Evidence for Conformational Changes in *Escherichia coli* Porphobilinogen Deaminase during Stepwise Pyrrole Chain Elongation Monitored by Increased Reactivity of Cysteine-134 to Alkylation by *N*-Ethylmaleimide†

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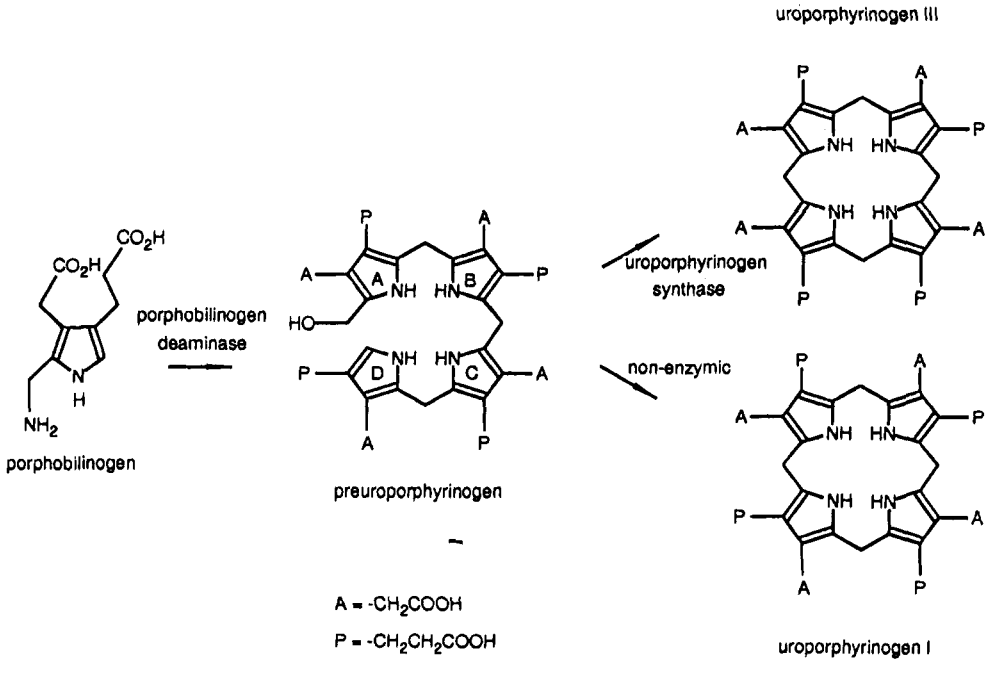
ABSTRACT: Porphobilinogen deaminase from *Escherichia coli* becomes progressively more susceptible to inactivation by the thiophilic reagent *N*-ethylmaleimide (NEM) as the catalytic cycle proceeds through the enzyme−intermediate complexes ES, ES_2, ES_3, and ES_4. Site-directed mutagenesis of potentially reactive cysteines has been used to identify cysteine-134 as the key residue that becomes modified by the reagent and leads to inactivation. Since cysteine-134 is buried at the interface between domains 2 and 3 of the *E. coli* deaminase molecule, the observations suggest that a stepwise conformational change occurs between these domains during each stage of tetrapyrrole assembly. Interestingly, mutation of the invariant active-site cysteine-242 to serine leads to an enzyme with up to a third of the catalytic activity found in the wild-type enzyme. Electrospray mass spectrometry indicates that serine can substitute for cysteine as the dipyrromethane cofactor attachment site.

The biosynthesis of uroporphyrinogen III from porphobilinogen involves two enzymes, porphobilinogen deaminase and uroporphyrinogen III synthase (Jordan, 1991, 1994). The deaminase constructs a 1-hydroxymethylbilane called preuroporphyrinogen by polymerizing four molecules of porphobilinogen, while the synthase cyclizes and rearranges this linear intermediate to give uroporphyrinogen III (Leeper, 1994) (Scheme 1). Uroporphyrinogen III is the progenitor tetrapyrrole for the heme and siroheme prosthetic groups required by hemoproteins and the sulfite and nitrite reductase enzymes (Warren & Scott, 1990) as well as providing the macrocyclic ring system of the chlorophylls, bacteriochlorophylls, and corrins (Jordan, 1991; Chadwick & Ackrill, 1994).

The cloning, sequencing, and expression of the *Escherichia coli hemC* gene (Thomas & Jordan, 1986), which encodes

Scheme 1: Formation of Uroporphyrinogen III from Porphobilinogen Catalyzed by Porphobilinogen Deaminase and Uroporphyrinogen Synthase



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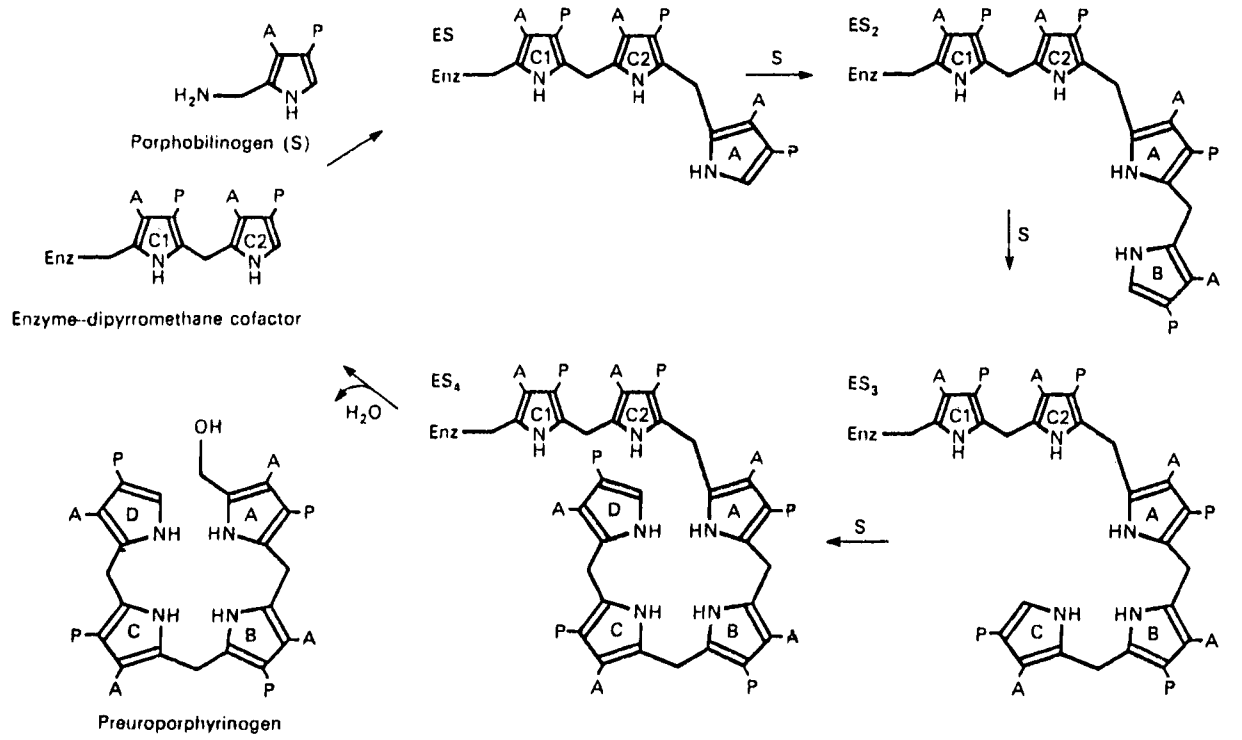
Scheme 2: Catalytic Cycle of Porphobilinogen Deaminase[a]



[a] The cycle shows the sequential addition of four substrate molecules to the enzyme-bound dipyrromethane cofactor and the release of the product. A = CH_2-CO_2H; P = -CH_2-CH_2-CO_2H.

porphobilinogen deaminase, has permitted the isolation of milligram amounts of the enzyme (Jordan *et al.*, 1988a). The enzyme utilizes a novel dipyrromethane cofactor that is covalently attached to the enzyme active site through a thioether link with cysteine-242 and which acts as a reaction primer for the polymerization reaction (Jordan & Warren, 1987). The assembly of preuroporphyrinogen involves the elongation of this dipyrromethane primer by the stepwise addition of four molecules of the substrate, porphobilinogen, through the enzyme−intermediate complexes ES, ES_2, ES_3, and ES_4 (Scheme 2). Thus the first molecule of substrate is deaminated at the active site of the enzyme and is attached covalently to the free α-position of the dipyrromethane cofactor, generating the first enzyme−intermediate complex, ES. The process is repeated with three further molecules of substrate, giving rise to ES_2, ES_3, and finally ES_4 (Warren & Jordan, 1988; Aplin *et al.*, 1991). ES_4 is particularly labile and is rapidly hydrolyzed to give the product preuroporphyrinogen to regenerate the enzyme, still containing the dipyrromethane cofactor (Scheme 2). The other enzyme−intermediate species (ES, ES_2, and ES_3) are, however, stable enough to be isolated by anion-exchange chromatography and can be studied individually (Warren & Jordan, 1988).

E. coli porphobilinogen deaminase is a small monomeric protein of subunit M_r 34 272 with 313 amino acids. The X-ray structure of *E. coli* porphobilinogen deaminase has been solved at 1.76-Å resolution (Louie *et al.*, 1992) and this has contributed greatly to our understanding of the reaction mechanism. The crystal structure (Figure 1) reveals that the enzyme is made up of three domains of approximately equal size which girdle a large active-site cleft (Louie *et al.*, 1992). The dipyrromethane cofactor is located within this active-site cleft, covalently attached through the

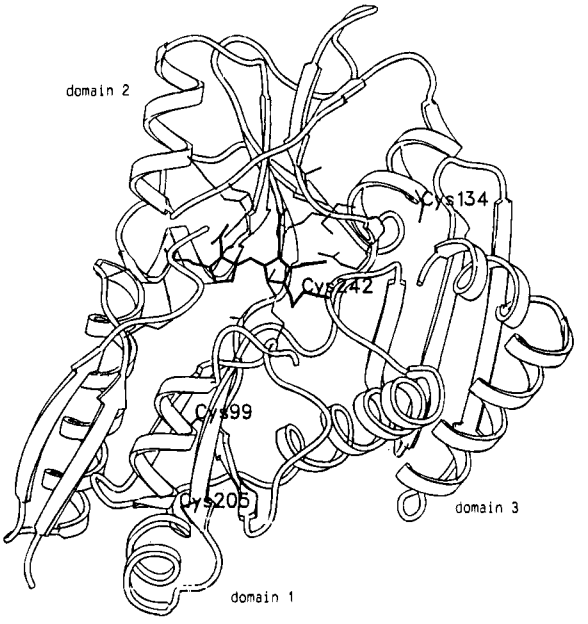


FIGURE 1: Three-dimensional structure of *E. coli* porphobilinogen deaminase indicating the four cysteine residues. Cysteine-99 occupies a partially exposed position between domains 1 and 2. Cysteine-205 is partially exposed to the surface of domain 1. Cysteine-134 occupies a buried position between domains 2 and 3. Cysteine-242 forms a thioether linkage with the dipyrromethane cofactor in the catalytic cleft.

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sulfur atom of cysteine-242 (Jordan *et al.*, 1988b; Miller *et al.*, 1988). The crystal data (Louie *et al.*, 1992) together with detailed mechanistic studies (Warren & Jordan, 1988) suggest that the protein contains a single active site with aspartate-84 playing a central role in the catalytic process (Woodcock & Jordan, 1994).

The X-ray structure of the *E. coli* porphobilinogen deaminase (Louie *et al.*, 1992) suggests that the three domains of the enzyme interact through flexible hinge regions reminiscent of the periplasmic binding proteins to which the domains 1 and 2 of the deaminase are topologically related (Louie, 1993). By analogy with the binding mechanism of these proteins (Louie, 1993) and from experimental observations (Scott *et al.*, 1988; Jordan & Woodcock, 1991), major protein conformational changes are expected on assembly of the cofactor. Further conformational changes during chain elongation are envisaged as the polypyrrole chain is manipulated through the active site. Preliminary evidence for such a conformational change during catalysis is indicated by the increased susceptibility of the deaminase to inactivation by thiophilic reagents in the presence of substrate (Warren & Jordan, 1988). These observations suggest that a change in the protein conformation occurs during the catalytic cycle whereby a cysteine group, previously buried within the protein structure, becomes exposed to react with the modifying reagent. The availability of the three-dimensional structure of the deaminase now permits a rational and detailed study of this "reporter" cysteine. This paper describes the effects of site-directed mutagenesis on the two interdomain cysteine residues, at positions 99 and 134 (Figure 1), which have been substituted by serine residues. In addition, mutagenesis of the covalent binding site of the dipyrromethane cofactor, cysteine-242 (Figure 1), to serine has provided surprising information about the nature of the cofactor assembly process.

MATERIALS AND METHODS

Chemicals. Porphobilinogen was synthesized from 5-aminolevulinic acid using purified 5-aminolevulinic acid dehydratase as previously described (Jordan & Seehra, 1986). DEAE-Sephacel was purchased from Pharmacia. All other chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.).

Strains and Construction of pBG101. All DNA manipulations were carried out by standard techniques (Maniatis *et al.*, 1982). The bacterial strain, *Escherichia coli* TB1, is a derivative of JM83 (F^- *ara* Δ(*lac*−*proAB*) *rpsL* [ϕ*80dlac*Δ-(*lacZ*)M15]) except that it is *hsdR*(r^-_k m^+_k) and is used as a host for pUC plasmids (Baldwin *et al.*, 1984). *E. coli* strains CJ 236 (*dut*$^-$ *ung*$^-$) and MV1190 (*dut*$^+$ *ung*$^+$) and the vector pTZ18U were purchased from Bio-Rad. Plasmid pBG101 was constructed as previously described (Scott *et al.*, 1988) by ligation of a 1.6-kb *Bam*HI−*Sal*I restriction fragment from pLC41-04 (Clarke & Carbon, 1976) containing the *hemC* gene (Thomas & Jordan, 1986) into the *Bam*HI−*Sal*I sites of pUC18. Bacteria were grown at 37 °C on LB medium or LB agar plates containing 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl/L of water. Ampicillin, when required, was added at 100 μg/mL.

Isolation of Wild-Type Porphobilinogen Deaminase. Wild-type porphobilinogen deaminase was isolated from a genetically engineered strain of *E. coli* K-12 ST1048 (Thomas & Jordan, 1986; Jordan *et al.*, 1988a) harboring plasmid pST48 that was constructed by cloning the *Bam*HI−*Sal*I fragment from pLC41-04 into these restriction sites of a pBR322 derivative as previously described (Thomas & Jordan, 1986). The purification generated homogeneous enzyme, as judged by a single band on polyacrylamide gels in the presence of sodium dodecyl sulfate, corresponding to a M_r of 34 000. The enzyme was freeze-dried from water and in this state remained stable for several months when stored at −20 °C. Prior to use, the enzyme was dissolved in 100 mM Tris-HCl, pH 8.2.

Assay of Porphobilinogen Deaminase. Enzyme assays were carried out by preincubating porphobilinogen deaminase (10 μg) for 2 min at 37 °C in a volume of 450 μL containing 250 μL of 0.1 M Tris-HCl buffer, pH 8.0, and 200 μL of distilled water. The reaction was initiated by the addition of 50 μL of 2 mM porphobilinogen. At time intervals of 5, 10, 15, and 20 min, samples (80 μL) were removed from the assay and mixed with 20 μL of 5 N HCl to terminate the reaction and to cyclize preuroporphyrinogen to uroporphyrinogen I. To each sample a further 900 μL of 1 N HCl containing 0.01% benzoquinone was added, and 20 min was allowed for oxidation to uroporphyrin I. The absorbance was measured against a blank with no enzyme at 405.5 nm. The amount of uroporphyrin I formed was calculated using an extinction coefficient ϵ of 5.48×10^5 cm^{-1} M^{-1}. The specific activity of the wild-type enzyme (Jordan et al., 1988a) is 40 μmol of uroporphyrin I formed h^{-1} mg^{-1}. Protein was determined by the method of Bradford (1976).

Site-Directed Mutagenesis of the hemC Gene. Mutant deaminases C99S, C134S, and C242S were generated by the method of Kunkel *et al.* (1987) using a kit purchased from Bio-Rad. The *hemC* gene from pBG101 was subcloned into the vector pTZ18U to construct pTZ18U:*hemC*, which was transformed into strain CJ236 (*dut*$^-$ and *ung*$^-$) for the production of single-stranded phagemid DNA containing uracil. A 40-base oligonucleotide primer was synthesized which was designed to change the codon for amino acid 99 from TGT (Cys) to TCT (Ser) and to change the codon for amino acid 105 from CGC to CGG, eliminating one of the two *Nru*I restriction enzyme sites found in the gene while conserving the amino acid (Arg). Similarly, a mutagenic primer was synthesized which was designed to change the codon for amino acid 134 from TGT (Cys) to TCT (Ser) and a further primer was used to change the codon for amino acid 242 from TGT (Cys) to TCT (Ser), at the same time changing the codon for amino acid 244 from GTG to GTC, eliminating a *Ban*I restriction enzyme site in the gene while conserving the amino acid (Val). Isolation of single-stranded DNA, annealing of the primers, complementary strand synthesis, and transformations of the double-stranded DNA into strain MV1190 (*dut*$^+$ *ung*$^+$) were performed according to the directions provided by Bio-Rad. Plasmid DNA from transformed cells was screened by restriction analysis. Plasmids in which the *Nru*I or *Ban*I restriction enzyme sites had been eliminated were sequenced through the region of the primer to confirm the described base changes. *E. coli* strain MV1190 bearing pTZ18U:*hemC* or the mutagenized plasmids were grown for 18−24 h in 50 mL of LB medium containing ampicillin at 50 μg/mL. The mutations were confirmed by complete DNA sequence analysis. The mutant bacterial strains were grown overnight in LB medium and the proteins were purified by the method of Jordan *et al.*

(1988a) except for the C242S mutant, which was heat labile. The C242S mutant was purified from 6 g wet weight of bacterial cells harvested from 2 L of culture. Sonication and ammonium sulfate fractionation and DEAE chromatography was carried out as described previously by Jordan *et al.* (1988) except that no heat treatment was used. The protein collected from the DEAE cellulose was concentrated to 5 mL and applied to a column of G-75 Sephadex (100 cm × 2.5 cm) and developed in 100 mM Tris-HCl buffer, pH 8, containing 1 mM 2-mercaptoethanol. The enzyme was concentrated to 2−3 mL and desalted using a PD10 column (Pharmacia). The protein solution was freeze-dried and the residue (30 mg) was stored at −20 °C until required.

Inactivation of Porphobilinogen Deaminases with N-Ethylmaleimide (NEM). Inhibitor studies were carried out by adding NEM (5 mM) to 0.25 unit of either enzyme or enzyme−intermediate complex in 0.1 M Tris-HCl buffer, pH 8.2, in a final volume of 450 μL. After incubation with inhibitor for 20 min at 20 °C, the inhibitor was removed by gel filtration using a PD10 (Pharmacia) column that had been equilibrated in the above buffer. The enzyme activity was determined as described above.

Formation and Purification of Enzyme−Intermediate Complexes. The enzyme−intermediate complexes were generated at 4 °C by mixing stoichiometric quantities of the enzyme (30 nmol) and substrate (30−90 nmol) in a rapid-mixing device (Berry *et al.*, 1981). The individual complexes were then isolated and purified by ion-exchange chromatography using a MonoQ HR 5/5 column attached to a Pharmacia FPLC system as previously described (Jordan *et al.*, 1988a).

Electrospray Mass Spectrometry. Electrospray mass spectral data were collected using a triple-quadrupole atmospheric mass spectrometer equipped with an electrospray interface (VG Biotech, Altringham, Cheshire, U.K.). The analyses were carried out using 10 μL of deaminase (20 pmol/μL) in methanol−water (1:1) containing 4% formic acid and injected into the electrospray source via a Rheodyne 7125 injection valve. The source voltage was 50 V, the source temperature was 50 °C, and the injector flow rate was 10 μL/min. Typically 50 scans were used to accumulate data. Sample solutions were prepared immediately prior to analysis. The mass spectrometer was scanned over a range of 1100−1450 Da. The instrument was calibrated using horse heart myoglobin (M_r 16 951.5) at a concentration of 20 pmol/μL. Typical cone voltage values were between 50 and 70 V.

RESULTS AND DISCUSSION

Reaction of E. coli Porphobilinogen Deaminase with N-Ethylmaleimide in the Presence of Substrate. Native *E. coli* porphobilinogen deaminase was found to be relatively unreactive to thiophilic reagents such as NEM and only concentrations of these reagents above 5 mM caused significant inactivation (Warren & Jordan, 1988). However, when such reagents were reacted with the enzyme in the presence of porphobilinogen, there was a dramatic increase in the level of inactivation. When the reaction of NEM was investigated in more detail with the purified enzyme−intermediate complexes, ES, ES_2, and ES_3, the inactivation was more pronounced as the number of bound substrate molecules increased (Figure 2). These observations suggested that during the catalytic cycle a sulfhydryl group that was previously unreactive becomes accessible to the reagent.

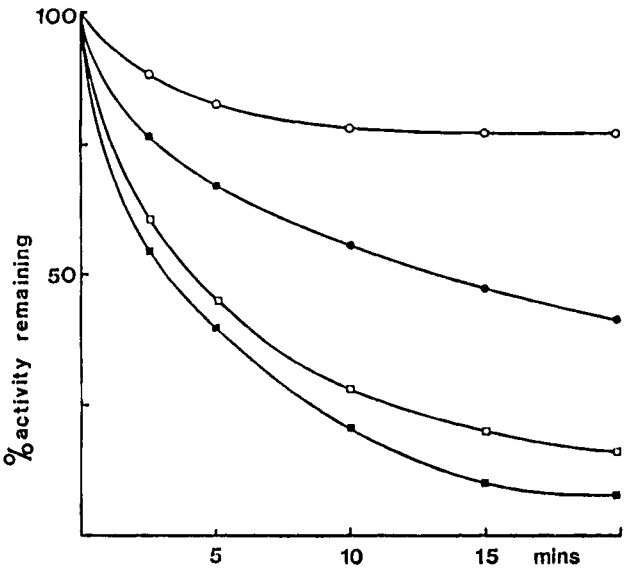

FIGURE 2: Activity of *E. coli* porphobilinogen deaminase and enzyme intermediate complexes after treatment with NEM. Time course to show the inactivation of native porphobilinogen deaminase and enzyme−intermediate complexes ES, ES_2, and ES_3 by NEM. Reaction conditions are as described in the Materials and Methods section. Native enzyme (0); enzyme−intermediate complexes ES (●), ES_2 (□), and ES_3 (■).

E. coli porphobilinogen deaminase possesses a total of four cysteine residues (Thomas & Jordan, 1986) occurring at positions 99, 134, 205, and 242 (Figure 1). Cysteine-99 occupies a partially exposed position between domains 1 and 3, whereas cysteine-134 is located at the domain 2/domain 3 interface. Both of these residues are thus potentially susceptible to major environmental changes during the catalytic reaction. Of the other two cysteine residues, cysteine 205 is located in domain 1 and is likely to maintain the same partially exposed position throughout the catalytic cycle, whereas cysteine 242 is blocked, in the form of a thioether, by virtue of its role as the covalent attachment site for the dipyrromethane cofactor (Jordan *et al.*, 1988b; Miller *et al.*, 1988). Neither cysteine-205 nor cysteine-242 is therefore a realistic candidate for the observed substrate-dependent modification by NEM.

Properties of Wild-Type and Mutant Porphobilinogen Deaminases. To study the nature of the inactivation by NEM, three site-directed mutants were generated in which cysteines at positions 99, 134, and 242 were substituted by structurally related serine residues (see Materials and Methods). The wild-type porphobilinogen deaminase and the three cysteine mutants, termed C99S, C134S, and C242S, were purified to homogeneity using published purification methods (Jordan *et al.*, 1988a). The enzymes all migrated on SDS−PAGE as proteins of the same M_r (34 000 ± 3000) as shown in Figure 3. The specific activities of C99S and C134S mutants were also determined and compared favorably with the wild-type enzyme (Table 1). The activity of the mutant protein C242S, however, was only about a third that of the wild-type.

Electrospray Mass Spectrometric Analysis of Mutant Deaminases. To confirm the identity of the mutated species, their molecular masses were determined by electrospray mass spectrometry (Figure 4). All the mutants (Figure 4b−d) and

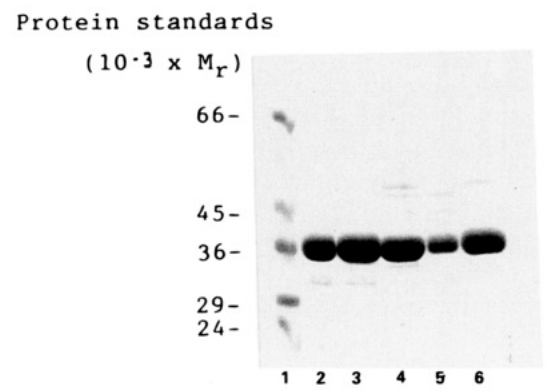

FIGURE 3: SDS−polyacrylamide electrophoresis gel of wild-type and cysteine mutants of *E. coli* porphobilinogen deaminase. Molecular weight markers (Sigma), lane 1; wild type (i), lane 2; C99S mutant, lane 3; C134S mutant, lane 4; C242S mutant, lane 5; wild type (ii), lane 6.

Table 1: Specific Activity, M_r from Electrospray Mass Spectrometry, and Susceptibility of Wild-Type and Mutant Deaminases to NEM in the Absence and Presence of Substrate

	specific activity	M_r from ESMS	NEM-modified E[a] (% activity)	NEM-modified ES[b] (% activity)
WT	38	34 272 ± 5	78	20
C99S	36	34 257 ± 6	72	22
C134S	35	34 256 ± 3	85	94
C242S	15	33 836 ± 2 34 260 ± 8	88	11

[a] In the absence of substrate. [b] In the presence of substrate.

the wild type (Figure 4a) show major protein peaks with small amounts of impurity. However the C242S mutant (Figure 4d) shows a second peak representing approximately a third of the protein sample. A summary of the results obtained by this analysis is shown in Table 1. Since the expected M_r of the wild-type holodeaminase is 34 272 ± 5 (Figure 4a), a cysteine → serine mutation would be expected to result in a lowering of the M_r by 16 mass units to 34 256 ± 5. The analysis shows that C99S mutant (Figure 4b) and C134S mutant (Figure 4c) do indeed have the expected M_rs of the mutant holoenzymes, within experimental error.

When the C242S mutant was analyzed by electrospray mass spectrometry, the major mass peak was 33 836 ± 5 (Figure 4d), consistent with the presence of the mutant apoenzyme, the dipyrromethane cofactor accounting for 419 mass units. Since this mutation involves the invariant cysteine 242, which acts as the covalent attachment site for the dipyrromethane cofactor, this result reinforces previous observations that cysteine-242 has a vital mechanistic role in the process of cofactor assembly and product formation (Jordan, 1991). However, in addition to the major peak, a second peak of M_r = 34 256 was also detected, suggesting that some mutant holoenzyme was also present in the sample. The significance of this observation is discussed later.

Inhibition Studies with N-Ethylmaleimide on Cysteine → Serine Mutants. The effect of NEM was investigated with the purified mutant proteins to compare their susceptibility to the reagent with that of the wild-type enzyme. Analysis of the reactivity of the mutant proteins in both their free form (E) and enzyme−intermediate forms (ES, ES_2, and ES_3) were performed in the presence of NEM. The results of this investigation (Table 1) show that, like the wild-type enzyme, the mutant C99S is inhibited only weakly (approximately 20%) by NEM. Addition of porphobilinogen to the C99S mutant, which converts the enzyme into enzyme−intermediate complexes, led to a large inactivation (approximately 80%) resembling the behavior of wild-type enzyme under similar conditions.

When similar experiments were carried out with the C134S mutant, however, the results were completely different. This mutant enzyme was not only insensitive to the inhibition with NEM in the absence of substrate but the addition of porphobilinogen failed totally to enhance the susceptibility to the reagent. The results therefore point strongly to cysteine-134 as the site at which NEM modification leads to inactivation of the *E. coli* porphobilinogen deaminase. The location of cysteine-134 in a buried position between domains 2 and 3 is consistent with its low reactivity in the holoenzyme. An increase in the susceptibility of this residue to modification by NEM suggests that, as a result of sequential substrate binding during the catalytic cycle, domains 2 and 3 must separate from one another to allow the reagent access.

The above results are supported by studies with NEM on other deaminases. For instance, it is notable that the naturally occurring residue equivalent to cysteine-134 in the *Arabidopsis thaliana* deaminase is serine 141 (Witty *et al.*, 1993), similar to the C134S *E. coli* mutation generated in the present study. The *Arabidopsis* deaminase is completely insensitive to the effect of NEM (Jones & Jordan, 1994). However, the porphobilinogen deaminase from *Euglena gracilis*, which like the *E. coli* enzyme contains a cysteine residue at the equivalent position, is susceptible to inactivation by the reagent (Williams *et al.*, 1981).

Properties of E. coli Porphobilinogen Deaminase Modified by N-Ethylmaleimide. The experiments described above indicate that NEM is only able to gain access to cysteine-134 during the polymerization reaction. Thus modification of the enzyme by the reagent during the catalytic cycle would be expected to prevent the enzyme from regaining its resting structure by trapping the conformation adopted during the reaction. To determine if further evidence for such conformational changes could be obtained, the effect of NEM on the enzyme and enzyme−intermediate complexes were followed using nondenaturing PAGE (Laemmli, 1970).

Wild-type enzyme exhibits microheterogeneity (Jordan *et al.*, 1988a) and migrates as three protein bands (Figure 5, lane 1a), all of which are fully active. When substrate is added to the enzyme (Figure 5, lane 1b), the enzyme−intermediate complexes migrate with higher mobility because of their greater negative charge. Addition of NEM to the wild-type deaminase has no apparent effect on the mobility of the free enzyme (Figure 5, land 2a); however, the presence of the reagent has an interesting effect on the distribution of enzyme intermediate complexes, with ES_2 becoming the major band (Figure 5, lane 2b). This suggests that the enzyme, after reacting with substrate, is modified by the reagent and that further reaction with substrate is prevented. The band pattern on the gel suggests that ES_2 is trapped predominantly.

To determine the effect of the modification by NEM after the bound substrates had been removed, the enzyme from the experiment shown in Figure 5, lane 1b, was treated with hydroxylamine (Figure 5, lane 3a). Treatment of enzyme−substrate complexes with hydroxylamine has been shown to cause the release of bound substrate with regeneration of

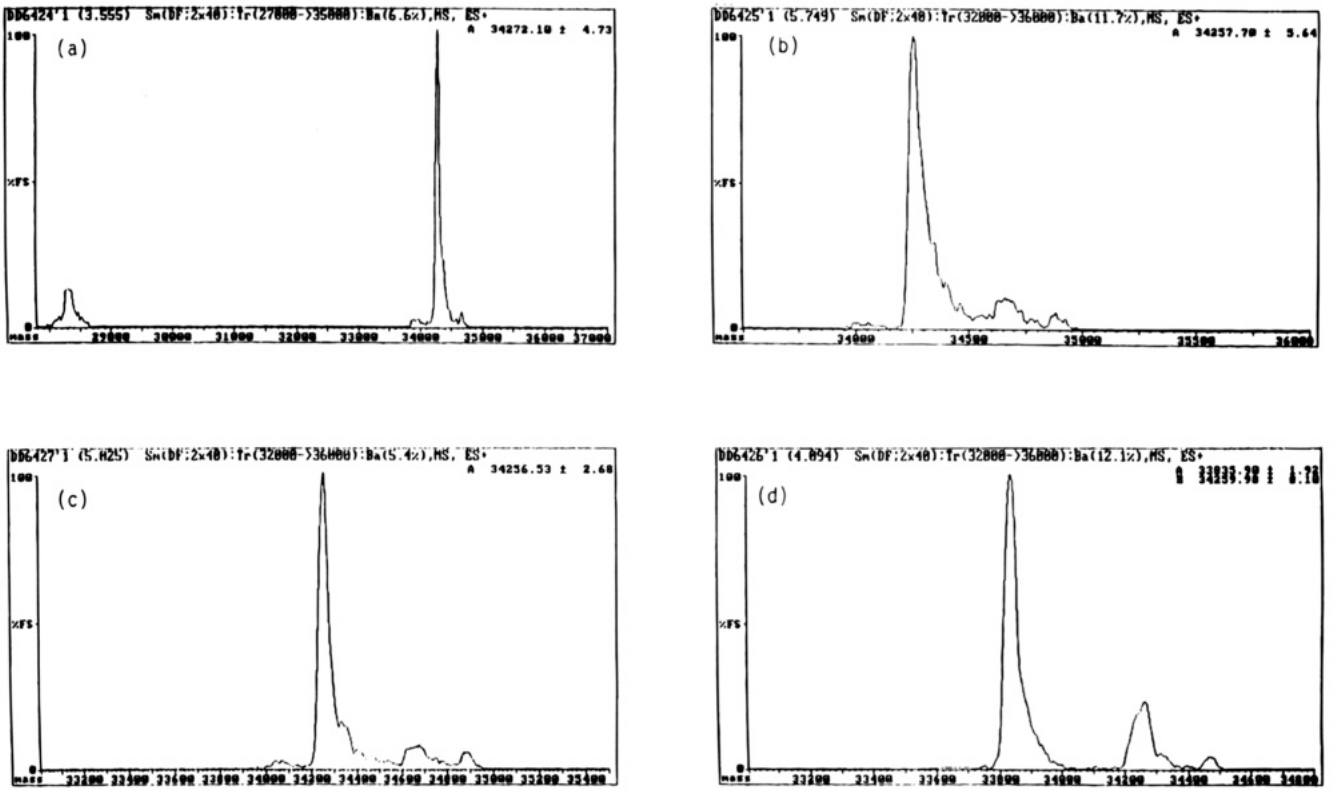

FIGURE 4: Electrospray mass spectrometric data for porphobilinogen deaminase wild type and cysteine mutants. (a) Wild type; (b) C99S mutant; (c) C134S mutant; (d) C242S mutant. Spectra were determined as described in Materials and Methods section.

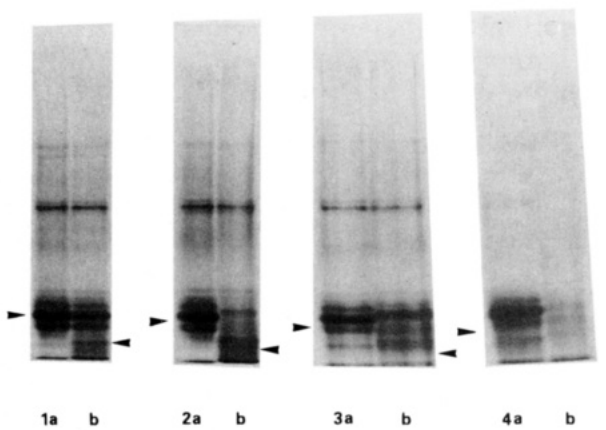

FIGURE 5: Nondenaturing PAGE of wild-type porphobilinogen deaminase after treatment with NEM under different conditions. Enzyme, lane 1a; enzyme + substrate, lane 1b; enzyme + NEM, lane 2a; enzyme + substrate + NEM, lane 2b; enzyme + substrate + hydroxylamine, lane 3a; enzyme + substrate + NEM + hydroxylamine, lane 3b; enzyme + substrate + heat treatment, lane 4a; enzyme + substrate + NEM + heat treatment, lane 4b. Please note that only the center band of the three deaminase bands is shown for clarity. The higher M_r species are aggregates.

free enzyme (Warren & Jordan, 1988). The modified enzyme−intermediate complexes after treatment with hydroxylamine migrated with faster mobility (Figure 5, lane 3b), presumably reflecting the effect of cysteine 134 modification. A further interesting observation was that the inactivation of the enzyme−intermediate complexes by NEM caused the deaminase to become more susceptible to heat treatment. The unmodified enzyme is able to withstand heating to 70 °C without appreciable loss of activity or precipitation (Figure 5, lane 4a), whereas the modified enzyme is precipitated at only 40 °C (Figure 5, lane 4b). These results therefore suggest that the alkylating reagent traps the enzyme in a less stable conformation than that found with the free enzyme, possibly because the stabilizing contacts between domains 2 and 3 are lost. It cannot be discounted, of course, that the introduction of a hydrophobic ligand such as NEM to a position between two domains has itself led to the instability of the enzyme rather than to the conformational change alone.

Reactions of Other Cysteine Residues with N-Ethylmaleimide. The experiments above highlight that reaction of NEM with cysteine-134 leads to inactivation of the deaminase. However, there is evidence that the reagent also reacts with other cysteine residues in the primary structure, although these modifications do not lead to inactivation. This has been deduced from the observation that when wild-type enzyme−intermediate complexes were reacted with ^{14}C-labeled NEM (2−10 mCi/mmol), some 3 mol equiv of radioactivity were incorporated. Furthermore, when the ^{14}C-labeled enzyme was subjected to formic acid cleavage at the single aspartyl-proline, 103−104, linkage (Thomas & Jordan, 1986), both peptides were labeled with ^{14}C radioactivity, suggesting that cysteine-99, -134, and -205 are all labeled. Cysteine-242 is not available for modification since it is attached to the dipyrromethane cofactor (data not shown).

These observations were confirmed by electrospray mass spectrometry (data not shown). This technique has proved useful to investigate the binding of substrates and inhibitors at the active sites of enzymes (Aplin *et al.*, 1990, 1991, 1993). Thus, when both the free form of the deaminase (E) and the enzyme−intermediate forms (ES complexes) were reacted with *N*-ethylmaleimide and the resulting modified protein was then subjected to electrospray mass spectrometry, up to 3 molecules of the inhibitor were found to have reacted, as molecular weight species of 34 395, 34 525, and 34 644

were detected. These experiments suggest that NEM also reacts at cysteine-99 and -205 but that modification at these sites does not lead to inactivation of the enzyme. It is notable that, during X-ray studies, mercury derivatives of cysteine-99 and -205, but not cysteine-134, were formed, consistent with these two cysteine residues being partially exposed (Louie *et al.*, 1992).

Activity of the C242S Mutant and Mechanism of Cofactor Assembly. Preliminary studies with the purified C242S mutant indicated that it was essentially inactive (Jordan *et al.*, 1988b; Scott *et al.*, 1988) and behaved as an apoenzyme devoid of the dipyrromethane cofactor. However, an alternative purification method used in the current study that did not involve heat treatment yielded a protein with significant catalytic activity (Table 1), suggesting that some mutant holoenzyme was present. When a detailed analysis of the purified C242S protein was performed by electrospray mass spectrometry, in addition to the large peak of M_r 33 836, a smaller peak at 34 260 was present, representing about a third of the sample (Figure 4d). These M_r values are consistent with those expected for the C242S apoenzyme and holoenzyme, respectively, since they differ by the expected mass of the dipyrromethane cofactor (419), within experimental error.

Analysis of the C242S mutant protein by nondenaturing gel electrophoresis also confirms this distribution. The C242S holoprotein migrates with an R_f and double-band profile characteristic of the holoenzyme (data not shown); however, there is also a large amount of protein that runs as a smear with a much reduced R_f, typically observed with apodeaminase (Warren & Jordan, 1988; Scott *et al.*, 1988, 1989). Moreover, when the purified protein is heat-treated, the heat-labile apoenzyme is denatured, leaving only a band corresponding to the holoenzyme.

To confirm that the C242S mutant deaminase contains the dipyrromethane cofactor, the purified protein was reacted with Ehrlich's reagent, which gives a characteristic color reaction with pyrroles and their derivatives. The dipyrromethane cofactor also gives an Ehrlich's positive reaction (Jordan & Warren, 1987; Warren & Jordan, 1988). The positive color reaction (data not shown) indicated that a proportion of the C242S mutant contained the dipyrromethane cofactor. This observation together with the nondenaturing PAGE and the electrospray mass spectrometric analysis (Figure 4d) confirms that 25−50% of the C242S mutant contains the dipyrromethane cofactor covalently attached to the enzyme protein.

The ability of the C242S mutant to assemble the dipyrromethane cofactor is rather surprising since this would involve the rather unreactive serine OH making an oxygen ether linkage with the C1 ring of the cofactor. The fact that the reaction occurs to yield approximately a third of the C242S mutant as a holoenzyme reflects the reactive nature of porphobilinogen. Although it is remarkable that the serine OH has formed an ether linkage with the C1 ring of the cofactor, it is not surprising that, once reacted, the second ring (C2) of the cofactor is assembled and that the normal catalytic cycle of the enzyme can operate. Indeed, the C242S mutant holoenzyme has properties almost identical to those of the wild-type deaminase.

Conclusions. The identification of cysteine-134 as the thiol group which becomes progressively more susceptible to alkylation with NEM during the catalytic cycle allows important conclusions to be deduced with respect to the molecular events which take place during chain elongation. Cysteine-134 is located on an α-helix of domain 2 that is protected from the solvent by close proximity to domain 3 (Figure 1). It can be envisaged that the position of domain 3 with respect to the α-helix bearing cysteine-134 must change progressively during the catalytic cycle to allow incremental exposure of cysteine-134.

Although ligand binding to both deaminase and the related family of periplasmic binding proteins involves major changes in conformation between domains 1 and 2 (Louie, 1993; Baker *et al.*, 1984), in the case of porphobilinogen deaminase it is considered likely that domain 3, to which the dipyrromethane cofactor is attached, plays an imortant role in the process of polymerization. From the above studies it is clear that the elongation mechanism results in a change in the relative positions of domain 2 with respect to domain 3, suggesting that substantial movement of all three domains with respect to one another may need to occur in order to accommodate the large tetrapyrrole product (Louie *et al.*, 1992). X-ray analysis of the ES complexes is at an advanced state (Lambert *et al.*, 1994) to elucidate the precise mechanism of assembly of preuroporphyrinogen and the role that these protein conformational changes play in the reaction mechanism.

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REFERENCES

Aplin, R. T., Baldwin, J. E., Schofield, C. J., & Waley, S. (1990) *FEBS Lett. 277*, 211−214.

Aplin, R. T., Baldwin, J. E., Pichon, C., Roessner, C. A., Scott, A. I., Schofield, C. J., Stolowich, N. J., & Warren, M. J. (1991) *Bioorg. Med. Chem. Lett. 1*, 503−507.

Aplin, R. T., Robinson, C. V., Schofield, C. J., & Wetwood, N. J. (1993) *Tetrahedron 49*, 10903−10912.

Baker, E. N., Rumball, S. V., & Anderson, B. F. (1987) *Trends Biochem. Sci. 12*, 350−353.

Baldwin, T. O., Berends, T., Bunch, T. A., Holtzman, T. F., Rausch, S. K., Shamansky, L., & Treat, M. L. (1984) *Biochemistry 23*, 3663−3667.

Berry, A., Jordan, P. M., & Seehra, J. S. (1981) *FEBS Lett. 129*, 220−224.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−252.

Chadwick, D. G., & Ackrill, K., Eds. (1994) *The Biosynthesis of the Tetrapyrrole Pigments*, Ciba Foundation Symposium *180*, Wiley, Chichester, U. K.

Clark, L., & Carbon, J. (1976) *Cell (Cambridge Mass.) 9*, 91−99.

Jones, R. M., & Jordan, P. M. (1994) *Biochem. J. 299*, 895−902.

Jordan, P. M. (1991) *New Compr. Biochem 19*, 1−66.

Jordan, P. M. (1994) in *The Biosynthesis of the Tetrapyrrole Pigments* (Chadwick, D. G., & Ackrill, K., Eds.) Ciba Foundation Symposium *180*, 70−96, Wiley, Chichester, U.K.

Jordan, P. M., & Seehra, J. S. (1986) *Methods Enzymol. 123*, 427−434.

Jordan, P. M., & Warren, M. J. (1987) *FEBS Lett. 225*, 87−92.

Jordan, P. M., Thomas, S. D., & Warren, M. J. (1988a) *Biochem. J 254*, 427−435.

Jordan, P. M., Warren, M. J., Williams, H. J., Stolowich, N. J., Roessner, C. A., Grant, S. K., & Scott, A. I. (1988b) *FEBS Lett. 235*, 189−193.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Lambert, R., Brownlie, P. D., Woodcock, S. C., Louie, G. V., Cooper, J. B., Warren, M. J., Jordan, P. M., Bluendell, T. L., & Wood, S. P. (1994) in *The Biosynthesis of the Tetrapyrrole Pigments* (Chadwick, D. G., & Ackrill, K., Eds.) Ciba Foundation Symposium *180*, pp 97−110.

Leeper, F. J. (1994) in *The Biosynthesis of the Tetrapyrrole Pigments* (Chadwick, D. G., & Ackrill, K., Eds.) Ciba Foundation Symposium *180*, pp 111−130, Wiley, Chichester, U.K.

Louie, G. V. (1993) *Curr. Opin. Struct. Biol. 3*, 401−408.

Louie, G. V., Brownlie, P. D., Lambert, R., Cooper, J. B., Blundell, T. L., Wood, S. P., Warren, M. J., Woodcock, S. C., & Jordan, P. M. (1992) *Nature (London) 359*, 33−39.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Miller, A. D., Hart, G. J., Packman, L. C., & Battersby, A. R. (1988) *Biochem. J. 254*, 915−918.

Scott, A. I., Roessner, C. A., Stolowich, N. J., Karuso, P., Williams, H. J., Grant, S. K., Gonzales, M. D., & Hushino, T. (1988) *Biochemistry 27*, 7984−7990.

Scott, A. I., Clemens, K. R., Stolowich, N. J., Santander, P. J., Gonzalez, M. D.,& Roessner, C. A. (1989) *FEBS Lett. 242*, 319−324.

Thomas, S. D., & Jordan, P. M. (1986) *Nucleic Acids Res. 14*, 6215−6226.

Warren, M. J., & Jordan, P. M. (1988) *Biochemistry 27*, 9020−9030.

Warren, M. J., & Scott, A. I. (1990) *Trends Biochem. Sci. 180*, 486−491.

Williams, D. C., Morgan, G. S., McDonald, E., & Battersby, A. R. (1981) *Biochem. J. 193*, 301−310.

Witty, M., Lim, S. H., Wallace-Cook, A. D. M., & Smith, A. G. (1993) *Plant Physiol. 103*, 139−147.

Woodcock, S. C., & Jordan, P. M. (1994) *Biochemistry 33*, 2688−2695.

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