Purification, properties and synthesis of δ -aminolaevulinate dehydratase from *Neurospora crassa*

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δ -Aminolaevulinate dehydratase, the second and rate-limiting enzyme of the haem-biosynthetic pathway, was purified 300-fold from induced cultures of *Neurospora crassa*. The native enzyme has a mol.wt. of about 350 000, whereas the salt-treated enzyme after incubation at 37°C for 10 min has a mol.wt. of about 232 000. The mol.wt. of the subunit is about 38 000. Antibodies to the purified enzyme were raised in rabbits. By using radiolabelling and immunoprecipitation techniques it was shown that addition of iron and laevulinate to iron-deficient cultures brings about a significant increase in the synthesis of the enzyme, and protoporphyrin, the penultimate end product of the pathway, represses enzyme synthesis.

The second enzyme of the haem-biosynthetic pathway, δ -aminolaevulinate dehydratase (EC 4.2.1.24), which catalyses the conversion of δ -aminolaevulinate into porphobilinogen, has previously been shown to be the regulatory and rate-limiting enzyme of the pathway in *Neurospora crassa* (Muthukrishnan *et al.*, 1968, 1969). In bacterial and animal systems δ -aminolaevulinate synthase (EC 2.3.1.37), the first enzyme of the pathway, is regulatory and rate-limiting, and δ -aminolaevulinate dehydratase is present in considerable excess in these systems (Lascelles, 1964; Tschudy *et al.*, 1965).

In *N. crassa*, the activity of the enzyme in normal cultures is very low compared with the activities detected in animal and plant systems. In addition, the enzyme activity is almost undetectable in iron-deficient cultures, and the enzyme is strikingly induced by the addition of iron. This induction is blocked by protoporphyrin, the penultimate end product of the haem-biosynthetic pathway. On the basis of activity assays it has been suggested that iron induces the enzyme and protoporphyrin acts as the 'repressor' (Muthukrishnan *et al.*, 1969). To substantiate this point and to study the properties of the enzyme protein, δ -aminolaevulinate dehydratase was purified from induced cultures. Antibody was raised to the purified protein, and by using labelled amino acid precursors and immunoprecipitation techniques the rate of the enzyme synthesis has been followed under conditions of induction and repression.

Abbreviations used: SDS, sodium dodecyl sulphate; IgG, immunoglobulin G.

Materials and methods

Materials

^{14}C -labelled *Chlorella* protein hydrolysate (27 mCi/mg-atom of C) was purchased from Bhabha Atomic Research Centre, Bombay, India. Haemin, protoporphyrin IX dimethyl ester, coproporphyrin tetraethyl ester and other biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Growth conditions

Neurospora crassa Em 5297a (wild-type) was grown in 250 ml flasks containing 25 ml of basal medium at 30°C for 40 h, unless otherwise specified. Iron-deficient medium was prepared by treating the major salts with 8-hydroxyquinoline and glucose with Dowex-50 (H^+ form) resin. The detailed procedures have been described by Padmanaban & Sarma (1965).

Assay of δ -aminolaevulinate dehydratase

δ -Aminolaevulinate dehydratase was assayed in the 0–40%-satd.-(NH_4)₂SO₄ fraction of the mycelial buffer extract by the procedure of Muthukrishnan *et al.* (1972). Briefly, the assay mixture in a total volume of 2 ml contained: δ -aminolaevulinate hydrochloride, 2.5 μmol ; reduced glutathione, 7.5 μmol ; ZnSO₄·7H₂O, 0.2 μmol ; potassium phosphate buffer, pH 8.0, 100 μmol ; and protein, 5–7 mg. After incubation for 2 h at 37°C, the reaction was stopped by the addition of 1 ml of 12.5% (w/v) trichloroacetic acid containing 0.1 M-mercuric chloride. An equal volume of modified Ehrlich reagent was added

to the supernatant after centrifugation and the A_{550} was measured after 15 min (Granick & Mauzerall, 1958). The enzyme activity is expressed in nmol of porphobilinogen formed/2h per mg of protein. Protein was measured by the method of Lowry *et al.* (1951).

Purification of δ -aminolaevulinate dehydratase

The enzyme was induced in 40h-old iron-deficient cultures by the addition of laevulinate (160 μ g/ml) and ferric ammonium citrate (1 μ g of iron/ml). The cultures were shaken for 2h, when maximal induction of the enzyme occurred. Mycelia from 120 flasks were pooled in batches and processed for enzyme purification.

The mycelia (2g fresh wt. per batch) were ground in a pre-cooled pestle and mortar with glass powder and 20ml of 0.05M-potassium phosphate buffer, pH 8.0, containing 5mM-cysteine and 1mM-phenylmethanesulphonyl fluoride. Phenylmethanesulphonyl fluoride was routinely used in all buffers during the entire purification procedure to inhibit proteinase activity. The homogenates were centrifuged at 10000g for 10min. The supernatants were pooled and subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation, and the precipitate formed between 0 and 40% saturation was collected by centrifugation (10000g for 10min) and suspended in about 100ml of phosphate buffer (pH 7.0, 0.05M) containing 5mM-cysteine. Protamine sulphate (8ml of 1%, w/v, solution) was then added drop by drop with stirring and the precipitated nucleoproteins were removed by centrifugation at 10000g for 10min. The supernatant was subjected to a second $(\text{NH}_4)_2\text{SO}_4$ fractionation and the precipitate formed between 0 and 40% saturation was collected by centrifugation and suspended in 16ml of potassium phosphate buffer (pH 8.0, 0.05M) containing 5mM-cysteine. The preparation was subjected to a third $(\text{NH}_4)_2\text{SO}_4$ fractionation and the precipitate formed between 10 and 25% saturation was collected by centrifugation. The precipitate was dissolved in 1ml of potassium phosphate buffer (pH 8.0, 0.02M) containing 0.2mM-cysteine. This preparation was loaded on a 2ml column of *p*-aminophenylmercuric acetate-Sepharose equilibrated with the same buffer. *p*-Aminophenylmercuric acetate-Sepharose was prepared basically as described by Sluyterman and Wijdenes (1974). CNBr-activated Sepharose 4B (50ml packed volume) was suspended in 100ml of dimethyl sulphoxide at 0°C. Phenylmercuric acetate (750mg) dissolved in 12.5ml of dimethyl sulphoxide was then added. No buffer was added at this stage, since salts tended to precipitate the mercurial. The suspension was stirred gently for 20h at 0°C. The suspension was then warmed to 35°C for 20min and filtered. The Sepharose was resuspended in 50ml of 20% (v/v) dimethyl sulphoxide at 35°C for

15 min and filtered. This treatment was repeated four times. The Sepharose was then packed into a column and washed with about 500ml of 20% dimethyl sulphoxide for 4–5h at room temperature. These washings served to remove the free mercurial. The Sepharose was next suspended in 50ml of 0.1M-ethylenediamine adjusted to pH 8.0 with dilute acetic acid and gently stirred overnight at room temperature. The Sepharose was then washed and packed into a column (3 cm $\times$ 1 cm). It was then treated with 5,5'-dithiobis-(2-nitrobenzoic acid) to eliminate all residual reactive groups of the activated Sepharose as well as to determine the capacity of the column. The coupling obtained was about 4 μ mol of *p*-aminophenylmercuric acetate/ml of Sepharose. After the enzyme was loaded, the column was kept closed for 1h to facilitate binding of the enzyme, and then extensively washed with the loading buffer. About 0.7 ml of 50mM-dithiothreitol was then added and the column was kept closed for a further 1h to facilitate desorption of the enzyme. The enzyme was finally eluted with 10ml of 50mM-dithiothreitol. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to give 30% saturation, and the precipitate obtained was collected by centrifugation at 10000g for 15 min. The precipitate was dissolved in potassium phosphate buffer (pH 8.0, 0.02M) containing 0.2mM-cysteine, and the thiol-specific affinity chromatography was repeated a second time and the enzyme eluate concentrated by $(\text{NH}_4)_2\text{SO}_4$ (30%-satn.) fractionation.

The enzyme pellet was dissolved in about 0.5 ml of 0.05M-Tris/HCl buffer, pH 7.0, and further purified by preparative gel electrophoresis. Polyacrylamide gels (4%) were run under non-denaturing conditions by the procedure of Laemmli (1970). After electrophoresis, one of the gels was stained for δ -aminolaevulinate dehydratase activity. For this purpose, the gel was incubated with 10ml of the δ -aminolaevulinate dehydratase assay mixture for 6h and the gel was quickly rinsed twice with water. The gel was then placed in 10ml of Ehrlich reagent and a pink band was obtained about 0.5 cm from the top of the gel. A slice corresponding to the position of the pink band obtained after activity staining was cut out from an identical gel, placed on another tube gel and electrophoresis was carried out as before.

Methods of affinity chromatography

CNBr was prepared by the method of Hartman & Dreger (1950) and the coupling of amino group-containing ligands to Sepharose was carried out by the procedure of March *et al.* (1974).

Production of antibodies

Antibodies were raised in rabbits by giving a primary injection of 400 μ g of purified δ -aminolaevulinate dehydratase in complete Freund's adjuvant intradermally at multiple sites on the back.

After about 3 weeks, two booster injections were given at 15-day intervals. The rabbits were bled 1 week after the third injection. Subsequently the animals were injected with 200 μ g of the protein every 4 weeks and bled 1 week after every injection. The antisera were pooled and the IgG fraction was prepared by using $(\text{NH}_4)_2\text{SO}_4$ fractionation and DEAE-cellulose chromatography as described by Campbell *et al.* (1970).

Characterization of the antibody

The specificity of the interaction of the antibody with δ -aminolaevulinate dehydratase in purified preparations and crude extracts was first checked by the Ouchterlony double-diffusion technique (Thomas *et al.*, 1976). The antibody gave a precipitin line with the purified enzyme, which fused with that obtained with the crude extract from iron-induced cultures. There was no reaction with solubilized *Neurospora* mitochondrial proteins, cytochrome *c* and bovine serum albumin. Then the optimal concentration of the antibody required to precipitate quantitatively a given amount of the antigen was determined by quantitative immunoprecipitation analysis. For this purpose, the $(\text{NH}_4)_2\text{SO}_4$ fraction obtained after the protamine sulphate precipitation step [II $(\text{NH}_4)_2\text{SO}_4$ fraction; Table 1] was dialysed against 10 mM-potassium phosphate buffer, pH 7.6, containing 15 mM-NaCl and 1 mM-phenylmethanesulphonyl fluoride, and used as the antigen. Fixed concentrations of the antibody were added to various concentrations of the antigen. Separate antibody and antigen controls were included. The final volumes were made up to 0.5 ml to give final concentrations of 10 mM-potassium phosphate, 15 mM-NaCl, 1% Triton X-100 and 1 mM-phenylmethanesulphonyl fluoride (buffer A). The tubes were incubated at 4°C for 4 h. The immunoprecipitates were collected by centrifugation at 10000 *g* for 15 min and the supernatant was used for δ -aminolaevulinate dehydratase assay. The precipitate was washed three times with buffer A and finally with buffer A modified by omission of Triton X-100. The precipitate was dissolved in 0.01 M-NaOH and used for protein determination by the method of Lowry *et al.* (1951).

Measurement of the rate of synthesis of δ -aminolaevulinate dehydratase *in vivo*

To measure the rate of synthesis of δ -aminolaevulinate dehydratase *in vivo*, control (iron-deficient) and induced mycelia (250 mg fresh wt.) were incubated for 3 min with 5 μ Ci of ^{14}C -labelled *Chlorella* protein hydrolysate per 25 ml culture. For enzyme induction the iron-deficient cultures were treated simultaneously with ferric ammonium citrate and laevulinate, and 5 μ Ci of ^{14}C -labelled *Chlorella* protein hydrolysate was added 10 min after the

addition of the inducers; 3 min later the mycelia were thoroughly washed with cold water and identically treated mycelia were pooled. Protoporphyrin was added to another batch of mycelia 5 min before the addition of the inducers, and mycelia were then incubated in ^{14}C -labelled *Chlorella* protein hydrolysate in the above manner for 3 min. As a control, iron-deficient mycelia were incubated in ^{14}C -labelled *Chlorella* protein hydrolysate medium lacking added ferric ammonium citrate for 3 min and processed for immunoprecipitation.

Immunoprecipitation of radioactive samples

The antigen was prepared from the labelled mycelia essentially as described under 'Characterization of the antibody'.

For purposes of immunoprecipitation, 500 μ g of the labelled partially purified enzyme preparation and 3.5 mg of anti- $(\delta$ -aminolaevulinate dehydratase) IgG fraction were mixed. In order to check for non-specific precipitation, bovine serum albumin and anti-(bovine serum albumin) IgG fraction were added in place of anti- $(\delta$ -aminolaevulinate dehydratase) antibody. The mixtures were incubated overnight at 4°C. They were then thoroughly washed as described under 'Characterization of the antibody'. The immunoprecipitates were dissociated by suspension in 0.05 ml of 0.05 M-Tris/HCl buffer, pH 6.8, containing 2% SDS and 5% mercaptoethanol and heating the samples in a boiling-water bath for 2 min. The samples were then loaded on SDS/polyacrylamide (6%) gel for electrophoresis.

In order to measure the total soluble protein radioactivity after labelling *in vivo*, portions of the partially purified preparation used for immunoprecipitation were directly transferred to Whatman filter-paper discs and air dried. These discs were washed once with 5% trichloroacetic acid at 90°C for 15 min, twice with cold 5% trichloroacetic acid, once with ethanol/diethyl ether (3:1, v/v) and finally once with ether. For measurement of radioactivity in the amino acid pool, portions of the crude extract were precipitated with trichloroacetic acid and the supernatant after neutralization to pH 6.0 was passed through a Dowex (H^+ form) column (8 cm $\times$ 1 cm). The total amino acid fraction was eluted from the column with 2 M- NH_3 . The eluate was evaporated to dryness *in vacuo*, dissolved in water and portions were used for measurement of radioactivity and amino acid content by using ninhydrin.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis with and without SDS was carried out by the procedure of Laemmli (1970). δ -Aminolaevulinate dehydratase was detected on the gel by incubating the gel with the enzyme assay mixture and then staining the gel

with Ehrlich reagent. Protein staining on the gel was carried out with Coomassie Blue.

Solubilization of haemin and porphyrins

Crystalline haemin was dissolved in 0.1 ml of 0.1 M-KOH and then made up to volume with 0.1 M-phosphate buffer, pH 8.0. Protoporphyrin and coproporphyrin esters were hydrolysed with 5 M-HCl for 40 h at room temperature (25°C) in the dark and the acid was removed *in vacuo*. Protoporphyrin was dissolved in alkali as described for haemin. Coproporphyrin was reduced with sodium amalgam as described by Poulson & Polglase (1974). The coproporphyrinogen solution was finally adjusted to pH 8.0 with phosphoric acid.

Radioactivity measurements

Polyacrylamide gels meant for radioactivity determination were cut into 2 mm slices with a mechanical gel slicer. The gel slices were digested with 0.5 ml of 30% (w/v) H₂O₂ at 55°C for 15 h.

Samples on filter discs were suspended in a toluene/2,5-diphenyloxazole 'cocktail' [0.5% (w/v) 2,5-diphenyloxazole in toluene] for assay of radioactivity. For aqueous samples toluene/Triton X-100/2,5-diphenyloxazole 'cocktail' (2 vol. of toluene, 1 vol. of Triton X-100 and 0.5% 2,5-diphenyloxazole) was used. Radioactivity was assayed in a Beckman LS-100 liquid-scintillation counter. The counting efficiency for ¹⁴C was about 35%.

Results and discussion

The purification of δ -aminolaevulinate dehydratase is summarized in Table 1. The enzyme was

purified 300-fold from induced cultures of *N. crassa*. Conventional methods such as ion-exchange chromatography and gel-filtration techniques could not be used for the purification of the enzyme, since the enzyme lost 90% of its activity in these procedures. Zinc-chelate affinity chromatography with bisoxiran-Sepharose (Porath *et al.*, 1975) and substrate affinity chromatography with δ -aminolaevulinate-Sepharose were also unsuccessful. Other problems faced with the purification and gel analysis of δ -aminolaevulinate dehydratase from *N. crassa* have been the associated proteolytic activity and poor staining of the protein in gels. The interference by the high proteolytic activity of *N. crassa* extracts, leading to the partial cleavage of the multienzyme complexes from this organism, has been realized for some years (Gaertner & Cole, 1973; Lampkin *et al.*, 1976).

The purified enzyme obtained in the present study shows a single band in polyacrylamide gels run under non-denaturing conditions (Fig. 1a). The enzyme on dissociation shows a doublet in SDS/polyacrylamide gels, with a mol.wt. around 38 000 (Fig. 1b). Two very faint bands (not visible in Fig. 1) in the upper part of the gel were also seen. It was thought that this doublet could have arisen because of partial degradation of the subunit by proteolysis. The subunit molecular weight obtained is similar to that recorded for δ -aminolaevulinate dehydratase from other sources (Nandi *et al.*, 1968; Doyle, 1971; Wu *et al.*, 1974).

Since the mobility of the enzyme is low on 4%-acrylamide gels run under non-denaturing conditions, it is suspected that the enzyme might exist as a complex with other proteins. The enzyme was

Table 1. *Purification of δ -aminolaevulinate dehydratase from Neurospora crassa*

The experimental details are given in the text. The enzyme activity could not be assayed in the crude extract owing to the presence of an inhibitor (Muthukrishnan *et al.*, 1972).

Preparation	Total protein (mg) (A)	δ -Aminolaevulinate dehydratase specific activity (nmol of porphobilinogen/ 2 h per mg of protein) (B)	Recovery of enzyme activity (A $\times$ B)
I (NH ₄) ₂ SO ₄ pellet fraction (0–40% satn.)	990	2.10	2079
II (NH ₄) ₂ SO ₄ pellet fraction after protamine sulphate treatment	279.5	5.98	1671
III (NH ₄) ₂ SO ₄ pellet fraction (10–35% satn.)	100	15.20	1520
First thiol affinity chromatography	9	146.8	1321
Second thiol affinity chromatography	1.1	628.0	691

treated separately with 0.5% Triton X-100, 0.2% deoxycholate, 1 M-urea, 0.1% SDS or 50 mM-NaCl to check whether any of these treatments increased the mobility of the enzyme without decreasing the activity. After the enzyme preparation containing 50 mM-NaCl was incubated at 37°C for 10 min, the mobility of the enzyme increased slightly in 4% gels, as detected by staining for enzyme activity. The difference could not be picked up clearly because of diffusion of porphobilinogen in the gel. This treatment also led to partial inactivation of the enzyme. Higher concentrations of NaCl completely inactivated the enzyme. Treatment with detergents did not induce any change in the mobility of the enzyme.

Fig. 1. Polyacrylamide-gel electrophoresis of purified δ -aminolaevulinate dehydratase from *N. crassa*

The preparation obtained after preparative gel electrophoresis was re-run (a) under non-denaturing conditions on 4%-acrylamide gels and (b) after dissociation with SDS on 6%-acrylamide gels; (c) depicts the mobilities of standard proteins: (1) bovine serum albumin, (2) ovalbumin and (3) cytochrome c, run under conditions described in (b).

In further investigations of this phenomenon, the elution behaviour of the native and salt-treated enzyme was compared on Sephadex G-200 columns. As mentioned above, the enzyme loses 90% of the activity when loaded on these columns. The activity could not be restored by adding metal ions and other cofactors or by concentrating the enzyme by using $(\text{NH}_4)_2\text{SO}_4$ to precipitate the enzyme. By using the residual activity to determine the elution profile, the molecular weights obtained for the native and salt-treated enzyme have been determined. Fig. 2 shows that the native enzyme has a mol.wt. of about 350 000, whereas the enzyme incubated with 50 mM-NaCl at 37°C for 10 min has a mol.wt. around 232 000. It is possible that the two very faint bands detected in SDS/polyacrylamide gels in addition to the enzyme subunits could have arisen from other proteins complexed with δ -aminolaevulinate

Fig. 2. Molecular-weight determination of native and salt-treated δ -aminolaevulinate dehydratase by Sephadex G-200 chromatography

The purified native or salt-treated enzyme (300-fold) was loaded on a Sephadex G-200 column (30 cm $\times$ 1.8 cm) equilibrated with potassium phosphate buffer (pH 8.0, 0.05 M) containing 5 mM-cysteine, and 0.5 ml fractions were collected. For salt treatment, the enzyme was incubated with buffer containing 0.05 M-NaCl at 37°C for 10 min. The standards were run under identical conditions: A, ferritin; B, native enzyme; C, catalase and salt-treated enzyme; D, bovine immunoglobulin; E, cytochrome c.

dehydratase. The molecular weights of δ -aminolaevulinate dehydratase from other sources have been reported to range from 250000 to 280000 (Nandi *et al.*, 1968; Doyle, 1971; Wu *et al.*, 1974).

The other properties of the enzyme are essentially identical with those reported by Muthukrishnan *et al.* (1972) for a 6-fold purified enzyme. The enzyme requires thiol groups for activity. *p*-Hydroxymercuribenzoate (1 mM final concn.) inhibits the enzyme activity completely. The pH optimum for the enzyme reaction is 8–8.4 and the temperature optimum is 55°C. Metal-chelating agents such as *o*-phenanthroline (1 mM) and EDTA (1 mM) inhibit the enzyme activity. The K_m for δ -aminolaevulinate is 0.1 mM. Protoporphyrin (0.1 mM) and haemin (0.1 mM) do not inhibit the activity of the enzyme, but coproporphyrinogen III (0.1 mM) inhibits the activity completely.

Antibodies to δ -aminolaevulinate dehydratase were raised in rabbits and characterized. The 300-fold purified enzyme obtained after the second thiol affinity chromatography was used as the antigen. The IgG fraction shows cross-reaction in Ouchterlony plates (Thomas *et al.*, 1976) with the purified enzyme and with crude extracts obtained from induced cultures. Fig. 3 shows the quantitative immunoprecipitation analysis of anti-(δ -aminolaevulinate dehydratase) IgG fraction with the partially purified enzyme preparation. At the equivalence point, 250 μ g of the partially purified δ -aminolaevulinate dehydratase was neutralized by 1.7 mg of the antibody, as shown by the protein analysis of the immunoprecipitate and estimation of enzyme activity in the supernatant. Efforts to raise antibodies to the preparation after gel electrophoresis, by injecting the gel slice containing the enzyme protein, were not successful.

The δ -aminolaevulinate dehydratase antibody was used to measure enzyme synthesis under different

conditions in the iron-deficient cultures of *N. crassa*. The results presented in Table 2 indicate that addition of iron salts and laevulinate to iron-deficient cultures results in about 6-fold and 4-fold increase in enzyme activity in 30 min respectively. The effect of iron salts + laevulinate is additive, and the increase in enzyme activity is blocked by cycloheximide and protoporphyrin.

Fig. 3. Quantitative immunoprecipitation of δ -aminolaevulinate dehydratase with anti-dehydratase antibody

The enzyme preparation obtained after the protamine sulphate step was used as the antigen. Increasing amounts of the antigen were added to a fixed amount (1.7 mg) of the IgG fraction in a total volume of 0.5 ml. The samples were incubated at 4°C for 4 h. The immunoprecipitates were collected, washed and used for protein determination (O). The supernatant was used for the assay of enzyme activity (Δ). Each point had a separate antigen control where the incubation was carried out without the addition of antibody. Other experimental details are given in the text.

Table 2. Effect of various additives on the induction of δ -aminolaevulinate dehydratase in iron-deficient cultures of *Neurospora crassa*

Iron-deficient *N. crassa* mycelia (grown for 40 h) were shaken for 1 h and then treated where indicated with ferric ammonium citrate (1 μ g of iron/ml of medium) and laevulinate (160 μ g/ml of medium). Protoporphyrin (2 μ g/ml) and cycloheximide (1 μ g/ml) when used were added 5 min before the addition of the inducers. The flasks were shaken for 30 min, at the end of which mycelia were washed and homogenized. The enzyme activity was assayed in the 0–40%-satd.-(NH₄)₂SO₄ fraction.

Treatment	δ -Aminolaevulinate dehydratase activity (nmol of porphobilinogen/2 h per mg of protein)
Iron-deficient (control)	0.5
+ Ferric ammonium citrate	2.9
+ Laevulinate	2.4
+ Ferric ammonium citrate + laevulinate	5.2
+ Ferric ammonium citrate + laevulinate + cycloheximide	0.4
+ Ferric ammonium citrate + laevulinate + protoporphyrin	0.3

To measure enzyme synthesis under these conditions, the mycelia were labelled with ^{14}C -labelled *Chlorella* protein hydrolysate for 3 min as described in the Materials and methods section. The enzyme was partially purified as shown in Table 1 up to the step of adding protamine sulphate and then immunoprecipitated with excess antibody. The partial purification of the enzyme preparation before immunoprecipitation was necessary because of the

tendency of the crude extract to precipitate under conditions of immunoprecipitation described, even in the absence of added antibody. Non-specific precipitation after protamine sulphate treatment was about 9–11%, as indicated by the radioactivity detected in the immunoprecipitate obtained by the addition of bovine serum albumin and its antibody to the labelled preparation. The immunoprecipitates were subjected to SDS/polyacrylamide-gel electro-

Fig. 4. *Synthesis of δ -aminolaevulinate dehydratase in *N. crassa**

Iron-deficient mycelia were treated with iron salt + laevulinate or iron salt + laevulinate + protoporphyrin as described in Table 2. The mycelia were labelled for 3 min, with ^{14}C -labelled *Chlorella* protein hydrolysate ($5\mu\text{Ci}/\text{flask}$) and the label was added 10 min after the addition of iron salt + laevulinate. The enzyme was partially purified up to the protamine sulphate step and then immunoprecipitated as described in the text. The immunoprecipitates were analysed by SDS/polyacrylamide-gel electrophoresis. The gel slices (2 mm) were digested with H_2O_2 and, radioactivity was measured. The radioactivity profiles correspond to the following treatments: - - - - , iron-deficiency; - · - · - , iron salt + laevulinate; —, iron salt + laevulinate + protoporphyrin. The radioactivity in the total soluble protein (c.p.m./mg of protein) under these conditions was: 92 155 (iron-deficient); 90 966 (iron salt + laevulinate); 93 116 (Iron salt + laevulinate + protoporphyrin).

Table 3. *Effect of an iron salt and protoporphyrin on the synthesis of δ -aminolaevulinate dehydratase in iron-deficient cultures of *N. crassa**

Incorporation of ^{14}C -labelled *Chlorella* protein hydrolysate into the δ -aminolaevulinate dehydratase subunit was calculated by integrating the area under the peak shown in Fig. 4 and expressing the data as a percentage of incorporation into the total soluble proteins. The radioactivity in the total amino acid pool was calculated by measuring the radioactivity and the total amino acid content by using ninhydrin in the trichloroacetic acid supernatant of the crude extract of the mycelia as described in the text. Concentrations of additives were those given in the legend for Table 2.

Treatment	$10^{-5} \times$ Radioactivity in amino acid pool (c.p.m./mg of amino acid pool)	Radioactivity in soluble protein (c.p.m./mg of protein)	Radioactivity in δ -aminolaevulinate dehydratase	
			(c.p.m./mg of soluble protein)	(% of that in total soluble protein)
Nil (iron-deficient)	6.45	92 155	251	0.27
+Ferric ammonium citrate + laevulinate	6.17	90 966	1026	1.13
+Ferric ammonium citrate + laevulinate + protoporphyrin	6.51	93 116	286	0.31

phoresis and the radioactivity profile of the gels is shown in Fig. 4. Addition of iron salt + laevulinate to iron-deficient cultures results in a striking labelling of the enzyme subunit, and this is blocked by protoporphyrin. The results presented in Table 3 indicate that there is no appreciable change in the labelling of the total amino acid pool or total soluble proteins. However, incorporation into δ -aminolaevulinate dehydratase expressed as a percentage of total soluble protein radioactivity increases by 5-fold on addition of iron to the iron-deficient cultures, under the conditions of labelling described. This increase is blocked by protoporphyrin. Thus the increase in enzyme activity in iron-deficient cultures caused by the addition of iron salt + laevulinate is due to enhanced enzyme synthesis, and protoporphyrin addition represses enzyme synthesis. The presence of other peaks in the radioactivity profile could be due to the fact that the antigen used to raise the antibodies was not completely homogeneous and that the enzyme exists as a complex with certain other proteins.

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References

- Campbell, H. H., Garvey, T. S., Cramer, N. E. & Sussdorf, D. H. (1970) *Methods in Immunology*, 2nd edn., pp. 189–198, W. A. Benjamin, New York
- Doyle, D. (1971) *J. Biol. Chem.* **246**, 4965–4972
- Gaertner, F. H. & Cole, K. W. (1973) *Arch. Biochem. Biophys.* **177**, 566–573
- Granick, S. & Mauzerall, D. (1958) *J. Biol. Chem.* **232**, 1119–1140
- Hartman, W. W. & Dreger, E. E. (1950) in *Organic Synthesis* (Blatt, A. H., ed), Collective Vol. 2, pp. 150–151, John Wiley and Sons, New York
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lampkin, S. L., IV, Cole, K. W., Vitto, A. & Gaertner, F. H. (1976) *Arch. Biochem. Biophys.* **177**, 561–565
- Lascelles, J. (1964) *Tetrapyrrole Biosynthesis and its Regulation*, W. A. Benjamin, New York
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- March, S. C., Parikh, I. & Cuatrecasas, P. (1974) *Anal. Biochem.* **60**, 149–152
- Muthukrishnan, S., Padmanaban, G. & Sarma, P. S. (1968) *Biochem. Biophys. Res. Commun.* **31**, 333–339
- Muthukrishnan, S., Padmanaban, G. & Sarma, P. S. (1969) *J. Biol. Chem.* **244**, 4241–4246
- Muthukrishnan, S., Malathi, K. & Padmanaban, G. (1972) *Biochem. J.* **129**, 31–37
- Nandi, D. L., Baker-Cohen, K. F. & Shemin, D. (1968) *J. Biol. Chem.* **243**, 1224–1235
- Padmanaban, G. & Sarma, P. S. (1965) *Arch. Biochem. Biophys.* **111**, 147–152
- Porath, J., Carlsson, J., Olsson, I. & Belfrage, G. (1975) *Nature (London)* **258**, 598–599
- Poulson, R. & Polglase, W. J. (1974) *J. Biol. Chem.* **249**, 6367–6371
- Sluyterman, L. A. E. & Wijdenes, J. (1974) *Methods Enzymol.* **34**, 544–552
- Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B., Kawalek, J. & Levin, W. (1976) *J. Biol. Chem.* **251**, 1385–1391
- Tschudy, D. P., Perlroth, M. G., Marver, H. S., Collins, A., Hunter, G. & Rechcigl, M. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **53**, 841–846
- Wu, W. H., Shemin, D., Richards, K. E. & Williams, R. C. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1767–1770