Evidence that pyridoxal phosphate modification of lysine residues (Lys-55 and Lys-59) causes inactivation of hydroxymethylbilane synthase (porphobilinogen deaminase)*

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A recombinant strain of *Escherichia coli* has been constructed that produces approx. 200 times the amount of hydroxymethylbilane synthase found in wild-type *E. coli* [Hart, Abell & Battersby (1986) *Biochem. J.* **240**, 273–276]. Enzyme purified from this strain is shown to be permanently inactivated by pyridoxal 5'-phosphate/ $\text{NaB}^1\text{H}_3^3\text{H}_1$. The inactivation is not complete despite the fact that approx. 1 mol of lysine residues is modified per mol of enzyme. Evidence is gained showing that (a) modification of one of two conserved lysine residues (Lys-55 or Lys-59) results in inactivation of hydroxymethylbilane synthase and (b) these lysine residues are present in or close to the active site.

INTRODUCTION

Hydroxymethylbilane synthase (HMBS, EC 4.3.1.8) [also known as porphobilinogen (PBG) deaminase], together with uroporphyrinogen III synthase (EC 4.2.1.75), catalyse the conversion of PBG (I) into uroporphyrinogen III (III) (Scheme 1), the precursor for haem, chlorophyll and vitamin B-12 (Battersby *et al.*, 1980; Leeper, 1985*a,b*, 1987). HMBS is an assembly enzyme that constructs hydroxymethylbilane (II) from PBG (I) (Battersby *et al.*, 1982), and a lysine residue has been demonstrated to be important for activity in enzyme from *Euglena gracilis* and *Rhodospseudomonas sphaeroides* (Hart *et al.*, 1984) and from wheat germ (Russell *et al.*, 1984).

The present paper describes the development of an HMBS-overproducing strain of *Escherichia coli*, partial inactivation of the isolated enzyme by pyridoxal 5'-phosphate/ $\text{NaB}^1\text{H}_3^3\text{H}_1$ and the subsequent identification of two heavily modified lysine residues.

EXPERIMENTAL

Materials

Trifluoroacetic acid (h.p.l.c. grade) was from Fisons Chemicals, Loughborough, Leics., U.K. Pyridoxal 5'-phosphate and iminodiacetic acid epoxy-activated Sepharose 6B (IDA-Sepharose) were from Sigma Chemical Co., Poole, Dorset, U.K. Acetic acid (A.R. grade) was from Koch-Light Ltd., Haverhill, Suffolk, U.K. Tosyl-L-phenylalanylchloromethane-treated trypsin, chymotrypsin and pepsin were from the Millipore Corp., Freehold, NJ, U.S.A. $\text{NaB}^1\text{H}_3^3\text{H}_1$ (7 Ci/mmol) was from Amersham International, Amersham, Bucks., U.K. Other chemicals were from the sources previously given (Miller *et al.*, 1988).

Enzyme assays

HMBS activity and M_r together with protein concentrations for purified samples of HMBS were determined as described by Hart *et al.* (1984, 1988).

Buffer solutions, growth of cells, spectral measurements and radioactivity assays

Buffer solutions were made and organisms were grown as described previously (Miller *et al.*, 1988). Fluorescence measurements were made with a Perkin-Elmer 3000 fluorescence spectrometer at room temperature with an excitation wavelength of 325 nm (slit width 5 nm) and an emission wavelength of 395 nm (Kempe & Stark, 1975) (slit width 10 nm). Radioactivity was assayed by liquid-scintillation counting on a Packard 2000 CA Tri-Carb or an LKB Rackbeta liquid-scintillation counter.

Bacteria

All cloning experiments used *E. coli* TG1 *recO*, obtained from Dr. P. Oliver, Department of Genetics, University of Cambridge. The *recO* mutation reduces the frequency of plasmid rearrangement (Morrison & Kolodner, 1986).

Construction of plasmids

Plasmid pPA119 was constructed (Fig. 1) by inserting a 1.7 kb *Bam*HI–*Sal*I fragment of pLC41-4 (Clarke & Carbon, 1976), containing the *hemC* gene (coding for HMBS), into the *tet*^R gene of pAT153 (Twigg & Sherratt, 1980) previously cut with the same two enzymes. A 270 bp *Bam*HI fragment of pKK223-3 (obtained from Pharmacia) containing the *tac* promoter was inserted into the *Bam*HI site of pPA119 to give pPA219, which was used to transform *E. coli* TG1 *recO* strain. HMBS assays were performed to identify a transformant con-

Abbreviations used: HMBS, hydroxymethylbilane synthase; PBG, porphobilinogen; IDA-Sepharose, iminodiacetic acid epoxy-activated Sepharose 6B.

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Scheme 1. Biosynthesis of uroporphyrinogen III

A = $\text{CH}_2\text{CO}_2\text{H}$; P = $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$.

taining a plasmid with the *Bam*HI fragment inserted in the required direction.

Purification of enzyme from the HMBS-overproducing strain

HMBS was purified as described previously (Hart *et al.*, 1986, 1988), and assay showed that the enzyme had been produced in approx. 200 times the amount present in wild-type cells.

Modification of HMBS with pyridoxal 5'-phosphate

HMBS (3.22 mg/ml) was dialysed against several changes of 0.1 M-sodium phosphate buffer, pH 7.0, containing EDTA (0.6 mM) and dithiothreitol (0.1 mM). The enzyme (now 2.86 mg/ml; 2 ml) was made 5 mM in pyridoxal 5'-phosphate and the solution was kept on ice for 1 h in the dark. A portion (75 μl) of freshly prepared 357 mM- NaBH_4 (containing $\text{NaB}^3\text{H}_1^1\text{H}_2$) in the same buffer as above was added, causing complete decolorization. After a further 30 min at 0 °C, the enzyme activity was assayed. Modified HMBS was dialysed against several changes of 15 mM-sodium phosphate buffer, pH 8.2, and then finally against 0.1 M-sodium phosphate buffer, pH 8.2; both buffers contained EDTA (0.6 mM) and dithiothreitol (0.1 mM).

Stoichiometry

Absorption spectra of samples of enzyme that had been inactivated, reduced and extensively dialysed as described above were recorded with a Uvikon 810P recording spectrophotometer. The extent of incorporation of pyridoxal 5'-phosphate was calculated by using a molar absorption coefficient of $1.07 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 325 nm for the reduced Schiff base derived from pyridoxal 5'-phosphate and the N^6 -amino group of lysine (Fischer *et al.*, 1963).

Carboxymethylation of modified HMBS

Pyridoxal 5'-phosphate-modified HMBS (2.13 mg/ml; 3.3 ml) was carboxymethylated as described by Miller *et al.* (1988). The extent of retention of the modifying pyridine residue was determined again as described above.

Digestion of modified HMBS with Endoproteinase Glu-C

Pyridoxal 5'-phosphate-modified and carboxymethylated HMBS (1.04 mg/ml; 4 ml; $1.45 \times 10^5 \text{ d.p.m./nmol}$) was digested with Endoproteinase Glu-C as described previously (Miller *et al.*, 1988).

Purification of cleavage peptides

Peptides were separated at a low light intensity by f.p.l.c. on a PEP RPC HR 5/5 column equilibrated with 0.1% (v/v) trifluoroacetic acid, and eluted with a 40 ml linear gradient of 0–80% (v/v) acetonitrile (far-u.v. grade) in 0.1% (v/v) trifluoroacetic acid. Elution of peptides was monitored at 214 nm, and fractions (1 ml) were collected at a flow rate of 0.7 ml/min. Appropriate portions were removed from selected fractions for assay of ^3H radioactivity, and the same fractions were then diluted to a final volume of 1.5 ml for fluorescence measurements. Two fractions eluted between 34.5 and 38.5% (v/v) acetonitrile, containing both significant radioactivity and fluorescent material, were combined. Three column runs were needed to process all the material from the above digest, and the total combined fractions were freeze-dried.

These peptides were further fractionated by immobilized-metal-ion (Fe^{3+}) affinity chromatography (Andersson & Porath, 1986) based upon the procedures of Michel & Bennett (1987) and Michel *et al.* (1988). A column of IDA-Sepharose (1 cm $\times$ 3 cm) was saturated with Fe^{3+} , by using 0.5 M- FeCl_3 , and equilibrated with 0.1 M-acetic acid (6 ml). The freeze-dried peptides were dissolved in 0.1 M-acetic acid (3 ml) and applied to the column, which was washed with 0.1 M-acetic acid (12 ml), 0.1 M-sodium acetate buffer, pH 5.0 (12 ml), and 1% (w/v) ammonium acetate buffer, pH 7.0 (12 ml). Finally the column was washed with 20 mM-sodium phosphate buffer, pH 8.0 (19 ml), to elute pyridoxal 5'-phosphate-modified peptides. Fractions (1.5 ml) were collected throughout and appropriate portions were removed for assay of ^3H radioactivity before fluorescence measurement. Fractions containing significant amounts of modified peptides (identified by high ^3H content and a strong fluorescent response) were pooled for final purification.

Modified peptides were purified by reverse-phase f.p.l.c. on the PEP RPC HR 5/5 column equilibrated with 24% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid and eluted at 0.7 ml/min with a linear gradient (20 ml) of 24–34% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. Elution of peptides was monitored at 214 nm, and five column runs were needed to process all the foregoing material. All major peaks (showing both appreciable fluorescence and ^3H radioactivity) were individually collected and concentrated, then freeze-dried in separate Eppendorf tubes, with the use of a Savant Speedvac Concentrator, ready for further analysis.

Amino acid analysis and sequence determination

Analysis for the amino acid composition of the modified peptides and their amino acid sequence (on an Applied Biosystems 477A/120A pulsed-liquid sequencer) was carried out as described previously (Miller *et al.*, 1988). For sequence analysis the sample size was typically 8–30 pmol, and at each cycle of the degradation 40% of the phenylthiohydantoin derivative was used for identification and the remainder assayed for ^3H radioactivity. Enzymic digestions of pyridoxal 5'-phosphate-modified peptides were carried out with pepsin (3%, w/w, in 35 μl of 2.5% formic acid at 37 °C for 2 h), trypsin (5%, w/w, in 12 μl of 0.5% NH_4HCO_3 at 37 °C for 3 h) or chymotrypsin (conditions as for trypsin). The resulting peptides were purified by reverse-phase h.p.l.c. on an Aquapore RP 300 column (2 mm $\times$ 3 cm) that had been equilibrated with 0.1% (v/v) trifluoroacetic acid and eluted at 0.1 ml/min with a linear gradient of 0–60% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid during 30 min. Monitoring was at 220 nm and 300 nm with a diode-array detector through a Hewlett-Packard 1090 L liquid chromatograph. The recovery of loaded peptide was generally 50–65%.

RESULTS AND DISCUSSION

The HMBS content of an *E. coli* strain containing the plasmid pPA119 (Fig. 1) was about 30-fold greater than that of cells without the plasmid. Insertion of a *tac* promoter into pPA119 upstream of the *hemC* gene gave a new plasmid pPA219, which led to approx. 200-fold over-expression of HMBS. Approx. 50-fold over-expression of HMBS has also been reported by Thomas & Jordan (1986). This new strain of *E. coli* (TG1 *recO*) containing pPA219 has been important also for our

other studies (Hart *et al.*, 1987, 1988; Beifuss *et al.*, 1988; Miller *et al.*, 1988).

E. coli HMBS was found to be inhibited by pyridoxal 5'-phosphate in a way qualitatively similar to the behaviour of enzymes from other sources (Hart *et al.*, 1984; Russell *et al.*, 1984). Inhibition of the *E. coli* enzyme required 50 min to reach equilibrium, and the maximum inhibition was obtained at a concentration of 5 mM-pyridoxal 5'-phosphate. In order to identify which amino acid residues of HMBS had been modified by the pyridoxal 5'-phosphate, the Schiff base intermediate was treated at the equilibrium stage with NaB^3H_4 . Activities of $30 \pm 5\%$ of the control (unmodified enzyme) were observed, which were unchanged after extensive dialysis, showing the modification to be permanent. The u.v.-visible-absorption spectrum of the modified HMBS showed two absorption maxima: the normal absorbance at 280 nm for protein together with a band at 325 nm corresponding to N^6 -phosphopyridoxyl-L-lysine residues (Fischer *et al.*, 1963). The latter absorbance corresponded to 0.8–0.9 mol of pyridoxyl 5'-phosphate/mol of enzyme, a value unaffected by subsequent carboxymethylation and dialysis procedures outlined above.

Digestion of modified HMBS with successive portions of Endoproteinase Glu-C (Miller *et al.*, 1988) caused almost complete cleavage provided that a fresh batch of proteinase was used each time. The initial f.p.l.c. purification of the cleavage peptides gave a very similar result to previous digestions (Miller *et al.*, 1988); only peptides that were eluted between 34.5 and 38.5% (v/v) acetonitrile were consistently fluorescent and strongly ^3H -labelled. Further purification of these labelled peptides was achieved by immobilized-metal-ion (Fe^{3+}) affinity chromatography (Andersson & Porath, 1986) (Fig. 2); this shows elution of peptides carrying phosphopyridoxyl

Fig. 1. Construction of plasmid pPA219

The *hemC* gene (coding for HMBS) from pLC41-4 was inserted into pAT153, giving pPA119. Inclusion of a *tac* promoter, from pKK223-3, upstream of the *hemC* insert in pPA119 gave pPA219, which was used to transform *E. coli*.

residues, which was carried out after all normal peptides had been washed through. The column affinity for N^6 -phosphopyridoxyl-lysyl peptides was higher than for phosphopeptides (Michel *et al.*, 1988), so that a sodium phosphate buffer was required, in addition to alkaline

Fig. 2. Purification of peptides from Endoproteinase Glu C digestion of modified HMBS on an immobilized-metal-ion (Fe^{3+}) affinity column

Fractions were collected as described in the text and both fluorescence ($\circ$) and ^3H radioactivity ($\bullet$) were determined.

pH, to elute the former peptides. To our knowledge, peptides modified by pyridoxyl 5'-phosphate have not previously been purified in this way. A final reverse-phase f.p.l.c. step further separated the labelled products (Figs. 3a and 3b). In an analytical experiment (Fig. 3a), at least 60% of the total ^3H recovered in the labelled fractions was associated with one broad peak (designated A) with the remainder distributed over the other fractions. Of these, peak B was the next most strongly labelled (at least 7% of the total recovered ^3H activity) (Fig. 3a); it was further characterized along with peak A.

Amino acid composition analysis of peak A suggested a single peptide corresponding to the section Leu-38-Glu-67 (Fig. 4) of the gene-derived amino acid sequence (Thomas & Jordan, 1986; Alefounder *et al.*, 1988). This peptide should contain three lysine residues, but only two were detected by amino acid analysis, suggesting that one had been modified by pyridoxal 5'-phosphate. The three lysine residues are located in the last 13 residues of this 30-residue peptide, so further cleavage was desirable. Digestion of a sample (500 pmol) with pepsin and fractionation of the products by reverse-phase h.p.l.c. gave one major peptide (AP1), which absorbed at 300 nm (phosphopyridoxyl-lysine absorption maximum in acid) and contained 72% of the recovered ^3H . The remaining ^3H was accounted for in undigested peptide (11%) and five other minor peaks (each 2–7%). Sequence analysis of peptide AP1 (Fig. 4) identified this peptide as Asp-50-Phe-62 of the amino acid sequence. Both lysine residues in this peptide gave their corresponding phenylthiohydantoin derivatives in low yield (20–30% of the expected value), which suggested that both residues had been partly modified by reaction with pyridoxal 5'-phosphate/ $\text{NaB}^1\text{H}_3^3\text{H}_1$. At this stage it was clear that only a very small amount at most of pyridoxyl 5'-phosphate could be associated with Lys-64.

Fig. 3. Final separation of phosphopyridoxyl-peptides, from Endoproteinase Glu-C digestion of modified HMBS, on PEP RPC HR 5/5

(a) The illustrated elution pattern (.....) was obtained by measuring A_{214} , with the trifluoroacetic acid buffers as described in the text, with approx. 1 nmol of peptides. The bold line (—) shows the acetonitrile gradient. Fractions were collected (1 ml) and analysed for fluorescence ($\bullet$) and ^3H radioactivity ($\circ$). (b) Typical elution profile (—) obtained, with the same buffers as in (a), with approx. 10 nmol of peptides during preparative-scale purification of peaks A and B. The bold line (—) shows the acetonitrile gradient.

Fig. 4. Sequence analysis of pyridoxal 5'-phosphate-modified peptides from HMBS

The phosphopyridoxyl-peptides were purified as described in the text. The identity of peak A was deduced from its amino acid composition referenced to the amino acid sequence of HMBS (Thomas & Jordan, 1986; Alefounder *et al.*, 1988). Other sequences ($\longleftrightarrow$) were determined by automated Edman degradation and identification of amino acid phenylthiohydantoin derivatives; (K) represents the site of a lysine residue in the HMBS sequence but which from peak A gives a low yield of lysine phenylthiohydantoin derivative; X represents a site from which no recognizable amino acid phenylthiohydantoin derivative was observed in sufficient yield to be identified.

In order to confirm that both Lys-55 and Lys-59 were labelled, peptide AP1 (190 pmol) was treated with trypsin. Four major products were isolated by h.p.l.c., one of which corresponded to undigested material. Of the remainder, one (AT1) was neither radioactive nor showed the characteristic 325 nm absorbance (300 nm in acid) of phosphopyridoxyl-lysine. Sequence analysis showed peptide AT1 to be Asp-50–Lys-55, and it gave a normal yield of lysine phenylthiohydantoin derivative from the unlabelled lysine residue that had been the site of cleavage of trypsin. The two remaining peptides, AT2 and AT3, were each labelled with ^3H . Peptide AT2 was found to be Val-56–Phe-62; the very low yield of lysine phenylthiohydantoin derivative (2–3 % of the expected value) together with a small release of ^3H at the fourth sequence cycle confirmed that Lys-59 carried a phosphopyridoxyl residue. Barely 3 % of the loaded ^3H was recovered because the anilinothiazolinone derivative of phosphopyridoxyl-lysine was not extracted from the Polybrene-coated sample disc. As expected, the modified Lys-59 had not been a site of attack by trypsin. Peptide AT3 was shown, by similar experiments, to be Asp-50–Lys-59 in which Lys-55 carried the modifying pyridoxyl 5'-phosphate group whereas the C-terminal Lys-59 did not.

The foregoing results can only be explained if the peptide fraction AP1 is a mixture of two singly modified peptides (Fig. 4), one containing phosphopyridoxyl-Lys-55 and the other phosphopyridoxyl-Lys-59, which, because of their closely similar properties, failed to separate under the h.p.l.c. conditions used. However, the selectivity of trypsin for unmodified lysine residues allowed the presence of the two peptides to be detected.

It thus follows that peak A (Figs. 3a and 3b) is a mixture of two peptides with identical primary sequences (Fig. 4) but singly modified at either Lys-55 or Lys-59. These two residues are by far those most heavily modified when HMBS is treated with pyridoxal 5'-phosphate/ $\text{NaB}^1\text{H}_3^3\text{H}_1$ (Fig. 3a), and the results from the sequence analyses above indicate that both residues are modified to similar extents. Hence the observed inactivation of HMBS by pyridoxal 5'-phosphate/ $\text{NaB}^1\text{H}_3^3\text{H}_1$ is either a result of modification of one specific residue (Lys-55 or Lys-59) or due to the fact that modification of either one causes inactivation.

Substrate protection experiments involved preparation of HMBS–PBG₂ di-complex and HMBS–PBG₃ tri-complex (Hart *et al.*, 1987, 1988; Jordan *et al.*, 1988), which

were treated separately with pyridoxal 5'-phosphate/ $\text{NaB}^1\text{H}_3^3\text{H}_1$ as above. The two products still retained respectively 60 % and 70 % of the enzymic activity of the corresponding untreated di- and tri-complexes, and the incorporation of phosphopyridoxyl groups was in each case approx. 50 % of the level observed when HMBS not carrying bound substrate was subjected to the lysine-modifying conditions. Enzymic degradation of the two pyridoxal-modified complexes, as above, followed by h.p.l.c. fractionation showed in each case a substantial decrease in the size of peak A relative to the other peaks in the chromatogram; the earlier results for digestion of lysine-modified HMBS showing peak A are illustrated in Figs. 3(a) and 3(b). These results interlock to indicate that enzyme-bound substrate protects one or both of Lys-55 and Lys-59 from modification.

Bearing in mind the initial incorporation of approx. 1 mol of pyridoxyl 5'-phosphate/mol of enzyme and the firm identification of two singly modified peptides as the major products from Endoproteinase Glu-C digestion of modified HMBS, it appears that modification of both Lys-55 and Lys-59 cannot occur to any significant extent in the same enzyme molecule. Presumably steric congestion around their location prevents attack at the second lysine residue once one has been modified. There are interesting possibilities for the roles of Lys-55 and Lys-59. One or both may play a direct part in the HMBS catalytic cycle, but the indirect effect of the presence of one on the pK_a value of the other could also be important.

Characterization of peak B (Figs. 3a and 3b) was carried out as for peak A. By amino acid composition, this peptide was Asn-4–Glu-37 of the gene-derived sequence, and it was subdigested once with chymotrypsin. Sequence analysis of the labelled peptides from the chymotryptic digest showed peptide B to be modified by pyridoxal 5'-phosphate at Lys-24 only. No significant amount of label was detected on Lys-26, the other lysine residue in this peptide. Peak B (cf. Figs. 3a and 3b) was not significantly decreased in the substrate protection experiments described above, and so became the dominant labelled peak in the chromatogram. Lys-24 cannot be in the PBG-binding site and a role in catalysis is therefore unlikely.

CONCLUSIONS

HMBS isolated from a new overproducing strain of *E. coli* is found to be permanently inactivated by pyridoxal

5'-phosphate/ $\text{NaB}^1\text{H}_3^3\text{H}_1$. There is only a 60–65 % loss in enzyme activity despite the fact that approx. 1 mol of lysine residues is modified per mol of enzyme. A similar observation has been reported recently by Warren & Jordan (1988). Pyridoxal 5'-phosphate/ $\text{NaB}^1\text{H}_3^3\text{H}_1$ modifies several lysine residues in the enzyme but two residues are modified far more extensively than any others; they are identified as Lys-55 and Lys-59 and substrate protection experiments indicate that both are in the active site or close to it. Inactivation of HMBS results from modification of one or possibly of either one of these lysine residues. Their apparent significance for the catalytic cycle is in keeping with their being conserved in HMBS from *E. coli* (Thomas & Jordan, 1986; Alefounder *et al.*, 1988), human (Raich *et al.*, 1986) and rat (Stubnicer *et al.*, 1988).

The observed minor modification of Lys-24, the lack of protection of it in the di- and tri-complexes with PBG and the fact that Lys-24 is not a conserved residue all indicate that it does not have an important catalytic role.

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