Investigation of putative active-site lysine residues in hydroxymethylbilane synthase

Preparation and characterization of mutants in which (a) Lys-55, (b) Lys-59 and (c) both Lys-55 and Lys-59 have been replaced by glutamine*

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A new construct carrying the *hemC* gene was transformed into *Escherichia coli*, resulting in approx. 1000-fold over-expression of hydroxymethylbilane synthase (HMBS). This construct was used to generate HMBS in which (a) Lys-55, (b) Lys-59 and (c) both Lys-55 and Lys-59 were replaced by glutamine (K55Q, K59Q and K55Q–K59Q respectively). All three modified enzymes are chromatographically separable from wild-type enzyme. Kinetic studies showed that the substitution K55Q has little effect whereas K59Q causes a 25-fold decrease in k_{cat}^{app}/K_m^{app} . Treatment of K55Q, K59Q and K55Q–K59Q separately with pyridoxal 5'-phosphate and NaBH₄ resulted in incomplete and non-specific reaction with the remaining lysine residues. Pyridoxal modification of Lys-59 in the K55Q mutant caused greater enzymic inactivation than similar modification of Lys-55 in K59Q. The results in sum show that, though Lys-55 and Lys-59 may be at or near the active site, neither is indispensable for the catalytic activity of HMBS.

INTRODUCTION

Hydroxymethylbilane synthase (HMBS, EC 4.3.1.8, also known as porphobilinogen deaminase) catalyses the conversion of porphobilinogen (PBG) into hydroxymethylbilane (Scheme 1), which is subsequently cyclized by uroporphyrinogen III synthase (EC 4.2.1.75) to uroporphyrinogen III, the precursor for haem, chlorophyll and vitamin B-12 (Battersby *et al.*, 1980). HMBS has previously been purified from *Escherichia coli* (Hart *et al.*, 1986; Jordan *et al.*, 1988) and the corresponding gene *hemC* has been sequenced (Thomas & Jordan, 1986; Alefounder *et al.*, 1988).

E. coli HMBS has been shown to be partially inactivated by treatment with pyridoxal 5'-phosphate (PLP) and NaBH₄ (Miller *et al.*, 1989). PLP specifically modifies amino groups (Karpeisky & Dixon, 1986; Eyzaguirre, 1987), and is therefore expected to react with several of the eight lysine residues and possibly with

the free α -amino group of the terminal methionine residue. Analysis of cleavage peptides from wild-type HMBS modified by PLP/NaBH₄ revealed that Lys-55 and Lys-59 are the most heavily modified positions (Miller *et al.*, 1989), and the fact that protection was afforded by bound PBG against PLP modification (Hart *et al.*, 1984) indicated that these lysine residues are at or close to the active site.

The present paper describes experiments to assess the importance of these lysine residues for catalysis. Three modified proteins with lysine-to-glutamine changes, K55Q, K59Q and the double mutant K55Q–K59Q, were prepared by using oligonucleotide-directed mutagenesis *in vitro*. The mutant proteins were characterized kinetically, and their modification with PLP/NaBH₄ was studied.

EXPERIMENTAL

General

Reagents were obtained as reported previously (Hart *et al.*, 1986, 1988; Miller *et al.*, 1989). Plasmids pUC18 and pIH223-3 were obtained from Pharmacia. The oligonucleotides for use in site-directed mutagenesis were prepared either by Dr. B. Langlois d'Estaintot in this Laboratory or by Mike Weldon, Department of Biochemistry, University of Cambridge. U.v.-absorption spectra were recorded on a Perkin–Elmer Lambda 2 spectrophotometer attached to an Amstrad computer equipped with the PECSS software (Perkin–Elmer).

Bacteria, construction of plasmids and site-directed mutagenesis

All cloning experiments used *E. coli* strain TG1 *recO* (Miller *et al.*, 1989). Plasmid pPA102 was constructed from a *PvuII* fragment of pUC18 containing the origin of replication, but not the multiple cloning region, plus an *HaeIII* fragment of pIH223-3 containing the *tac* promoter and the multiple cloning region. The latter plasmid is a derivative of pKK223-3 lacking the *BamHI* site upstream of the *tac* promoter. The absence of this

Scheme 1. Reaction catalysed by hydroxymethylbilane synthase (HMBS)

A = CH₂CO₂H; P = CH₂CH₂CO₂H.

Abbreviations used: HMBS, hydroxymethylbilane synthase; PBG, porphobilinogen; PLP, pyridoxal 5'-phosphate.

* This paper is Part 36 of the series 'Biosynthesis of Porphyrins and Related Macrocycles'. Part 35 is Hart *et al.* (1990).

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site from pPA102 allows the *Bam*HI site in the multiple cloning region to be used. Since the *lacIⁿ* gene of pUC18 contains one of the *Pvu*II sites, it will have been inactivated during the construction of pPA102. An unmodified *hemC* gene was cloned into pPA102 to give pPA410.

The Amersham system (version 2) was used for oligonucleotide-directed mutagenesis *in vitro*. The original template bacteriophage contained the *hemC* gene coding strand (*Bam*HI–*Sal*I fragment) cloned into M13mp8. Oligonucleotides were used for the generation of mutants and to act as internal primers for sequencing potentially mutant bacteriophage (clones were reversed by removal of the insert by using *Bam*HI and *Hind*III and its subsequent insertion into M13mp19 cut with the same two enzymes). Following the mutagenesis procedure, a few plaques were picked and screened for the presence of the required mutation by sequencing the region of interest. In each case, when a mutant had been selected, it was checked by sequencing the entire gene on both strands. The double mutation K55Q–K59Q was made by using the K55Q mutant bacteriophage as template.

Plasmids pPA413 (K55Q), pPA448 (K59Q) and pPA453 (K55Q–K59Q) were obtained after cloning the corresponding mutant *hemC* genes into pPA102.

Growth of cells, purification of HMBS species and enzyme assays

Buffers, growth conditions and the protein purification were as previously described (Hart *et al.*, 1988). Protein concentrations were determined by A_{280} measurements. Enzyme assays were carried out as described by Hart *et al.* (1984) with PBG concentrations of 270 μ M at pH 8.0 (standard assays) and 3–400 μ M at pH 7.4 (K_m^{app} determinations); 25 units of enzyme solution were used in the latter assays. One unit of enzyme activity is defined as the amount of enzyme necessary to produce 1 nmol of porphyrinogen in 1 h.

Modification of HMBS species with PLP/NaBH₄

All buffers used in these experiments contained EDTA (0.6 mM) and dithiothreitol (0.1 mM). PLP (13.3 mg/ml; 178 μ l) and NaBH₄ (13.5 mg/ml; 60 μ l) were added to samples of enzyme solution (2.4 mg/ml; 1.6 ml) in 0.1 M-sodium phosphate buffer, pH 7.0, as described previously (Miller *et al.*, 1989). As a control, samples containing enzyme were treated in the same way with NaBH₄ but not PLP. The enzyme specific activities were not reduced in the controls. Residual activities for the PLP/NaBH₄-treated samples were determined with respect to controls (Table 1). Three sample portions were then combined, and the buffer was exchanged against 15 mM-Tris/HCl buffer, pH 8.3, by using NAP-25 columns (Pharmacia). Of the resulting mixture, 7.0 ml

(corresponding to 10.4 mg of protein starting material) was applied to a Mono Q HR 10/10 column and chromatographed at 4 ml/min with a linear gradient of 0–0.4 M-NaCl in 300 ml of the same buffer. The corresponding elution profiles are shown in Fig. 2(a). Fractions (2–4 ml) were collected at the apex of representative peaks and assayed. U.v.-absorption spectra of these fractions were recorded and corrected for eluent composition at the position of the corresponding peaks. By using data from the appropriate control (see below) the plots in Figs. 2(b) and 2(c) were generated.

Control samples were combined similarly, and after buffer exchange 1.00 ml (corresponding to 1.49 mg of protein starting material) was chromatographed. The specific activity of the eluted material was 1.00 ± 0.10 with respect to the mean of the values for individual HMBS species given in Table 1. The area obtained in these chromatographs was used to estimate the amount of protein left unmodified in the PLP/NaBH₄-treated samples (Table 1).

In separate experiments, control and sample portions were combined similarly, and buffer exchange was performed three times in series by using NAP-25 columns and an elution volume of 3.00 ml to remove completely excess reduced PLP. The u.v.-absorption spectrum of the control (unmodified protein) was then subtracted from the spectrum of the sample (PLP/NaBH₄-modified protein) to get an average u.v.-absorption spectrum of the modifying residues. For HMBS and its mutants, these spectra are remarkably similar, the absorbance maximum being at 323 nm (wild-type), 324 nm (K59Q), 325 nm (K55Q) and 326 nm (K55Q–K59Q). The spectra exhibit a minimum at 273 nm (20–28% of the maximum) and a shoulder at 293 nm. Within experimental error, the absorbance contribution at 280 nm is independent of the type of HMBS [$31.5 \pm 3.2\%$ ($n = 4$) of the maximum] and was used to calculate the specific activities of representative fractions (Fig. 2c).

RESULTS AND DISCUSSION

A convenient vector for cloning and over-expression of mutant *hemC* genes, plasmid pPA102, has been constructed (see the Experimental section). Mutant *hemC* genes corresponding to the substitutions K55Q, K59Q and K55Q–K59Q have been generated by using the Amersham system and introduced into pPA102 along with unmodified *hemC* as a control. The HMBS enzymes were over-expressed in *E. coli* TG1 *recO* and purified by following previously established procedures (Hart *et al.*, 1988). The enzymes were characterized according to their specific activity in the standard assay (Table 1), their f.p.l.c. elution properties on MonoQ (Fig. 1), their kinetic parameters (Table 2) and their behaviour when subjected to modification with PLP/NaBH₄ (Table 1 and Fig. 2). Where duplicate data are given, these represent results obtained from separate fermentations of the same strain.

Compared with the plasmid pPA219 (Miller *et al.*, 1989), the use of vectors derived from pPA102 lead to much higher levels of over-expression of HMBS enzymes. The level is independent of whether wild-type or modified HMBS is expressed and was found to be 920 ± 420 ($n = 8$) with respect to wild-type *E. coli* K12.

When assayed under standard conditions, i.e. with a sufficiently high PBG concentration to saturate wild-type HMBS, the mutant enzymes show a lower specific activity than wild-type HMBS (Table 1). Of the two substitutions, K59Q has a more pronounced effect than K55Q, and with both substitutions to give (K55Q–K59Q)-HMBS the two effects appear to combine. When the four over-produced HMBS enzymes (wild-type and the three mutant forms) were analysed by f.p.l.c., a single protein peak was obtained in each case. This is in marked contrast with the results for wild-type HMBS reported by Jordan *et al.* (1988), who, using

Table 1. Properties of HMBS enzymes: wild-type, K55Q, K59Q and K55Q–K59Q

Nature of HMBS	<i>E. coli</i> strain TG1 <i>recO</i>	Specific activity in standard assay		Modification with PLP/NaBH ₄		
		(units/mg)	(%)	Residual activity (%)	Portion of protein left unmodified (%)	
Wild-type	pPA410	10500*	100	30,† 29	< 1.9	
		10900, 9430				
K55Q	pPA413	7600, 8230	77 ± 7	33, 37	< 6.0	
K59Q	pPA448	2750, 2940	28 ± 3	47, 52	< 4.9	
K55Q–K59Q	pPA453	2430, 2550	24 ± 2	54, 62	~ 6.6, ~ 5.6	

* Taken from Hart *et al.* (1986).

† Taken from Miller *et al.* (1989).

Fig. 1. Chromatography of HMBS enzymes on MonoQ HR 10/10

(a) With 20 mM-Tris/HCl buffer, pH 7.5, and 0.5 mg each of wild-type and K55Q-HMBS; (b) 15 mM-Bistris/HCl buffer, pH 6.0, and 0.4 mg, 0.7 mg and 1.0 mg of wild-type, K59Q- and (K55Q-K59Q)-HMBS respectively; in both cases a linear gradient (0–350 mM-NaCl in 240 ml of the same buffer) was used at a flow rate of 4 ml/min.

both a different over-producer and isolation procedure, obtained a mixture of five closely related proteins all showing similar HMBS activity. The mixture was used for their work. Our analytical conditions (column, buffer etc.) were identical with those reported by Jordan *et al.* (1988).

All three mutant enzymes are separable from wild-type HMBS (Fig. 1), and K55Q and K59Q can be separated from K55Q-K59Q. The substitution of glutamine for lysine increases the overall negative charge of the protein, and the elution order

is as expected on that basis. A clean separation of K59Q from K55Q has not been achieved, but the latter is always eluted slightly later than the former when they are run separately.

Determination of the kinetic parameters of the HMBS enzymes at pH 7.4 showed that $k_{cat}^{app.}$ is hardly affected by the substitutions, whereas $K_m^{app.}$ is substantially increased when K59Q is introduced. This is illustrated by an approx. 25-fold lower $k_{cat}^{app.}/K_m^{app.}$ of enzymes carrying Gln-59 instead of Lys-59.

Lys-55 and Lys-59 were chosen as targets for site-directed mutagenesis because these were the residues most heavily labelled upon treatment of wild-type HMBS with PLP/NaBH₄³H₁ (Miller *et al.*, 1989), and therefore similar modification of the mutant enzymes was of interest. In addition to the determination of residual activities after PLP/NaBH₄ treatment of the mutant enzymes (Table 1), the mixtures were chromatographed at pH 8.3 on MonoQ, and representative peaks were characterized according to specific activity and absorbance maxima A_{λ} above 300 nm (Fig. 2) due to reduced *N*⁵-phosphopyridoxyl-L-lysine residues. It is known that the peak position for this chromophore and its absorption coefficient are strongly dependent on its environment in the protein, the ϵ values varying by up to 2-fold (Eyzaguirre, 1987; Fischer *et al.*, 1963; Blackburn & Schachman, 1976; Paech & Tolbert, 1978). Thus the stoichiometric loading cannot be reliably calculated; we used an ϵ value of 4600 M⁻¹·cm⁻¹.

The results obtained by treatment of HMBS with PLP and NaBH₄ are summarized as follows. In all cases $\geq 94\%$ of the original protein is modified by treatment with PLP/NaBH₄. So the residual activity of the products must be largely due to enzyme molecules carrying one or more modifying residues. More heavily modified protein molecules with more negatively charged phosphate groups are retained by the anion-exchanger more strongly than lightly loaded ones (Fig. 2b). In addition, the elution profiles suggest that individual lysine residues are modified incompletely and rather non-specifically, producing mainly mixtures of partially loaded proteins. Fig. 2(c) indicates that an increasing degree of modification leads to a larger decrease in specific activity. This is in keeping with results obtained previously with HMBS from other sources (Hart *et al.*, 1984).

Partial PLP/NaBH₄ modification of Lys-59 in K55Q reduces the enzymic activity more than partial modification of Lys-55 in K59Q (cf. residual activities, Table 1). This is in keeping with the observation that the substitution lysine to glutamine has a stronger effect at the position 59 than at the position 55 of wild-type HMBS.

CONCLUSIONS

Use of the vector pPA102 led to an approx. 1000-fold over-expression of HMBS and to modified forms of HMBS having lysine-to-glutamine substitutions at sites 55 and/or 59. The K55Q substitution leads to a slight decrease of the specific activity at pH 8.0, whereas $K_m^{app.}$ and $k_{cat}^{app.}$, measured at pH 7.4, remain as for the wild-type enzyme. The K59Q substitution has more pronounced effects. While $k_{cat}^{app.}$ is unaffected, $K_m^{app.}$ increases by more than one order of magnitude. This leads to a low specific activity (approx. 28% with respect to wild-type enzyme), since the concentration of PBG used in the standard assay is no longer high enough to saturate K59Q-HMBS. The double substitution K55Q-K59Q shows that the effects of each single substitution on the specific activity and the kinetic parameters combine without mutual interference. The three mutant enzymes can be separated chromatographically (Mono Q) from the wild-type protein, but K55Q was inseparable from K59Q. Treatment of the enzymes with PLP/NaBH₄ caused $\geq 94\%$ of the original protein to be modified in each case. Individual lysine residues are modified

Table 2. Kinetic parameters of wild-type-, K55Q-, K59Q-, and K55Q-K59Q-HMBS from *E. coli* at pH 7.4 and 37 °C

Nature of HMBS	$K_m^{app.}$ (μ M)	$k_{cat}^{app.*}$ (s ⁻¹)	$k_{cat}^{app.}/K_m^{app.}$ (M ⁻¹ ·s ⁻¹)
Wild-type	5.2	0.106	20300
	9.7	0.104	10700
K55Q	7.0	0.108	15400
	4.3	0.102	23700
K59Q	167	0.107	639
	186	0.134	720
K55Q-K59Q	116	0.0825	714
	175	0.112	641

* With respect to formation of product.

Fig. 2. F.p.l.c. of mixtures obtained after treatment of wild-type and mutant HMBS enzymes with PLP/NaBH₄.

(a) Elution profiles corresponding to 10.4 mg of protein starting material. The arrows mark the gradient volume for elution of unmodified protein; (b) ratio of absorbances at λ_{max} and 280 nm (A_{λ}/A_{280}) of representative peaks versus gradient volume; (c) A_{λ}/A_{280} versus specific activity of representative peaks. ●, Wild-type HMBS; ○, K55Q-HMBS; △, K59Q-HMBS; □, K55Q-K59Q-HMBS. For further details see the Experimental section.

incompletely and apparently independently, higher modification causing greater loss of activity. Partial modification of Lys-59, similar to the substitution K59Q, has a stronger effect than (partial) modification of Lys-55.

Finally, although the modification study with PLP and NaBH₄ (Miller *et al.*, 1989) indicated that Lys-55 and Lys-59 may be at or near the active site, the current study shows that neither is indispensable for the catalytic activity of HMBS.

We thank the Science and Engineering Research Council and Roche Products Ltd. for financial support, the Stiftung für Stipendien auf dem Gebiete der Chemie (Switzerland) for a postdoctoral grant to A. H.), the Wellcome Trust for supporting the Cambridge Oligonucleotide Synthesis Facility, and Dr. B. Langlois d'Estaintot and Mike Weldon, Cambridge, for their help in preparing oligonucleotides for use in site-directed mutagenesis.

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Received 21 March 1990/4 May 1990; accepted 23 May 1990