Mutagenesis of arginine residues in the catalytic cleft of *Escherichia coli* porphobilinogen deaminase that affects dipyrromethane cofactor assembly and tetrapyrrole chain initiation and elongation

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Substitutions of conserved arginine residues in the catalytic cleft of *Escherichia coli* porphobilinogen deaminase were constructed by site-specific mutagenesis of the *hemC* gene. Mutant proteins exhibited a range of defects including the failure to assemble the dipyrromethane cofactor and the inability to initiate and propagate the tetrapolymerization reaction. Mutations of arginine residues at positions 11, 131, 132 and 155, all of which interact with the carboxylic acid side chains of the dipyrromethane cofactor, were the most disruptive.

INTRODUCTION

Uroporphyrinogen III, the first cyclic intermediate of the tetrapyrrole biosynthesis pathway, is assembled in two enzymic steps from four molecules of porphobilinogen. Initially, porphobilinogen deaminase (EC 4.3.1.8) catalyses the tetrapolymerization of porphobilinogen to give preuroporphyrinogen, a highly unstable 1-hydroxymethylbilane intermediate (Burton *et al.*, 1979; Battersby *et al.*, 1979b). Preuroporphyrinogen then acts as the substrate for uroporphyrinogen III synthase and is transformed into uroporphyrinogen III (Jordan *et al.*, 1979). Porphobilinogen deaminase assembles the four rings of preuroporphyrinogen in a stepwise fashion in which the pyrrole ring A is first bound to the deaminase followed by rings B, C and finally D (Jordan & Seehra, 1979; Battersby *et al.*, 1979a). Enzyme-intermediate complexes with one, two or three molecules of bound substrate (ES, ES₂ and ES₃) may be isolated and their individual properties studied (Berry *et al.*, 1981; Warren & Jordan, 1988).

Porphobilinogen deaminase possesses a novel prosthetic group, the dipyrromethane cofactor (Jordan & Warren, 1987; Hart *et al.*, 1987), which arises from the autocatalytic coupling of two molecules of porphobilinogen (rings C1 and C2 in Scheme 1) to the apo-deaminase protein. The dipyrromethane cofactor is covalently linked to cysteine-242 in the *Escherichia coli* enzyme (Jordan *et al.*, 1988a; Hart *et al.*, 1988), and is not itself incorporated into the product but functions as a primer to which the four substrate molecules are sequentially attached (Jordan & Warren, 1987; Warren & Jordan, 1988). Thus, at the point before the release of the tetrapyrrole product from the deaminase, a total of six pyrrole units (C1, C2 and substrates A, B, C and D) are attached to the protein. The negatively charged acetate and propionate side chains (A and P in Scheme 1) of these pyrrole units would be expected to interact with positively charged amino acid side chains in the protein. Comparison of the derived primary structure of the *Escherichia coli* enzyme (Thomas & Jordan, 1986) with those from human (Raich *et al.*, 1986), rat (Stubnicer *et al.*, 1988) and *Euglena gracilis* (Sharif *et al.*, 1989) identified 12 conserved arginine residues at positions 7, 11, 101, 131, 132, 149, 155, 176, 182, 206, 232 and 277, several of which were proposed as candidates for the acetate (A)- and propionate (P)-binding groups.

The generation of arginine mutants by site-directed mutagenesis of the *Escherichia coli* porphobilinogen deaminase *hemC* gene was therefore expected to provide information about the participation of these groups in the reaction sequence. To minimize the potential structural disruption that could be caused by the introduction of a hydrophobic or negatively charged amino acid, replacement of arginine by another basic amino acid, histidine, was chosen as the main mutagenic strategy. Histidine also offers advantages as an n.m.r. probe and may be modified by chemical reagents for further investigations. This paper presents a preliminary account of the effects of mutations at arginine-7, -11, -101, -131, -132, -149, -155, -176, -182, -206, -232 and -277 on the autocatalytic assembly of the cofactor and on the tetrapyrrole chain initiation and the stages of chain elongation during the tetrapolymerization reaction. The roles of these arginine residues are considered in the light of their mutational effects and on their position in the tertiary structure of the enzyme currently under investigation by X-ray analysis (S. P. Wood, unpublished work).

EXPERIMENTAL

Materials

Porphobilinogen was synthesized from 5-aminolaevulinic acid by using homogeneous 5-aminolaevulinic acid dehydratase (Jordan & Seehra, 1986). All laboratory reagents were purchased from BDH Chemicals or Sigma Chemical Co., both at Poole, Dorset, U.K. F.p.l.c. requisites and PD10 columns were from Pharmacia, Milton Keynes, Bucks., U.K. Mutagenesis kits, restriction enzymes and [α -³⁵S]thio[d]ATP were purchased from Amersham International, Amersham, Bucks., U.K. Mutagenic primers were prepared by using an Applied Biosystems model 381A DNA synthesizer with Applied Biosystems reagents.

Bacterial strains used for mutagenesis and expression

Escherichia coli K12 strain TB1 was employed for the expression of wild-type and mutant porphobilinogen deaminase genes. Mutagenesis was carried out with the *Bam*HI–*Hind*III fragment obtained from plasmid pST46, which contains the *hemC* gene (Thomas & Jordan, 1986) cloned into the appropriate location in M13mp19. The *hemC* mutants were generated by using single-base-mismatched oligonucleotides of 20–22 bases by

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Scheme 1. Assembly of the tetrapyrrole attached to the dipyrromethane cofactor of porphobilinogen deaminase

the method of Nakamaye & Eckstein (1986). After identification of mutants by sequencing, their DNAs were subcloned into the *Bam*HI-*Sal*I site of plasmid pUC18 and the mutant proteins were expressed from strain TB1 harbouring the recombinant plasmids.

Purification of wild-type and mutant porphobilinogen deaminase proteins

Wild-type and mutant strains were grown in 5-litre amounts of LB medium containing ampicillin (50 µg/ml). Cells were harvested and then disrupted by sonication, and the proteins in the cell-free extract were analysed by PAGE in the presence of SDS (Laemmli, 1970). Wild-type porphobilinogen deaminase was purified to homogeneity from *Escherichia coli* as previously described (Jordan *et al.*, 1988b). Mutant deaminases were purified to homogeneity by using a similar method. Purification of inactive deaminases was monitored by PAGE in the presence of SDS.

Determination of kinetic properties of the mutant porphobilinogen deaminases

Enzyme activity was determined as µmol of uroporphyrin formed/h per mg of protein (Jordan *et al.*, 1988b). The K_m and V_{max} values were determined for each active mutant and were compared with those of the wild-type enzyme.

Determination of the presence of the dipyrromethane cofactor in the deaminase enzymes

Crude cell-free extracts were passed initially through a PD10 gel-filtration column to separate the deaminase from Ehrlich-positive small molecules. The resulting protein solutions were treated with an equal volume of modified Ehrlich's reagent (Jordan & Warren, 1987) and the absorbance was determined at 555 nm after 10 min (Warren & Jordan, 1988). Similar determinations were made at all stages of the purification.

Analysis of wild-type and mutant proteins for the formation of enzyme intermediate complexes

The abilities of purified mutant proteins to form the intermediate complexes ES, ES₂ and ES₃ were investigated by PAGE (Berry *et al.*, 1981) and by f.p.l.c. (Warren & Jordan, 1988).

RESULTS AND DISCUSSION

Substitutions of conserved arginine residues of *Escherichia coli* porphobilinogen deaminase by site-directed mutagenesis of the *hemC* gene

During the catalytic cycle (Scheme 1) up to four molecules of substrate and the two rings of the dipyrromethane cofactor, each with two negatively charged side chains (A and P), are covalently bound to porphobilinogen deaminase, making a total of 12 negatively charged carboxylate groups. Strong candidates for the binding of these groups are several of the positively charged conserved arginine residues, mutations of which would be expected to have effects on specific stages of the enzyme reaction. Thus 12 mutants were constructed, in 11 of which the arginine residues (underlined in Fig. 1) were changed to histidine residues: R11H, R101H, R131H, R132H, R149H, R155H, R176H, R182H, R206H, R232H and R277H. Arginine-7 was mutated to lysine (R7K). The position in the primary structure was derived from the nucleotide sequence of the *hemC* gene (Thomas & Jordan, 1986).

The mutant *hemC* genes in bacteriophage M13mp19 were identified by sequencing and subcloned into plasmid pUC18 for expression of the proteins in strain TB1.

General properties of mutant proteins

Each of the 12 expressed mutant proteins was visible as a band at 35000 Da when cell-free extracts were subjected to PAGE in the presence of SDS. Although the amounts of each mutant

MLDNVLRIATRSPLALWQAHYVKDKLMASHPGLVVELVPMVTRGDVILDTPAKVGGKGLFVKELEVALLENRA
 DIAVHSMKDVPEFPQGLGLVTICEREDPRDAFVSNNYDSLDPAGSIVGTSSLRQCQLAERRPDLIIRSLRG
 NVGTRLKLDNGEYDAIILAVAGLKRGLGLESRIIRAALPPEISLPAVGQGAVGIECRLLDDSRRELLAALNHETA
 LRVTAERAMNTRLEGGCQVPFGSYAELIDGEITWLRALVGAPDGSQIIRGERRGAPQDAEQMGISLAEELLNNGAR
 EILAEVYNGDAPA

Fig. 1. Amino acid sequence of *Escherichia coli* porphobilinogen deaminase

The arginine residues mutated are underlined.

protein varied slightly, no evidence for degraded protein bands was found, indicating that none of the active mutants is significantly susceptible to proteinases. The active mutants exhibited similar thermal stability to the wild-type enzyme at 50 °C in crude extracts, suggesting that the proteins had folded normally. Preliminary screening for activity showed that mutants R131H and R132H were inactive and that mutants R11H and R155H were extremely low in activity compared with TB1 wild-type controls. The activities of mutants R149H, R176H and R232H were markedly diminished, whereas mutants R7K, R101H, R182H, R206H and R277H appeared to have essentially normal activity (Table 1).

All the mutant proteins were purified to homogeneity and subjected to an analysis of their physical and kinetic properties. The purified mutant enzymes (except R7K) migrated faster towards the anode compared with the wild-type recombinant enzyme when subjected to PAGE at pH 8.0, reflecting the loss of the strong positive charge of arginine (results not shown). After the mutants had been constructed and expressed, nucleotide sequences for mouse (Beaumont *et al.*, 1989) and *Bacillus subtilis* (Petricek *et al.*, 1990) porphobilinogen deaminases became available. Three of the arginine residues, at positions 7, 182 and 277, were found to be non-conserved when these sequences were considered. Since these three mutants as well as mutants R101H and R206H were all found to have kinetic properties essentially similar to those of the wild-type enzyme, they are not considered further in this paper, although they act as useful controls.

Mutations that affect the assembly of the dipyrromethane cofactor

The dipyrromethane cofactor may be identified in porpho-

bilinogen deaminase by using Ehrlich's reagent, which gives a characteristic purple colour (Jordan & Warren, 1987). Mutants R131H and R132H were both negative in the Ehrlich's reaction and were also completely devoid of any catalytic activity. From their behaviour during purification, in which they are precipitated at lower concentrations of $(\text{NH}_4)_2\text{SO}_4$ compared with the wild-type enzyme, these mutants appear to be in a different conformation to that of the holoenzyme and resemble more the state of the apoenzyme (Warren & Jordan, 1988; Scott *et al.*, 1989). Mutations of arginine-131 and -132 to the hydrophobic amino acid leucine also lead to proteins devoid of the dipyrromethane cofactor (Lander *et al.*, 1991). Arginine-131 and -132 are located in the active-site cleft close to the dipyrromethane cofactor in the highly conserved sequence ...GTSSLRR... and provide the sites for binding the negatively charged A and P side chains of the substrate and cofactor at the deamination site. These arginine residues are situated at the end of a short helix with their side chains pointing into the active-site cleft (S. P. Wood, unpublished work).

Mutations that affect chain initiation and elongation

The ability of the mutant enzymes to catalyse the complete tetrapolymerization reaction may be investigated by examining the distribution of the enzyme-intermediate complexes ES, ES₂ and ES₃ by using f.p.l.c. (Warren & Jordan, 1988). The accumulation of such enzyme intermediate complexes would suggest that mutations affecting arginine residues important in the translocation process had occurred. The formation of these enzyme-intermediate complexes were studied with 10 μM -porphobilinogen and 200 μM -porphobilinogen concentrations with both wild-type and mutant enzymes (Fig. 2).

Table 1. Properties of porphobilinogen deaminases with mutations at conserved arginine residues

Enzyme activity was determined as μmol of uroporphyrin formed/h per mg of protein (Jordan *et al.*, 1988b). The formation of enzyme-intermediate complexes with 10 μM - or 200 μM -porphobilinogen was monitored by f.p.l.c. as previously described by Warren & Jordan (1988) (see also Fig. 2). Abbreviation: N.D., not determinable.

Mutation	Specific activity ($\mu\text{mol/h}$ per mg)	K_m (μM)	Cofactor assembly	Enzymic stage affected	
				Low substrate (10 μM)	High substrate (200 μM)
Wild-type	43	17	+	None	None
R11H	0.1	N.D.	+	E→ES	E→ES
R101H	20	20	+	Normal	Normal
R131H	Inactive	—	—	Cofactor assembly	Cofactor assembly
R132H	Inactive	—	—	Cofactor assembly	Cofactor assembly
R149H	11.1	200	+	ES→ES ₂	All
R155H	0.5	N.D.	+	E→ES	ES ₃ →product
R176H	6.0	30	+	ES→ES ₂	ES ₂ →ES ₃
R206H	43	19	+	Normal	Normal
R232H	4.0	150	+	ES→ES ₂	ES→ES ₂ and ES ₃ ES ₃ →product

(a) $E \rightarrow ES$. Mutant R11H is a severely crippled enzyme with only a small trace of enzymic activity. Although it assembles the dipyrromethane cofactor, the binding and attachment of the first substrate is grossly affected and no enzyme-intermediate complexes can be detected at low or high substrate concentrations (Fig. 2b). Arginine-11 is situated with its side chain pointing into the interior of the active-site cleft and is close to the negatively charged carboxylate groups on ring C2 of the dipyrromethane cofactor. Mutant R155H, like R11H, exhibits only a trace of activity except at substrate concentrations of $200 \mu\text{M}$, where ES_3 accumulates (Fig. 2c). The turnover of the enzyme is, however, very low and the further conversion of ES_3 into ES_4 and product is very seriously affected. *Escherichia coli* R11L and R155L mutants have been generated that also exhibit greatly diminished specific activity; however, in contrast with mutant R155H, the R155L mutant accumulates ES at $200 \mu\text{M}$ -porphobilinogen (Lander *et al.*, 1991). In the holoenzyme, arginine-155 is spatially located between arginine-11 and -132 with its side chain pointing into the active-site cleft near the negatively charged side chains of the cofactor ring C2. It is interesting that a natural mutant affecting the equivalent arginine residue, R173Q in the ubiquitous human isoenzyme, has been reported in a patient suffering from acute intermittent porphyria in which the deaminase activity is also severely depressed (Delfau *et al.*, 1990). The mutant protein cross-reacts with antiserum raised to the normal human deaminase, indicating that it is likely to be stable and correctly folded with the cofactor assembled, as is also the case with the *Escherichia coli* R155H mutant.

(b) $ES \rightarrow ES_2$. Mutant R232H has a specific activity less than 10% of that of the wild-type and accumulates ES at low porphobilinogen concentrations (Fig. 2e), in contrast with the wild-type, where ES_2 is the predominant species (Fig. 2a). The

conversion of ES into ES_2 and of ES_2 into ES_3 is hindered, suggesting that the normal translocation mechanism of the substrate may be affected. At higher porphobilinogen concentrations this mutant formed ES_3 as the major species. Arginine-232 is located on the surface of the α -helix carrying the dipyrromethane cofactor.

Mutant R149H exhibits about one-quarter of the wild-type activity and, like R232H, also accumulates ES at low substrate concentrations (Fig. 2d). At higher porphobilinogen concentrations ES, ES_2 and ES_3 are formed in approximately equal amounts. This contrasts with the wild-type, where E and ES_3 predominate. Arginine-149 is situated close to arginine-155 in the tertiary structure with its side chain pointing into the active-site cleft. R232L and R149L mutants also show diminished activity (Lander *et al.*, 1991); however, their properties have not been studied in detail. It is interesting that a human deaminase mutant affecting the equivalent arginine residue, R167Q in the ubiquitous human enzyme, has been described that causes acute intermittent porphyria due to the low activity of the deaminase (Delfau *et al.*, 1990).

(c) $ES_2 \rightarrow ES_3$. Arginine mutant R176H was particularly interesting since its affinity for the substrate was only marginally affected. At low substrate concentrations it accumulates ES, similarly to mutants R149H and R232H; however, at higher concentrations of substrate it is the only mutant that accumulated ES_2 (Fig. 2f), indicating that both the $ES \rightarrow ES_2$ and the $ES_2 \rightarrow ES_3$ steps are affected. Similar behaviour has been observed with R176L (Lander *et al.*, 1991). Arginine-176 is located in the vicinity of the active-site cleft near arginine-11 and may be important in the translocation mechanism.

(d) $ES_3 \rightarrow ES_4$ and $ES_4 \rightarrow \text{preuroporphyrinogen}$. No mutant was defective in the $ES_3 \rightarrow ES_4$ stage alone, although, like the wild-

Fig. 2. Accumulation of enzyme-intermediate complexes by arginine→histidine mutants of *Escherichia coli* porphobilinogen deaminase

Homogeneous enzyme (3 nmol) was mixed with a low concentration ($10 \mu\text{M}$; —) or a high concentration ($200 \mu\text{M}$; ----) of porphobilinogen at 4°C . The resulting enzyme-intermediate complexes were chromatographed through a MonoQ 5/SHR f.p.l.c. column, which was developed in 20 mM-Tris/HCl buffer, pH 7.5, with a linear gradient of 0–0.4 M-NaCl (—). Protein was detected by the absorbance at 280 nm. (a) Wild-type enzyme; (b) mutant R11H; (c) mutant R155H; (d) mutant R149H; (e) mutant R232H; (f) mutant R176H.

type, mutants R149H, R155H and R232H all accumulated ES₃ at 200 μ M-porphobilinogen. No arginine→histidine mutant accumulated ES₄, suggesting that once this enzyme–intermediate complex is formed it is rapidly broken down to product and free enzyme. This contrasts with the finding that mutant R155L accumulates ES₄ in the presence of very high concentrations (10 mM) of porphobilinogen (Lander *et al.*, 1991). In this case, however, the substitution of a polar side chain by one with hydrophobic properties may have caused additional effects.

Conclusions

The porphobilinogen deaminase apoenzyme catalyses the assembly of the dipyrromethane cofactor by the deamination of two 'non-substrate' molecules of porphobilinogen (C1 and C2 in Scheme 1). The resulting deaminase, after assuming the holo-enzyme conformation, then catalyses the sequential deamination and coupling of four 'substrate' molecules in the catalytic cycle proper (Warren & Jordan, 1988). Finally, the tetrapyrrole product, preuroporphyrinogen, is released by hydrolysis, leaving the dipyrromethane cofactor intact (Jordan & Warren, 1987). All these reactions belong to the same generic class and were predicted to take place at a single catalytic site capable of binding the two pyrrole rings between which the new carbon–carbon bond is formed. These two binding sites have been designated cofactor (C) and substrate (S) sites (Warren & Jordan, 1988).

The recent determination of the preliminary X-ray structure of *Escherichia coli* porphobilinogen deaminase at 0.3 nm (3.0 Å) resolution (S. P. Wood, unpublished work) has established the spatial position of all the arginine side chains. Mutations R131H and R132H give deaminase proteins unable to assemble the dipyrromethane cofactor, since arginine-131 and -132 provide the positively charged groups necessary for interacting with the negatively charged A and P side chains at the (C) binding site (Warren & Jordan, 1988). The finding that both R11H and R155H mutants, although able to assemble the cofactor, form the ES complex with great difficulty at low substrate concentrations suggests that arginine-11 and -155 are crucial for binding the carboxylate groups at the (S) site. These residues may also be involved in the binding of subsequent substrate molecules and for manoeuvring the bound cofactor (or intermediate) through the catalytic machinery. Since the E→ES step is largely prevented, the activities of these mutants are extremely low. In the preliminary crystal structure of the deaminase (S. P. Wood, unpublished work) the side chains of arginine-11 and -155 are quite close to one another and interact with the C2 ring of the cofactor in the holoenzyme. Mutations of arginine-176, -232 and -149 to histidine are less severe and affect predominantly the later stages of the assembly process, reflecting their more peripheral location with respect to the catalytic site.

One of the most interesting aspects arising from this study is the finding that a relationship exists between the enzyme specific activity and the enzyme–intermediate complexes that accumulate. Thus mutations that result in zero or very low specific activities (R131H, R132H, R11H and R155H) involve residues that interact intimately with the two rings of the dipyrromethane cofactor and interfere with the earliest stages in the enzyme reaction. Mutations resulting in enzymes with specific activities nearer to that of the wild-type enzyme (R149H, R176H and

R232H) affect later stages of the tetrapolymerization process. Most interestingly, no mutant in this study was found that accumulated ES₄, providing further evidence for the participation of a single catalytic site. Presumably any mutant capable of executing the many stages necessary for the formation of ES₄ would also be able to catalyse the hydrolysis of this final enzyme–intermediate complex to yield the product. Although the conservative arginine→histidine mutants generated for this study provide insight into the mechanism of the tetrapolymerization process, the crystallization of individual enzyme–intermediate complexes of selected mutant deaminases will be necessary to provide a full picture of this remarkable and complex polymerization reaction.

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