activity (%)
	Control	100
Group A	<i>p</i> -Chloromercuribenzoate (1 mM)	27
	<i>N</i> -Ethylmaleimide (1 mM)	29
	Iodoacetamide (1 mM)	27
	5,5'-Dithiobis-(2-nitrobenzoic acid) (0.1 mM)	39
	Iodosobenzoate (1 mM)	28
Group B	KCN (10 mM)	81
	KCN (1 mM)	93
	SO ₃ ²⁻ (10 mM)	71
	SO ₃ ²⁻ (1 mM)	98
	Thiourea (10 mM)	104
	Cysteine (0.1 M)	10

nents were separated and identified by the procedure of A. M. del C. Batlle, H. A. Sancovich & M. Grinstein (unpublished work). The overall yield of porphobilinogen was greater than 60%.

DISCUSSION

From the behaviour of the dehydratase during purification, the effect of dialysis and its molecular weight, the enzyme seems to have the properties of a globulin, as stated by Gibson *et al.* (1955). Final purification of the dehydratase yielded a solution that behaved as a single protein under the accepted standard tests, suggesting the presence of only one protein component. There was no indication that more than one enzyme was involved in the conversion of δ -aminolaevulinic acid into porphobilinogen, although estimations of molecular weight at very low concentrations of protein by the gel-filtration method did suggest that there might be some dissociation of the enzyme into sub-units; several attempts to obtain further evidence on this point were unsuccessful. It must remain a possibility, as yet undecided, that the enzyme could dissociate into sub-units under a particular set of conditions.

Several other workers who have studied this enzyme have concluded that the reaction involves essential thiol groups. This has been confirmed for the purified enzyme, as thiol reagents such as iodoacetamide, *p*-chloromercuribenzoate, *N*-ethylmaleimide, 5,5'-dithiobis-(2-nitrobenzoic acid) and iodosobenzoate inhibited the enzyme. The activating effect of cysteine or GSH on the enzyme supports the view that thiol groups are necessary for activity; moreover, by using the disulphide 5,5'-dithiobis-(2-nitrobenzoic acid) the total number of thiol groups of the dehydratase was determined: 11 thiol groups/enzyme molecule were found.

As already stated, the presence of disulphide groups could be envisaged, since some reagents known to cleave dithiol bonds produced 20–30% inhibition of the dehydratase.

All attempts to detect the existence of a prosthetic group failed, nor was there any good evidence for the presence of a light-absorbing cofactor from the absorption spectrum of the purified enzyme. However, some experiments, not reported here, seem to indicate that the enzyme might contain a bound metal.

All efforts to elucidate the enzymic mechanism involved in this reaction have so far been fruitless, though one could speculate on the possibility of the

enzyme being a dimer, perhaps joined by disulphide bridges.

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