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Purification, *N*-terminal amino acid sequence and properties of hydroxymethylbilane synthase (porphobilinogen deaminase) from *Escherichia coli*

Graham J. HART,* Christopher ABELL and Alan R. BATTERSBY
University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K.

Hydroxymethylbilane synthase (porphobilinogen deaminase) was purified to apparent homogeneity from *Escherichia coli*. The enzyme is a monomer of M_r approx. 40000. The K_m for porphobilinogen and relative $V_{max.}$ values have been obtained at various pH values over the range 6.2–8.8, enabling pK values for ionizable groups important for activity to be determined. The *N*-terminal amino acid sequence is presented.

INTRODUCTION

Hydroxymethylbilane synthase, EC 4.3.1.8 [usually called 'porphobilinogen (PBG) deaminase'], together with uroporphyrinogen III synthase (EC 4.2.1.75), catalyse the transformation of PBG into uroporphyrinogen III (Battersby *et al.*, 1980). This macrocycle is a precursor for a family of pigments including haem, chlorophyll and vitamin B-12. Some of the properties of PBG deaminases from a number of sources have been investigated (Davies & Neuberger, 1973; Jordan & Shemin, 1973; 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 many potentially important studies are restricted by the small amounts of enzyme that can be conveniently purified. Thus studies are in progress to clone the PBG deaminase gene of *Escherichia coli*. We report here a purification of the enzyme, its *N*-terminal amino acid sequence and some of its properties.

EXPERIMENTAL

Bistris, Tris (Trizma grade), aprotinin, benzamidine hydrochloride, pepstatin, phenylmethanesulphonyl fluoride, dithiothreitol, bovine serum albumin, ovalbumin, carbonic anhydrase and cytochrome *c* were from Sigma Chemical Co., Poole, Dorset, U.K. Bactotryptone and bactopeptone were from Difco Laboratories, East Molesey, Surrey, U.K. 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.

Enzyme assays

PBG deaminase activity was determined as described by Battersby *et al.* (1983*a*). Protein concentrations were determined by A_{280} measurements, assuming an $A_{1cm}^{1\%}$ value of 10.

Buffer solutions

pH values for buffer solutions were adjusted at room temperature, irrespective of the temperature at which they were subsequently used.

Spectra

U.v.–visible absorption spectra were recorded on a Uvikon 810 recording spectrophotometer (Kontron Instruments).

Organisms

Two stains of *E. coli*, K12 and JA 200/pLC 41-4, were used. The latter was obtained from the *E. coli* Genetic Stock Centre, Department of Human Genetics, Yale University School of Medicine, New Haven, CT, U.S.A., and is a derivative of K12. This strain was found to produce approx. seven times the PBG deaminase activity produced by K12.

Growth of *E. coli*

K12 cells were grown at 37 °C in medium of the following composition: glucose (10 g/l), $(NH_4)_2SO_4$ (2 g/l), $MgSO_4$ (0.8 g/l) and KH_2PO_4 (10 g/l), adjusted to pH 7.1 with NaOH. Strain JA 200/pLC 41-4 was grown in the above medium supplemented with bactotryptone (1.6 g/l), bactoyeast extract (1 g/l) and NaCl (0.5 g/l). A 200 ml portion of an overnight shake culture was used to inoculate 20 l of growth medium in a 25 l fermenter vessel. During growth, the suspension was stirred at 250 rev./min, sterile air was slowly bubbled into the medium, and the pH was maintained at 7.1 by automatic titration with 5 M-NaOH. Cells were harvested after 24 h (while they were still in the exponential phase of growth) by using a Sharples continuous-flow centrifuge, were washed with 0.1 M-sodium phosphate buffer, pH 7.6, containing 0.1 mM-dithiothreitol, and, if not used immediately, were stored at −18 °C. A typical 20 l growth gave 65 g wet weight of cells.

Purification of PBG deaminase

All solutions used during the purification of the enzyme contained the following additions, unless

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

* To whom correspondence and reprint requests should be addressed.

Table 1. Purification of PBG deaminase from 65 g wet weight of *E. coli* JA 200/pLC 41-4

Step	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Initial extract	130	45000	12500	3.6	100	1
After heat treatment	116	42000	11630	3.6	93	1
After $(NH_4)_2SO_4$ precipitation, dialysis and gel filtration	162	36200	207	175	80	49
After Mono Q HR 10/10 f.p.l.c. at pH 8.5	16	23800	4.3	5530	53	1540
After Mono Q HR 5/5 f.p.l.c. at pH 6.0	2	9800	0.54	18100	22	5030
After Mono Q HR 5/5 f.p.l.c. at pH 8.3	1.7	3800	0.17	22300	8.5	6190

otherwise stated: dithiothreitol (0.1 mM), EDTA (0.6 mM), aprotinin (1 mg/l), benzamidine hydrochloride (1 mM), pepstatin (1 mg/l), phenylmethanesulphonyl fluoride (0.6 mM, previously dissolved in ethanol, 5 ml/l of final solution). When proteinase inhibitors were not included, large losses of activity and irreproducible behaviour were often observed during ion-exchange chromatography.

Operations were performed at 0–4 °C, except for the f.p.l.c. steps, which were at ambient temperature (generally 12–16 °C), but fractions from these columns were collected on ice.

Washed cells (thawed if previously frozen) were resuspended in 0.1 M-sodium phosphate buffer, pH 8.0 (2 ml/g wet wt. of cells) and sonicated in approx. 70 ml batches by using a Dawe Soniprobe (type 7530A) operated at 7A for 4 × 1 min each batch. The supernatant liquid from centrifugation (20000 *g*, 30 min) was divided into approx. 20 ml portions and heated in tubes (1.6 cm × 15 cm) in a water bath at 55 °C for 10 min, and then cooled in ice. Precipitated protein was removed by centrifugation (20000 *g*, 15 min). $(NH_4)_2SO_4$ (430 g/l) was added, and after 30 min the precipitate was collected by centrifugation (20000 *g*, 15 min), resuspended in a minimum volume of 15 mM-Tris/HCl buffer, pH 8.5, and dialysed against the same buffer for 1.5–2 h. Insoluble protein was removed by centrifugation (40000 *g*, 10 min) and the clear solution was fractionated on a column (4.6 cm × 112 cm) of Sephadex G-75 equilibrated with the last-mentioned buffer. Fractions (14 ml) were collected at a flow rate of 75 ml/h. PBG deaminase-containing fractions were pooled and applied to a Mono Q HR 10/10 column attached to a Pharmacia f.p.l.c. system. The column had previously been equilibrated with 15 mM-Tris/HCl, pH 8.5, and was eluted at 2 ml/min with a linear gradient of 40–250 mM-NaCl in the same buffer; the gradient volume was 210 ml. PBG deaminase was eluted in four to five 4 ml fractions at a NaCl concentration of approx. 190 mM. [On some occasions a small second peak of PBG deaminase activity was found to be eluted immediately after the main peak of enzyme activity. This peak, which contained approx. 15% of the activity of the main peak, is probably a PBG deaminase–PBG complex; such complexes have been found for PBG deaminases from human erythrocytes (Anderson & Desnick, 1980) and *Rhodopseudomonas spheroides* (Berry *et al.*, 1981). This minor peak has not been characterized.] Combined fractions were dialysed overnight against 15 mM-Bistris/HCl, pH 6.0, and applied to a Mono Q HR 5/5 f.p.l.c. column equilibrated with the same buffer. This buffer, in conjunction with a NaCl gradient (0–250 mM in 40 ml), was also used for elution at a flow rate of 1 ml/min. PBG deaminase was eluted at approx. 180 mM-NaCl and the combined fractions were dialysed against 15 mM-Tris/HCl, pH 8.3. At this stage SDS/polyacrylamide-gel electrophoresis showed the presence of three minor impurities. These were removed by further f.p.l.c. (Mono Q HR 5/5; 0–330 mM-NaCl in 15 mM-Tris/HCl, pH 8.3; 35 ml gradient at 1 ml/min). PBG deaminase was eluted at a NaCl concentration of approx. 220 mM. The final product was dialysed against appropriate buffers as required.

M_r determination

The M_r value for PBG deaminase was determined by

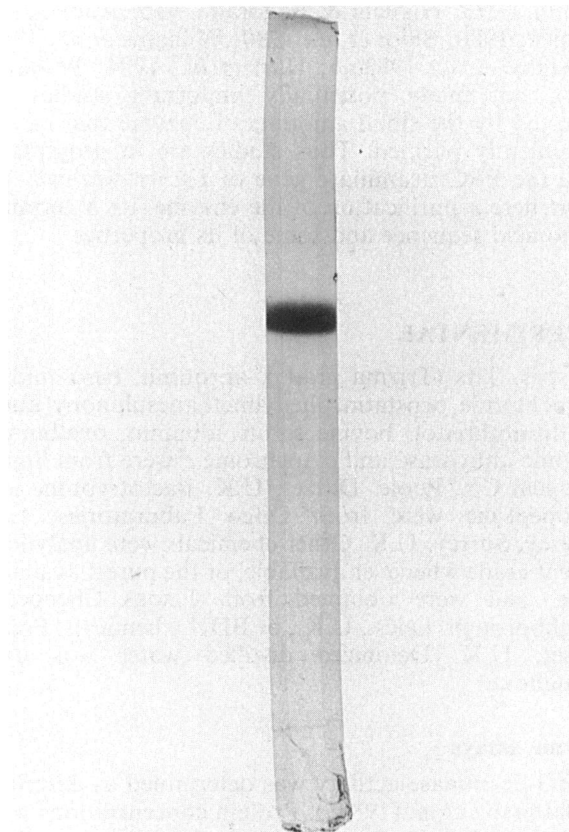

Fig. 1. Analysis of PBG deaminase from *E. coli* by SDS/polyacrylamide-gel electrophoresis

Electrophoresis was performed as described in the text with 7.5 μg of purified PBG deaminase.

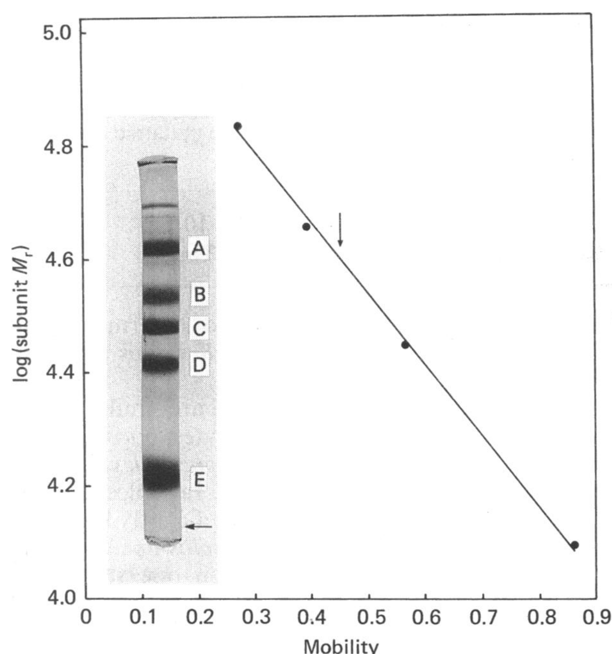

Fig. 2. Determination of minimum M_r for PBG deaminase from *E. coli* by SDS/polyacrylamide-gel electrophoresis

The mobilities of the peptide chains relative to a Bromophenol Blue marker are plotted against log (subunit M_r). The standards used are given above. The relative mobility of PBG deaminase is indicated by an arrow; this corresponds to an M_r of 39500. Inset: SDS/polyacrylamide-gel electrophoresis was performed as described in the text. The protein bands indicated are: A, bovine serum albumin (M_r 68000); B, ovalbumin (M_r 45000); C, PBG deaminase; D, carbonic anhydrase (M_r 29000); E, cytochrome *c* (M_r 12400). The arrow indicates the mobility of the Bromophenol Blue marker.

gel filtration on a Superose 12 HR 10/30 f.p.l.c. column equilibrated with 0.05 M-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl, or with 0.1 M-$NaHCO_3$, pH 8.2. The standards used (Sigma Chemical Co.) were bovine serum albumin, ovalbumin, carbonic anhydrase and cytochrome *c*. The void volume of the column was determined by using Blue Dextran. Samples were applied in a volume of 0.2 ml, the flow rate was 0.5 ml/min and the eluate was continuously monitored at 280 nm.

The M_r value for PBG deaminase under denaturing conditions was determined by using SDS/polyacrylamide-gel electrophoresis by the method of Weber *et al.* (1972). The protein standards used were those listed above.

Amino acid sequencing

The *N*-terminal amino acid sequence was determined at the S.E.R.C. sequencing service, Department of Biochemistry, University of Aberdeen, Aberdeen, Scotland, U.K., by Professor J. Fothergill and Mr. B. Dunbar. Enzyme purified from both strains of *E. coli* was sequenced with identical results.

RESULTS AND DISCUSSION

Table 1 summarizes a typical purification. The final product, which showed only one band on SDS/polyacrlamide-gel electrophoresis (Fig. 1), loses no more than 5% of its activity on storage on ice in 15 mM-Bistris/HCl buffer, pH 6.0, or in 0.1 M-sodium phosphate buffer, pH 7.0, over 28 days. The behaviour of the enzyme during the purification was identical for both strains of *E. coli* used. The final products were apparently identical as judged by M_r value, K_m for PBG and *N*-terminal amino acid sequence (see below).

The u.v.–visible absorption spectra of enzyme samples recorded in 0.1 M-sodium phosphate buffer, pH 7.0, containing EDTA (0.6 mM) and dithiothreitol (0.1 mM) as the only additions showed only one absorption maximum, at 276–278 nm, with a small shoulder at 290–295 nm; there was no absorbance above 320 nm. There is thus no evidence for bound cofactors.

Gel-filtration experiments on a calibrated Superose 12 column indicated an M_r value in the range 34000–40000. The lower value was obtained when the column was run in 0.05 M-sodium phosphate buffer (pH 7.0)/0.15 M-NaCl, the higher value when the column was run in 0.1 M-$NaHCO_3$. SDS/polyacrylamide-gel-electrophoretic experiments, one of which is shown in Fig. 2, indicated a minimum M_r value of 39100 ± 1600 (mean ± S.D. for five determinations). PBG deaminase from *E. coli* is clearly monomeric, and the enzyme is similar in size to those from human erythrocytes (Anderson &

Table 2. Results from kinetic experiments at various pH values for PBG deaminase from *E. coli*

Assays were carried out at 37 °C over a range of PBG concentrations in 0.2 M-sodium phosphate buffers (pH 6.2–8.0) or in 0.2 M-sodium pyrophosphate buffers (pH 8.0–8.8), and apparent values for K_m and $V_{max.}$ were obtained from plots of [PBG]/initial velocity against [PBG]. Mean values of $V_{max.}^{app.}$ for each pH value are expressed relative to the mean value (assigned as 1.00) obtained at pH 7.4.

pH	$K_m^{app.}$ (μM)	$V_{max.}^{app.}$ (arbitrary units)	Relative $V_{max.}^{app.}$
6.2	14	0.048	0.21
6.5	12	0.080	0.34
6.7	8, 6	0.085, 0.085	0.37
7.0	15.5, 13, 13.5	0.129, 0.172, 0.143	0.64
7.4	25, 20.5	0.243, 0.222	1.00
7.7	21	0.232	1.00
8.0 (phosphate)	14.5, 17.5, 16.5, 16.5	0.203, 0.207 0.224, 0.178	0.88
8.0 (pyrophosphate)	19	0.183	0.79
8.3	16	0.127	0.55
8.65	22	0.052	0.22
8.8	18.5	0.026	0.11

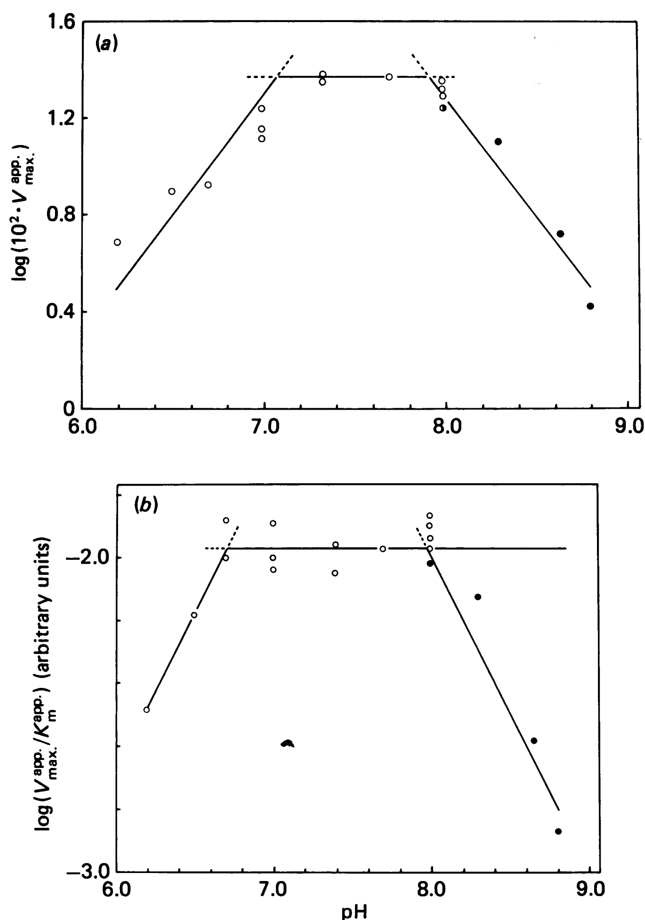

Fig. 3. Effect of pH on (a) $\log(V^{app.}_{max.})$ and (b) $\log(V^{app.}_{max.}/K^{app.}_m)$ for PBG deaminase from *E. coli*

Values for $V^{app.}_{max.}$ and $V^{app.}_{max.}/K^{app.}_m$ were obtained from plots of [PBG]/initial velocity against [PBG] made at several pH values in 0.2 M-sodium phosphate buffers (○) or 0.2 M-sodium pyrophosphate buffers (●). Logarithms of the values obtained are plotted against pH.

Desnick, 1980), spinach (Higuchi & Bogorad, 1975), *Rhodopseudomonas spheroides* (Davies & Neuberger, 1973; Jordan & Shemin, 1973), *Chlorella regularis* (Shioi *et al*., 1980), rat spleen (Williams, 1984) and *Euglena gracilis* (Williams *et al*., 1981).

Kinetic experiments were made at several pH values and values for $V^{app.}_{max.}$ and $K^{app.}_m$, obtained from plots (not shown) of [PBG]/initial velocity against [PBG], are given in Table 2. Plots of log ($V^{app.}_{max.}$), and log ($V^{app.}_{max.}/K^{app.}_m$) against pH were then constructed (Fig. 3); the intersection points for the straight-line sections of the plots, which must have slopes of +1, 0 and −1, are the pH values corresponding to pK_1^{ES}, pK_2^{ES} and pK_1^{E}, pK_2^{E} respectively (see, e.g., Cornish-Bowden, 1979), where pK^{ES} values represent ionizations in enzyme–substrate complexes and pK^{E} values represent ionizations in free enzyme. The values determined for pK_1^{ES}, pK_2^{ES}, pK_1^{E} and pK_2^{E} were 7.07, 7.92, 6.70 and 7.98 [cf. those determined for PBG deaminase from *Euglena gracilis* by Williams *et al*. (1981), which were 6.1, 8.9, < 5 and 8.2 respectively]. It is noteworthy that binding of PBG to *E. coli* PBG deaminase has virtually no effect on pK_2, but pK_1 is increased by 0.37 pH unit, presumably reflecting a substrate-induced conformational change.

Table 3. *N*-Terminal amino acid sequence for PBG deaminase from *E. coli*

One preparation gave small amounts of glycine, alanine or methionine at position 1 in addition to glutamic acid. The remaining sequence was identical.

1				5					10
Glu-	Leu-	Asp-	Asn-	Val-	Leu-	Arg-	Ile-	Ala-	Thr-

Further work will be necessary to determine which critical ionizations give rise to the pK values mentioned above.

The above values of $K^{app.}_m$ for PBG are similar to those for deaminase from human erythrocytes, 6 μM at pH 8.2 (Anderson & Desnick, 1980), *Rps. spheroides*, 13–20 μM at pH 7.6 (Jordan & Shemin, 1973), rat spleen, 1 μM at pH 7.5 (Williams, 1984), but are significantly lower than those for algal enzymes; *Euglena gracilis* has a K_m value for PBG of 195 μM at pH 8.0 when operating in the absence of uroporphyrinogen III synthase (Battersby *et al*., 1979, 1983*b*) and *Chlorella regularis* gave values of 85–90 μM at pH 7.4 (Shioi *et al*., 1980).

The *N*-terminal amino acid sequence of *E. coli* PBG deaminase given in Table 3 represents the first amino acid sequence information to be published for any PBG deaminase. Preliminary results indicate that this sequence is very different from the *N*-terminal sequence for PBG deaminase from *Euglena gracilis* (G. J. Hart & A. R. Battersby, unpublished work).

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