Purification and properties of uroporphyrinogen III synthase (co-synthetase) from *Euglena gracilis**

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Uroporphyrinogen III synthase (co-synthetase) purified from *Euglena gracilis* is a monomer of M_r 38 500 by gel-filtration studies and 31 000 by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The pI is apparently in the range 4.8–5.1. No evidence for any cofactors was found, and folate derivatives were shown to be absent; no metal ions appear to be present in the enzyme. The K_m for hydroxymethylbilane is in the range 12–40 μM , and the product, uroporphyrinogen III, is an inhibitor. Modification studies suggest that arginine residues are essential for the activity of co-synthetase; lysine residues may also be essential, but histidine, cysteine and tyrosine residues are not.

INTRODUCTION

The pigments of life, including haem, cytochromes, chlorophylls and vitamin B-12, all have as a common precursor the tetrapyrrolic macrocycle uroporphyrinogen III [(3) in Scheme 1] (Battersby *et al.*, 1980). The biosynthesis of this macrocycle from porphobilinogen (PBG) (1) requires two enzymes, hydroxymethylbilane

synthase (PBG deaminase, EC 4.3.1.8) and uroporphyrinogen III synthase (commonly called co-synthetase, EC 4.2.1.75). The role of PBG deaminase is to join together four molecules of PBG to form a linear unrearranged tetrapyrrole, which is released from the enzyme as the hydroxymethylbilane (2). This bilane is the substrate for co-synthetase, the role of which is to ring-close the linear bilane with concomitant intramolecular rearrangement of ring D to form uroporphyrinogen III (Battersby *et al.*, 1982*a,b*, and references cited therein). Others independently detected the intermediate released by deaminase and showed its role as substrate for co-synthetase, but the structure they deduced for it differed from hydroxymethylbilane (2) and was incorrect (Burton *et al.*, 1979, and references cited therein).

PBG deaminase has been purified from a number of different sources and studied in detail (Jordan & Shemin, 1973; Frydman & Feinstein, 1974; Higuchi & Bogorad, 1975; Anderson & Desnick, 1980; Shioi *et al.*, 1980; Williams *et al.*, 1981; Battersby *et al.*, 1983*a,b*; Hart *et al.*, 1984; Williams, 1984), but less is known about co-synthetase. Partial purifications of various co-synthetase preparations have been carried out by Sancovich *et al.* (1969), Levin (1971), Frydman & Feinstein (1974), Higuchi & Bogorad (1975) and Clement *et al.* (1982), but few studies have been made of the properties of these materials. More recently Kohashi *et al.* (1984) purified co-synthetase from rat liver to a high degree and suggested that a folate derivative is an important component of the enzyme.

The present paper reports our isolation of high-quality co-synthetase from *Euglena gracilis* together with studies of the properties of this enzyme. This work complements the work on PBG deaminase from *E. gracilis* (Williams *et al.*, 1981; Battersby *et al.*, 1983*a,b*; Hart *et al.*, 1984) and also the earlier investigation of the substrate specificity of co-synthetase from this source (Battersby *et al.*, 1981).

Scheme 1. Biosynthesis of uroporphyrinogen III

Abbreviations used: PBG, porphobilinogen; f.p.l.c., fast protein liquid chromatography.

* This paper is considered as part 26 of the series 'Biosynthesis of Porphyrins and Related Macrocycles'. Part 25 is Robinson *et al.* (1985).

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EXPERIMENTAL

Materials

Tris (Trizma grade), Bistris, butane-2,3-dione, phenylglyoxal, methyl methanethiosulphonate, iodoacetamide, pyridoxal 5'-phosphate, diethyl pyrocarbonate, Rose Bengal, phenylmethanesulphonyl fluoride, pepstatin, pteroylglutamic acid, tetrahydropteroylglutamic acid and 5-methyltetrahydropteroylglutamic acid were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Tetranitromethane was from Aldrich Chemical Co., Gillingham, Dorset, U.K. Hydroxylamine was obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K., and HA-Ultrogel from LKB, Croydon, Surrey, U.K. PBG was prepared as described previously (Battersby *et al.*, 1977). Formylbilane octamethyl ester and hydroxymethylbilane octamethylester were prepared, purified and characterized as described by Battersby *et al.* (1982b). These esters were hydrolysed overnight at room temperature in N_2 -saturated 2 M-KOH (0.1 ml/mg of bilane) under N_2 in screw-cap vials. Immediately before use the solutions were diluted with N_2 -saturated ice-cold deionized distilled water to adjust the final concentration of KOH to 0.8 M, and were then stored on ice and protected from light with metal foil; they were freshly prepared for all enzymic experiments. Other chemicals were analytical-reagent grade whenever available, or the purest available grade, and were obtained from Fisons Chemicals, Loughborough, Leics., U.K., or BDH Chemicals, Poole, Dorset, U.K. Deionized distilled water was used throughout.

Rapid assay for comparing relative amounts of co-synthetase

When PBG deaminase acts alone on PBG (1), the released hydroxymethylbilane (2) accumulates (the lag phase) and only relatively slowly ring-closes non-enzymically to uroporphyrinogen I (5), which is determined spectrophotometrically (Battersby *et al.*, 1982a). In contrast, excess co-synthetase in the assay causes a rapid enzymic ring closure of the hydroxymethylbilane (2) to uroporphyrinogen III (3), and the plot of uroporphyrinogen III formed against time is linear with no lag phase (Battersby *et al.*, 1982a,b). This difference led to a so-called 'lag assay', which allowed the relative amounts of co-synthetase in a number of fractions to be quickly determined. A similar approach has been used by Jordan *et al.* (1980) to assay for co-synthetase activity in extracts from *Rhodospseudomonas spheroides*.

The conditions used in the 'lag assay' were as follows. PBG deaminase (sufficient to give in the absence of co-synthetase an absorbance at 406 nm of 0.1–0.2 when the assay mixture was treated as described below), co-synthetase (such that ΔA_{406} due to added co-synthetase was less than 0.25) and PBG (550 μ M) were incubated at 37 °C for 3 min in 0.2 M-sodium phosphate buffer, pH 8.0, that contained 0.6 mM-EDTA in a final volume of 0.75 ml. The assay was initiated by the addition of PBG, and after 3 min of incubation the reaction was stopped and porphyrinogens were oxidized to porphyrins by the addition of I_2/KI [0.4 ml of 0.5% (w/v)/1% (w/v) solution]. Excess of I_2 was reduced 3 min later by sodium metabisulphite solution [0.1 ml of freshly prepared 1% (w/v) solution], 1.75 ml of 7.1% (w/v) trichloroacetic acid solution was added and the porphyrin content was

determined by measuring A_{406} . The control value (A_{406} for assays that were 'deaminase alone') was subtracted from the values obtained when co-synthetase was present in assays.

Co-synthetase was assayed quantitatively and kinetic assays were performed by monitoring uroporphyrinogen formation (determined as the porphyrin) with synthetic hydroxymethylbilane as substrate (Battersby *et al.*, 1982b). Assays were performed at 25 °C (unless otherwise stated) in 0.5 ml of 0.2 M-Tris/HCl buffer, pH 8.25 (containing 0.6 mM-EDTA), and were initiated by addition of hydroxymethylbilane at 83 μ M final concentration for the standard assays or as appropriate for the kinetic assays. These additions of substrate were made as 10 μ l of a solution in 0.8 M-KOH for the standard assay or as required for kinetic assays. Before addition of enzyme and hydroxymethylbilane, an appropriate quantity of 0.8 M-HCl was added to the above buffer to neutralize the KOH added with the bilane. Samples (four or five of 50 μ l over 2 min) were removed after the initiation of the reactions, and added to 0.4 ml of I_2 solution (as above) in 0.7 ml of water. Sodium metabisulphite solution and trichloroacetic acid were added 3 min later (as above), and A_{406} values were determined. The initial rates of porphyrin production were determined from plots of A_{406} against time of sampling. Assays were also performed in the absence of co-synthetase to determine the non-enzymic rate of ring closure of the hydroxymethylbilane, and subtraction from the rate observed in the presence of co-synthetase gave the true enzymic rate. The amount of co-synthetase used was such that the change in A_{406} per min was not more than 0.1, which ensured that progress plots were linear for at least 2 min; thus initial rates could be accurately determined.

One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 nmol of uroporphyrinogen/h under the conditions specified.

Determination of protein concentrations

Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin as standard, the latter being determined in solution by using an $A_{1\text{cm}, 279}^{1\%}$ value of 6.67 (Janatova *et al.*, 1968).

Spectra

U.v.-visible-absorption spectra were recorded on a Pye-Unicam SP8-100 recording spectrophotometer, and fluorescence spectra on a Perkin-Elmer 3000 fluorescence spectrometer equipped with a Servoscribe recorder.

Purification of PBG deaminase

PBG deaminase was purified from *E. gracilis* essentially as described by Battersby *et al.* (1983a), except that the heat step, which destroys co-synthetase activity, was omitted. This allowed PBG deaminase and co-synthetase to be isolated from the same broken-cell extract.

Purification of co-synthetase

All steps were performed at 0–4 °C, and all buffers contained 0.6 mM-EDTA and 0.1 mM-dithiothreitol unless otherwise stated. Deaminase/co-synthetase preparations were obtained essentially as described previously (Battersby *et al.*, 1982a), except that the gel filtration was performed in 15 mM-Tris/HCl buffer, pH 8.75 (the pH was adjusted at 4 °C) on Sephadex G-75. Active fractions

from gel filtration (representing material from 80–160 litres of *E. gracilis* culture) were then applied, and washed on with more buffer (30 ml), to a column (1.7 cm × 23 cm) of Whatman DE-52 DEAE-cellulose equilibrated with the same buffer. Elution at 40 ml/h was with a linear gradient made by running 400 ml of 15 mM-Tris/HCl buffer, pH 8.75, containing 65 mM-NaCl into 400 ml of 15 mM-Tris/HCl buffer, pH 8.75, containing 13.3 mM-NaCl and then continued with the buffer containing 65 mM-NaCl; 14 ml fractions were collected. Deaminase appeared in five to seven fractions within fractions 22–32, and co-synthetase was eluted (in about 15 fractions) in the region of fractions 40–60. These conditions for the separation of deaminase and co-synthetase up to this stage and the 'lag assay' above were developed by Dr. G. W. J. Matcham for earlier work (Battersby *et al.*, 1981, 1982b, 1983b). The pooled co-synthetase fractions were immediately concentrated to 8–10 ml in an Amicon ultrafiltration apparatus with a UM 10 membrane, dialysed against 10 mM-sodium acetate buffer, pH 5.45, and then applied to a column of DE-52 DEAE-cellulose (1.4 cm × 14 cm) equilibrated with the same buffer. The column was washed with a gradient made by running 250 ml of 8 mM-sodium acetate buffer, pH 4.85, into 250 ml of 10 mM-sodium acetate buffer, pH 5.45, and 11 ml fractions were collected at a flow rate of approx. 30 ml/h. The peak of co-synthetase activity was eluted at pH 4.95 ± 0.1 (mean $\pm$ S.D. for eight preparations) and the active fractions were combined, adjusted to approx. pH 7.5 by the addition of 1 M-Tris and concentrated to 2–3 ml by ultrafiltration. The concentrated enzyme was dialysed against buffer of the appropriate pH and ionic strength before use. For storage, dialysis against 0.1 M-sodium phosphate buffer, pH 7.0, was used. At 0–4 °C in this buffer, at a protein concentration of 1–2 mg/ml, the enzymic activity had a half-life of about 2 weeks, but was stable for several months when stored deep-frozen. Repeated freezing and thawing rapidly inactivated the enzyme.

Further purification of enzyme from the DEAE-cellulose column (up to 25 mg total protein) has been achieved by chromatofocusing on a Mono P HR 5/20 column attached to a Pharmacia f.p.l.c. system after dialysis of the enzyme and equilibration of the column against 15 mM-BisTris/HCl buffer, pH 5.95. When the protein had been loaded and washed on to the column with a further 5–10 ml of equilibration buffer, the column was developed at a flow rate of 1 ml/min with a 1:10 dilution of Polybuffer 74/HCl, pH 4.2. The chromatography was run at room temperature, but the fractions (1 ml) collected on ice. Co-synthetase activity was eluted at pH 4.9 ± 0.1 . This method is quicker than the pH-gradient chromatography, but it gave the same yield, and enzyme of only slightly higher specific activity.

To obtain larger amounts of co-synthetase, several preparations (as above) were carried up to and through the DE-52 DEAE-cellulose step. The concentrated enzyme samples (in 65 mM-NaCl/15 mM-Tris/HCl buffer, pH 8.75) were stored frozen. The combined material from 550 litres of *E. gracilis* culture was thawed and taken through pH-gradient chromatography as described above, except that all buffers here and for later steps contained 1 mM-phenylmethanesulphonyl fluoride, added from an ethanolic 0.1 M solution, and pepstatin (1 mg/l). The active fractions were adjusted (as above) to pH 7.5, and concentrated by ultrafiltration to approx.

6 ml, dialysed overnight against 10 mM-sodium phosphate buffer, pH 6.8, and applied to a column of HA-Ultrogel (1.4 cm × 10 cm) previously equilibrated with the same buffer. This was also used for elution at a flow rate of 20 ml/h; 6 ml fractions were collected. The fractions containing co-synthetase were combined, concentrated to about 8 ml by ultrafiltration and dialysed overnight against 15 mM-Tris/HCl buffer, pH 8.75.

Further purification was achieved by f.p.l.c. with a Pharmacia system and a Mono Q HR 5/5 column. The gradient used was 0–120 mM-NaCl in 15 mM-Tris/HCl buffer, pH 8.75 (at 4 °C), the gradient volume was 20 ml and the flow rate was 2 ml/min. The column was run at approx. 15 °C, but fractions were collected on ice. Co-synthetase activity was eluted in a volume of 2.4 ml at an NaCl concentration of about 95 mM; this solution was concentrated to approx. 1 ml in a dialysis bag surrounded with solid sucrose (2.5 h at 4 °C). The outside of the bag was rinsed with 50 mM-sodium phosphate buffer, pH 7.0, containing dithiothreitol (0.1 mM), and then the enzyme solution was dialysed against several changes of the last-mentioned buffer. The enzyme showed one major band (estimated by eye to account for 60–70% of the total protein) and two minor ones on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (see below); it was used for spectroscopic analysis (see below).

M_r determination

The M_r value for co-synthetase was determined by gel filtration on a Sephadex G-100 column (2.5 cm × 54 cm) equilibrated at 4 °C with 50 mM-sodium phosphate buffer, pH 7.0, containing 0.2 M-NaCl and 0.1 mM-dithiothreitol. The standards used (with their M_r values in parentheses) were bovine serum albumin (68000), ovalbumin (45000), chymotrypsinogen (25000), ribonuclease A (13360) and cytochrome *c* (12400). The void volume of the column (V_0) was obtained by using Blue Dextran. In all runs, 0.6 ml samples were applied to the column, 5 ml fractions were collected and protein concentrations for the standards were monitored by A_{280} measurements. Blue Dextran was monitored by A_{600} , and co-synthetase was determined by its activity in the 'lag assay' (see above). The elution volume (V_e) for each sample was taken as that of the peak in the elution profile, and M_r for co-synthetase was assessed by interpolation in a plot of $\log M_r$ against V_e/V_0 (Andrews, 1964).

The M_r value for co-synthetase under denaturing conditions was determined by using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by the method of Weber & Osborn (1969) modified such that samples were denatured in the presence of sodium dodecyl sulphate by incubation at 100 °C for 2 min. The standards used, except for ribonuclease, were those listed above for the gel-filtration experiment, and M_r for co-synthetase was determined from a plot of $\log$ (apparent M_r) against mobility relative to a Bromophenol Blue marker.

Modification of amino acid residues and effect of metal chelators

Appropriate controls were carried out for all experiments below, and co-synthetase was shown to be at least 96% stable under the conditions used.

(a) **Butane-2,3-dione and phenylglyoxal.** Mixtures of co-synthetase (1.9×10^4 units/ml) and various concentrations of butanedione (up to 40 mM) were made in 50 mM-sodium borate buffer, pH 9.0, at 25 °C. Samples (normally 15 μ l) were removed at intervals and assayed in the standard way. Modification of co-synthetase with phenylglyoxal was carried out at 25 °C in 0.1 M-sodium bicarbonate buffer, pH 8.4.

(b) **Methyl methanethiosulphonate and iodoacetamide.** Enzyme samples for these experiments were freed from dithiothreitol by dialysis against 50 mM-sodium phosphate buffer, pH 8.0. Mixtures of co-synthetase (1.4×10^4 units/ml) and methyl methanethiosulphonate (0.2 mM) were incubated on ice in 50 mM-sodium phosphate buffer, pH 8.0, and 5 μ l samples were assayed. Co-synthetase (1.5×10^4 units/ml) and iodoacetamide (0.36 mM) were incubated in 50 mM-sodium phosphate buffer, pH 8.0, at 25 °C, and 10 μ l samples were assayed.

(c) **Pyridoxal 5'-phosphate.** Co-synthetase (1.9×10^3 units/ml) and various concentrations of pyridoxal 5'-phosphate (up to 8 mM) were incubated in the dark at 25 °C in 0.1 M-sodium phosphate buffer, pH 7.0, and 40 μ l samples were assayed. Mixtures of co-synthetase and pyridoxal 5'-phosphate were reduced by the addition of the amount of freshly prepared 80 mM- NaBH_4 solutions in ice-cold 0.1 M-phosphate buffer, pH 7.0, that decolorized the solutions within 2–3 s. The reduced samples were dialysed against 0.1 M-phosphate buffer, pH 7.0, for 3 h before assay.

(d) **Diethyl pyrocarbonate.** Co-synthetase (5.2×10^3 units/ml) and various concentrations of diethyl pyrocarbonate (up to 0.41 mM) were incubated at 25 °C in 0.1 M-sodium phosphate buffers, pH 6 and pH 7, and 20 μ l samples were assayed. Diethyl pyrocarbonate in propan-2-ol solution was assayed by the addition of portions to 10 mM-imidazole/HCl buffer, pH 7.0, and was found to be 80–90% pure; $\epsilon = 2.95 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 240 nm was used for *N*-ethoxycarbonylimidazole (Dickenson & Dickinson, 1975). The incubation mixtures contained 3–5% (v/v) of propan-2-ol, a concentration shown not to effect co-synthetase activity.

(e) **Light and Rose Bengal.** Co-synthetase (1.4×10^4 units/ml) and Rose Bengal (7 μM) in 0.1 M-sodium phosphate buffer, pH 7.0, or in 15 mM-Tris/HCl buffer, pH 8.75, were incubated at 20 ± 1 °C in tubes (1 cm $\times$ 10 cm) held in a beaker (1 litre) of water and illuminated by a 40 W tungsten bulb, centred 8 cm from the tubes; 10 μ l samples were assayed.

(f) **Tetranitromethane and iodine.** Co-synthetase (1.3×10^4 units/ml) and tetranitromethane (0.26 mM or 0.51 mM) were incubated at 25 °C in 50 mM-Tris/HCl buffer, pH 8.0 (measured at 20 °C), and 20 μ l samples were assayed. Tetranitromethane was prepared in 95% ethanol and the ethanol carried over into the mixtures was 2.5% or 5% (v/v), amounts shown not to affect co-synthetase activity. Various amounts of aqueous I_2/KI (0.45 mM/2 mM) were added to co-synthetase (0.1 ml/740 units) in 50 mM-sodium phosphate buffer, pH 7.0, at 0 °C. After the I_2 had been decolorized (within 1 min), samples (15–20 μ l) were assayed. With the highest

I_2 concentration (185 μM), the I_2 colour remained after 10 min.

(g) **1,10-Phenanthroline and 2,2'-bipyridyl.** Co-synthetase was dialysed before use against 0.1 M-sodium phosphate buffer, pH 7.0, from which EDTA had been omitted, and portions (7.3×10^3 units/ml) were incubated in the dark at 0 °C with 1,10-phenanthroline (3 mM) or 2,2'-bipyridyl (3 mM) before assay (30 μ l samples).

(h) **Hydroxylamine, azide and cyanide.** The effects of NH_2OH , NaN_3 and KCN were studied by performing assays in the presence of various concentrations of each of these reagents.

RESULTS AND DISCUSSION

Purification and molecular properties

A large-scale purification of co-synthetase is summarized in Table 1. The final enzyme rapidly lost activity on storage, but when assayed immediately after the f.p.l.c. step it had a specific activity of 4.8×10^5 units/mg. When assayed under the conditions for deaminase (0.2 M-phosphate buffer, pH 8.0, and 37 °C, Williams *et al.*, 1981), co-synthetase had a specific activity of approx. 8×10^5 units/mg. This value, a minimum because of the presence of two minor impurities (see above), is to be compared with 3×10^4 units/mg for homogeneous deaminase assayed under identical conditions (Battersby *et al.*, 1983a); both enzymes were assayed with substrate concentrations giving about the same $[\text{S}]/K_m$ value, where $[\text{S}]$ is the substrate concentration used. Thus co-synthetase is far more active, by more than an order of magnitude, than deaminase both on a weight-for-weight basis and on a molar one (see below for M_r results). Since co-synthetase need only be present in amounts relative to deaminase to ensure complete formation of uroporphyrinogen III, the small molar quantity found in *E. gracilis* need be no surprise.

Gel-filtration experiments with co-synthetase on a calibrated Sephadex G-100 column led to an M_r value of $38\,500 \pm 800$ (mean $\pm$ s.d. for five determinations on different preparations). In addition, two experiments were performed with the column equilibrated with 15 mM-Tris/HCl buffer, pH 8.75, and essentially no difference in V_e for co-synthetase was observed, thus indicating no interactions between native enzyme and the gel.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated a subunit M_r for co-synthetase of $31\,000 \pm 800$ (mean $\pm$ s.d. for five determinations on different preparations). Co-synthetase is clearly monomeric, but the reason for the difference between M_r values found by using the two methods is not clear. That the reason is not partial proteolytic degradation of the samples during preparation for the polyacrylamide-gel electrophoresis was shown by elution of active co-synthetase from such gels (by using the method of Hager & Burgess, 1980) for running on the calibrated Sephadex G-100 column; they were eluted at V_e values corresponding to an M_r of approx. 39 000. The M_r for the *E. gracilis* enzyme is much less than the value of 62 000 found for wheat-germ co-synthetase by Higuchi & Bogorad (1975), but our M_r value from gel filtration is similar to the value of 42 000 determined, by gel filtration, for the rat liver

Table 1. Purification of co-synthetase from 550 litres of *E. gracilis* culture

'Deaminase/co-synthetase' was prepared in four separate batches (with 100–150 litres of culture per batch) as described by Battersby *et al.* (1982a), and the two enzymes were separated by ion-exchange chromatography as described in the text. After concentration of the co-synthetase-containing fractions, the four preparations were combined for the remaining steps. Only when deaminase had been removed could co-synthetase be quantitatively assayed. Yields and fold purifications are quoted both for the individual step and in square brackets as yield/fold purification from the crude deaminase-free material. Analysis of the porphyrins produced by 'deaminase/co-synthetase' preparations showed a minimum of 98% type III isomer; co-synthetase activity is therefore still present at least in equivalent amounts relative to deaminase activity. Deaminase yields to this stage were approx. 60%. [The values for the steps marked with an asterisk (*) are summations of values from separate preparations.]

Step	Volume (ml)	$10^{-6} \times$ Activity (units)	Protein (mg)	$10^{-6} \times$ Specific activity (units/mg)	Yield (%)	Purification (fold)
* Initial cell extract	3500		7.7×10^4			
* After $(\text{NH}_4)_2\text{SO}_4$ fractionation and dialysis	200		2.5×10^4			
* After gel filtration on Sephadex G-75 (i.e. 'deaminase/co-synthetase')	1400		2300			
* After DE-52 DEAE-cellulose chromatography of deaminase/co-synthetase and concentration	21	3	336	0.009	100	1
After pH-gradient chromatography	88	1.05	9.5	0.11	35	12.2
After HA-Ultrogel chromatography and concentration	8.1	0.5	3.04	0.164	47.6, [16.7]	1.5, [18.2]
After f.p.l.c. at pH 8.75	2.4	0.12	0.25	0.48	24, [4]	2.9, [53.3]

enzyme (Kohashi *et al.*, 1984). It is relevant that inclusion of phenylmethanesulphonyl fluoride and pepstatin in all buffers for the entire isolation process did not affect the observed M_r .

The pH (4.95 ± 0.1) at which co-synthetase was eluted at 4 °C from anion-exchange columns developed with a pH gradient marked the pH at which the enzyme was no longer sufficiently negatively charged to bind to the column. This pH would be expected to be close to, but not lower than, the isoelectric point (pI). Similarly, when the co-synthetase was chromatofocused at 15–18 °C on a column of Mono P HR5/20 connected to a Pharmacia f.p.l.c. system, it was eluted at pH 4.9 ± 0.1 . These findings point to a range of 4.8–5.1 for the pI of co-synthetase from *E. gracilis* over the range 4–18 °C. Wheat-germ co-synthetase has a pI of 6.1–6.7 (Higuchi & Bogorad, 1975).

The purest co-synthetase preparation was dialysed against 50 mM-sodium phosphate buffer, pH 7.0, containing 0.1 mM-dithiothreitol, and its u.v.-visible-absorption spectrum then showed only one absorption maximum, at 280 nm, with no absorption above 360 nm; the A_{280}/A_{260} ratio was 1.61:1. Over the range 240–360 nm, the absorption spectrum resembled very closely those recorded for ovalbumin and bovine serum albumin under the same conditions at similar concentrations. Thus co-synthetase from *E. gracilis* does not contain any significant quantity of a bound cofactor that absorbs radiation in the 240–360 nm range. It was therefore of interest that Piper *et al.* (1983) and Kohashi *et al.* (1984) have reported that co-synthetase isolated from rat liver contains a tetrahydrofolate derivative and that this may act as a coenzyme for the biosynthesis of uroporphyrinogen III. Since such a cofactor should be readily detected spectroscopically, the following experiments were done.

(a) A solution of bovine serum albumin (A_{280} approx. 0.2) was mixed with that quantity of dihydropteroylglutamate (7 μ M) that corresponded to the molar concentration of co-synthetase used for the above spectroscopic measurements. The measured spectrum was markedly different from that of bovine serum albumin alone, with significantly higher absorption over 300–350 nm. When dihydropteroylglutamate was replaced by tetrahydropteroylglutamate (6 μ M), this was also readily detected. These two experiments indicated that stoichiometric amounts of folate derivatives are not present in co-synthetase from *E. gracilis*, assuming that bound folate does not show vastly different absorption characteristics from unbound material.

(b) There were pointers in the work of Kohashi *et al.* (1984) that it is a 5-methyltetrahydropteroylpolyglutamate derivative that is associated with rat liver co-synthetase. Accordingly, 5-methyltetrahydropteroylglutamate was first shown to be readily detected by fluorescence spectroscopy in amounts corresponding to less than 0.1 mol of folate/mol of co-synthetase (excitation at 290 nm gave an emission maximum at 353 nm, with 5 nm band widths). Co-synthetase (0.12 mg in 2.4 ml of 50 mM-Tris/HCl buffer, pH 7.6, containing 0.2 M-2-mercaptoethanol) was heated for 10 min at 95 °C (the conditions used by Kohashi *et al.*, 1984), then cooled on ice and centrifuged; the supernatant was filtered through a 0.2 μ m-pore-size filter before immediate analysis in the spectrofluorimeter. No fluorescence for tetrahydrofolate was detected.

(c) We reported above that co-synthetase could be re-activated after denaturation by sodium dodecyl sulphate followed by polyacrylamide-gel electrophoresis. This would remove any essential cofactor not covalently bound, and therefore activity would not be recovered. In a further experiment, a large sample of enzyme (8.6×10^4 units) was denatured by boiling for 3 min with 1% sodium dodecyl sulphate and then was thoroughly dialysed against several changes of 0.1 M-sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulphate. On renaturation of the protein, 60% of the original co-synthetase activity was restored. A covalently bound cofactor would not be removed in this way; however, it would be present in stoichiometric amounts (relative to the protein) and, as shown above, stoichiometric folate is not detectable in *E. gracilis* co-synthetase.

(d) Kohashi *et al.* (1984) used a folate-AH-Sepharose 4B column as the final purification step for rat liver co-synthetase. The enzyme was bound to the column when applied in 50 mM-Tris/HCl buffer, pH 7.8, and was eluted with a gradient of KCl in the same buffer. We studied this process with co-synthetase-containing fractions from the DE-52 DEAE-cellulose column (see Table 1). These were dialysed against 50 mM-Tris/HCl buffer, pH 7.2, and a sample (5 mg of total protein) was applied to a folate-AH-Sepharose column (1 cm $\times$ 10 cm) prepared as described by Kohashi *et al.* (1984). All the proteins in the sample (at least ten different ones by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) were bound to the column, and all were eluted with 1 M-KCl, indicating that the proteins are binding through non-specific interactions.

Finally, when pteroylglutamate (up to 6 mM), tetrahydropteroylglutamate (up to 5 mM) or 5-methyltetrahydropteroylglutamate (up to 2.4 mM) was included in assays for co-synthetase, there was no effect on the activity of the enzyme. If co-synthetase had a specific folate-binding site, some detectable effect would have been expected.

The conclusion from the four studies just described is that co-synthetase from *E. gracilis* does not contain nor does it require a folate cofactor. It is conceivable that co-synthetase from rat liver differs in its mechanism and does use folate. However, the amount of folate derivative isolated from rat liver co-synthetase was only 14–50 ng/mg of protein from an enzyme having an M_r of 42000 (Kohashi *et al.*, 1984). This corresponds to approx. 1 mmol of folate derivative associated with 1 mol of enzyme. It is difficult to see how such a small amount could be mechanically significant for the biosynthesis of uroporphyrinogen III.

Comment is needed finally on the experiments of Kohashi *et al.* (1984) in which tetrahydropteroylglutamate or tetrahydropteroylpolyglutamate was added to a mixture of co-synthetase-free deaminase and PBG (1). They reported that the folates caused formation of some uroporphyrinogen III (3) whereas only uroporphyrinogen I (5) was formed (as is usual) without the tetrahydrofolate. This does not occur with co-synthetase-free deaminase from *E. gracilis* (G. J. Hart, unpublished work); addition of tetrahydropteroylglutamate (1 mM) or 5-methyltetrahydropteroylglutamate (1 mM) to this enzyme and PBG produced only uroporphyrinogen I (5), formed by non-enzymic ring closure of the hydroxymethylbilane (2). Further, neither tetrahydropteroylglutamate nor 5-methyltetrahydropteroylglutamate had any effect on

Fig. 1. Initial-rate measurements for the formation of uroporphyrinogen III by co-synthetase

The initial rates were measured at 25 °C in 0.2 M-Tris/HCl buffer, pH 8.25, containing 0.6 mM-EDTA, with various concentrations of hydroxymethylbilane. The rates of uroporphyrinogen formation in the absence of enzyme were subtracted from the rates observed in the presence of enzyme before the values were plotted. Results are shown (● and ○) from two different experiments with different preparations of enzyme and of hydroxymethylbilane.

the initial lag phase characteristic of the action of co-synthetase-free deaminase on PBG (Battersby *et al.*, 1982a). Thus, again, no evidence for a role for folate derivatives could be found.

Kinetic studies

Steady-state kinetic experiments with co-synthetase at 25 °C in 0.2 M-Tris/HCl, pH 8.25, led to values in the range 12–40 μM for K_m (hydroxymethylbilane), the variation arising (a) when one enzyme preparation was used with different batches of synthetic hydroxymethylbilane and (b) when different enzyme preparations were used on a single batch of bilane. Results from two different experiments are shown in Fig. 1. A previously determined value of 11 μM for K_m (hydroxymethylbilane) (Battersby *et al.*, 1981) is close to the lowest value obtained in the present work.

A limited study of product inhibition was carried out in which uroporphyrinogen III (prepared by sodium-amalgam reduction of uroporphyrin III) was incorporated into assays. Uroporphyrinogen III acted as an inhibitor apparently competitively with respect to hydroxymethylbilane, with a K_i value of similar magnitude to the K_m value for hydroxymethylbilane. Fuller experiments were not carried out because of the difficulty of measuring small changes in large uroporphyrinogen concentrations. However, co-synthetase from mouse spleen is inhibited to about 80% by 20 μM -uroporphyrinogen III (Levin, 1971).

Effect of butane-2,3-dione and of phenylglyoxal

Treatment of co-synthetase with butane-2,3-dione caused a rapid decline in enzymic activity (Fig. 2a) by a pseudo-first-order process, the half-time for the reaction being approx. 18 min (Fig. 2b). The presence of 1 mM-formylbilane (4) afforded almost total protection.

Fig. 2. Time course of inactivation of co-synthetase by butane-2,3-dione

(a) Co-synthetase (1.93×10^4 units/ml) was incubated at 25 °C with butanedione (40 mM) in 50 mM-sodium borate buffer, pH 9.0. Samples were removed at intervals and assayed for activity of co-synthetase as described in the text. Results are shown (● and ○) for two different preparations of co-synthetase. Also shown (Δ) are results from experiments in which formylbilane (4) (1 mM) was added to co-synthetase before the addition of butanedione (40 mM). (b) Semi-logarithmic plot of the data shown in (a).

Phenylglyoxal also inactivated co-synthetase, but much more rapidly than did butanedione. At 25 °C, in 0.1 M-sodium bicarbonate buffer, pH 8.4, 30 mM-phenylglyoxal totally abolished co-synthetase activity within 6 min. With 0.3 mM-phenylglyoxal 50% of the initial activity was lost within 1 min.

Under the conditions used, both butane-2,3-dione and phenylglyoxal are specific modifiers of arginine residues (Riordan, 1973). To determine directly the number of residues modified, the kinetic method of Levy *et al.* (1963) was used. Pseudo-first-order rate constants (k_{app}) were determined over a range of butanedione concentrations

Fig. 3. Kinetic analysis of inactivation of co-synthetase by butane-2,3-dione at 25 °C in 50 mM-sodium borate buffer, pH 9.0

Pseudo-first-order rate constants ($k_{app.}$) for the modification of co-synthetase by various concentrations of butanedione were determined from semi-logarithmic plots of the type shown in Fig. 2(b). The logarithms of these pseudo-first-order rate constants are plotted against the logarithms of the butanedione concentrations.

between 1 mM and 40 mM from semi-logarithmic plots of the type shown in Fig. 2(b). A plot of $k_{app.}$ against butanedione concentration (not shown) enabled a rate constant of $0.90 \text{ M}^{-1} \text{ s}^{-1}$ to be derived for the reaction between co-synthetase and butanedione. Fig. 3 shows the logarithms of the derived pseudo-first-order rate constants plotted against the logarithms of the butanedione concentrations. The slope of this plot gives the number of molecules (n) of modifier reacting/molecule of enzyme and affecting the activity; the value for n was 0.95. This suggests that modification of one arginine residue per enzyme molecule is sufficient to inactivate co-synthetase. The protection of co-synthetase against 40 mM-butanedione by the formylbilane (4) (Fig. 2) suggests strongly that the modifiable arginine residue in co-synthetase is closely involved in the binding of the natural substrate, hydroxymethylbilane.

Effect of methyl methanethiosulphonate and of iodoacetamide

Neither reagent had any effect on the activity of co-synthetase, results that argue against the presence of essential thiol groups.

Effect of pyridoxal 5'-phosphate

Incubation of co-synthetase with pyridoxal 5'-phosphate caused a rapid decline in enzymic activity (Fig. 4a), which was less rapid when the formylbilane (4) was included in the incubation mixture. With all tested concentrations of modifier, activity fell within 20 min to

Fig. 4. Time course of inactivation of co-synthetase by pyridoxal 5'-phosphate and effect of pyridoxal 5'-phosphate concentration on the final residual activity at equilibrium

(a) Co-synthetase (1.9×10^3 units/ml) was incubated at 25 °C with pyridoxal 5'-phosphate (7.5 mM) in 0.1 M-sodium phosphate buffer, pH 7.0. Samples were removed at intervals and assayed for activity of co-synthetase as described in the text. Results are shown (● and ○) from two separate experiments. Also shown (△) are results from experiments in which formylbilane (4) (0.64 mM) was added to co-synthetase before the addition of pyridoxal 5'-phosphate (7.5 mM). (b) Co-synthetase (1.9×10^3 units/ml) was incubated at 25 °C with various concentrations of pyridoxal 5'-phosphate in 0.1 M-sodium phosphate buffer, pH 7.0. Each point represents the activity remaining at equilibrium in such an incubation determined in most cases after 20 min of incubation.

a steady value that depended on the concentration of modifier used (Fig. 4b).

When a mixture of partially inactivated enzyme and residual pyridoxal 5'-phosphate was dialysed overnight against several changes of 0.1 M-phosphate buffer, pH 7.0, 92% of the initial enzymic activity was restored. However, when the mixture was treated with sufficient freshly prepared 80 mM- NaBH_4 solution to decolorize the solution within about 3 s, followed by dialysis against 0.1 M-phosphate buffer, pH 7.0, there was no re-activation, the final activity being 9% of its initial value. A second treatment with pyridoxal 5'-phosphate/ NaBH_4 decreased the activity to about 5% of the original starting activity (cf. Chen & Engel, 1975; Hart *et al.*, 1984), but treatment of co-synthetase with NaBH_4 alone followed by dialysis

had no significant effect on the enzymic activity. These results strongly implicate the involvement of one or more lysine residues in the mechanism of action of co-synthetase.

Effect of diethyl pyrocarbonate and of Rose Bengal and light

Diethyl pyrocarbonate at 0.41 mM decreased the activity of co-synthetase to 10% of the original value after 5 min of incubation at 25 °C; lower concentrations of reagent led to lower rates of inactivation, and pretreatment of co-synthetase with the formylbilane (4) afforded partial protection. Under the conditions used, diethyl pyrocarbonate is normally considered to be a reasonably specific modifier of histidine residues (Ovadi *et al.*, 1967), but the fact that the modified co-synthetase was found not to be re-activated by NH_2OH under conditions that would restore histidine residues (Miles, 1977) indicates that this is probably not true in the present case.

Photo-oxidation of histidine residues by using Rose Bengal resulting in loss of enzymic activity has been used to detect essential histidine residues in several enzymes, e.g. enolase (Westhead, 1965) and yeast alcohol dehydrogenase (Dickenson & Dickinson, 1975). Rose Bengal (7 μM) and light had no effect on co-synthetase activity even after irradiation times of up to 60 min.

The above results are in keeping with the conclusion from the experiments with diethyl pyrocarbonate that co-synthetase does not have essential histidine residues, as found for the human erythrocyte enzyme (Frydman & Feinstein, 1974).

Effect of other reagents

(a) Incubation of co-synthetase with tetranitromethane (cf. Sokolovsky *et al.*, 1966) had no effect on the activity of the enzyme even after 46 min.

(b) Addition of I_2 to co-synthetase resulted in small decreases in enzymic activity, the maximum loss, produced by an I_2 concentration of approx. 75 μM , being only 20% of the initial activity. These results together suggest that tyrosine residues are not essential to the activity of co-synthetase.

(c) EDTA at the concentrations routinely used in buffers and assays (0.6 mM) had no significant effect on the activity of co-synthetase, nor did 1,10-phenanthroline (3 mM) or 2,2'-bipyridyl (3 mM) (cf. Dickinson & Berrieman, 1977; Cremona & Singer, 1964). It is thus unlikely that metal ions are essential components of co-synthetase.

(d) When NH_2OH (0.1 M, added as the hydrochloride) was incorporated into co-synthetase assays, the small effect was matched by using 0.1 M-NaCl.

NaN_3 was inhibitory to co-synthetase, but even at very high (10 mM) concentrations 25–30% of the control activity remained. Similarly, 25% of the initial activity remained with 25 mM-KCN. Clearly no essential residues are being destroyed by any of these reagents.

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