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

A ROLE FOR TRISULPHIDES

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1. The aminolaevulinate synthetase activator activity of fresh extracts of semi-anaerobically grown *Rhodospseudomonas spheroides* was resolved into two fractions by ion-exchange chromatography. One fraction was identified as cystine trisulphide (CySSSCy). Analysis of the other fraction indicated the presence of about equal amounts of glutathione trisulphide (GSSSG) and the mixed trisulphide of glutathione and cystine (GSSSCy). 2. Four further fractions with activator activity were observed on ion-exchange chromatography of extracts prepared by methods similar to those described earlier [Neuberger *et al.* (1973) *Biochem. J.* **136**, 491–499]. These activators were generated by the extraction procedure. Two of them have been identified as trisulphanedisulphonate ($S_3O_6^{2-}$) and additional cystine trisulphide. 3. For the series of polysulphanedisulphonates ($^-O_3S-S_n-SO_3^-, n \geq 1$), activator activity at μM concentrations was exhibited only by compounds with $n \geq 3$. This, together with the results described above, indicates that for a compound $R-S_n-R'$ (where R and R' are organic or inorganic groups) the only structural requirement for activity is $n \geq 3$. 4. Oxygenation of a semi-anaerobic culture of *R. spheroides* for 1.5 h before harvesting the cells produced a decrease of more than 90% in the cellular content of cystine trisulphide and glutathione trisulphides. 5. Chromatography on DEAE-Sephadex confirmed the presence of multiple forms of aminolaevulinate synthetase in extracts of *R. spheroides* [Tuboi *et al.* (1970) *Arch. Biochem. Biophys.* **138**, 147–154]. Oxygenation of a semi-anaerobic culture resulted in the disappearance of high-activity enzyme (a-form) and the accumulation of low-activity enzyme (b-form) in the cell. Spontaneous activation [Marriott *et al.* (1969) *Biochem. J.* **111**, 385–394] and activation by cystine trisulphide both resulted in the almost complete conversion of the b-form into the a-form.

Rhodospseudomonas spheroides growing semi-anaerobically in the light has a high rate of bacteriochlorophyll synthesis and a high activity of 5-aminolaevulinate synthetase, the first enzyme specific to tetrapyrrole biosynthesis. When O_2 is bubbled through such a culture, bacteriochlorophyll synthesis ceases immediately and there is no accumulation of precursors of this pigment, consistent with the observed fall in the activity of 5-aminolaevulinate synthetase. This loss of enzyme activity is associated with marked changes in the sulphur metabolism of the cells, and it appears that the enzyme is controlled *in vivo* by a mechanism involving sulphur compounds of low molecular weight, although the details of this mechanism remained unknown (Davies *et al.*, 1973). The purification and properties of endogenous activators of aminolaevulinate synthetase in *R. spheroides* have been described (Neuberger *et al.*, 1973b). Neuberger *et al.* (1973c) and Davies *et al.* (1973) indicated that these compounds are trisulphides of cystine or glutathione.

The present paper gives the evidence for the structures of the endogenous activators and provides details of their isolation from cells grown on [^{35}S]-sulphate. The use of various extraction methods has revealed a number of other compounds with activator activity. These compounds are generated during extraction and are therefore of doubtful physiological importance. Identification of some of these compounds has led to a study of the structural requirements for activator activity. The amounts of the trisulphides of cystine and glutathione found in cells grown under different conditions are consistent with trisulphides being involved in the control of aminolaevulinate synthetase in *R. spheroides*.

Tuboi *et al.* (1970) have described multiple forms of aminolaevulinate synthetase in *R. spheroides* that are resolved by chromatography of extracts on DEAE-Sephadex. We have confirmed the presence of multiple forms of the enzyme, and we have studied the effect of growth conditions, spontaneous activation of the enzyme (Marriott *et al.*, 1969) and activation

with cystine trisulphide on the chromatographic properties of aminolaevulinate synthetase on DEAE-Sephadex.

Materials and Methods

Chemicals

Potassium trithionate (potassium monosulphane-disulphonate) was prepared as described by Krause & Busch (1958). Potassium pentathionate (potassium trisulphanedisulphonate) and potassium hexathionate (potassium tetrasulphanedisulphonate) were prepared as described by Goehring & Feldmann (1948). Cystine trisulphide [bis-(2-amino-2-carboxy-ethyl) trisulphide] was prepared as described by Fletcher & Robson (1963). Sodium salts of other oxy-acids of sulphur were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.; other chemicals were obtained from the sources described by Neuberger *et al.* (1973a).

Methods

The growth and harvesting of *Rhodopseudomonas spheroides* (N.C.I.B. 8253), the preparation of cell-free extracts, the determination of dry weight and protein, the assay of enzymic activities, the determination of aminolaevulinate and the procedures for high-voltage electrophoresis and radioautography were as described previously (Neuberger *et al.*, 1973a), as was the dialysis method for the extraction of the activator (Neuberger *et al.*, 1973b). The activator activity of extracts was determined as described by Neuberger *et al.* (1973b), except that the enzyme preparation used was the upper supernatant from step 2 of the purification procedure for 'low'-activity enzyme. Assays of aminolaevulinate synthetase in fractions from chromatography on DEAE-Sephadex were supplemented with succinic thiokinase (EC 6.2.1.5) (35 μ g of protein; activity 18000 nmol of succinohydroxamate/h per mg of protein) prepared from *R. spheroides* by $(\text{NH}_4)_2\text{SO}_4$ fractionation (50–65% satn.) and gel filtration on Sephadex G-200 in 0.05 M-potassium phosphate buffer, pH 7.4, containing 5 mM- β -mercaptoethanol.

Determination of cyanide-labile sulphur. The method is based on the formation and isolation of 1 mol of [^{35}S]thiocyanate per mol of trisulphide treated with alkaline cyanide. To a dried sample of [^{35}S]trisulphide (at least 200 c.p.m./nmol of S) was added HCl (25 μ mol), NH_3 (100 μ mol), KCN (12.5 μ mol) and water, to a total volume of 1.1 ml. After incubation at 25°C for 45 min, HCl (100 μ mol) and 0.35 ml of a 50% (v/v) suspension of Dowex 50 (H^+ form) in water were added. The suspension was mixed, centrifuged briefly at 1000 g for 1 min and the supernatant was retained. The pellet was washed with 1 ml of

water, and the total radioactivity in supernatant plus water wash was determined. In the controls, cyanide was omitted from the reaction mixture but added after the final acidification. The amount of cyanide-labile sulphur (as thiocyanate) was calculated from the known specific radioactivity of the treated compound.

Preparation of samples of aminolaevulinate synthetase which were chromatographed on DEAE-Sephadex.

(1) Experiments on spontaneous activation. A suspension of semi-anaerobically grown *R. spheroides* (600 mg dry wt.; 6 ml) was disrupted by sonication, and centrifuged at 25000 g for 10 min (Neuberger *et al.*, 1973a). The clear supernatant (5.5 ml) was diluted with 0.02 M-potassium phosphate buffer, pH 7.4 (11 ml), and treated in one of two ways. In experiment (a) the sample was immediately subjected to gel filtration in three portions on columns (0.9 cm $\times$ 15.0 cm) of Sephadex G-50 (coarse grade), which were eluted with the same buffer. The first 4 ml of pigmented material eluted from each column were combined, diluted to 25 ml in 0.02 M-phosphate buffer, pH 7.4, and centrifuged at 400000 g for 90 min. In experiment (b) the sample was immediately treated with a further 9 ml of phosphate buffer and stored at 4°C for 90 min to allow spontaneous activation of aminolaevulinate synthetase, and centrifuged at 400000 g for 90 min. To the clear supernatant solutions (23 ml) was added β -mercaptoethanol (5 mM), and the samples were fractionated by chromatography on DEAE-Sephadex.

A unit of enzyme activity is defined as that which catalyses the formation of 1 nmol of amino-laevulinate/h per mg of protein. Sample (a) had 9900 units of activity when assayed alone; 20700 units when assayed with cystine trisulphide (5 μ M); 69 mg of protein. Sample (b) had 16800 units when assayed alone; 19800 units when assayed with cystine trisulphide (5 μ M); 103 mg of protein.

(2) Experiments on activation with cystine trisulphide. Cells (850 mg dry wt.; 8 ml) from an oxygenated culture of semi-anaerobically grown *R. spheroides* (Neuberger *et al.*, 1973a) were disrupted by sonication and centrifuged at 25000 g for 15 min. The clear supernatant was treated in one of two ways. In experiment (a) the supernatant was diluted to 25 ml in 0.05 M-potassium phosphate buffer, pH 7.4. In experiment (b) the supernatant was diluted in 0.02 M-phosphate buffer, pH 7.4, and incubated with cystine trisulphide (50 μ M) for 90 min at 4°C. Both samples were then centrifuged at 400000 g for 90 min. To the clear supernatant solutions (20 ml) was added an equal volume of water and β -mercaptoethanol (5 mM), and the solutions were fractionated by chromatography on DEAE-Sephadex. Sample (a) had 7300 units of activity when assayed alone; 19700 units when assayed with cystine trisulphide (5 μ M); 98 mg of protein. Sample (b) had 16800 units when assayed alone; 14600 units when

assayed with cystine trisulphide ($5\mu\text{M}$); 96mg of protein.

Chromatography on DEAE-Sephadex. Samples of aminolaevulinate synthetase (about 100mg of protein) were applied to a column ($2\text{cm}\times 17\text{cm}$) of DEAE-Sephadex A-25 previously equilibrated at 1°C with 0.03M -phosphate buffer, pH 7.4, containing 5mM - β -mercaptoethanol. The column was eluted at 20ml/h with 0.03M -phosphate buffer, pH 7.4 (10ml), followed by a linear gradient formed from 180ml each of 0.03M -phosphate buffer, pH 7.4, and 0.15M -phosphate buffer, pH 7.4; fractions (4.5ml) were collected. All buffers contained 5mM - β -mercaptoethanol. Samples ($100\mu\text{l}$) were assayed (90min, 37°C) for aminolaevulinate synthetase in the absence and in the presence of cystine trisulphide ($5\mu\text{M}$).

Results

Purification of the activators

The activators were purified by a modification of the method described previously (Neuberger *et al.*, 1973b). The earlier procedure involved six steps, and resulted in considerable loss of activity, owing to exposure of activator to neutral and alkaline pH values. The present procedure is rapid, involves only

three steps, and yields sufficient active product for analysis.

R. spheroides was grown semi-anaerobically in the light in medium containing [^{35}S]sulphate ($5.5\mu\text{Ci/mg-atom of S}$). Cells were harvested, washed and suspended to 100mg dry wt./ml in 1mM -potassium phosphate buffer, pH 7.4. Immediately after suspension the cells (6g) were disrupted by sonication, water (25ml) was added, and the preparation was centrifuged at $400000g$ for 90min at 4°C in an MSE 65 high-speed centrifuge. The clear supernatant (60ml) was stored at -20°C for 16h and then ultrafiltered on an Amicon UM-10 membrane at 1°C , and the ultrafiltrate (50ml) was fractionated on Dowex 50 by stepwise elution with HCl (Fig. 1). Two clearly resolved peaks of activator activity (AI and AII) were eluted with 2M -HCl. This activity was resolved from the bulk of the radioactivity, which was eluted as a single peak in fractions 36–50, and which consisted largely of GSSG and cystine. The active fractions under peak AI (fractions 53–62) were combined, as were those under peak AII (fractions 65–74), and the acid was removed by rotary evaporation at 40°C . Preparations of fractions AI and AII were rechromatographed on the same Dowex 50 system to remove the remaining traces of GSSG and cystine.

Fig. 1. Purification of activator extract on Dowex 50

Ultrafiltrate (50ml) was applied to a column ($0.9\text{cm}\times 10.0\text{cm}$) of Dowex 50 (X4; H^+ form; 200–400 mesh). The column was eluted at 36ml/h with (1) water (10ml), (2) 0.5M -HCl (50ml), (3) 1.0M -HCl (50ml), (4) 1.5M -HCl (50ml), (5) 2.0M -HCl (100ml) and (6) 4.0M -HCl (50ml), and fractions (3ml) were collected. Fractions were dried under vacuum in a desiccator containing NaOH, and portions (equivalent to $500\mu\text{l}$) were assayed for activator activity (●) and radioactivity (○).

Fractions AI and AII were further purified by preparative high-voltage electrophoresis at pH 2.0 for 50 min as described previously (Neuberger *et al.*, 1973b). Radioactive compounds were detected on the paper by radioautography. The fraction-AI preparation at this stage generally contained three major ^{35}S -labelled compounds with mobilities relative to alanine in this system of 0.45, 0.65 and 0.80. The fraction-AII preparation contained only one major ^{35}S -labelled compound, with a mobility relative to that of alanine of 0.50. All radioactive compounds were recovered from the paper by elution with water (Neuberger *et al.*, 1973b) and were assayed for activator activity. In the fraction-AII preparation the single major compound was active, whereas in the fraction-AI preparation only the compound with a mobility relative to alanine of 0.45 was active. The activator activity of the radioactive compounds after electrophoresis could also be assayed by adding a small square of the paper directly to the enzyme assay.

The material that was not adsorbed on the Dowex 50 (Fig. 1) and the fractions that were eluted with water were combined and dried by rotary evaporation at 40°C, followed by storage *in vacuo* for 20 h. This extract contained no significant activator activity. In some experiments, fractions which were eluted from the Dowex 50 column (Fig. 1) before application of 0.5 M-HCl were applied directly to a column of Dowex 1 (X2; Cl^- form; 200–400 mesh; 1 cm $\times$ 5 cm), and this column was developed by stepwise elution with acid (0.05–9.0 M-HCl). No significant activity was recovered from this column by this procedure.

Purity of the activator preparations

Both fractions AI and AII gave a single ^{35}S -labelled band when re-run on high-voltage electrophoresis at pH 2.0; the radioactivity corresponded exactly to a single band of ninhydrin reactivity. Both fractions AI and AII were analysed on a Locarte Mini amino acid analyser, which involved the elution of a 23 cm column with sodium citrate buffers (Moore & Stein, 1963), pH 3.25 (0.2 M) for 55 min, pH 4.25 (0.2 M) for 100 min and pH 6.65 (1.0 M) for 140 min at 50°C. The flow rate was 30 ml/h, and an SnCl_2 -ninhydrin system was used for colour development. Fraction AI was eluted as a single peak at 142.5 min (at the pH 4.25 buffer front), and fraction AII was eluted as a single peak at 154.5 min. These analyses showed that, in both preparations, contaminating amino acids contributed less than 5% of the total ninhydrin reactivity.

Identification of the activators

Analysis of fraction AI after oxidation with performic acid indicated that the preparation contained two compounds in approximately equal amounts. Attempts to separate these compounds were precluded by the small amount of material available. The analyses were consistent with an

equimolar mixture of the trisulphide of glutathione (GSSSG) and the mixed trisulphide of glutathione and cystine (GSSSCy). Thus when fraction AI was oxidized with performic acid, and fractionated by high-voltage electrophoresis at pH 2.0, fraction AI had disappeared and two new acidic, ninhydrin-positive, radioactive products were formed. These products were identified, by procedures described previously (Neuberger *et al.*, 1973a), as cysteic acid and the compound (GSO_3H) obtained on oxidation of glutathione with performic acid; the intensity of the radioactive bands on radioautography indicated that significantly greater amounts of GSO_3H than of cysteic acid had been formed.

Fraction AI was oxidized with performic acid, hydrolysed with 6 M-HCl (20 h, 110°C), and amino acids were determined on the Locarte amino acid analyser; the amounts of each product, relative to glutamic acid, which were obtained on two separate preparations are: glutamic acid 1.00 (1.00); glycine 1.08 (1.08); cysteic acid 1.41 (1.34). Parallel experiments were performed with GSH, and the values obtained were: glutamic acid 1.00 (1.00); glycine 1.08 (0.97); cysteic acid 0.98 (0.93). Thus the amounts of constituent amino acids formed on hydrolysis of fraction AI were consistent with 1 mol of glutamic acid or glycine per mol of trisulphide and between 1 and 2 mol of cysteic acid per mol of trisulphide. Analysis of fraction AI for cyanide-labile sulphur as a percentage of the total sulphur (see the Materials and Methods section) gave for three separate preparations 29.0, 32.1 and 34.6%. This value, together with the high activator activity of fraction AI per mol of sulphur (see below), makes it very unlikely that the preparation was contaminated with any GSSG or cystine.

Fraction AII behaved identically with synthetic cystine trisulphide (CySSSCy) in the following systems: (i) high-voltage electrophoresis at pH 2.0; (ii) the Locarte amino acid analyser system described above; (iii) fractionation on Dowex 50 (X4; 200–400 mesh; H^+ form) by stepwise elution with HCl as described above.

Further, when fraction AII was oxidized with performic acid it was converted into a single new compound, which was identified as cysteic acid by the procedure described previously (Neuberger *et al.*, 1973a). Also, analysis of fraction AII for cyanide-labile sulphur as a percentage of the total sulphur gave for three separate preparations 31.4, 33.0 and 33.7%. Analysis of synthetic $\text{U-}^{35}\text{S}$ -labelled cystine trisulphide (Neuberger *et al.*, 1973c) gave for three separate determinations 31.4, 31.5 and 32.4%.

Activator activity of fractions AI and AII

The activity of fractions AI, AII and synthetic cystine trisulphide is shown in Fig. 2. The concentrations of fractions AI and AII which were added to

Fig. 2. Activator activity of fractions AI and AII

Activators AI (●), AII (▲) and cystine trisulphide (■) were added, at a range of concentrations, to tubes containing substrate mixture, low-activity aminolaevalinate synthetase (about 0.25 mg of protein) and water (total volume 1.0 ml). Incubation was at 37°C for 90 min and the aminolaevalinate formed was determined (see the Materials and Methods section).

assays were determined from radioactivity measurements, assuming 3 mol of S/mol of activator. Synthetic cystine trisulphide and fractions AI and AII all produced a maximum of 4–5-fold activation at a concentration of 3 μM and also significant activations at 0.3 μM.

Activator activity extracted by other methods

During the development of the rapid extraction procedure given above, a number of modifications of the original method (Neuberger *et al.*, 1973b) were used. We found that if the procedure involved dialysis of whole or broken cells, the freeze-drying of these diffusates or the storage of samples in acid, then up to six active compounds could be separated by ion-exchange chromatography. Fractions AI and AII were always present in amounts which varied with the method of extraction.

Thus in one set of experiments sonicated fresh cells were extracted by dialysis against water. The diffusate was applied to a column (1 cm × 10 cm) of Dowex 50 (X4; H⁺ form; 200–400 mesh) and the effluent from this column was applied directly to another column (1 cm × 5 cm) of Dowex 1 (X2; Cl⁻ form; 200–400 mesh). Elution of the Dowex 50 column with HCl, as described in the legend to Fig. 1, produced fractions AI and AII and a further peak of activity (AIII) which was eluted with 4M-HCl. Fraction AIII was not identified; however, its elution behaviour is similar to that of cystine tetrasulphide

(CySSSSCy) (cf. Fletcher & Robson, 1963). Elution of the Dowex 1 column with HCl, as described in the legend to Fig. 3, produced one active compound (AIV) which was eluted with 0.5M-HCl immediately after the peak of inorganic sulphate. Fraction AIV was unstable on rechromatography in this system, and has not been identified.

In another series of experiments, whole cells were extracted by dialysis against water. The diffusate was concentrated by freeze-drying and applied to a column of Dowex 50. Elution of this column with HCl, as described in the legend to Fig. 1, produced fractions AI and AII only. The fractions eluted from this column before application of 0.5M-HCl were combined and, when assayed as a dilute solution, found to contain low, but significant, activator activity. When this solution (about pH 1.8) was concentrated by rotary evaporation at 40°C and stored *in vacuo* for 20 h, an increase in activator activity of approximately 20-fold was observed. If the dilute acid solution was neutralized before concentration, the product was virtually inactive, and if this inactive extract was subsequently stored in 1M-HCl at 22°C for 20 h, it became highly active. Such a preparation was fractionated on Dowex 1 (Fig. 3) and the activity was resolved into two peaks, AV and AVI.

Fraction AV was rechromatographed on the Dowex 50 system described in Fig. 1 and was eluted at the position for cystine trisulphide. Identification of fraction AV as cystine trisulphide was made by the methods described above for fraction AII.

Fraction AVI was rechromatographed on the Dowex 1 system, described in the legend to Fig. 3, with the application of an additional 25 ml of 6M-HCl. The activity was recovered with 70 ml of 6M-HCl and was associated with a peak of radioactivity which was eluted at the same position. This strong binding of fraction AVI to Dowex 1 suggested that it was an inorganic sulphur compound. The chromatography of oxy-acid anions of sulphur on anion-exchange resins was studied by Pollard *et al.* (1964b), and polysulphanedisulphonates ($S_nO_6^{2-}$, $n \geq 3$) were found to be eluted with high concentrations of HCl.

Fraction AVI behaved identically with authentic trisulphanedisulphonate ($S_3O_6^{2-}$) and could be distinguished from other polysulphanedisulphonates in each of the following systems: (i) Dowex 1 chromatography as described in the legend to Fig. 3 (cf. Pollard *et al.*, 1964b); (ii) high-voltage electrophoresis at pH 5.0 in the system described by Aminuddin & Nicholas (1973); (iii) t.l.c. on cellulose F in the solvent described by Pollard *et al.* (1955), $R_F = 0.43$; (iv) t.l.c. on cellulose F in butanol–acetone–water (2:2:1, by vol.); $R_F = 0.41$.

Fraction AVI also exhibited activator activity at μM concentrations, in agreement with the activity curve determined for authentic trisulphanedisulphonate (see below).

Fig. 3. Purification of activator extract on Dowex 1

The water wash from the chromatography on Dowex 50 of a diffusate of 7 g dry wt. of cells (see the text) was concentrated to a final volume of 5 ml and was adjusted with NaOH to pH 3.5. This was applied to a column (0.9 cm × 5.0 cm) of Dowex 1 (X2; Cl⁻ form; 200–400 mesh). The column was eluted at 40 ml/h with (1) water (30 ml), (2) 0.05 M-HCl (25 ml), (3) 0.10 M-HCl (25 ml), (4) 0.50 M-HCl (25 ml), (5) 1.0 M-HCl (25 ml), (6) 3.0 M-HCl (25 ml), (7) 6.0 M-HCl (25 ml) and (8) 9.0 M-HCl (50 ml), and fractions (4.5 ml) were collected. Fractions were dried under vacuum in a desiccator containing NaOH, and portions (equivalent to 100 μ l) were assayed for activator activity (●) and radioactivity (○).

These results show that, during dialysis of fresh sonicated cells, reactions occur leading to the formation of an unidentified activator (AIV). Also, when whole cells are dialysed and the diffusate is concentrated by freeze-drying, acidic compounds are generated which are not adsorbed to Dowex 50 in water and which, on exposure to acid, react or decompose to form trisulphanedisulphonate (AVI) and cystine trisulphide (AV).

The precursor of trisulphanedisulphonate (AVI), and of small amounts of disulphanedisulphonate, which was also identified in extracts, may be thio-sulphane (see below) or an organic sulphopolysulphate. The precursor of cystine trisulphide (AV) must be an acidic derivative of cystine, since it is eluted from Dowex 50 with water. It cannot be cysteic acid, since this is stable in HCl, but it may be an alanine sulphopolysulphane, since these compounds are known to be decomposed by strong acid (Waley, 1959). *S*-Sulphocysteine (Segal & Johnson, 1963) and alanine sulphodisulphane (Roy & Trudinger, 1970) were prepared, and samples were chromato-

graphed on Dowex 1 as described in the legend to Fig. 3. The compounds were eluted with 0.05 M-HCl and 0.10 M-HCl respectively. Neither of these compounds was an activator of aminolaevulinate synthetase before or after treatment with HCl, so that the precursor of cystine trisulphide in these extracts remains unknown. It is of interest, however, that these compounds have ion-exchange properties similar to those of fraction AIV described above. However, fraction AIV is a more acidic compound, so that if it were an alkylsulphopolysulphane it would have to be a higher homologue of alanine sulphodisulphane or perhaps a sulphopolysulphane derivative of glutathione.

Activator activity of other polysulphanes

The finding that cystine trisulphide and glutathione trisulphides had similar activating activity (Fig. 2) suggested that the trisulphane group alone is essential for activity and that the nature of the side chains is relatively unimportant. To test this further, we ex-

Fig. 4. Activator activity of inorganic sulphur compounds

Freshly prepared solutions of salts of the sulphur compounds $\text{S}_2\text{O}_3^{2-}$ ($\circ$), $\text{S}_2\text{O}_4^{2-}$ (Δ), $\text{S}_2\text{O}_6^{2-}$ ($\square$), $\text{S}_3\text{O}_6^{2-}$ (∇), $\text{S}_4\text{O}_6^{2-}$ ($\blacksquare$), $\text{S}_5\text{O}_6^{2-}$ ($\blacktriangle$) and $\text{S}_6\text{O}_6^{2-}$ ($\bullet$) were added, at a range of concentrations, to tubes containing substrate mixture, low-activity aminolaevulinate synthetase (about 0.25 mg of protein) and water (total volume 1.0 ml). Incubation was at 37°C for 90 min and the aminolaevulinate formed was determined.

amined a number of inorganic sulphur compounds, including the series of polysulphanedisulphonates ($^-\text{O}_3\text{S}-\text{S}_n-\text{SO}_3^-, n \geq 1$) for activity (Fig. 4). It is clear that only compounds with $n \geq 3$ produce significant activation of aminolaevulinate synthetase at concentrations below $10 \mu\text{M}$.

The greater the number of sulphur atoms in the molecule, the more effective is the compound as an activator. $\text{K}_2\text{S}_6\text{O}_6$ at $0.5 \mu\text{M}$ is as effective as cystine trisulphide or $\text{K}_2\text{S}_5\text{O}_6$ at $3 \mu\text{M}$. However, a 10% saturated aqueous solution of elemental sulphur (S_8) had no effect on the assay of aminolaevulinate synthetase. At concentrations greater than $20 \mu\text{M}$ most of these inorganic sulphur compounds interfere with the determination of aminolaevulinate by the method described earlier (Neuberger *et al.*, 1973a). In experiments with $\text{S}_2\text{O}_3^{2-}$, $\text{S}_2\text{O}_4^{2-}$ and $\text{S}_4\text{O}_6^{2-}$, an excess of anions was therefore removed from the incubation mixture by adsorption on to a column ($0.9 \text{ cm} \times 3 \text{ cm}$) of Dowex 1 (acetate form) and the aminolaevulinate was measured in the eluate. With this method we found that thiosulphate produced activation of aminolaevulinate synthetase at concentrations above $10 \mu\text{M}$ (Fig. 4). When, however, a solution of $\text{Na}_2\text{S}_2\text{O}_3$ ($10 \mu\text{M}$) was treated with 1 M-HCl for several hours, and the acid was removed, the sample now produced up to sevenfold activation at 1 – $10 \mu\text{M}$. This is clearly

Fig. 5. Fractionation on Dowex 50 of an extract of oxygenated cells

An ultrafiltrate (50 ml) prepared from 6 g of oxygenated semi-anaerobically grown cells (see under 'Purification of activator') was applied to a column ($0.9 \text{ cm} \times 10.0 \text{ cm}$) of Dowex 50 (X4; H^+ form; 200–400 mesh) at 36 ml/h . The column was eluted as described in the legend to Fig. 1, and fractions (3 ml) were collected. Fractions were dried under vacuum in a desiccator containing NaOH, and portions (equivalent to $500 \mu\text{l}$) were assayed for activator activity ($\bullet$) and radioactivity ($\circ$).

due to the formation of polysulphanedisulphonates under these conditions (Pollard *et al.*, 1964a), and it may explain the generation of trisulphanedisulphonate (AVI) in extracts of *R. spheroides* as described above.

We have also found that, when the trisulphane group is replaced by a selenodisulphane group, there is only a slight decrease in the activator activity. A sample of glutathione selenotrisulphide (GSSeSG)

was prepared, isolated and concentrated by the method described by Vernie *et al.* (1974), which was based on the method of Ganther (1971). A stable preparation of this compound (1 mM, in 0.1 M ammonium acetate, pH 5) when added at concentrations of 1–10 μ M increased the activity of aminolaevulinate synthetase threefold; this is similar to the effect of cystine trisulphide at these concentrations.

Fig. 6. Chromatography on DEAE-Sephadex of a preparation of aminolaevulinate synthetase (a) in which spontaneous activation had been prevented and (b) in which spontaneous activation had occurred

Preparation of enzyme samples and details of the chromatography are described in the Materials and Methods section. Samples of aminolaevulinate synthetase were chromatographed on DEAE-Sephadex with a linear concentration gradient of potassium phosphate buffer, pH 7.4. Fractions were assayed for aminolaevulinate synthetase in the absence ($\circ$) and in the presence ($\bullet$) of 5 μ M-cystine trisulphide. ----, Protein concentration, estimated by measurement of E_{280} ; —, phosphate concn.

Effect of growth conditions on the cellular content of trisulphides

Organisms grown semi-anaerobically in the light were estimated to contain 20–30 nmol of cystine trisulphide/g dry wt. and 5–10 nmol of glutathione trisulphides/g dry wt. These values were calculated from the amount of radioactivity in the purified activators recovered from preparative high-voltage electrophoresis, and are probably underestimates of the amounts present in whole cells. Oxygenation of a semi-anaerobic culture for 1 h resulted in a fall in the cellular content of trisulphides to undetectable amounts, that is to less than 0.5 nmol/g dry wt. The fractionation on Dowex 50 (Fig. 5) of an extract of oxygenated cells gave no activity in any of the eluted fractions (cf. Fig. 1). High-voltage electrophoresis at pH 2.0 of fractions which were eluted with 1.0M-, 1.5M- and 2.0M-HCl showed no radioactive compounds with rates of migration consistent with cystine trisulphide or glutathione trisulphides. The major sulphur compound of low molecular weight in oxygenated cells (fractions 54–64, Fig. 5) was identified as homolanthionine (cf. Neuberger *et al.*, 1973a). Homolanthionine does not inhibit activation of aminolaevalinate synthetase by trisulphides. Further, no activator activity was recovered from extracts of oxygenated cells in the fractions eluted from Dowex 50 before the application of 0.5M-HCl. It can be concluded that, during oxygenation of a semi-anaerobic culture of *R. spheroides*, trisulphides are either metabolically degraded or excreted into the culture medium.

Multiple forms of 5-aminolaevalinate synthetase in R. spheroides

Evidence for interconvertible low-activity and high-activity forms of aminolaevalinate synthetase in *R. spheroides* was first provided by Marriott *et al.* (1969), who described a spontaneous activation of the enzyme present in crude cell-free extracts. Subsequent work (Marriott *et al.*, 1970; Neuberger *et al.*, 1973a) suggested the importance of the spontaneous activation for an understanding of the control of the enzyme *in vivo*. Different forms of the enzyme were separated by chromatography on DEAE-Sephadex (Tuboi *et al.*, 1970), and evidence was presented by Hayasaka & Tuboi (1974) for two low-activity forms (FIb and FIIb) and two high-activity forms (FIa and FIIa) of aminolaevalinate synthetase. Activation of the enzyme (b-form to a-form) occurred on incubation with cystine and activating enzyme, whereas inactivation (a-form to b-form) was achieved by incubation with a thiol and a particulate fraction from *R. spheroides*.

To clarify the role of these multiple forms in the control of the enzyme *in vivo* in relation to our own findings, we have studied the effect of spontaneous

activation and of activation with cystine trisulphide on the relative proportions of a-form and b-form enzyme as determined by chromatography on DEAE-Sephadex A-25.

The elution profile shown in Fig. 6(a) was obtained with an extract of semi-anaerobically grown cells when spontaneous activation was prevented by gel filtration of the extract immediately after its preparation (Marriott *et al.*, 1969). The profile shown in Fig. 6(b) was obtained in a parallel experiment in which a sixfold spontaneous activation of the enzyme was allowed before chromatography. Fractions from both columns were assayed with and without cystine trisulphide (5 μ M) and the recovery of enzyme activity was between 85 and 110% in each experiment. In the first experiment (Fig. 6a) three peaks of enzyme activity were observed. They have been labelled Ia, IIb and Ib to indicate their probable identity with the forms described by Tuboi & Hayasaka (1973). Form Ia is a 'high-activity' form, which is not affected by the addition of trisulphide to the assay, whereas form Ib is a 'low-activity' form, which is activated up to fivefold by the addition of cystine trisulphide. Form IIb is also a low-activity form, which is activated about 1.5–2.0-fold by trisulphide. In the second experiment (Fig. 6b), only one peak of activity was observed, and the activity of this form was not affected by trisulphide in the assay. Further, its elution position was consistent with it being form Ia. It appears that spontaneous activation is the result of the conversion of forms Ib and IIb into form Ia. It is possible, however, that peak Ia observed in our experiments contains more than one high-activity form of the enzyme (cf. Hayasaka & Tuboi, 1974).

When O₂ is bubbled through a culture of *R. spheroides* growing semi-anaerobically, and samples are taken at intervals, there is a progressive fall in the degree of spontaneous activation observed in cell-free extracts (Marriott *et al.*, 1969). After oxygenation, the aminolaevalinate synthetase is in a stable low-activity form which does not show spontaneous activation but which can be activated by the addition of cystine trisulphide (Neuberger *et al.*, 1973c). The profile shown in Fig. 7(a) was obtained for the enzyme extracted from semi-anaerobically grown cells which had been oxygenated for 1.5 h immediately before they were harvested. The results shown in Fig. 7(b) were obtained on fractionation of an identical preparation which had been activated with cystine trisulphide before chromatography. The untreated sample (Fig. 7a) contained forms Ib and IIb but not form Ia, whereas the sample treated with trisulphide contained mainly form Ia, but not forms Ib and IIb. It is therefore clear that the activation of low-activity aminolaevalinate synthetase with cystine trisulphide results in the conversion of forms Ib and IIb into form Ia. This appears to be the same conversion as is observed during the spontaneous activation.

Fig. 7. Chromatography on DEAE-Sephadex of low-activity aminolaevulinate synthetase (a) untreated or (b) after treatment with cystine trisulphide

Preparation of enzyme samples and details of the chromatography are described in the Materials and Methods section. Samples of low-activity aminolaevulinate synthetase were chromatographed on DEAE-Sephadex with a linear concentration gradient of potassium phosphate buffer, pH 7.4. Fractions were assayed for aminolaevulinate synthetase in the absence ($\circ$) and in the presence ($\bullet$) of 5 μ M-cystine trisulphide. ----, E_{280} ; —, phosphate concn.

Discussion

Trisulphides as natural activators of aminolaevulinate synthetase

In a previous paper (Neuberger *et al.*, 1973b) we discussed the control of aminolaevulinate synthetase

in *R. spheroides* in terms of endogenous activators of the enzyme. The structures of the activators were unknown at that time. They have now been identified as cystine trisulphide and the mixed trisulphide of cystine and glutathione. To our knowledge this is only

the second report of an organic trisulphide being isolated from a biological source. Lenthionine (1,2,3,5,6-pentathiepane), a cyclic polysulphide, was isolated from the edible mushroom *Shiitake lentinus edodes* (Morita & Kobayashi, 1966). This compound appears to be formed enzymically from a glutathione-type precursor present in dried extracts (Yasumoto *et al.*, 1971).

We modified our original extraction procedure for the activators (Neuberger *et al.*, 1973b) in order to eliminate the possibility of trisulphides being formed during extraction. Trisulphides are known to be formed in a variety of non-enzymic reactions. For example, both the oxidation of glutathione in air, catalysed by traces of Fe^{3+} or Cu^{2+} at alkaline pH values (Massey *et al.*, 1971), and the reaction of cystine with sodium sulphide in 0.1M-NaOH (Villarejo & Westley, 1963), generate persulphides, and therefore possibly trisulphides. Such reactions are unlikely to have occurred during centrifugation and ultrafiltration of our extracts at neutral pH values. In addition, treatment of cystine with acid generates cystine trisulphide (Fletcher & Robson, 1963). The trisulphides isolated from *R. spheroides*, however, could not have been formed in this way, since the extracts were not treated with acid before chromatography on Dowex 50. Further, we have found that fractionation of cystine in HCl on Dowex 50 did not generate trisulphide (Neuberger *et al.*, 1973c). Although a non-enzymic synthesis of these compounds seems very unlikely, we cannot exclude the possibility of their enzymic formation during the extraction procedure. Thus we have demonstrated (Wider de Xifra *et al.*, 1975) that cystathionase (EC 4.4.1.1) from *R. spheroides* converts cystine into its trisulphide. It seems likely that the trisulphides described are normal constituents of *R. spheroides*, and that at least one function of these compounds is the control of the activity of aminolaevulinate synthetase. The preponderance of cystine trisulphide over other trisulphides in extracts of *R. spheroides* might indicate that this is the regulator of aminolaevulinate synthetase *in vivo*. Glutathione trisulphides may be formed by exchange reactions of the cystine trisulphide with glutathione.

Multiple forms of aminolaevulinate synthetase

We showed previously that aminolaevulinate synthetase from *R. spheroides* can exist in low-activity and high-activity forms, and that activation occurs in the presence of cystine trisulphide (Neuberger *et al.*, 1973c). Tuboi & Hayasaka (1972) separated low-activity and high-activity forms of the enzyme by chromatography of extracts on DEAE-Sephadex. Activation of the low-activity form was achieved by disulphides in the presence of an endogenous activating enzyme. To compare the two methods of activating the enzyme, we have used this DEAE-Sephadex

procedure to investigate the forms present in our extracts before and after activation, and to study the effect of cystine trisulphide on these different forms.

We have confirmed the presence of at least three forms of aminolaevulinate synthetase in *R. spheroides*, and have obtained an elution pattern for enzyme extracted from semi-anaerobic cells similar to that previously reported (Fig. 5a of Tuboi & Hayasaka, 1971). It appears that the b-forms of the enzyme can be converted into the a-form by treatment with cystine trisulphide. This conversion seems to be identical with that obtained by Tuboi & Hayasaka (1972, 1973), who used cystine and activating enzyme. Cystine trisulphide can be formed from cystine on incubation with cystathionase purified from *R. spheroides* (Wider de Xifra *et al.*, 1975), and it is therefore possible that the activating enzyme purified by Hayasaka & Tuboi (1974) is cystathionase. The chromatographic properties of the two enzymes on DEAE-Sephadex are similar, but the apparent molecular weight of the activating enzyme (90000) is higher than that of bovine serum albumin, whereas that of cystathionase appears to be much lower (Wider de Xifra *et al.*, 1975).

The mechanism by which cystine trisulphide activates aminolaevulinate synthetase is unknown. It seems likely, however, that activation results from a direct interaction of enzyme with trisulphide (Neuberger *et al.*, 1973b). Both organic trisulphides and inorganic polysulphanes have been found to activate aminolaevulinate synthetase, and there appears to be no strict structural requirement for an efficient activator molecule, apart from the presence of a trisulphane group. The S atoms in these polysulphane compounds are very reactive towards various nucleophilic reagents, such as cyanide, thiols and sulphite, and such reactions lead to the transfer of S to the nucleophile. Activation of aminolaevulinate synthetase by cystine trisulphide, or by any other polysulphane, may therefore involve a transfer of a sulphane S atom from the activator into the enzyme. Such an interaction would be expected to produce an enzyme-bound persulphide (E-S-SH) or trisulphide

Alternatively the trisulphide may convert adjacent thiols on the enzyme into an intramolecular disulphide bridge (cf. Tuboi & Hayasaka, 1973). Such a reaction may either involve the release of the sulphane S atom of the trisulphide as sulphide, or occur catalytically. Both of these latter reactions have a precedent in the oxidation of GSH to GSSG by cytochrome c (Massey *et al.*, 1971), which is very effectively catalysed by μM concentrations of cystine trisulphide or

glutathione trisulphide. It is also possible that a thiol-catalysed exchange reaction may occur between the trisulphide and an enzyme disulphide, producing an enzyme trisulphide. To decide which, if any, of these activation mechanisms occurs with aminolaevulinate synthetase will require further experimental work.

There are several reports of sulphane S being a normal constituent of enzyme proteins. Both xanthine oxidase (Massey & Edmondson, 1970) and aldehyde oxidase (Branzoli & Massey, 1974) appear to contain a persulphide group. Both enzymes are inactivated when S is removed from the protein by treatment with cyanide. Oxidation of ferredoxin and several other iron-sulphur proteins can lead to the conversion of the enzyme-bound 'labile' sulphide into a protein-bound trisulphide (Petering *et al.*, 1970). Further, the reconstitution of inactive ferredoxin by the insertion of S has been achieved with rhodanese in the presence of thiosulphate (Finazzi-Agrò *et al.*, 1971). Rhodanese itself appears to contain sulphane sulphur either as a persulphide or as a trisulphide (Westley, 1973).

Control of aminolaevulinate synthetase in R. sphaeroides

A major aim of the present work was to investigate the role of the different forms of the enzyme in the control of aminolaevulinate synthetase *in vivo*. We were particularly interested in the changes taking place during the rapid fall in enzyme activity which occurs when a semi-anaerobic culture is oxygenated. Isolation of the enzyme, from cells grown semi-anaerobically, by the three-step method of Tuboi & Hayasaka (1971) gave almost exclusively the a-form. The small amount of b-form that was present initially in crude extracts was always converted into the a-form during the purification, generally after fractionation with $(\text{NH}_4)_2\text{SO}_4$. The greater amount of b-form present in crude extracts of oxygenated cells was also partially converted into the a-form during purification. Thus, when a crude extract, which gave the pattern shown in Fig. 7(a), was purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation, and Sephadex G-200 and DEAE-Sephadex column chromatography, the product then showed a pattern similar to that given in Fig. 6(a). It is clear, therefore, that analysis of extracts after this purification procedure does not give an accurate indication of the forms present in the extracts immediately after disruption of the cells. To overcome these difficulties, supernatants from the high-speed centrifugation of freshly disrupted cells were fractionated directly on DEAE-Sephadex, both with and without β -mercaptoethanol. The patterns obtained were essentially the same whether or not β -mercaptoethanol was present (Figs. 6 and 7), except that the recoveries of activity were very poor in its absence. It therefore seems unlikely that β -mercaptoethanol has converted any of the a-form into the b-form in these

extracts (cf. Tuboi & Hayasaka, 1973). Consequently the patterns shown in Figs. 6 and 7 probably describe the mixture of forms in the crude extracts.

The enzyme pattern shown in Fig. 6(b), for an extract after spontaneous activation, probably represents the form of the enzyme present in cells growing semi-anaerobically and synthesizing bacteriochlorophyll at a high rate. This has been discussed in detail in a previous paper (Neuberger *et al.*, 1973a). Comparison of Figs. 6(b) and 7(a) would indicate that the loss of aminolaevulinate synthetase activity, produced by oxygenation of a growing culture, is the result of the disappearance of a-form enzyme in the cell. There are several possible explanations for this loss.

First, the present work shows that there is a coincidental loss of cystine trisulphide. This may result from either the depletion in the cellular content of cystine (Neuberger *et al.*, 1973a), which is a substrate for the synthesis of trisulphide (Wider de Xifra *et al.*, 1975), or the removal of a sulphane sulphur from the trisulphide (see below). This loss of trisulphide would retard any further conversion of b-form into a-form enzyme. Secondly, in addition to removal of trisulphide, there appears to be a conversion of the a-form into the b-form. Inactivation of the a-form has been demonstrated in the presence of a high concentration of thiol reagent (Tuboi & Hayasaka, 1973) and apparently also occurs under reducing conditions during harvesting of cells (Neuberger *et al.*, 1973a). It is difficult, however, to understand how oxygenation could produce such reducing conditions in the cell. The loss of the a-form and its apparent conversion into the b-form during oxygenation may not, however, be a reductive process but may result from the removal of sulphane S. This reaction, and the equivalent reaction with cystine trisulphide, would require the presence of a thiophile, such as sulphite.

Thus, if there is a dynamic equilibrium between the b-form and the a-form of the enzyme in the cell, the relative amounts of the two forms would be determined by the cellular content of cystine and also possibly by that of the thiophile (Fig. 6 of Wider de Xifra *et al.*, 1975).

Such processes do not, however, fully explain the observed decrease in enzyme activity during oxygenation, since there always appears to be an irreversible loss of about 50% of the total activity (cf. Marriott *et al.*, 1969). Possible explanations, such as repression of enzyme synthesis, the conversion of the enzyme into an inactive form and the accumulation of an inhibitor of the enzyme during oxygenation, have not been investigated in this work.

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