Control of 5-Aminolaevulinate Synthetase Activity in *Rhodopseudomonas spheroides*

PURIFICATION AND PROPERTIES OF THE HIGH-ACTIVITY FORM OF THE ENZYME

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1. The high-activity form of aminolaevulinate synthetase has been prepared from extracts of semi-anaerobically grown cells of *Rhodopseudomonas spheroides*, which were allowed to become activated in air. Specific activity was 130000–170000 nmol of aminolaevulinate/h per mg of protein at 37°C. 2. Enzyme fraction Ia prepared on DEAE-Sephadex was a mixture of four active enzymes, pI 5.55, 5.45, 5.35 and 5.2, when prepared in either Tris or phosphate buffers and when extracts were activated by air or by cystine trisulphide. 3. The enzyme was further purified by preparative polyacrylamide-gel electrophoresis in imidazole/veronal buffer, pH 7.6, followed by gel filtration on Sephadex G-100 and concentration with DEAE-Sephadex. 4. The most active enzyme, pI 5.55, ran as a single protein band, mol.wt. 49000, in sodium dodecyl sulphate and 2-mercaptoethanol. The apparent molecular weight under non-denaturing conditions was 62000–68000 on Sephadex G-100 or G-200, pH 7.5, and on polyacrylamide-gel electrophoresis, pH 8.5, at enzyme concentrations below 10000 units/ml, i.e. <60 µg of protein/ml, and the enzyme was mainly monomeric. 5. The enzyme was homogeneous by gel disc electrophoresis at pH 8.9 and 7.6, but a slightly more diffuse band of protein was obtained during electrophoresis in glycine buffer, pH 7.4. 6. Enzyme samples possessed an intrinsic yellow fluorescence when viewed under u.v. light and this fluorescence coincided exactly with enzymic activity on gel electrophoresis. Fluorescence maxima were 420 nm (excitation) and 495 nm (emission). 7. Radioactive ³⁵S-labelled enzyme had 14 atoms of sulphur/mol of protein (or /40 leucine residues) of which 5–6 residues were cyst(e)ine and 8–9 residues were methionine. 8. No carbohydrate was detected apart from glucose, which prevented accurate determination of tryptophan with methanesulphonic acid and tryptamine.

The purification of 5-aminolaevulinate synthetase from many sources has proved very difficult owing to the instability of the enzyme and to the small amounts of enzyme that appear to be present in organisms and tissues. Several methods have been reported for the isolation of the enzyme, e.g. from rat liver (Scholnick *et al.*, 1972), from chick liver mitochondria after induction (Whiting & Granick, 1976), from photosynthetic bacteria and from non-photosynthetic bacteria (Tait, 1973). The more stable forms of the enzyme have been isolated from *Rhodopseudomonas spheroides* (Warnick & Burnham, 1971; Nandi & Shemin, 1977), and from a mutant strain Y of this organism (Fanica-Gaignier & Clement-Metral, 1973). It is not clear, however, whether the same molecular forms were being investigated in the different strains and in the different preparations. To understand better the possible regulation of

5-aminolaevulinate synthetase by trisulphides (Sandy *et al.*, 1975) and the interconversions of different forms of the enzyme, which are probably mediated by changes in the status of the sulphur groups on the protein, the physical and chemical properties of the various forms of 5-aminolaevulinate synthetase affected by the activator molecules, cystine trisulphide and the mixed trisulphide of cystine and glutathione, need to be examined. The present paper describes the purification of the high-activity form of 5-aminolaevulinate synthetase, albeit in small amounts (300 µg per preparation, specific activity 170000 nmol of 5-aminolaevulinate/h per mg of protein at 37°C), in batches obtained over a period of 8 days. The identification and behaviour of the enzyme on several analytical systems is discussed. Several properties of the enzyme were investigated and the findings are discussed here and in the following

paper (Davies & Neuberger, 1979) in the light of possible dimerizations and different modes of binding pyridoxal phosphate.

Materials and Methods

Chemicals

L-2-Oxindol-3-ylalanine was prepared from L-tryptophan by the method of Wieland *et al.* (1955), and methionine sulphone was prepared from DL-methionine by the method of Lepp & Dunn (1955). Cystine trisulphide was prepared by the method of Fletcher & Robson (1963). L-Cysteic acid (Light and Co., Colnbrook, Bucks., U.K.) was re-crystallized from hot water and dried at 50°C; the optical rotation showed it was the anhydrous compound. L-Cysteine-sulphinic acid, β -alanine, DL-kynurenine, imidazole (grade III), pyridoxamine, pyridoxamine phosphate and pyridoxal phosphate were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. CoA and ATP were from Boehringer-Mannheim G.m.b.H., Mannheim, Germany. Methanesulphonic acid was a product of Fluka A.G., Buchs SG, Switzerland, and tryptamine was prepared as the free base from the hydrochloride sold by Sigma. Acrylamide, bisacrylamide, Coomassie Blue, 2-mercaptoethanol, sucrose, glycine and standard amino acids were from BDH Chemicals, Poole, Dorset, U.K. or from laboratory stock. Phenol was re-distilled under N₂ under reduced pressure. HCl was diluted from re-distilled constant-boiling HCl (6.0M). Other chemicals were obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K.

Growth and harvesting of organisms

R. spheroides (N.C.I.B. 8253; National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, U.K.) was grown semi-anaerobically in the light in 3 litre conical flasks at 32–34°C for 18–24 h in the malate/glutamate medium S described by Lascelles (1956). The medium was supplemented with 10 μ M-Fe(II) ion added as (NH₄)₂Fe(SO₄)₂ (4 mg/l) and with MnCl₄·4H₂O (1 mg/l). Growth and harvesting of the organisms and estimation of dry weight were as described previously (Neuberger *et al.*, 1973).

Growth on [³⁵S]sulphate

Organisms were grown as described above, but at the time of inoculation 0.8 mCi of [³⁵S]sulphate (carrier-free) (The Radiochemical Centre, Amersham, Bucks., U.K.) was added to the medium in each flask. The amount of sulphate per flask was 2.1 mmol.

Radioactivity

³⁵S radioactivity was determined in a model 3390 Packard Tri-Carb liquid-scintillation spectrometer in

mixtures of 2,5-diphenyloxazole (2 g), 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.05 g) plus Triton X-100 (250 ml) in toluene (500 ml) with an external standard to correct for quenching. ³⁵S-labelled amino acids after electrophoresis were determined on paper in 12.5 ml of scintillation fluid lacking Triton X-100. The counting efficiency was determined by comparison with known amounts of ³⁵SO₄²⁻ added to blank regions of the paper.

Cell extracts containing unactivated enzyme

Cells from semi-anaerobically grown cultures, aerated cultures and oxygenated cultures were prepared as described previously (Sandy *et al.*, 1975). Cell suspensions were disrupted by ultrasonic disintegration as described by Neuberger *et al.* (1973), or by passage through a French pressure cell as used in the present paper for the preparation of high-activity enzyme. The extract was centrifuged at 25000g for 15 min to remove unbroken cells and cell debris, diluted and then centrifuged at 100000g for 90 min to give crude extracts containing unactivated enzyme. Protein was determined by the method of Lowry *et al.* (1951) with bovine plasma albumin as standard.

Enzyme assay

Enzyme was assayed by the method described earlier with succinic thiokinase added to the assay mixture (Sandy *et al.*, 1975). Samples suspected of having low-activity enzyme were also assayed in the presence of 5 μ M-cystine trisulphide, which was added last to the assay tube. With partially purified and highly purified samples of enzyme the total volume of the assay solution was decreased from 1 ml to 0.5 ml and the reaction was stopped by the addition of 0.5 ml of 10% (w/v) trichloroacetic acid. 5-aminolaevulinate was then determined in the whole reaction mixture (Mauzerall & Granick, 1956). One unit of enzyme activity corresponds to the formation of 1 nmol of 5-aminolaevulinate/h at 37°C.

Gel disc electrophoresis

Samples were analysed with the Shandon apparatus (model SAE 2717; Shandon Scientific Instruments, Camberley, Surrey, U.K.). Samples of enzyme were concentrated with dry Sephadex G-200 and dialysed against 1 mM-potassium phosphate, pH 7.4, 5 mM-2-mercaptoethanol, 0.1 mM-pyridoxal 5'-phosphate. Enzyme samples (>1000 units, <200 μ l) were applied in solutions of 1% sucrose or 10% glycerol to the top of 7.5% (w/v) polyacrylamide gels followed by layers of water and electrode buffer. Gels were prepared in three sets of buffers to give working conditions of pH 8.5, 7.4 or 7.6. Gels in glycine and imidazole buffers were subjected to a pre-run for 1–1.5 h before the sample was applied and the electrode buffers were renewed every 1.25 h. The buffers used

were: (a) 0.375M-Tris/HCl gel buffer, pH8.9, with 10mM-Tris/76mM-glycine electrode buffer, pH8.5 (Jovin *et al.*, 1964); (b) 1.25M-glycine/KOH gel buffer, pH8.3, with 36mM-glycine/72mM-lutidine electrode buffer, pH8.3 (Davies & Neuberger, 1973); (c) 63mM-imidazole/HCl gel buffer, pH7.0, with 2.5mM-imidazole/30mM-veronal electrode buffer, pH6.7. Enzyme was located by the yellow fluorescence observed under long-wavelength u.v. light and the $R_{\text{Bromophenol Blue}}$ values were recorded. Protein was stained with Coomassie Blue for 3h or more. The conditions of electrophoresis, the pH recorded within the gel and the $R_{\text{Bromophenol Blue}}$ values for enzyme were: (a) Tris, 200V, 2mA/gel, pH8.5, $R_{\text{Bromophenol Blue}}$ 0.39–0.41; (b) glycine, 250V, 0.5mA/gel, pH7.45, $R_{\text{Bromophenol Blue}}$ 0.30–0.32; (c) imidazole, 250V, 1.5mA/gel, pH7.6, $R_{\text{Bromophenol Blue}}$ 0.32–0.36.

Enzyme activity eluted from the gels

Enzyme activity could be detected in slices of gel if the gels were pre-run for 1.5h before the sample was applied. Slices (2mm) were macerated and were incubated with substrate mixture plus succinic thiokinase. Slices were also treated overnight with substrate mixtures lacking succinic thiokinase and were assayed the next day after the addition of succinic thiokinase. The recovery of activity from the gel was greatest at pH7.4 (50%), but appreciable amounts of activity were also detected at pH8.5 in Tris buffer. Only one region of enzyme activity was ever found in all gels.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Enzyme was incubated with 1% sodium dodecyl sulphate/5mM-2-mercaptoethanol at 37°C for 2h and the molecular weight of 5-aminolaevulinate synthetase was determined by the method of Weber & Osborn (1969) with bovine serum albumin (mol.wt. 65 000), ovalbumin (43 000), chymotrypsinogen (25 000) and lysozyme (14 600) as standards.

Slab-gel electrophoresis

This was carried out with the slab-gel-electrophoresis apparatus made by Raven Scientific Co., Haverhill, Suffolk, U.K. A slab of polyacrylamide gel (7.5%, w/v; 14cm×14cm×0.3cm) was prepared between glass plates in the buffer systems developed for gel disc electrophoresis. 2-Mercaptoethanol (5mM) was added to the electrode buffers for either imidazole/veronal or glycine/lutidine. The gel was pre-run in the cold room at 1°C for 5h at 200V with four changes of electrode buffer and left overnight. Electrophoresis was continued for 1h the next day. Approx. 4ml of enzyme solution in 1mM-potassium

phosphate buffer containing 1% sucrose and Bromophenol Blue was applied to the four slots in the gel. The enzyme was covered by layers of water and electrode buffer and electrophoresis was carried out at 250V and 10–16mA for 5–6h. The buffers were again renewed every 1.25h. With glycine buffers electrophoresis was continued for 5.5h at 340V and 7mA for both the pre-run and the test run. The electrode buffers were renewed after 3h. The marker dye ran approx. 12cm as a very sharp band. At the end of the experiment the gel was viewed under u.v. light and the very sharp fluorescent band of enzyme was cut out. The rest of the gel was stained for protein with Coomassie Blue.

Isoelectric focusing

Samples of form Ia of the enzyme (see Fig. 6 of Sandy *et al.*, 1975) were dialysed against 1mM-potassium phosphate buffer/0.1mM-pyridoxal 5'-phosphate, and focused with an Isco model 630 electrophoresis apparatus (Instrumentation Specialties Co. Inc., Lincoln, NE, U.S.A.). Focusing was achieved in a 150ml gradient of 0–40% (w/w) sucrose containing 1% ampholytes (LKB-Producter A.B., Stockholm, Sweden) and 5mM-2-mercaptoethanol at 3°C over 48–72h. Both wide (pH3–10) and narrow (pH4–6) pH gradients were used. The electrode vessels contained 0.16M-NaOH in water (top) and 43mM-phosphoric acid in 45% sucrose (bottom). A known weight of enzyme solution was made 20% (w/w) in sucrose and applied as the centre fractions of the sucrose gradient. When the narrow pH gradient was used, the pH of the standard solution of ampholyte was first adjusted to 5.6 with KOH before enzyme was added to avoid precipitation of the enzyme sample. The voltage was increased from 300V to 700V after 24h and to 900V for the last 24h. The focused solution was pumped from the column at a rate of 3ml/min and 2.4ml fractions were collected. The pH was determined at room temperature, the enzyme was assayed and the ^{35}S radioactivity was determined. Ampholytes were removed from the fractions containing enzyme by gel filtration on a column (2.2cm×60cm) of Sephadex G-100 in 25mM-potassium phosphate, pH7.4, 5mM-2-mercaptoethanol, 0.1mM-pyridoxal 5'-phosphate.

Oxidation with performic acid

Samples of purified enzyme (100–200µg) or lysozyme (200µg) (Sigma) were treated with 250µl of formic acid and 50µl of performic acid (prepared by reacting 5vol. of 30% H_2O_2 with 95vol. of formic acid for 2h at 20°C) at 0°C for 2h. Water (1ml) was added and the solution was rapidly dried under vacuum; this was repeated three times. The residue was dissolved in constant-boiling HCl.

Hydrolysis

Samples of enzyme were dialysed extensively against water and either freeze-dried or taken to dryness under vacuum over P_2O_5 and NaOH. A small amount was taken for the determination of ^{35}S radioactivity and the rest was hydrolysed with 0.5 ml or 1.0 ml of 6M-HCl, at 110°C for 24 h. Samples of lysozyme and of dialysis solution were treated similarly. For analysis of tyrosine 1 mg of phenol was added to each sample before hydrolysis. The hydrolysate was dried under vacuum over P_2O_5 and NaOH. The residue was dissolved in water and analysed. If polyacrylamide was removed previously from the samples of enzyme by chromatography on DEAE-Sephadex, then the hydrolysed samples were dissolved in 0.2M-sodium citrate loading buffer, pH 2.2, and analysed. For the determination of tryptophan, the sample of enzyme was hydrolysed under vacuum with 300 μ l of 4M-methanesulphonic acid plus 0.2% tryptamine at 115°C for 24 h (Liu, 1972; Simpson *et al.*, 1976). The cooled hydrolysate was treated with 300 μ l of 3.5M-NaOH and 600 μ l of water and up to 0.9 ml of this solution was analysed on the amino acid analyser.

Amino acid analysis

The acid hydrolysate (equivalent to 10–40 μ g of protein) was analysed on the long column (24 cm) of a Locarte amino acid analyser. The following analytical programmes with citrate buffers were used: (a) pH 2.80 (120 min), 4.40 (100 min), $t = 50^\circ C$; (b) pH 3.25 (33 min), 4.40 (134 min), 6.65 (150 min), $t = 50^\circ C$ (33 min), then increased to $60^\circ C$; (c) pH 4.25 (66 min), 5.28 (200 min), $t = 50^\circ C$; and, for the short (15 cm) column, (d) pH 5.28, $t = 60^\circ C$. A standard amount (25 nmol) of *p*-fluorophenylalanine was added to each run as an internal standard. Areas were drawn by hand and the amino acid composition was determined by comparison with a standard amino acid mixture.

High-voltage electrophoresis

A sample of amino acid hydrolysate in water (250 μ l; 9.5 nmol of ^{35}S ; 38 μ g of protein) was diluted with 500 nmol of cysteic acid and 500 nmol of methionine sulphone and applied as a 2 cm line to Whatman 3MM paper (57 cm $\times$ 11.5 cm). The technique was essentially that of Atfield & Morris (1961). Electrophoresis was carried out in 1.25% (v/v) formic acid/21.7% (v/v) acetic acid, pH 2.0, at 3 kV and 40 mA, for 50 min on a Shandon model L24 high-voltage-electrophoresis apparatus. Standards of cysteic acid, methionine sulphone, methionine and alanine were used as markers. The paper was dried and sprayed with a very dilute solution (0.01%) of ninhydrin in acetone, which was just sufficient to detect the position of the amino acids. The paper was

dried in the dark and the spots (3 cm $\times$ 3.5 cm) on paper were cut out and counted in 12.5 ml of scintillation fluid. The counting efficiency was found by application of a standard amount of [^{35}S]sulphate both to blank regions of the paper and to sections containing amino acids and stained with ninhydrin. Standards were counted for radioactivity with 75% recovery on blank paper and with 72% recovery on spots of alanine and cysteic acid. For the hydrolysed enzyme only 7.7 nmol of ^{35}S was recovered, of which 61% was with methionine sulphone and 39% with cysteic acid plus cysteinesulphinic acid. After being counted for radioactivity, the paper was dried, washed with toluene (4 $\times$ 20 ml) until no more fluorescence was extracted, and was dried. The amino acids were eluted with 0.1M-HCl (40 ml), dried and dissolved in 1.5 ml of water. Half the sample was counted for ^{35}S radioactivity and 10% was used for the determination of amino acids.

Carbohydrate analysis

Neutral sugars were measured by g.l.c. with a Perkin-Elmer F-11 gas chromatograph fitted with dual columns and flame-ionization detection, after methanolysis and trimethylsilylation of 50 μ g of purified enzyme, to which 25 nmol of mannitol had been added (Chambers & Clamp, 1971). Lysozyme, ovomucoid (100 μ g) and a standard mixture of sugars were used as reference compounds.

Absorption spectra and fluorescence spectra

These were recorded on a Unicam SP.800 spectrophotometer and on a Perkin-Elmer model MPF-3 spectrofluorimeter that were thermostated at $0^\circ C$ for examination of samples of native enzyme. Fluorescence spectra were recorded with a band width of 8 nm (slits 1 mm) and with sensitivity settings from 10 to 100%, and were not corrected.

Purification of high-activity enzyme

Cell extract. Cells (10–12 g dry wt.) in 0.05M-potassium phosphate buffer, pH 7.4, were disrupted by passage of a cell suspension (100 mg dry wt./ml) through a French pressure cell at 110 MPa (16000 lb/in²) at $0-4^\circ C$. The extract was centrifuged at 20000g for 15 min in an MSE 18 refrigerated centrifuge. The clear supernatant solution (70–80 ml) was removed and diluted to 300 ml with phosphate buffer and stirred gently in air at $4^\circ C$ for 60 min. This procedure brought about the activation of the enzyme (Marriott, 1968). The solution was centrifuged at 100000g for 90 min in an MSE Superspeed 64 centrifuge.

(NH₄)₂SO₄ fractionation. The clear supernatant was treated with 2-mercaptoethanol (10 mM) and was brought to 40% saturation with (NH₄)₂SO₄ at $0^\circ C$. The mixture was stirred gently for 30 min and the

precipitate was removed by centrifugation and was stored at 0°C overnight.

Sephadex G-200. The pigmented material was dissolved in the minimum volume (about 24 ml) of 25 mM-potassium phosphate buffer, pH 7.4, containing 5 mM-2-mercaptoethanol, and was centrifuged. The clear solution was loaded on a column (6 cm × 55 cm) of Sephadex G-200 equilibrated with the same phosphate buffer and was gel-filtered at 24 ml/h (Tuboi & Hayasaka, 1971). The fractions (15 ml) containing the bulk of the enzyme (fractions eluting between 840 and 1040 ml) were pooled and assayed (6000 units/ml).

DEAE-Sephadex. Enzyme sample in 25 mM-potassium phosphate buffer was applied directly at 50 ml/h to a column (2 cm × 25 cm) of DEAE-Sephadex A-25 that had been equilibrated overnight with 25 mM-potassium phosphate/5 mM-2-mercaptoethanol, pH 7.4. The column was washed with 50 ml of buffer and the enzyme was eluted with a gradient formed from 240 ml each of 25 mM-potassium phosphate, pH 7.4, 5 mM-2-mercaptoethanol and 0.15 M-potassium phosphate, pH 7.4, 5 mM-2-mercaptoethanol at a rate of 15 ml/h at 1°C. Fractions (6 ml) were collected and assayed. Assays were carried out in the presence and in the absence of cystine trisulphide to check that only the high-activity form of 5-aminolaevulinate synthetase was present. The major amount of enzyme was in the Ia form (10000–20000 units/ml). We could not exclude the possibility that a small amount of form IIa was also present since the chromatographic pattern often showed a shoulder on the main peak (cf. Fig. 6b of Sandy *et al.*, 1975). The small amount of trailing fraction IIb was pooled separately.

Stock solutions of fraction Ia. The bulk of fraction Ia (5–6 fractions) was pooled, assayed and treated in one of two ways. (i) The pool of enzyme (30–40 ml in approx. 0.05 M-potassium phosphate/5 mM-2-mercaptoethanol) was treated with pyridoxal 5'-phosphate (0.1 mM) and the pH was adjusted to 7.0–7.2. It was diluted with an equal volume of cold water containing 2-mercaptoethanol and concentrated to about 4 ml under N₂ by ultra-filtration through an Amicon Diaflo UM-10 membrane. The yellow colour of the protein solution increased during this step. Enzyme was dialysed first against 25 mM-potassium phosphate, pH 7.0, and then against 1 mM-potassium phosphate, pH 7.0, 5 mM-2-mercaptoethanol, 0.1 mM-pyridoxal 5'-phosphate overnight. (ii) The preparation of fraction Ia was diluted with water and was concentrated by re-absorption on a small column (9 ml) of DEAE-Sephadex, followed by elution with 0.15 M-potassium phosphate, pH 7.4, containing 2-mercaptoethanol (Hayasaka & Tuboi, 1974). Fractions (1 ml) were collected and assayed for activity. Fractions containing most of the enzyme showed a strong yellow fluorescence. These were

pooled and were dialysed against several litres of 1 mM-potassium phosphate/5 mM-2-mercaptoethanol, plus 0.1 mM-pyridoxal 5'-phosphate if the enzyme was to be stored or was to be purified further, or alternatively was dialysed without pyridoxal 5'-phosphate if the fraction Ia preparations were to be examined.

Preparative slab-gel electrophoresis. Samples of form Ia (4 ml) were subjected to electrophoresis at 1°C and 250 V for 5.5 h. The sharp yellow fluorescent band of enzyme was cut out ($R_{\text{Bromophenol Blue}} = 0.32–0.35$) and macerated with a glass rod in 3 ml of 25 mM-potassium phosphate buffer, pH 7.4, containing 2-mercaptoethanol and pyridoxal 5'-phosphate (0.1 mM) and was left overnight at 0°C. Clear samples of gel from either side of the fluorescent area were also cut out and treated similarly. The rest of the gel was stained for protein with Coomassie Blue. The enzyme was extracted from the gel with six lots of buffer (1 ml) until most had been extracted and the gel became opaque. About 25–35% of the enzyme activity was usually recovered.

Sephadex G-100. The solution (7 ml) was gel-filtered on a column (2.2 cm × 60 cm) of Sephadex G-100 at 1°C in 25 mM-potassium phosphate, pH 7.4, 5 mM-2-mercaptoethanol, 0.1 mM-pyridoxal 5'-phosphate at a rate of 14 ml/h and 3.6 ml fractions were collected. Enzyme activity and ³⁵S radioactivity were always eluted at 109–110 ml. The major fractions of enzyme were pooled and were dialysed exhaustively against water adjusted to pH 7.0 before amino acid analysis, and were dialysed against dilute phosphate buffer for the determination of protein and enzyme activity. ³⁵S-labelled protein was used to check on the loss of material from these very dilute solutions of protein, and as a measure of the amount of protein hydrolysed for amino acid analysis. Appreciable amounts of soluble polyacrylamide were present in these samples even after gel-filtration, and on hydrolysis gave polyacrylic acid that was not soluble in citrate buffer, pH 2.2. Hence these polymers were separated from the enzyme by a second passage through a column of DEAE-Sephadex.

Concentration. Fractions off the gel were pooled and applied to a small column (9 ml) of DEAE-Sephadex. The column was washed with 25 ml of buffer, and the enzyme was eluted with 0.175 M-potassium phosphate buffer containing 2-mercaptoethanol. The enzyme was eluted after 4–7 ml and could be detected by its intense yellow fluorescence, by its enzymic activity and by its radioactivity; 50000–100000 units of activity were detected. The yellow pyridoxal 5'-phosphate was firmly bound to the column and was only eluted after 45 ml. Stock solutions of enzyme were stored in 0.2 mM-pyridoxal 5'-phosphate and were dialysed before use against 0.15 M-potassium phosphate, pH 7.4, containing pyridoxal 5'-phosphate. When ³⁵S-containing samples

were prepared the final concentrated solution was gel-filtered on Sephadex G-100 and exact correlations were found between enzymic activity, ^{35}S and fluorescence. The enzymic activity per nmol of radio-active sulphur was constant across the peak. For amino acid analysis the enzyme solutions were finally dialysed against water and were freeze-dried.

Results and Discussion

The purification stages for high-activity enzyme are shown in Table 1. Preparations of fraction Ia were usually of specific activity 50000–90000 units/mg of protein and were generally stabilized for a day or two with 0.1 mM-pyridoxal 5'-phosphate and 5 mM-2-mercaptoethanol in phosphate buffer, pH 7.4, at 0°C. Extra pyridoxal 5'-phosphate was not added when fraction Ia samples were analysed for cofactor, or when they were examined fluorimetrically and spectrophotometrically.

About 20 samples of purified enzyme have been prepared and analysed. Approx. 300 μg of enzyme were obtained from each batch of about 10 g dry wt. of cells. The samples had specific activities of 130000–170000 units/mg of protein and approx. 600 units of activity/nmol of ^{35}S giving 220–270 nmol of ^{35}S /mg of protein. Our best preparation had 200000 units/mg of protein, 700 units/nmol of ^{35}S , and 3.5 μg of protein/nmol of ^{35}S .

Molecular weight

We found no difference in apparent molecular weight between the high-activity form of the enzyme and the low-activity form that could be activated by cystine trisulphide when the enzymes were gel-filtered on Sephadex G-100 and G-200. Both forms of the enzyme, in fractions precipitated by $(\text{NH}_4)_2\text{SO}_4$, were eluted just behind bovine serum albumin

(mol.wt. 65000). When compared with standard proteins, bovine serum albumin and cytochrome c, on Sephadex G-200 in 25 mM-potassium phosphate/5 mM-2-mercaptoethanol, the high-activity form (Ia) had an apparent mol.wt. of 62000 when it was eluted at 10000 units/ml. The same value was obtained for enzyme that was eluted at 2000 units/ml from Sephadex G-100 (Andrews, 1964) in 50 mM-potassium phosphate, pH 7.4, 5 mM-2-mercaptoethanol, irrespective of whether or not the buffer contained 0.15 M-KCl, 0.5 M-NaCl or 0.1 mM-pyridoxal 5'-phosphate. Samples of highly purified enzyme were eluted from Sephadex G-100 in phosphate buffer containing 0.1 mM-pyridoxal 5'-phosphate at 110 ml, in exactly the same position as enzyme from less pure preparations. The enzyme activity correlated with the radioactivity and the specific activity of the enzyme across the peak was 600 units/nmol of ^{35}S .

Enzyme of pI 5.55 and the enzymically active yellow fluorescent protein (see below) in samples of fraction Ia were also examined by gel disc electrophoresis on polyacrylamide gels (5, 7, 9 and 11% polyacrylamide) at pH 8.9 by the method of Hedrick & Smith (1968). The apparent mol.wts. were 68000 ± 7000 when compared with the standard proteins bovine serum albumin (monomer, dimer and trimer), ovalbumin (two forms) and chymotrypsinogen.

Electrophoresis of pure high-activity enzyme and enzyme of pI 5.55 in sodium dodecyl sulphate and 2-mercaptoethanol gave a single protein subunit of mol.wt. 49000, in approximate agreement with values in the literature (cf. 57000, Warnick & Burnham, 1971; 53000, Jordan & Shemin, 1972; 41000–45000, Nandi & Shemin, 1977).

Various values of molecular weight have been reported for several forms of 5-aminolaevulinate synthetase under non-denaturing conditions. Haya-saka & Tuboi (1974) reported values around 53000–

Table 1. Representative purification scheme for aminolaevulinate synthetase from *R. spheroides*

Suspensions of cells (12 g dry wt.) were disrupted with a French press as described in the Materials and Methods section. Specific activity is measured as nmol of aminolaevulinate/h per mg of soluble protein at 37°C. N.d., not determined.

	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (μmol of 5-aminolaevulinate/h)	Specific activity	Purification (fold)
Disrupted cells	120	35.0	4200	240	56	1
Supernatant (25000g)	400	10.5	4200	1200	280	5
Supernatant (100000g)	268	6.5	1750	980	560	10
$(\text{NH}_4)_2\text{SO}_4$ fractionation (0–40% satn.)	25	20.4	510	800	1570	28
Sephadex G-200	225	0.51	115	600	5200	93
DEAE-Sephadex (fraction Ia)	37	0.18	6.7	435	65000	1160
Fraction Ia (concentrated and dialysed)	3.9	0.64	2.5	210	54000	965
Electrophoresis	6.5	N.d.	N.d.	100	N.d.	N.d.
DEAE-Sephadex A-25	4.0	0.045	0.180	31	170000	3000
Sephadex G-100 (pool)	21	0.013	0.275	36	130000	2300
(peak)	3.6	0.040	0.144	24	170000	3000

67000 for fractions I and II in both a- and b-forms on Sephadex G-100. For the most active form Yubisui & Yoneyama (1972) reported a mol.wt. of 61000, which is in approximate agreement with the value of 57000 given by Warnick & Burnham (1971), obtained in the presence and absence of sodium dodecyl sulphate. Tuboi *et al.* (1970), Fanica-Gaignier & Clement-Metral (1973) and Jordan & Shemin (1972) have also given values ranging from 80000 to 100000 in other preparations of forms I and II. Nandi & Shemin (1977) reported a value of 84000 for enzyme that we believe had become activated during the preparation. These authors showed that the molecule existed as a dimer in fairly concentrated solutions (approx. 0.4mg/ml), but at a concentration less than 0.16mg/ml, i.e. <24000units/ml, dissociation of the dimer appeared to occur. Since most of our samples were at concentrations <20000units/ml they may have been expected to contain both monomers and dimers and to exhibit intermediate values of molecular weight. Only for partially purified samples of fraction Ia were concentrations >50000units/ml available, so that only here could a large proportion of dimers be expected. Most of the highly purified samples had activities of 3000units/ml in which solutions the monomeric form would be expected to predominate.

Isoelectric focusing

Form Ia of the enzyme, which was eluted first from DEAE-Sephadex at pH7.4, appeared to be the least acidic of the various forms. In a wide pH gradient all the enzymic activity focused around pH5.4. In a narrow pH gradient samples of form Ia appeared to be a further mixture of molecular forms whose relative abundance decreased with their increased acidity (Fig. 1). The pI values were 5.55, 5.45, 5.35 and 5.2 in the presence of ampholytes. The same distribution of active forms was obtained in preparations of fraction Ia isolated after either spontaneous activation of enzyme or activation by added cystine trisulphide of the low-activity enzyme from oxygenated cells. The enzymic activity per nmol of ^{35}S for the species of pI5.55 was greater than that for the species of pI5.4, and in the spontaneously-activated preparation it was twice the value found in the trisulphide-activated preparation. Other activated fractions gave slightly different patterns (Fig. 2). For example, fraction Ia, which was obtained from rechromatography of 'aged' and apparently activated samples of fraction IIb, showed relatively more of the more acidic forms.

We confirmed the findings of Burnham & Lascelles (1963) that enzymes assayed in phosphate buffer only showed 60–70% of the enzymic activity observed in Tris buffer. Thus phosphate may have competed with pyridoxal phosphate for the enzyme. To test

Fig. 1. Isoelectric focusing of fraction Ia of aminolaevulinate synthetase

Radioactive ^{35}S -labelled aminolaevulinate synthetase was focused in a narrow pH gradient (—; pH4–6) in the presence of ampholytes (1%) for 72h at 0°C. Fractions (3ml) were collected and the pH values were determined. Fractions were assayed for aminolaevulinate synthetase activity (○, 25μl) and for radioactivity (●, 300μl).

Fig. 2. Isoelectric-focusing pattern for two samples of aminolaevulinate synthetase

Two preparations of fraction Ia, separated on DEAE-Sephadex, were focused as described in the legend to Fig. 1. Fractions (3ml) were collected, the pH values were determined and fractions (25μl) were assayed for aminolaevulinate synthetase activity. Preparations were: high-activity enzyme (fraction Ia; ○); sample of fraction Ia recovered from rechromatography of fraction IIb (●). For details, see the text. —, pH gradient.

whether bound phosphate ions produced a different form of the enzyme, samples of fraction Ia were prepared from cells and extracts in Tris buffer instead of phosphate buffer, and were focused. The same pattern of molecular forms was obtained for both buffer systems. The high-activity form, pI5.55, predominated in each sample analysed.

When the different molecular forms were filtered separately on Sephadex G-100 to remove the ampholytes the enzymic activity was always eluted in the

same place (110ml). This indicated that each of the forms had a similar molecular weight in phosphate buffer containing 2-mercaptoethanol and pyridoxal phosphate.

The major and most basic form, pI 5.55, showed an intrinsic yellow fluorescence when examined by gel disc electrophoresis at pH 7.4 and when viewed under long-wavelength u.v. light. Samples of the purified enzyme were also fluorescent. These samples showed only one band of protein on normal gel disc electrophoresis at pH 8.5, but showed two extremely close bands of protein around pI 5.5 when isoelectrically focused in ampholytes on polyacrylamide gels (4% w/v).

The focusing method could not be used for the preparation of 5-aminolaevulinate synthetase since precipitation of the enzyme and other proteins occurred and the recoveries of activity (10–25%, 10000–20000 units) were low. The method clearly showed that form Ia of 5-aminolaevulinate synthetase is a mixture of forms that have slightly different isoelectric points and different charges at pH 7.4. These forms may have interchanged disulphide bonds as was observed for bovine serum albumin (Walleik, 1976), or may have different modes and degrees of binding the cofactor pyridoxal 5'-phosphate, some of which may be removed from the enzyme by ampholytes. Alternatively, the forms may differ in the number of amide groups, in the conformation of the protein, or in the amino acid composition of the protein chain as a result of partial proteolysis. The important change in the chromatographic properties on DEAE-Sephadex of 5-aminolaevulinate synthetase after activation with trisulphide (form Ib into form Ia) is unlikely to be caused by partial proteolysis of the protein, but is probably caused by a change in charge and conformation.

Gel electrophoresis

Enzymic activity could be detected in gel slices if the sodium persulphate was first removed from the gels by electrophoresis for 1 h before the sample was applied. Greater amounts of enzymic activity were also detected when experiments were run at pH values lower than 8.9, e.g. at pH 7.5 or 6.6, as the enzyme was more stable at the lower pH values.

Gel disc electrophoresis in glycine buffer at pH 7.4 instead of Tris, pH 8.5, has shown that many enzyme samples contained more than one protein band if they were prepared from fraction Ia by variations of the column procedures described in the Materials and Methods section, e.g. chromatography on DEAE-Sephadex at pH 5.8 instead of pH 7.4 with either potassium phosphate or pyridoxal 5'-phosphate as eluent, or separations on columns of hydroxyapatite at pH 6.8 and 7.4. Therefore preparative gel electrophoresis at pH 7.4 seemed a suitable method for

purification of enzyme and provided a reasonable recovery (25–35%) of enzymic activity. Electrophoresis at pH 7.5 with suitable concentrations of imidazole/veronal buffers (pH 6.7) gave a pattern of protein bands similar to that obtained in glycine buffer and only one protein band was enzymically active. The enzymic activity could again be recovered from the gel. Preparative electrophoresis on slabs of polyacrylamide gel at pH 7.4 was used mostly with imidazole buffers (Fig. 3), though glycine buffers were used in the early preparations.

Samples of purified enzyme showed only one band of protein on disc gel electrophoresis in Tris buffer at pH 8.5 and this band was enzymically active. A slightly more diffuse band of protein was observed when purified enzyme was examined in the highly resolving conditions in glycine buffer. The glycine/lutidine system, pH 6.6, maintained a pH value of 7.4 within the gel (Davies & Neuberger, 1973).

Only one band of enzyme was found in gels at pH 8.9 and 7.5 and this was closely associated with a yellow fluorescent band of protein. In each step after the electrophoresis the amount of fluorescence correlated exactly with the amount of enzymic

Fig. 3. Schematic representation of the gel electrophoresis of fraction Ia of aminolaevulinate synthetase

Aminolaevulinate synthetase was subjected to either slab gel electrophoresis or disc gel electrophoresis in imidazole/veronal buffer, pH 7.6. For details, see the text. (a) Protein was stained with Coomassie Blue, and mobilities were recorded relative to the marker dye. (b) Sections of gel (0.4 cm slab; 0.2 cm cylinder) were cut out, incubated with substrate mixture for 1 h at 37°C and the aminolaevulinate formed was determined. The position of the yellow fluorescent protein observed under u.v. light is marked with an arrow.

activity and served as a convenient intrinsic marker for high-activity 5-aminolaevalinate synthetase.

Sulphur content

The sulphur content of separate batches of purified 5-aminolaevalinate synthetase was determined by the three following methods since the amount of protein available at any time was small: (a) measurement of ^{35}S radioactivity, which served as a measure of the amount of protein analysed, and that helped trace the loss of material on handling; (b) direct determination of the amino acids cysteic acid and methionine sulphone on the amino acid analyser; (c) isotope dilution of the acid hydrolysate with unlabelled cysteic acid and methionine sulphone. The results of method (c) are given here. Enzyme containing 250 nmol of ^{35}S /mg of protein was oxidized with performic acid and was hydrolysed. Amino acid analysis of half the sample showed 24.5 nmol of leucine was recovered from 38 μg of protein. The second half was diluted with approx. 100-fold molar excess (500 nmol) of unlabelled cysteic acid and methionine sulphone and the radioactive amino acids were separated on paper by high-voltage electrophoresis at pH 2. Only 81% of the total radioactivity was recovered (4500 d.p.m.). The distribution is shown in Fig. 4. Traces

of cysteinesulphinic acid followed the bulk of the cysteic acid. The ratio of the sum of cysteic acid plus cysteinesulphinic acid to methionine sulphone was 0.63:1. This corresponded to 5.0 residues of cysteine and 7.7 residues of methionine per 40 leucine residues and per 63 000 g of protein measured by the method of Lowry *et al.* (1951) (see below). Approx. 3 atoms of sulphur were unaccounted for in this experiment.

The cysteic acid and methionine sulphone were eluted from the spots on the paper and their specific radioactivities were determined. These were: cysteic acid, 1.8 nmol of ^{35}S in 335 nmol of amino acid; methionine sulphone, 3.9 nmol of ^{35}S in 422 nmol of amino acid. Thus 2.69 nmol of cysteic acid and 4.62 nmol of methionine sulphone were present in the original hydrolysate of 38 μg of protein. Therefore there were 5.1 residues of cysteic acid plus cysteinesulphinic acid and 7.6 residues of methionine sulphone per 40 residues of leucine, and per 62 000 g of protein, in agreement with the previous determination.

It is noteworthy that small amounts (17%) of cysteinesulphinic acid were observed both in samples of enzyme and in samples of lysozyme after mild oxidation with performic acid (Hirs, 1956). Cysteic acid and cysteinesulphinic acid are not resolved on the amino acid analyser, but show as a decreased colour factor since the colour factor for sulphinic acid is 70% that of cysteic acid. Perhaps this accounts for the low recoveries of cysteic acid (80–90%) obtained by Hirs (1967) with a 12-fold excess of performic acid.

Amino acid analysis

Very small amounts of protein were available at any one time, so protein concentrations of the dialysed preparations of pure enzyme were determined with only 10–20 μg of protein. Consequently the amounts oxidized with performic acid and subsequently hydrolysed were known only approximately. The hydrolysed samples of native 5-aminolaevalinate synthetase and performic acid-oxidized enzyme were analysed under three sets of analytical conditions and comparisons were made with known amounts of lysozyme treated in the same way. The results are shown in Table 2.

Since a protein subunit (mol.wt. 49 000) is composed of about 450 amino acids, the amount of each amino acid was recorded relative to 40 residues of leucine; values on the basis of mg of protein were less reliable. For eight analyses 40 nmol of leucine was associated with a range of 52 to 70 μg of protein and with a range of 12 to 16 nmol of ^{35}S . The enzyme has a low percentage of sulphur amino acids (3.4%) and a very low percentage of cysteine residues (1.3%). Each subunit (mol.wt. 49 000) has approx. 5–6 cysteine residues and 8–9 methionine residues and a

Fig. 4. High-voltage electrophoresis of oxidized and hydrolysed ^{35}S -labelled aminolaevalinate synthetase

Purified enzyme was oxidized with performic acid, hydrolysed with 6M-HCl, mixed with cysteic acid (500 nmol) and methionine sulphone (500 nmol) and subjected to high-voltage electrophoresis on paper at 3 kV and 40 mA, pH 2, for 50 min. Sections were stained with ninhydrin, dried, cut out and counted for radioactivity (shaded areas). For details, see the Materials and Methods section. (a) Radioactivity; (b) ninhydrin-positive compounds in the hydrolysate; (c) standard amino acid markers: A, cysteic acid; B, cysteinesulphinic acid; C, methionine sulphone; D, methionine; E, alanine.

Table 2. *Amino acid composition of the high-activity form of aminolaevulinate synthetase from R. spheroides*

Samples of enzyme were hydrolysed with 6M-HCl for 24h. The composition was determined on a Locarte amino acid analyser with either standard mixtures of amino acids (first column) or with lysozyme as standard (second column). The values and standard deviations are given relative to 40 residues of leucine and the number of determinations are given in parentheses.

Amino acid	Amino acid composition	
	(Residues/ 40 leucine residues)	(Whole number of residues/ 40 leucine residues)
Asp	43.5 ± 2.5 (7)	45
Thr	21.3 ± 2 (7)	23
Ser	21.6 ± 1 (6)	27
Glu	36.2 ± 1 (5)	34
Pro	23.1 ± 1 (5)	21
Gly	47.7 ± 1.3 (6)	48
Ala	44.5 ± 1.5 (6)	44
½Cys*	5.2 ± 0.6 (5)	5-6
Val	30.1 ± 1.2 (7)	30
Met*	9.2 ± 1 (3)	8-9
Ile	26.5 ± 1.2 (8)	27
Leu	40.0 (8)	40
Tyr	12.3 ± 0.2 (3)	13
Phe	13.3 ± 1 (8)	14
His	16.3 ± 1 (6)	17
Lys	21.4 ± 1.5 (7)	21
Arg	28.3 ± 0.8 (5)	29
Total		447

* Determined as cysteic acid or methionine sulphone after oxidation of protein with performic acid (see the text for details).

total of 14 sulphur amino acids per 40 leucine residues.

The pure unhydrolysed enzyme has a specific activity of 600 units/nmol of ³⁵S and 170000 units/mg of protein. These values correspond to 8400 units/nmol of enzyme and a subunit mol.wt. of 50000, in approximate agreement with the results reported above.

The amino acid analyses reported in the present paper give no definite indication of sulphur amino acids other than cysteine and methionine. Since the amounts of protein hydrolysed (100-200 µg) were small and the sulphur content of the different preparations did show some variation, we cannot exclude the possibility that a small amount of labile sulphur may be lost from the enzyme, as occurs for Fe-S proteins such as ferredoxin (Petering *et al.*, 1971). Experiments designed to detect the presence of tri-sulphide and labile sulphur have shown no loss of enzymic activity with cyanide, however (Davies & Neuberger, 1979).

Trace amounts of tryptophan (1.6 residues/40 residues of leucine) were found in samples of enzyme hydrolysed under vacuum with methanesulphonic acid and tryptamine, but large amounts of an unreported compound were always found in these hydrolysed samples. This compound was neither a known degradation product of tryptophan (i.e. 2-oxoindol-3-ylalanine or kynurenine) nor a component of 5-aminolaevulinate synthetase, but appeared to be formed by reaction of glucose with tryptamine.

Carbohydrate analysis

Analysis of 50 µg of a sample of purified protein (200 µg) showed that 8-10% (w/w) glucose was present. Glucosamine, galactosamine, mannose, galactose and fucose were absent. The glucose was probably present as dextran, a contaminant from the filtration on Sephadex, which was not removed on prolonged dialysis against water. In preparations of smaller amounts of 5-aminolaevulinate synthetase (90 µg) the amount of carbohydrate was sufficient to interfere with the determination of tryptophan.

Conclusion

The high-activity form of 5-aminolaevulinate synthetase, which is produced by activation of the low-activity form by reaction with air and thiols via the participation of cystine trisulphide, has been purified about 1600-fold (130000-170000 nmol of 5-aminolaevulinate/h per mg of protein at 37°C) from crude unactivated extracts of cells grown in medium containing 10 µM-Fe(II) ion. This activity is as high as the activity of samples prepared earlier by Warnick & Burnham (1971) (130000 units/mg) and by Nandi & Shemin (1977) (150000 units/mg). The activity is higher than the values found by Fanica-Gaignier & Clement-Metral (1973) (22500 units/mg at 22°C, i.e. calculated to be 56000 units at 37°C) for cells grown in medium containing 17 µM-Fe(II) ion. However, the activity is apparently not as high as the activity of enzyme isolated from cells grown in medium containing 1 µM-Fe(II) ion when the effect of temperature is considered (156000 units/mg at 22°C, i.e. calculated to be 390000 units at 37°C) (Clement-Metral & Fanica-Gaignier, 1975).

The enzyme has an intrinsic yellow fluorescence closely associated with it, which is similar to that reported by the latter workers for samples of both their purified enzymes. We found that this fluorescence served as a convenient marker for the location of high-activity enzyme on gel electrophoresis.

The enzyme has 14 sulphur groups per subunit (mol.wt. 49000) and has 5-6 cysteine and 8-9 methionine residues. The apparent molecular weight

on polyacrylamide and dextran gels is approx. 62000 at concentrations of enzyme <10000 units/ml (i.e. <60 µg of enzyme protein/ml) and probably indicates some dimerization of the largely monomeric enzyme. Taken with the findings of Clement-Metral & Fanica-Gaignier (1975) that 1 or 2 thiol groups are present per 100000 g of protein in enzyme I, it is possible that the high-activity enzyme has 4–5 disulphide groups per 100000 g of protein, or 2 disulphide groups per monomer even in the presence of excess of 2-mercaptoethanol. The unactivated b-form probably has a greater number of thiol groups, but this has not yet been measured. It would be compatible with what is known about activation and deactivation of b- and a-forms of 5-aminolaevalinate synthetase (Sandy *et al.*, 1975).

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