Rat liver uroporphyrinogen III synthase has similar properties to the enzyme from *Euglena gracilis*, including absence of a requirement for a reversibly bound cofactor for activity

Elizabeth SMYTHE* and D. Clive WILLIAMS†

Department of Biochemistry, Trinity College, Dublin 2, Ireland

Uroporphyrinogen III synthase purified from rat liver is a monomer of M_r 36 000 by gel filtration and 28 000 by SDS/polyacrylamide-gel electrophoresis. The enzyme exists in two interconvertible forms separable on h.p.l.c. Both forms of the enzyme could be renatured with full activity after SDS/polyacrylamide-gel electrophoresis, demonstrating the absence of a reversibly bound cofactor. The enzyme activity could be inhibited by pyridoxal 5'-phosphate in the absence and in the presence of NaBH_4 , consistent with (an) essential lysine residue(s). The enzyme thus shows great similarity to that from *Euglena gracilis*.

INTRODUCTION

A key reaction in tetrapyrrole biosynthesis requires two enzymes, namely hydroxymethylbilane synthase (EC 4.3.1.8), which condenses four molecules of porphobilinogen to form the linear unrearranged tetrapyrrole hydroxymethylbilane, and uroporphyrinogen III synthase (EC 4.2.1.75), which can ring-close hydroxymethylbilane with concomitant intramolecular rearrangement of ring D to form uroporphyrinogen III [see references quoted in Battersby *et al.* (1982a,b) and Hart & Battersby (1985)].

Many studies have been performed on hydroxymethylbilane synthase [see references cited in Hart & Battersby (1985) and Smythe & Williams (1988)], but it is only recently that uroporphyrinogen III synthase has been purified, totally from *Euglena gracilis* (Hart & Battersby, 1985) and human erythrocytes (Tsai *et al.*, 1987) and partially from rat liver (Kohashi *et al.*, 1984).

Kohashi *et al.* (1984) suggested that a folate derivative was essential to the activity of rat liver uroporphyrinogen III synthase, whereas Hart & Battersby (1985) demonstrated conclusively that folate derivatives were absent from the pure active enzyme from *Euglena gracilis* and that no reversibly bound cofactors were necessary for enzyme activity.

It remained possible that mammalian uroporphyrinogen III synthase required a folate or other cofactor for activity. This present paper shows some of the properties of the enzyme and addresses the question of whether rat liver uroporphyrinogen III synthase requires a folate or other reversibly bound cofactor.

MATERIALS AND METHODS

Materials

Porphobilinogen was prepared according to the Rapoport–Wurziger method (Battersby *et al.*, 1977). Hexylglutathione–Sephacrose was a gift from Dr. T. J. Mantle, Trinity College, Dublin. The Protein Pak 125 gel-filtration column was from Waters Associates, Hartford, Northwich, Cheshire, U.K.

All other reagents were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Partial purification of uroporphyrinogen III synthase from rat liver

All procedures were carried out at 4 °C. Rat (Wistar strain) livers were perfused *in situ* with ice-cold 0.9% NaCl. Homogenates (25%, w/v) were prepared, by using a Dounce homogenizer (ten strokes), in 50 mM-Tris/HCl buffer, pH 7.8, containing 0.1 mM-dithiothreitol and 0.6 mM-EDTA. After centrifugation at 9000 *g* for 20 min, the resultant supernatant was centrifuged at 45 000 *g* for 2 h. The supernatant (hepatic cytosol fraction) was adjusted to 60% $(\text{NH}_4)_2\text{SO}_4$ saturation by the slow addition of $(\text{NH}_4)_2\text{SO}_4$ (370 g/l) over 30 min. During the $(\text{NH}_4)_2\text{SO}_4$ fractionation the pH was maintained at 7.8 by the prior addition to the supernatant of one-quarter its volume of 500 mM-Tris/HCl buffer, pH 7.8. After 1 h stirring the suspension was centrifuged at 12 000 *g* for 10 min. The pellet was discarded and the remaining supernatant was adjusted to 90% $(\text{NH}_4)_2\text{SO}_4$ saturation (further addition of 227 g/l) and centrifuged as described above. The pellet was suspended in 50 mM-Tris/HCl buffer, pH 7.8, containing 0.1 mM-dithiothreitol and 0.6 mM-EDTA. This preparation was dialysed extensively against the same buffer (3 × 10 litres). At this stage the preparation was routinely assayed for hydroxymethylbilane synthase and uroporphyrinogen III synthase activities, but in all cases only uroporphyrinogen III synthase activity was found in the 60–90%-satn. $(\text{NH}_4)_2\text{SO}_4$ fraction.

Dialysed $(\text{NH}_4)_2\text{SO}_4$ fractions were applied to hexylglutathione–Sephacrose columns (0.5 cm × 10 cm), prepared by the method of Mannervik & Guthenberg (1981), pre-equilibrated in 25 mM-Tris/HCl buffer, pH 7.8, containing 200 mM-NaCl. Unbound material, containing 100% of the applied uroporphyrinogen III synthase activity, was collected, and samples (100–500 μ l) were gel-filtered on a Waters Associates High Performance Liquid Chromatograph with a Protein Pak 125 column (0.78 cm × 30 cm). The column was equilibrated in 50 mM-Tris/HCl buffer, pH 7.8, containing

* Present address: Department of Biochemistry, University of Dundee, Dundee DD4 1HN, Scotland, U.K.

† To whom correspondence and requests for reprints should be addressed.

Table 1. Typical purification scheme for rat liver uroporphyrinogen III synthase

For experimental details see the Materials and methods section. A 40 g wet wt. portion of rat liver was used in this purification.

Step	Volume (ml)	Protein (mg)	Total activity (units)	Sp. activity (units/mg)	Yield (%)	Purification (fold)
60–90 %-satn. (NH ₄) ₂ SO ₄ fraction	20	126	749	6	100	1
Hexylglutathione–Sephacrose fraction	22	24	730	30	97	5
Protein Pak 125 chromatography						
Peak 1	8	0.65	190	298	25	49
Peak 2	8	0.5	210	420	28	70

0.1 mM-dithiothreitol, 0.6 mM-EDTA and, in certain specified cases, 0.1 mM-phenylmethanesulphonyl fluoride. Protein concentration was determined by using a Waters model 440 absorbance detector, set at 280 nm, linked to a Phillips chart recorder. The flow rate during column elution was 0.5 ml/min.

Assay of uroporphyrinogen III synthase activity

Uroporphyrinogen III synthase was assayed by its ability to accelerate ring-closure of hydroxymethylbilane produced *in situ* by hydroxymethylbilane synthase. The assay was carried out in 50 mM-Tris/HCl buffer, pH 8.0, containing 60 mM-KCl, which contained 1 unit of human erythrocyte hydroxymethylbilane synthase prepared according to the method of Smythe & Williams (1988), 100 μ l of 1 mM-porphobilinogen and uroporphyrinogen III synthase-containing fractions over a range of volumes from 50 to 400 μ l. The total assay volume was 1 ml. Mixtures were incubated for 5 min at 37 °C and the reaction was stopped with I₂ [1 % (w/v) I₂ with sufficient KI to dissolve it, 30 μ l]. The addition of I₂ causes oxidation of any remaining linear pyrroles, i.e. di-, tri- and tetra-pyrroles, to compound(s) that are unable to ring-close. After incubation at 37 °C for 5 min, the excess of I₂ was removed with freshly prepared Na₂S₂O₃ (2 %, w/v; 50 μ l) and the protein was precipitated by the addition of trichloroacetic acid (55 %, w/v; 100 μ l). The fluorescence of the samples was measured in a Perkin–Elmer spectrofluorimeter with excitation wavelength at 405 nm and emission wavelength at 595 nm. A second assay was performed with no uroporphyrinogen III synthase and this assay was stopped after 5 min at 37 °C with acid followed by I₂ as described by Williams (1984). This second assay gives the total rate of hydroxymethylbilane formation as the acid rapidly ring-closes the bilane to uroporphyrinogen before its oxidation to uroporphyrin. The production of uroporphyrin III in this lag assay was verified by using h.p.l.c. according to a modification of the method of Lim *et al.* (1983) for the separation of uroporphyrins I and III. One unit of uroporphyrinogen III synthase is defined as that amount of enzyme which in the presence of 1 unit of hydroxymethylbilane synthase caused activation of the rate of porphyrinogen production at 5 min to 50 % of the maximum rate, i.e. the second-assay rate.

Elution of uroporphyrinogen III synthase from SDS/polyacrylamide gels and renaturation of enzyme activity

Samples of uroporphyrinogen III synthase after gel filtration were re-activated after denaturation on SDS/polyacrylamide gels according to the method of Hager & Burgess (1980). Samples in SDS sample buffer (Laemmli, 1970) were applied to SDS/12.5 % polyacrylamide slab gels with β -lactoglobulin (0.5 mg/ml) added to each sample. Tracks containing samples were cut from the gels and sliced to give pieces of gel 1.4 mm in width. Elution from the gel and renaturation were carried out as described in the cited reference.

Protein determination

Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin as standard.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out according to the method of Laemmli (1970).

Modification of amino acid residues by pyridoxal 5'-phosphate

Uroporphyrinogen III synthase (20 units/ml) from each of the two peaks obtained after gel filtration, or as the 60–90 %-satn. (NH₄)₂SO₄ fraction, was treated exactly as described by Hart & Battersby (1985).

RESULTS AND DISCUSSION

Table 1 shows the results of a typical purification of rat liver uroporphyrinogen III synthase. Enzyme activity was not assayed until after the (NH₄)₂SO₄ fractionation stage, when the bulk (85–90 %) of the uroporphyrinogen III synthase activity was found in the 60–90 %-satn. (NH₄)₂SO₄ fraction free of hydroxymethylbilane synthase activity, which otherwise interfered with the uroporphyrinogen III synthase assay. As the major protein constituents of rat liver cytosol are the glutathione S-transferases (Jakoby, 1978), a large degree of purification was achieved on their removal on hexylglutathione–Sephacrose affinity resin. Perfusion of livers *in situ* with cold saline was found to be essential, as 3 times more uroporphyrinogen III synthase activity was found at the (NH₄)₂SO₄ fractionation stage if perfusion was

Fig. 1. H.p.l.c. gel filtration of rat liver uroporphyrinogen III synthase

Samples (0.25 ml) of uroporphyrinogen III synthase after hexylglutathione-Sepharose chromatography were gel-filtered on a Protein Pak 125 column (30 cm $\times$ 0.78 cm) in 50 mM-Tris/HCl buffer, pH 7.8, containing 0.1 mM-dithiothreitol and 0.6 mM-EDTA. $\circ$, Uroporphyrinogen III synthase activity; protein was monitored by the absorbance at 280 nm (—).

Fig. 2. Rechromatography of uroporphyrinogen III synthase peaks after gel filtration

(a) $\circ$, Samples of uroporphyrinogen III synthase obtained after hexylglutathione-Sepharose chromatography were gel-filtered as described in Fig. 1 legend; (b) $\bullet$, peak 1 was applied separately to Protein Pak 125 as for the initial chromatography; (c) $\blacksquare$, peak 2 was applied separately to Protein Pak 125 as for the initial chromatography.

performed. This may have been due to the removal of blood proteinases, minimizing proteolysis of the enzyme.

It was also found that if during $(\text{NH}_4)_2\text{SO}_4$ fractionation pH was maintained with aq. NH_3 rather than with Tris buffer then 100% of the uroporphyrinogen III synthase activity was found with hydroxymethylbilane synthase activity in the 0–60% satn. $(\text{NH}_4)_2\text{SO}_4$ fraction. Maintenance of pH with Tris buffer thus afforded a ready separation of the two enzymes. A further purification was achieved by gel filtration on h.p.l.c. Two peaks of enzyme activity were found on gel filtration (Fig. 1), one corresponding to an apparent M_r of 36000 and the other to M_r 20000 as judged by comparison with a standard curve of the elution volumes of proteins of known M_r . As the inclusion of phenylmethanesulphonyl fluoride (0.1 mM) and/or EDTA and the avoidance of low pH had no effect on the two peaks of activity, it seemed unlikely that proteolysis was responsible for this result. It also would seem unlikely that in a protein of M_r 36000 a proteolytic fragment of M_r 20000 would retain enzyme activity. When both peaks were taken separately and rechromatographed on Protein Pak 125, each peak gave rise to two peaks of activity in positions identical with the original two peaks (Fig. 2). Thus uroporphyrinogen III synthase exists in two interconvertible forms that can be separated by gel filtration on h.p.l.c. It was confirmed that both peaks possessed uroporphyrinogen III synthase activity by showing, by analysis of the product on h.p.l.c. (Smythe & Williams, 1988), that these forms when activating the ring-closure of hydroxymethylbilane caused the formation of uroporphyrinogen III. The enzyme form with lower apparent M_r may have interacted with the charged surface of the column packing material, leading to increased elution volume. Such interactions are not unknown in this type of column chromatography (Barth, 1980; Smythe & Williams, 1988). This interpretation is supported by the evidence from re-activation experiments (see below) that both forms of the enzyme are identical in M_r on SDS/polyacrylamide-gel electrophoresis.

Our preparation of enzyme after h.p.l.c. showed either a 49-fold or a 70-fold purification (peaks 1 and 2 respectively) over the $(\text{NH}_4)_2\text{SO}_4$ fraction and appeared as many protein bands on SDS/polyacrylamide-gel electrophoresis with a major band at M_r 28500. Kohashi *et al.* (1984) reported that a 73-fold purification of rat liver uroporphyrinogen III synthase over the $(\text{NH}_4)_2\text{SO}_4$ fraction yielded enzyme that appeared as two bands (each with enzyme activity) at M_r approx. 42000 on non-denaturing gel electrophoresis and on Sephadex G-100 filtration.

Elution of uroporphyrinogen III synthase from SDS/polyacrylamide gels and renaturation of activity

When samples (12 units) from each peak of uroporphyrinogen III synthase after gel filtration were subjected to SDS/polyacrylamide-gel electrophoresis the enzyme activity could be re-activated (Fig. 3) with total recovery of applied activity. In each case the peak of activity was found at an R_f value corresponding to an M_r of 28500. This result thus supports two forms of the enzyme having identical subunit M_r being interconvertible forms. Our results also show that the rat liver enzyme is identical with the *Euglena* enzyme in having different apparent M_r values on gel filtration

Fig. 3. Re-activation of uroporphyrinogen III synthase after SDS/polyacrylamide-gel electrophoresis

Uroporphyrinogen III synthase (12 units) from each of peaks 1 (a) and 2 (b) from gel filtration was subjected to SDS/12.5%-polyacrylamide-gel electrophoresis. Each of the tracks containing enzyme was sliced into 1.4 mm fractions and the protein was eluted from the gel. After denaturation with 6 M-guanidinium chloride the proteins were allowed to renature and were assayed for uroporphyrinogen III synthase activity. The M_r of each fraction was determined by co-electrophoresis of M_r standards: A, ovalbumin (M_r 45000); B, carbonic anhydrase (M_r 29000).

and SDS/polyacrylamide-gel electrophoresis. Hart & Battersby (1985) reported M_r values of 39000 on gel filtration and 31000 on SDS/polyacrylamide-gel electrophoresis and concluded that the monomeric enzyme is probably asymmetric. In the present work we find M_r 36000 (for one of the forms) on gel filtration and 28500 for SDS/polyacrylamide-gel electrophoresis. Tsai *et al.* (1987) and Jordan *et al.* (1988) have reported identical M_r values for the human erythrocyte and *Escherichia coli* enzymes on gel filtration and on SDS/polyacrylamide-gel electrophoresis (M_r 29000–30000). Thus the rat liver enzyme appears to be more similar to the *Euglena* enzyme than the erythrocyte and *Escherichia coli* enzymes in this respect.

The importance of the renaturation experiment is that this treatment would remove any essential cofactor not covalently bound, and therefore activity would not be recovered. This result therefore rules out the possibility that non-covalently bound cofactor, including folates, is essential for activity.

Fig. 4. Time course of inactivation of uroporphyrinogen III synthase by pyridoxal 5'-phosphate

Uroporphyrinogen III synthase (20 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 uroporphyrinogen III synthase activity as described in the text. Results are shown from experiments with the 60–90%-satn. $(\text{NH}_4)_2\text{SO}_4$ fraction of enzyme (■) and peak 1 (●) and peak 2 (○) from gel filtration.

Effect of pyridoxal 5'-phosphate on uroporphyrinogen III synthase activity

When uroporphyrinogen III synthase (20 units/ml) was incubated with pyridoxal 5'-phosphate (7.5 mM) at 25 °C a time-dependent decrease in enzyme activity was observed (Fig. 4). This effect was seen both with enzyme from the 60–90%-satn. $(\text{NH}_4)_2\text{SO}_4$ fraction and with peaks 1 and 2 from gel filtration. When the partially inactivated enzyme and residual pyridoxal 5'-phosphate were dialysed extensively against 0.1 M-sodium phosphate buffer, pH 7.0, the inhibition of enzyme activity was reversed as compared with a control sample that was similarly treated (but without pyridoxal 5'-phosphate). When the solution was treated with 80 mM- NaBH_4 and then dialysed, only 15% of the initial enzyme activity was recovered. These results are similar to those found by Hart & Battersby (1985) for the *Euglena* enzyme. Thus it appears that the rat liver enzyme is similar to the *Euglena* enzyme in having one (or more) essential lysine residue(s).

Rat liver uroporphyrinogen III synthase is thus similar to the *Euglena* enzyme in being monomeric, asymmetric, having essential lysine residue(s) and possessing no reversibly bound essential cofactor.

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