Biochem. J. (1983) **215**, 45–55
Printed in Great Britain

Purification of uroporphyrinogen decarboxylase from human erythrocytes

Immunochemical evidence for a single protein with decarboxylase activity in human erythrocytes and liver

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(*Received 21 March 1983/Accepted 1 June 1983*)

Uroporphyrinogen decarboxylase (EC 4.1.1.37) has been purified 4419-fold to a specific activity of 58.3 nmol of coproporphyrinogen III formed/min per mg of protein (with pentacarboxyporphyrinogen III as substrate) from human erythrocytes by adsorption to DEAE-cellulose, $(NH_4)_2SO_4$ fractionation, gel filtration, phenyl-Sepharose chromatography and polyacrylamide-gel electrophoresis. Progressive loss of activity towards uroporphyrinogens I and III occurred during purification. Experiments employing immunoprecipitation, immunoelectrophoresis and titration with solid-phase antibody indicated that all the uroporphyrinogen decarboxylase activity of human erythrocytes resides in one protein, and that the substrate specificity of this protein had changed during purification. The purified enzyme had a minimum mol.wt. of 39 500 on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Gel filtration gave a mol.wt. of 58 000 for the native enzyme. Isoelectric focusing showed a single band with a pI of 4.60. Reaction with *N*-ethylmaleimide abolished both catalytic activity and immunoreactivity. Incubation with substrates or porphyrins prevented inactivation by *N*-ethylmaleimide. An antiserum raised against purified erythrocyte enzyme precipitated more than 90% of the uroporphyrinogen decarboxylase activity from human liver. Quantitative immunoprecipitation and crossed immunoelectrophoresis showed that the erythrocyte and liver enzymes are very similar but not identical. The differences observed may reflect secondary modification of enzyme structure by proteolysis or oxidation of thiol groups, rather than a difference in primary structure.

Uroporphyrinogen decarboxylase (porphyrinogen carboxy-lyase; EC 4.1.1.37) is a cytosolic enzyme that catalyses the decarboxylation of a large number of natural and synthetic acetic acid-substituted porphyrinogens (Jackson *et al.*, 1976*a*; Smith *et al.*, 1976). During the biosynthesis of protohaem, it catalyses the sequential decarboxylation of the four acetic acid groups of uroporphyrinogen III to produce coproporphyrinogen III, with the formation of intermediate hepta-, hexa- and penta-carboxyporphyrinogens (Mauzerall & Granick, 1958). This reaction sequence appears to proceed via a preferred route that starts at the acetic acid substituent on ring D and then continues clockwise around the macrocycle (Jackson *et al.*, 1976*b*), and that may involve up to four different active centres (Cornford, 1964; Tomio *et al.*, 1970; Garcia *et al.*, 1973; Rasmussen & Kushner, 1979; Smith & Francis, 1979; De Verneuil *et al.*, 1980; Straka *et al.*, 1980).

In all the tissues that have been investigated, one protein, or a group of related proteins with one or more polypeptide chains in common, appears to catalyse the decarboxylation of all acetic acid-substituted porphyrinogens. Thus humans with inherited uroporphyrinogen decarboxylase deficiency (De Verneuil & Nordmann, 1978; Elder *et al.*, 1981) and uroporphyrinogen decarboxylase-deficient mutants of *Escherichia coli* (Sasarman *et al.*, 1979) and *Saccharomyces cerevisiae* (Urban-Grimal & Labbe-Boise, 1981) all show absence or reduction of the complete reaction sequence, whereas partial purification of the enzyme from avian erythrocytes (Tomio *et al.*, 1970), human erythrocytes (Elder & Tovey, 1977) and bovine liver (Straka *et al.*, 1980) has failed to separate activities towards different substrates. However, purification to homogeneity with assignment of all decarboxylase activity to a single protein has not been reported.

Decreased activity of the uroporphyrinogen decarboxylase occurs in various pathological con-

ditions. It is the primary enzymic abnormality in porphyria cutanea tarda (Kushner *et al.*, 1976), the commonest form of human porphyria, and in the hepatic porphyria produced by various polyhalogenated aromatic hydrocarbons (Elder, 1978), including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Goldstein *et al.*, 1982). Activity may also be decreased in acute alcoholism (McColl *et al.*, 1980) and after prolonged exposure to mercury or cadmium (Woods *et al.*, 1981). In none of these has the molecular basis for decreased enzyme activity been defined.

We now describe the purification of a protein from human erythrocytes that catalyses the decarboxylation of uroporphyrinogens I and III to the corresponding coproporphyrinogens. Immunochemical evidence suggests that this protein accounts for greater than 90% of the uroporphyrinogen decarboxylase activity of human erythrocytes and liver.

Experimental

Materials

Synthetic pentacarboxyporphyrin III pentamethyl ester, with the esterified acetic acid substituent on ring C, and uroporphyrin I and III octamethyl esters were gifts from Professor A. H. Jackson, Department of Chemistry, University College, Cardiff, Wales, U.K. Porphyrin esters were hydrolysed and dissolved in 0.01 M-KOH for storage at 4°C (Elder & Wyvill, 1982). DEAE-cellulose (DE52) was from Whatman Biochemicals, Maidstone, Kent, U.K.; Sephadex G-150, Sephacryl S200 (Superfine grade) and phenyl-Sepharose were from Pharmacia Fine Chemicals AB, Uppsala, Sweden; Bio-Gel P-150 was from Bio-Rad Laboratories, Watford, Herts., U.K.; agarose for gel electrophoresis (type HSA) was from Litex, Glostrup, Denmark; Ampholine PAG plates (pH 4.0–6.5) were from LKB Instruments, Selsdon, Surrey, U.K.; dithiothreitol, *N*-ethylmaleimide and standard proteins were from Sigma Chemical Co., Poole, Dorset, U.K. Other chemicals were obtained from Fisons, Loughborough, Leics., U.K., or BDH Chemicals, Poole, Dorset, U.K., and were of the highest grade available.

Measurement of uroporphyrinogen decarboxylase

Uroporphyrinogen decarboxylase activity was assayed as described by Elder & Wyvill (1982) using pentacarboxyporphyrinogen III, uroporphyrinogen III or uroporphyrinogen I as substrates and measuring the rate of formation of coproporphyrinogen. The assay mixture contained porphyrinogen (2 nmol) and dithiothreitol (0.88 μmol) in 0.1 M-K_2HPO_4/KH_2PO_4 buffer (pH 6.8)/0.1 mM-EDTA to a final volume of 0.1 ml.

Preparation of haemolysates and liver homogenates

Heparinised human blood (750–1000 ml), obtained within 6 h of collection, was centrifuged at 12000 $g_{av.}$ for 15 min at 4°C and the plasma was discarded. The cells were washed twice with an equal volume of ice-cold 0.9% (w/v) NaCl, and haemolysed by resuspending in ice-cold water to the original volume of blood with gentle stirring at 4°C for 2 h.

Human liver was obtained at autopsy, within 8 h of death, or by wedge biopsy at laparotomy under general anaesthesia. Homogenates (10–20%, w/v, in 0.1 M-K_2HPO_4/KH_2PO_4 buffer (pH 6.8)/0.1 mM-EDTA) were centrifuged at 15000 $g_{av.}$ for 5 min to obtain a supernatant fraction, which was stored at −70°C. Tissue preparations showed no loss of enzyme activity when stored at −70°C for at least 6 months.

Purification of uroporphyrinogen decarboxylase from human erythrocytes

All procedures were carried out at 4°C. Potassium phosphate buffers, pH 7.0, were prepared by adjusting the pH of approx. 10 mM-KH_2PO_4 to 7.0 with KOH and diluting to the required molarity.

Removal of haemoglobin. DEAE-cellulose (DE52) was added (100 g/litre) to haemolysate (750–1000 ml) that had been diluted to twice its volume with 4 mM-potassium phosphate buffer, pH 7.0. The mixture was stirred gently for 16–20 h and then centrifuged to sediment the resin. The resin was resuspended in an equal volume of 2 mM-potassium phosphate buffer (pH 7.0)/2 mM-dithiothreitol, centrifuged and the supernatant discarded. After this procedure had been repeated twice, the resin was washed repeatedly in the same way with 2 mM-potassium phosphate buffer (pH 7.0)/2 mM-dithiothreitol/0.1 M-KCl until the absorbance of the supernatant at 280 nm was less than 0.15 cm^{-1}. The resin was then resuspended in an equal volume of 2 mM-potassium phosphate buffer (pH 7.0)/2 mM-dithiothreitol/0.5 M-KCl, stirred gently for 16 h, centrifuged and the supernatant, which contained uroporphyrinogen decarboxylase activity, was collected. Elution was repeated twice, with stirring for 15 min, and the three supernatants were pooled. Solid $(NH_4)_2SO_4$ (25 g/100 ml) was dissolved in the pooled eluates and the solution was left for 16 h. Precipitated protein was collected by centrifugation and discarded. Additional $(NH_4)_2SO_4$ was added to the supernatant (16 g/100 ml of eluate) and precipitated protein was collected by centrifugation after 2 h. This precipitate was dissolved in a minimum volume of 50 mM-K_2HPO_4/KH_2PO_4 buffer (pH 7.0)/2 mM-dithiothreitol and clarified by centrifugation at 15000 $g_{av.}$ for 5 min.

Gel filtration. Redissolved $(NH_4)_2SO_4$ precipitate (up to 8.0 ml) was applied to a Sephacryl S200

column (2.5cm × 83cm) equilibrated with 50mM-K_2HPO_4/KH_2PO_4 buffer (pH 7.0)/0.2M-KCl/1mM-dithiothreitol; 15min fractions were collected at a flow rate of 13–14ml/h. Enzyme activity was eluted in a single sharp peak. All fractions containing enzyme activity were pooled, except for those that contained less than one-third the activity of the most active fraction. Enzyme was then concentrated by repeating the $(NH_4)_2SO_4$ precipitation as described above and dissolving the second precipitate in a minimum volume (1–2ml) of 50mM-K_2HPO_4/KH_2PO_4 buffer (pH 7.0)/2mM-dithiothreitol.

Phenyl-Sepharose chromatography. The concentrated enzyme preparation (1–2ml) was mixed with an equal volume of 2mM-potassium phosphate buffer (pH 7.0)/2mM-dithiothreitol/2M-$(NH_4)_2SO_4$ and applied to a phenyl-Sepharose column (1.0cm × 9.0cm), equilibrated with 2mM-potassium phosphate buffer (pH 7.0)/2mM-dithiothreitol/1M-$(NH_4)_2SO_4$, at a flow rate of 40–45ml/h. The column was eluted at the same flow rate with 40ml of buffer/dithiothreitol/1M-$(NH_4)_2SO_4$, followed by a linear gradient formed from 20ml of buffer/dithiothreitol/1M-$(NH_4)_2SO_4$ and 20ml of buffer/dithiothreitol, followed by 20ml of buffer/dithiothreitol and finally with 20ml of 50% (v/v) ethanediol in buffer/dithiothreitol; 2min fractions were collected from the start of the gradient. Fractions containing a high ratio of enzyme activity to protein were pooled.

Polyacrylamide-disc-gel electrophoresis. $(NH_4)_2SO_4$ (440mg/ml) was dissolved in the pooled fractions from phenyl-Sepharose columns. After 2h, the precipitate was collected by centrifugation at 15000$g_{av.}$ for 5min and dissolved in 50mM-KH_2PO_4/K_2HPO_4 buffer (pH 7.0)/0.1mM-EDTA/2.0mM-dithiothreitol. This solution (0.05ml/gel) was applied to a series of disc gels and electrophoresis carried out (Davis, 1964). Separating gels (0.5cm diameter × 5cm) contained 6.2% (w/v) acrylamide and 0.16% (w/v) bisacrylamide and stacking gels contained 5% (w/v) acrylamide and 1.25% (w/v) bisacrylamide. After electrophoresis, the gels were frozen and sliced into 2.5mm slices. Each slice was eluted for 16–20h at 4°C with 0.5ml of 50mM-KH_2PO_4/K_2HPO_4 buffer (pH 7.0)/0.1mM-EDTA/2.0mM-dithiothreitol. Enzyme activity was measured in the eluates and the fraction containing maximum activity was identified for each gel. These fractions were then pooled to form the final purified enzyme preparation, which was stored at −70°C.

Preparation of antisera

Rabbits were immunized by intradermal injection with enzyme preparation/Freunds adjuvant (incomplete, except for the first injection) (1:1, v/v, 1.0ml per animal) at five to ten sites every 2 months for 10 months (antiserum I) or every month for 2 months (antiserum II). For the production of antiserum I, the enzyme preparation was a partially purified (730-fold) uroporphyrinogen decarboxylase fraction obtained by polyacrylamide-disc-gel electrophoresis of the material eluted from a Sephacryl S200 column that contained 9μg of protein/ml. For the production of antiserum II, the enzyme fraction from a phenyl-Sepharose column was fractionated by isoelectric focusing on Ampholine PAG plates, pH 4.0–6.5. The zone containing enzyme activity (approx. 17cm × 0.6cm × 0.1cm) was cut out, and dispersed by sonication in 10mM-phosphate buffer, pH 6.8 (3.0ml), to give an enzyme preparation that contained approx. 15μg of protein/ml.

Antibodies were detected by radial immunodiffusion and by immunoprecipitation of enzyme activity. An immunoglobulin fraction was prepared from antiserum I by precipitation with Na_2SO_4 and dissolved in 0.2M-sodium borate buffer (pH 8.2)/0.15M-KCl to 40% of the serum volume.

Immunochemical techniques

Crossed immunoelectrophoresis (Weeke, 1973) and rocket immunoelectrophoresis (Laurell, 1972) was carried out in 1% agarose/0.025M-sodium barbital/HCl buffer (pH 8.6) gel containing antiserum on 10cm × 10cm plates (15ml of gel/plate) for 16–20h at 16mA constant current. Immunoprecipitates were visually detected by staining with Coomassie Brilliant Blue. Solid-phase antibody was prepared by reacting the immunoglobulin fraction from antiserum I with a diazonium salt of reprecipitated cellulose (Hales & Woodhead, 1980). The final suspension of solid-phase antibody contained 1.05mg of solid-phase protein/ml. For immunoadsorption, haemolysates or enzyme fractions were centrifuged at 15000$g_{av.}$ for 2min. Supernatants (100–150μl), diluted with 0.1M-KH_2PO_4/K_2HPO_4 buffer (pH 6.8)/0.1mM-EDTA when necessary, were mixed with solid-phase antibody, prepared by centrifuging a measured volume of suspension, washing the pellet three times with approx. 1.0ml of buffer/EDTA and removing the supernatant. The mixture was incubated, with occasional resuspension, for 1h at room temperature followed by 16–20h at 4°C. Solid-phase antibody–enzyme conjugates were sedimented by centrifugation and washed three to five times with approx. 1.0ml of buffer/EDTA before resuspension in assay medium for measurement of enzyme activity. For immunoprecipitation, haemolysate or enzyme preparation (0.05–0.12ml) was mixed with antiserum or non-immune rabbit serum diluted in 0.9% (w/v) NaCl to give a final volume of 0.05–0.175ml. After 2h at room temperature followed by 16–20h at 4°C, the mixture was centrifuged at 15000$g_{av.}$ for 5min.

Reaction with N-ethylmaleimide

Haemolysate or enzyme preparations were mixed with an equal volume of 20mM-*N*-ethylmaleimide in 0.1M-KH_2PO_4/K_2HPO_4 buffer (pH6.80)/0.1mM-EDTA and incubated at 37°C for up to 30min, before removal of portions (5–10μl) for enzyme assay or immunoelectrophoresis. Solid-phase-antibody–enzyme conjugate (prepared from 45μl of solid-phase antibody suspension) was suspended in the above buffer/EDTA medium containing dithiothreitol (3mg/ml) for 15min at room temperature, washed three times with approx. 1.0ml of buffer/EDTA, and incubated with 4mM-*N*-ethylmaleimide in buffer/EDTA (0.05ml) at 37°C for up to 30min. The reaction was stopped by adding 0.5ml of dithiothreitol (3mg/ml) in buffer/EDTA, centrifuging and washing the pellet once with approx. 1.0ml of buffer/EDTA before resuspension for enzyme assay.

Other methods

Apparent molecular weights were determined by gel filtration on 2.5cm × 80cm columns of Sephacryl S200 or Sephadex G-150 equilibrated and run as described above, or on a 1.0cm × 102cm column of Bio-Gel P150 equilibrated as described above and run at a flow rate of 5ml/h with collection of 10min fractions. Columns were calibrated with Dextran Blue 2000, yeast alcohol dehydrogenase, bovine serum albumin, cytochrome *c* and ovalbumin and elution volumes were measured from the start of each run to the maximum of each peak of protein or enzyme activity.

Analytical isoelectric focusing was carried out with a Pharmacia FBE 3000 flat bed apparatus (Pharmacia Fine Chemicals, Hounslow, Middx., U.K.). Sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis was carried out in 10% (w/v) acrylamide gels (Ames, 1974) or 8–22.5% (w/v) acrylamide gradient gels (Arioso *et al.*, 1977). Protein was detected by staining with Coomassie Brilliant Blue or by the silver technique of Merril & Goldman (1982). For determination of molecular weight, gels were calibrated with rabbit muscle phosphorylase *b*, bovine serum albumin, ovalbumin, soya-bean trypsin inhibitor and α-lactalbumin.

Protein concentrations were measured by the method of Bensadoun & Weinstein (1976) with bovine serum albumin as standard.

Results

Purification of uroporphyrinogen decarboxylase

Uroporphyrinogen decarboxylase activity was monitored during the purification procedure by measuring the rate of decarboxylation of pentacarboxyporphyrinogen III. Table 1 summarizes the purification from human erythrocytes of activity towards this substrate.

Recoveries of enzyme activity during each stage of the purification procedure were high. Less than 5% of the enzyme activity remained in the supernatant after adsorption of haemolysates with DEAE-cellulose. No detectable enzyme activity was eluted until the eluent contained 0.2M-KCl. With 0.5M-KCl, up to 86% of the enzyme could be recovered from the resin. On $(NH_4)_2SO_4$ fractionation, over 80% of the recovered enzyme activity (approx. 70%) was present in the second precipitate. The enzyme was eluted as a single peak from gel filtration columns with recoveries of 68–74%. On phenyl-Sepharose chromatography there was partial dissociation of the main protein peak from the main peak of enzyme activity (Fig. 1). Only those fractions containing a high ratio of enzyme activity to protein concentration were processed further, which accounts for the low yield at this stage (Table 1), although up to 81% of enzyme activity was recovered from these columns.

The final fraction, which contained 3% of the enzyme activity in the haemolysate, was used for further studies.

Purity of final fraction

Fig. 2 shows the patterns obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis at different stages of purification. The final fraction contains a prominent protein band at M_r 39000, but is not homogeneous, minor components being present in the M_r 65000–70000 region. Crossed immunoelectrophoresis against antiserum I similarly

Table 1. *Purification of uroporphyrinogen decarboxylase from human erythrocytes*

Enzyme was purified from 820ml of human blood as described in the Experimental section and assayed using pentacarboxyporphyrinogen III (20μM) as substrate.

Step	10^{-3} × Total activity (pmol/min)	10^{-3} × Specific activity (pmol/min per mg of protein)	Purification (fold)	Yield (%)
Haemolysate	1468	0.0132	1.0	100
DEAE-cellulose/$(NH_4)_2SO_4$ fractionation	746	2.42	182	50.7
Gel filtration	218	4.39	333	24.0
Phenyl-Sepharose chromatography	30.5	18.60	1409	4.3
Polyacrylamide-gel electrophoresis	5.6	58.33	4419	3.0

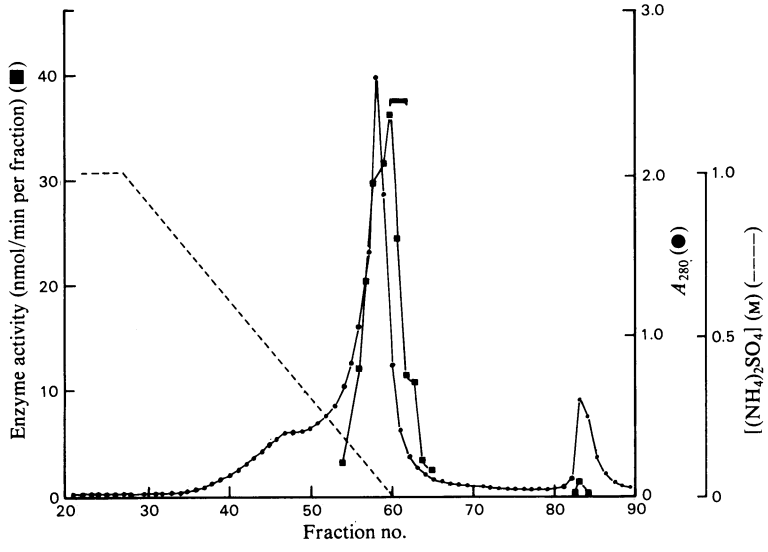

Fig. 1. *Phenyl-Sepharose chromatography of partially-purified uroporphyrinogen decarboxylase*
The flow rate was 42ml/h and 1.4ml fractions were collected. The horizontal bar indicates the fractions that were used for further purification. From fraction 70, the eluent contained 50% (v/v) ethanediol. Enzyme recovery was 77%.

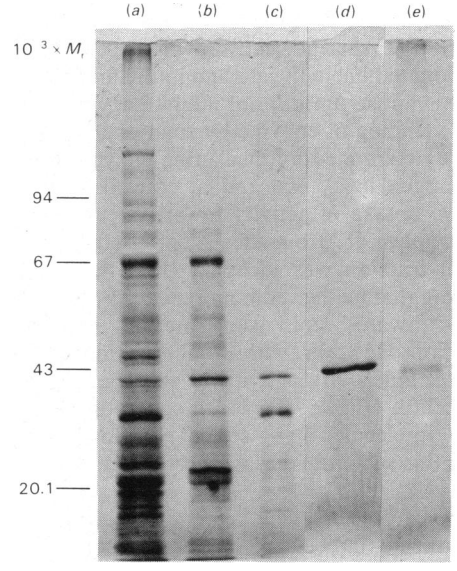

Fig. 2. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis at different stages of purification*
(*a*) After DEAE-cellulose/$(NH_4)_2SO_4$ fractionation; (*b*) after gel filtration; (*c*) after phenyl-Sepharose chromatography; (*d*) final fraction obtained by polyacrylamide-gel electrophoresis; (*e*) eluate obtained by isoelectric focusing. Samples (*a*)–(*c*) contained approx. 25pmol/min of enzyme activity (1.3–27μg of protein). Sample (*d*) contained 140pmol/min of enzyme activity (3μg of protein). The gel was stained by the silver technique.

showed progressive purification of a single component (Fig. 3). The major immunoprecipitate arc given by the final fraction was shown to represent enzyme by demonstrating enzyme activity in slices of gel containing immunoprecipitate (Fig. 4).

Further evidence in support of the identification of the protein with M_r 39000 as enzyme was obtained by isoelectric focusing. The fraction prepared by phenyl-Sepharose chromatography (Table 1) was focused on a narrow-range (pH4.0–6.5) slab gel, which was cut into 2mm slices. After elution, slices containing enzyme were identified by measurement of enzyme activity and by rocket immunoelectrophoresis with antiserum I. The eluate from the slice containing most enzyme gave a single protein band with M_r 39000 on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 2).

Specific activity and substrate specificity

Preliminary experiments, in which activities towards uroporphyrinogen III and pentacarboxyporphyrinogen III were determined for haemolysates prepared by different methods, showed that haemolysis of large volumes of erythrocytes by freeze–thawing (Elder & Tovey, 1977) led to a selective 6–8-fold decrease in enzyme activity towards uroporphyrinogen III, but that when cells were disrupted by hypo-osmotic lysis, the activities for each substrate were close to those reported for small volumes of fresh blood lysed by freeze–thawing (Elder *et al.*, 1978) (Table 2). However, during

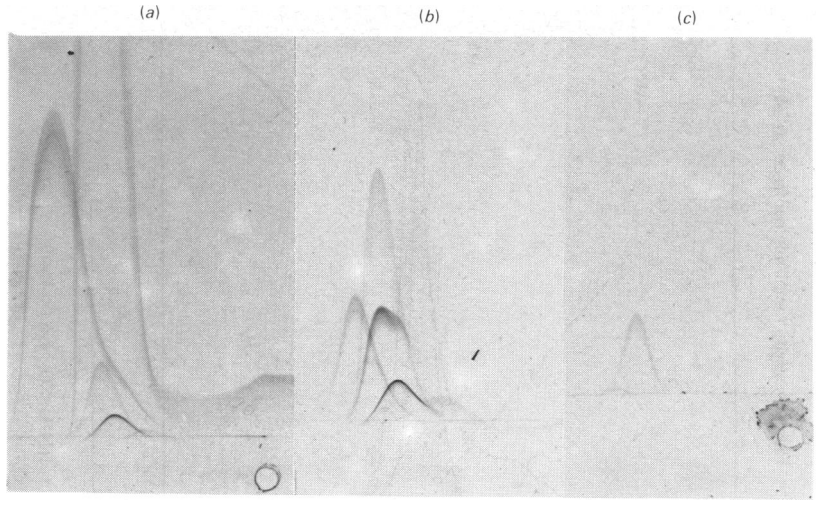

Fig. 3. *Crossed immunoelectrophoresis at different stages of purification*
(*a*) Haemolysate; (*b*) after phenyl-Sepharose chromatography; (*c*) final fraction obtained by polyacrylamide-gel electrophoresis. Wells contained 8 μl of sample. Amounts of enzyme activity applied were 28 pmol/min (*a* and *c*) and 98 pmol/min (*b*). The gel contained purified immunoglobulin (25 μl/ml) from antiserum I.

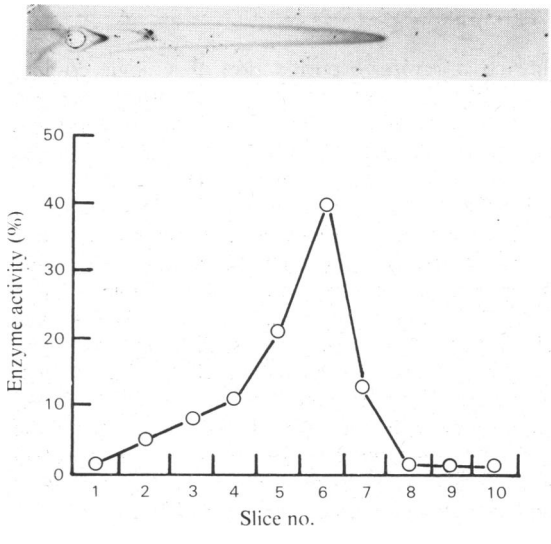

Fig. 4. *Rocket immunoelectrophoresis of final fraction*
Samples (5 μl) were run in duplicate. One rocket was stained with Coomassie Brilliant Blue, the other was cut into 5 mm slices. Assay medium (0.08 ml) was added to each slice and enzyme activity measured. Enzyme activity is shown as a percentage of the total activity recovered, which was 97% of that applied. The gel contained immunoglobulin (20 μl/1.5 ml) from antiserum I.

purification there was a relative loss of activity towards uroporphyrinogens I and III with the activity towards each isomer decreasing to a similar extent (Table 2). This change in substrate specificity was further investigated by using titration with solid-phase antibody prepared from antiserum I to compare the specific activity of the enzyme in unfractionated haemolysate and in the final fraction. Since this antiserum did not inhibit enzyme activity (Fig. 4), binding of enzyme by solid-phase antibody could be determined by measuring enzyme activity. With antigen in excess, and at equivalent enzyme activities, uptake of activity towards pentacarboxyporphyrinogen III from the haemolysate and from the final fraction was identical (Fig. 5; Table 2), suggesting that neither immunoreactivity nor specific activity towards this substrate is altered by purification. However, with uroporphyrinogen III as substrate, much less activity is adsorbed from the final fraction (Fig. 5; Table 2), suggesting that the enzyme molecule has selectively lost activity towards this substrate during purification.

Physical properties

The mean molecular weight of the enzyme estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis using gradient and 10% slab gels was 39500 (range 37000–42000; six determinations). The apparent molecular weight of the native enzyme estimated by gel filtration on Sepharose G-150, Sephacryl S-200 and Bio-Gel P-150 columns, all of which gave similar values, was 58000 (mean of six determinations; range 51000–64000).

Isoelectric focusing of the final fraction revealed a single major band with a pI of 4.60.

Table 2. *Substrate specificity of uroporphyrinogen decarboxylase preparations*

Enzyme activities were measured using 20 μM-pentacarboxyporphyrinogen III ($5CO_2^-$III), 20 μM-uroporphyrinogen I ($8CO_2^-$I) or 20 μM-uroporphyrinogen III ($8CO_2^-$III) as substrates. Haemolysates were prepared (*a*) by hypo-osmotic lysis of cells from 820 ml of blood or (*b*) by freeze–thawing 0.05 ml of blood as described by Elder & Wyvill (1982) from four normal subjects. Solid-phase-antibody–enzyme conjugate was prepared as described in the Experimental section and in the legend to Fig. 5. Under (*b*), the range of the ratios for four normal subjects is shown.

	Relative activities in solution		Solid-phase-antibody–enzyme conjugate	
Purification step	$8CO_2^-$III/$5CO_2^-$III	$8CO_2^-$I/$5CO_2^-$III	Relative activity $8CO_2^-$III/$5CO_2^-$III	Rate of decarboxylation of $5CO_2^-$III (pmol/min per 50 μl)
Haemolysate (*a*)	0.185	0.051	0.125	2.58
(*b*)	0.125–0.179	—		
Gel filtration	0.097	0.027	—	—
Final fraction	0.078	0.018	0.058	2.72

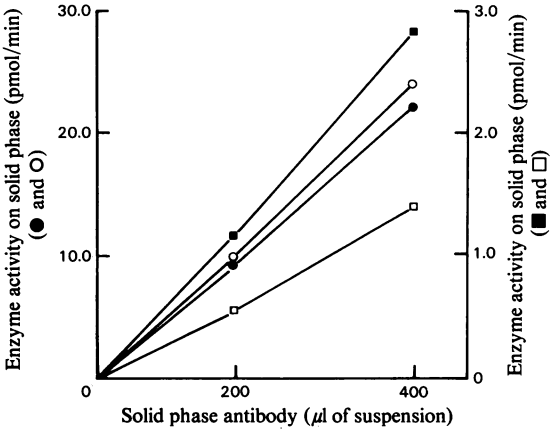

Fig. 5. *Titration of uroporphyrinogen decarboxylase with solid-phase antibody*

Haemolysate and final enzyme fraction were diluted to equivalent activity (15- and 30-fold respectively) with 0.1 M-potassium phosphate buffer, pH 6.80. Enzyme was adsorbed on to solid-phase antibody from diluted haemolysate (0.6 ml) (● and ■) or final fraction (○ and □) as described in the Experimental section. Enzyme activity was measured using pentacarboxyporphyrinogen III (● and ○) or uroporphyrinogen III (■ and □) as substrate. After adsorption 30–45% of activity remained in the supernatant.

Involvement of thiol groups in catalytic activity and immunoreactivity

Preliminary experiments showed that inclusion of glutathione or dithiothreitol (0.5–2.0 mM) in the buffers used for purification prevented loss of enzyme activity, particularly towards uroporphyrinogen III.

Incubation of either haemolysate or purified enzyme fraction with 10 mM-*N*-ethylmaleimide at 37°C for 30 min inhibited the decarboxylation of both pentacarboxyporphyrinogen III (greater than 97%) and uroporphyrinogen III (greater than 95%). The same treatment also abolished immunoreactivity as assessed by crossed immunoelectrophoresis against antiserum I. Crossed immunoelectrophoresis of haemolysate before and after reaction with *N*-ethylmaleimide showed that this loss of immunoreactivity was specific for the immunoprecipitate arc corresponding to purified enzyme, other arcs being little changed.

When antigenic determinants were protected by reaction with solid-phase antibody, the enzyme remained susceptible to inhibition by *N*-ethylmaleimide (Table 3). Inhibition at this site could be prevented by incubation with substrates or the corresponding porphyrins (Table 3).

Immunochemical properties of enzyme in human erythrocytes and liver

Antiserum II (see the Experimental section) was used for these studies. Immunoelectrophoresis with this antiserum against the final enzyme fraction, partially purified (333-fold) enzyme, haemolysate and mixtures of these gave one immunoprecipitate arc (results not shown), which was not formed when haemolysate treated with *N*-ethylmaleimide was used as the antigen. Fig. 6(*a*) shows that antiserum II precipitated greater than 95% of the enzyme activity from human haemolysate, with activities towards pentacarboxyporphyrinogen III and uroporphyrinogen III being precipitated in parallel. The small difference in the titre of antiserum required to precipitate 50% of the activity towards each substrate probably reflects the difficulty of obtaining accurate measurements of low rates of conversion of uroporphyrinogen III into coproporphyrinogen III in the presence of high concentrations of haemoglobin.

Fig. 6(*b*) shows that greater than 90% of the enzyme activity in human liver was also precipitated by antiserum II, with precipitation of

Table 3. *Effect of N-ethylmaleimide on solid-phase-antibody–uroporphyrinogen decarboxylase conjugate*
Solid-phase-antibody–enzyme conjugate was prepared by incubating solid-phase antibody (1.1 ml of suspension) with haemolysate [0.5 ml diluted to 2.0 ml with 0.9% (w/v) NaCl/10 mM-sodium phosphate buffer, pH 7.4] as described in the Experimental section. Portions of solid-phase-antibody–enzyme conjugate (45 μl) were incubated for 5 min at 37°C with porphyrin or porphyrinogen (both at 120 μM) before addition of 50 μl of 8 mM-*N*-ethylmaleimide in 0.1 M-potassium phosphate buffer, pH 6.80, or buffer alone and incubation for a further 20 min at 37°C, followed by addition of excess dithiothreitol, washing of the conjugate (see the Experimental section) and assay of the conjugate for uroporphyrinogen decarboxylase activity using pentacarboxyporphyrinogen III as substrate. Control tubes contained conjugate, buffer and 10 mM-KOH in place of porphyrin(ogen). Results are means and ranges for two (*) or four (†) measurements.

Final incubation mixture	Enzyme activity (pmol/min per 45 μl of conjugate)	Activity (% of mean control activity)
Control	0.79† (0.59–0.83)	100
N-Ethylmaleimide	0.08† (0.07–0.09)	10
Pentacarboxyporphyrinogen III/*N*-ethylmaleimide	0.88* ± 0.05	111
Pentacarboxyporphyrinogen III	0.67* ± 0.01	85
Pentacarboxyporphyrin III/*N*-ethylmaleimide	0.89* ± 0.02	113
Pentacarboxyporphyrin III	0.90* ± 0.02	114
Uroporphyrinogen III/*N*-ethylmaleimide	0.65* ± 0.01	82
Uroporphyrinogen III	0.62* ± 0.02	78
Uroporphyrin/*N*-ethylmaleimide	1.09* ± 0.05	138
Uroporphyrin	1.06* ± 0.04	134

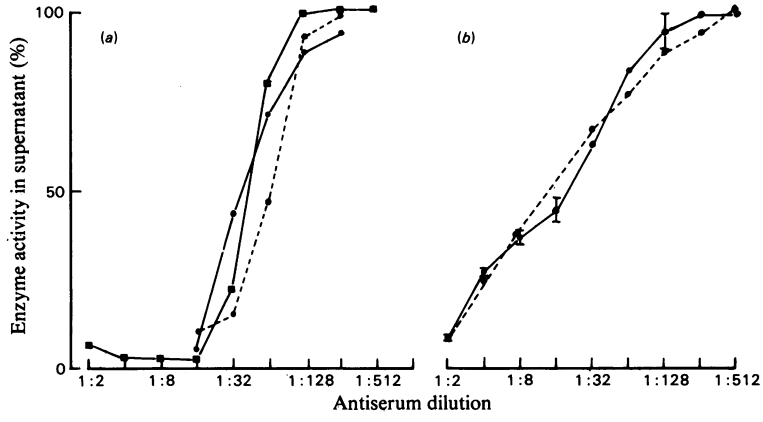

Fig. 6. *Immunoprecipitation of uroporphyrinogen decarboxylase activity*
(*a*) Shows immunoprecipitation of activity from haemolysate. Tubes contained diluted antiserum II (10 μl) and haemolysate (5.9 μl) (enzyme activity 16.8 pmol/min) in a total volume of 50 μl (■) or diluted antiserum II (75 μl) and haemolysate (75 μl) (●). (*b*) Shows immunoprecipitation of activity from liver extract. Tubes contained diluted antiserum II (10 μl) and liver extract (40 μl) (enzyme activity 9.9 pmol/min). Vertical bars indicate the range of results from two separate experiments. All tubes were incubated for 2 h at room temperature, 16 h at 4°C, centrifuged at 15000 $g_{av.}$ for 5 min, before assaying the supernatant for enzyme activity using either pentacarboxyporphyrinogen III (——) or uroporphyrinogen III (– – – –) as substrate. Enzyme activities are expressed as percentages of activity in control tubes containing non-immune rabbit serum.

activity towards each substrate showing close agreement. However, a higher concentration of antiserum was required to precipitate the liver enzyme than the erythrocyte enzyme (Fig. 6).

Crossed immunoelectrophoresis of haemolysate, liver extract and mixtures of the two (Fig. 7) also showed that the liver enzyme is very similar to, but not identical with, the erythrocyte enzyme. Although there is no detectable difference in charge, and Fig. 7(*d*) shows complete fusion of immunoprecipitates when the two samples are run together, there is a clear difference in the morphology of the immunoprecipitate produced by each tissue (Fig. 7). The same difference in immunoprecipitate morphology, without significant differences in peak height, was observed on rocket immunoelectrophoresis of haemolysate and liver extracts at equivalent enzyme activities.

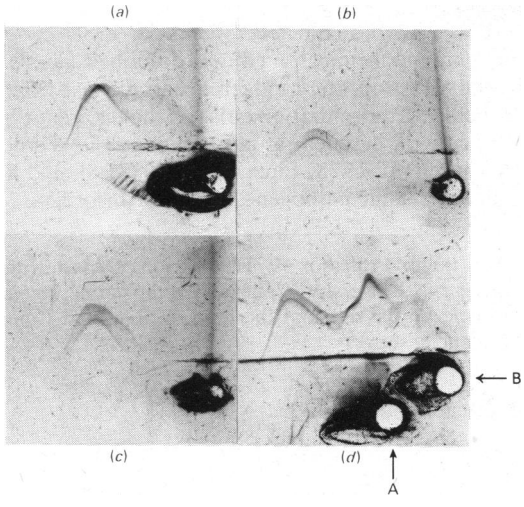

Fig. 7. *Crossed immunoelectrophoresis of haemolysate and liver extract*

Wells contained (*a*) 10 μl of haemolysate (enzyme activity 21.9 pmol/min), (*b*) 15 μl of liver extract (enzyme activity 4.4 pmol/min), (*c*) 5 μl of haemolysate and 10 μl of liver extract and (*d*) 15 μl of liver extract (A) and 5 μl of haemolysate (B). Agarose gels (24 ml) were poured on glass plates (10 cm × 10 cm); the antibody-containing zone (10 cm × 8.5 cm) contained antiserum II (1.5 μl/ml).

Discussion

The final fraction obtained from human erythrocytes by the purification procedure described above contained uroporphyrinogen decarboxylase at a higher specific activity, and purified to a greater extent, than has previously been described for vertebrate tissues (Tomio *et al.*, 1970; Romeo & Levin, 1971; Elder & Tovey, 1977; Straka *et al.*, 1980). Although the overall yield was low (Table 1), antiserum II, which was raised to purified enzyme, precipitated greater than 95% of the uroporphyrinogen decarboxylase activity from unfractionated haemolysate (Fig. 6), indicating that virtually all the enzyme in the haemolysate had the same antigenic determinants as the final material. Crossed immunoelectrophoresis of haemolysate with the same antiserum revealed only one peak of immunoprecipitate (Fig. 7), which was shown by rocket immunoelectrophoresis of mixtures to be immunochemically identical with purified enzyme. These findings suggest that the purified protein identified as uroporphyrinogen decarboxylase is responsible for all the uroporphyrinogen decarboxylase activity of human erythrocytes. Thus they substantiate previous evidence from studies of partially-purified enzyme (Tomio *et al.*, 1970; Elder & Tovey, 1977) and from investigation of the inheritance of this enzyme in eukaryotes (De Verneuil & Nordmann, 1978; Urban-Grimal & Labbe-Bois, 1981) that the sequential decarboxylation of uroporphyrinogen to coproporphyrinogen is catalysed by a single protein.

During purification, there was a relative loss of enzyme activity towards uroporphyrinogens I and III (Table 2). Previously we reported no change in substrate specificity during partial purification of this enzyme from haemolysates prepared by freeze–thawing (Elder & Tovey, 1977); but we have now shown that use of this method of haemolysis for large volumes of blood produces a marked loss of activity towards uroporphyrinogen before purification. Indirect immunochemical measurements of enzyme specific activity before and after purification (Fig. 5), together with the lack of evidence for more than one protein with uroporphyrinogen decarboxylase activity in human erythrocytes, suggest that the progressive loss of activity towards uroporphyrinogens I and III during purification (Table 2) indicates a change in the substrate specificity of the enzyme, rather than loss of a protein with preferential activity towards these substrates. Studies of the effect of heating and high salt concentrations on the reaction sequence (Cornford, 1964; Tomio *et al.*, 1970; Garcia *et al.*, 1973; Straka *et al.*, 1980), the rates of decarboxylation of different substrates (Garcia *et al.*, 1973; Rasmussen & Kushner, 1979; Smith & Francis, 1979, 1981) and mixed-substrate experiments (De Verneuil *et al.*, 1980) all suggest that more than one type of active centre is involved. Our results are consistent with this view and indicate that the uroporphyrinogen-binding site may be particularly susceptible to inactivation, perhaps by oxidation, during purification.

Reagents that react with thiol groups are known to inhibit uroporphyrinogen decarboxylase activity (Mauzerall & Granick, 1958; Romeo & Levin, 1971; Woods *et al.*, 1981). Our experiments with solid-phase-antibody–enzyme conjugate show that incubation with substrate prevents inactivation by *N*-ethylmaleimide (Table 3), thereby providing direct evidence that a thiol group is involved in binding both uroporphyrinogen III and pentacarboxyporphyrinogen III during decarboxylation. Protection was also provided by the corresponding porphyrins (Table 3), an observation that is consistent with the finding that uroporphyrin and some other porphyrins inhibit the decarboxylation reaction (Smith & Francis, 1981). The presence of a thiol group in the active centre is compatible with the protonation–deprotonation mechanism proposed for this enzyme (Barnard & Akhtar, 1975). The use of porphyrins and porphyrinogens to protect the enzyme from inactivation by thiol-group-specific reagents should allow direct determination of the

number of separate types of catalytic site involved in the reaction sequence.

Thiol groups also appear to be involved in the immunoreactivity of uroporphyrinogen decarboxylase. Inactivation of the enzyme by *N*-ethylmaleimide was accompanied by loss of reactivity towards both antisera. However, enzyme bound by a solid-phase-antibody reagent prepared from antiserum I retained catalytic activity that was susceptible to inhibition by *N*-ethylmaleimide (Table 3). Thus, it appears that the active centre and major antigenic determinants are at different sites on the enzyme molecule and that both contain thiol groups that are essential for their respective functions. Alternatively, formation of an ethylsuccinimide at the catalytic site when the enzyme is in solution may produce a change in conformation that leads to loss of immunoreactivity.

Measurements of the molecular weight of uroporphyrinogen decarboxylase have not previously been reported. It is not clear from our measurements whether the enzyme is a monomer or a dimer, since the molecular weight of the native enzyme was about 1.5 times that obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. In humans, an autosomal dominant mutation decreases uroporphyrinogen decarboxylase activity in erythrocytes and liver (Kushner *et al.*, 1976). Thus a monomeric enzyme would be expected to have the same structure in liver and erythrocytes, whereas at least one of the subunits of a dimer should be common to each tissue.

All the enzyme activity in human liver was precipitated by an antiserum raised against the erythrocyte enzyme (Fig. 6*b*). Evidence from quantitative immunoprecipitation and crossed immunoelectrophoresis (Figs. 6 and 7) indicates that the erythrocyte and liver enzymes are very similar but not immunochemically identical. The double precipitin arcs produced by the liver enzyme and, to a lesser extent, the erythrocyte enzyme (Fig. 6) may indicate the presence of closely related proteins, for example native and degraded enzyme (Laurell & McKay, 1981). The explanation of these minor differences is uncertain but, if the enzyme is a dimer, our findings do not exclude the possible presence of tissue-specific subunits or even mixtures of isoenzymes of similar charge. Alternatively, the differences may be explained by secondary alterations in structure, arising perhaps by partial proteolysis or modification of those thiol groups that are essential for immunoreactivity.

Little is known about the mechanisms that lead to decreased uroporphyrinogen decarboxylase activity in various pathological conditions. There is some evidence that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and hexachlorobenzene decrease catalytic activity without decreasing the concentration of immunoreactive enzyme (Elder & Sheppard, 1982). Use of the immunochemical methods described above to investigate uroporphyrinogen decarboxylase defects, particularly those that give rise to the different types of porphyria cutanea tarda in humans (De Verneuil & Nordmann, 1978; Elder *et al.*, 1978), should help to determine the pathogenesis of these disorders.

We thank Professor A. H. Jackson, Department of Chemistry, University College, Cardiff, for providing synthetic porphyrins and the Medical Research Council for financial support.

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