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# Investigation into the Nature of Substrate Binding to the Dipyrromethane Cofactor of *Escherichia coli* Porphobilinogen Deaminase

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ABSTRACT: The formation of the dipyrromethane cofactor of *Escherichia coli* porphobilinogen deaminase was shown to depend on the presence of 5-aminolevulinic acid. A *hemA*$^-$ mutant formed inactive deaminase when grown in the absence of 5-aminolevulinic acid since this strain was unable to biosynthesize the dipyrromethane cofactor. The mutant formed normal levels of deaminase, however, when grown in the presence of 5-aminolevulinic acid. Porphobilinogen, the substrate, interacts with the free $\alpha$-position of the dipyrromethane cofactor to give stable enzyme–intermediate complexes. Experiments with regiospecifically labeled intermediate complexes have shown that, in the absence of further substrate molecules, the complexes are interconvertible by the exchange of the terminal pyrrole ring of each complex. The formation of enzyme–intermediate complexes is accompanied by the exposure of a cysteine residue, suggesting that substantial conformational changes occur on binding substrate. Specific labeling of the dipyrromethane cofactor by growth of the *E. coli* in the presence of 5-amino[5-$^{14}$C]levulinic acid has confirmed that the cofactor is not subject to catalytic turnover. Experiments with the $\alpha$-substituted substrate analogue $\alpha$-bromoporphobilinogen have provided further evidence that the cofactor is responsible for the covalent binding of the substrate at the catalytic site. On the basis of these cummulative findings, it has been possible to construct a mechanistic scheme for the deaminase reaction involving a single catalytic site which is able to catalyze the addition or removal of either $NH_3$ or $H_2O$. The role of the cofactor both as a primer and as a means for regulating the number of substrates bound in each catalytic cycle is discussed.

Porphobilinogen deaminase (hydroxymethylbilane synthase, EC 4.3.1.8) catalyzes the formation of the (hydroxymethyl)bilane preuroporphyrinogen from four molecules of porphobilinogen (Burton et al., 1979; Battersby et al., 1979a). Preuroporphyrinogen is further transformed into uroporphyrinogen III (Jordan et al., 1979) and then into all other tetrapyrroles (Scheme I). Porphobilinogen deaminases have been purified and characterized from a large number of sources

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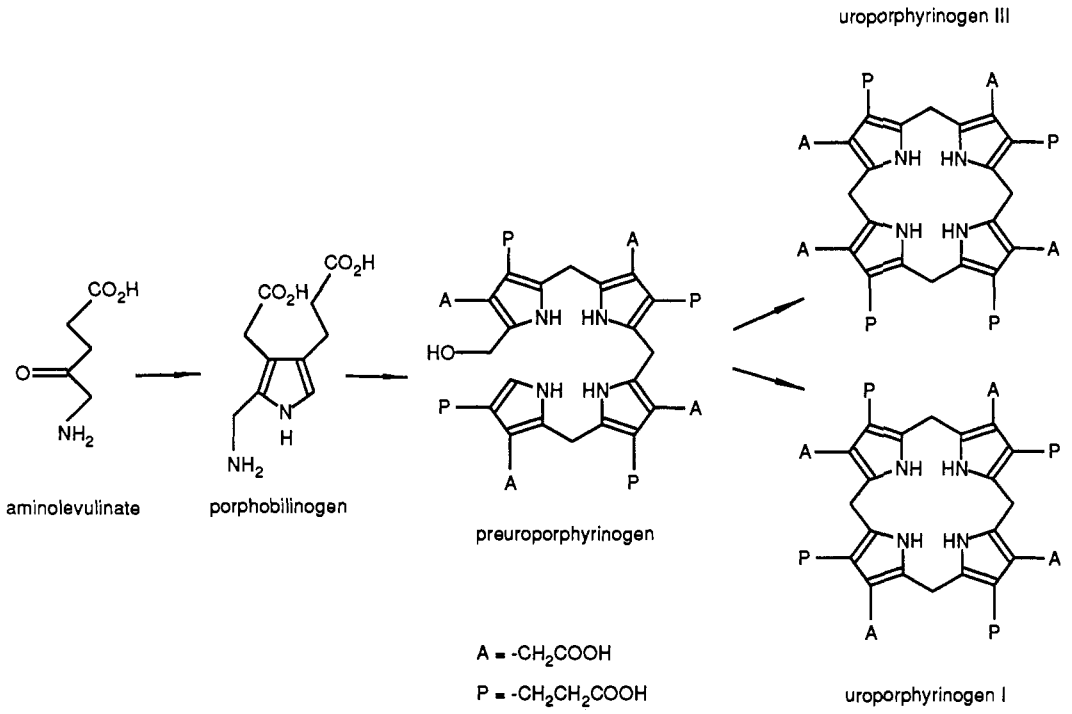
Scheme I: Biosynthesis of Uroporphyrinogen I and III from Porphobilinogen




and appear to be monomeric enzymes with molecular weights ranging from 34 000 to 44 000.

One of the most interesting features of the enzyme reaction is the existence of stable enzyme–intermediate complexes formed by the reaction of the enzyme with the substrate porphobilinogen. The enzyme–intermediate complexes were first described with the enzymes isolated from human erythrocytes (Anderson & Desnick 1980) and from *Rhodopseudomonas spheroides* (Berry et al., 1981). These enzyme–intermediate complexes represent sequential stages in the elaboration of the tetrapyrrole at the enzymic active site. More recently, the porphobilinogen deaminase isolated from genetically engineered strains of *Escherichia coli* (Jordan et al., 1988a) has also been shown to form enzyme–intermediate complexes.

Recent investigations using $^{14}C$ labeling have shown that the enzyme from *E. coli* contains a novel prosthetic group termed the dipyrromethane cofactor (DPMC) which is located at the catalytic site (Jordan & Warren, 1987). The enzyme exhibits a reaction with Ehrlich's reagent which is characteristic of a dipyrromethane and which is due to the enzyme-bound cofactor. When the enzyme, with its bound cofactor, is reacted with two molecules of substrate, an Ehrlich's reaction characteristic of a tetrapyrromethane occurs, establishing that the substrates actually interact covalently with the dipyrromethane cofactor. The cofactor is unique in that it is formed from the same pyrrole unit, porphobilinogen, which acts as the substrate for the reaction itself. By growth of the *E. coli* in the presence of 5-amino[5-$^{14}C$]levulinic acid, the direct precursor for porphobilinogen, the cofactor could be specifically labeled with $^{14}C$ radioactivity (Jordan & Warren, 1987). From these observations we proposed that the role of the dipyrromethane cofactor is to anchor the substrate molecules at the catalytic center and to direct the construction of the tetrapyrrole. Preliminary evidence for the existence of a pyrromethane group associated with the *E. coli* porphobilinogen deaminase has also been reported (Hart et al., 1987). The situation in the *Euglena* porphobilinogen deaminase is somewhat confusing since the substrate is reported to bind directly to a lysine residue at the catalytic site (Battersby et al., 1983; Hart et al., 1984), yet there is also evidence for the involvement of a pyrromethane group as well (Hart et al., 1987). More recently, we have confirmed the structure originally predicted for the dipyrromethane cofactor (Jordan & Warren, 1987) by the application of $^{13}C$ NMR and furthermore have established that the cofactor is attached to the *E. coli* porphobilinogen deaminase by means of a cysteine residue at position 242 in the sequence LEGACNVP (Jordan et al., 1988b).

The cloning and sequencing of the gene encoding porphobilinogen deaminase (*hemC*) in *E. coli* (Thomas & Jordan 1986) have permitted the construction of overproducing strains which yield milligram quantities of the deaminase enzyme (Jordan et al., 1988a) and which have greatly facilitated the study of the enzyme structure and mechanism. In this paper we report on the nature of the interaction of the substrate porphobilinogen with the dipyrromethane cofactor at the catalytic site of *E. coli* porphobilinogen deaminase. In addition, we present evidence for the exposure of a sulfhydryl group during the catalytic reaction.

## Experimental Procedures

### *Materials*

Porphobilinogen and [2,11-$^{14}C$]porphobilinogen (11.1 mCi/mmol) were prepared as described in Berry et al. (1981). 2-Bromoporphobilinogen was a generous gift from Professor A. I. Scott, Texas A&M University. 5-Amino[5-$^{14}C$]levulinic acid (49 mCi/mmol) was purchased from New England Nuclear. [4,5-$^{14}C_2$]Pyridoxal phosphate (1.93 mCi/mmol) was a trial synthesis sample from Amersham International, Buckinghamshire, England. *E. coli* strain ST1048 (Thomas & Jordan, 1986) was grown in 2-L flasks containing 1 L of Luria broth supplemented with 0.1% glucose and ampicillin, 30 μg/mL. Pyridoxal phosphate was purchased from Sigma Chemical Co. Mercury chloride, *p*-(chloromercuri)benzoate, 5,5′-dithiobis(2-nitrobenzoic acid), and *N*-ethylmaleimide were purchased from BDH, as were all other analytical reagents. *E. coli* strain A1004a (*mel, ilv, lacI, metE, hemA*) was a gift from A. Smith, Department of Biochemistry, University of Southampton, and was grown as above but with 2% glucose

and under anaerobic conditions.

*Methods*

Porphobilinogen deaminase was isolated from a genetically engineered strain of *E. coli* K-12 ST 1048 (Jordan et al., 1988a) harboring plasmid pST48 constructed by cloning the *Bam*HI–*Sal*I fragment from pLC41-4 into the *Bam*HI and *Sal*I restriction sites of a pBR322 derivative as previously described (Thomas & Jordan, 1986). The purification generated homogeneous enzyme as judged by a single band corresponding to a $M_r$ of 35 000 on polyacrylamide gels in the presence of sodium dodecyl sulfate. 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.* Porphobilinogen deaminase was assayed in a final volume of 450 μL of 0.1 M Tris-HCl buffer with 100 nmol of porphobilinogen at 37 °C as previously described (Jordan et al., 1988a). Protein was assayed by the method of Bradford (1976).

*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).

*Inhibitor Studies.* Inhibitor studies were carried out by adding inhibitor 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, and the enzyme activity was determined.

Pyridoxal 5′-phosphate inhibition was investigated by incubating either enzyme or enzyme–intermediate complex with pyridoxal 5′-phosphate followed by reduction with $NaBH_4$. A freshly prepared solution of 100 mM $NaBH_4$ in 0.1 M sodium phosphate buffer, pH 8.2, was slowly titrated with the incubation mixture until the solution just became colorless. Duplicate samples of enzyme or enzyme–intermediate complexes were incubated with pyridoxal 5′-phosphate but were not reduced with $NaBH_4$.

*Reaction with 5,5′-Dithiobis(2-nitrobenzoic acid).* About 1 mg (30 nmol) of either porphobilinogen deaminase or porphobilinogen deaminase–intermediate complexes was reacted with a final concentration of 5 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 0.1 M Tris-HCl buffer, pH 7. The absorbance of the liberated thionitrobenzoate was measured at 412 nm ($E_{412}$ = 12 800) and was followed over a period of 16 h at 10 °C. The activity of the enzyme in the reaction vial was also recorded simultaneously over the same period of time.

*Radioactivity Measurements.* All samples to be counted were made up to a final volume of 1 mL with water, and Tritoscint (9 mL) was added. The samples were then counted in an Intertechnique SL 40 scintillation counter programmed with the appropriate quench correction to convert cpm to dpm.

## Results and Discussion

*Dependence on 5-Aminolevulinic Acid for Porphobilinogen Deaminase Activity in a hemA Mutant of E. coli.* Previous studies in our laboratory established that porphobilinogen, biosynthesized from amino[5-$^{14}$C]levulinate, is incorporated specifically into the dipyrromethane cofactor. These findings thus established not only that porphobilinogen acts as the

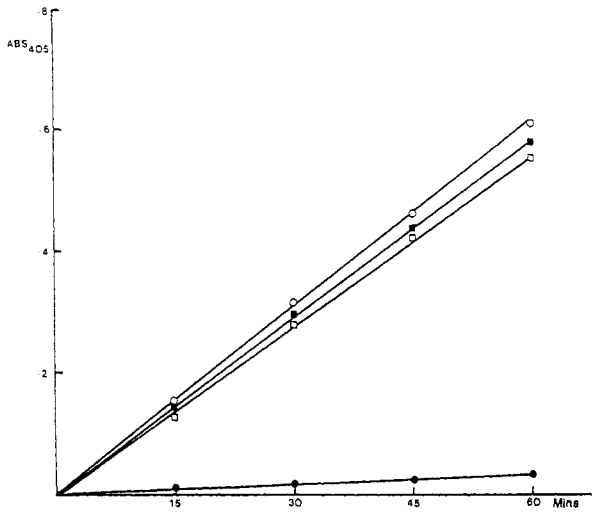



FIGURE 1: Time course for the generation of uroporphyrin I in extracts of *hemA* and wild type *E. coli*. Bacteria were grown anaerobically as described under Methods. Cells were harvested after growth in the absence or presence of 5-aminolevulinic acid. Porphobilinogen deaminase levels in cell-free extracts are as follows: *hemA*$^-$ mutant in the absence (●) or presence (○) of 5-aminolevulinic acid. Wild type in absence (■) or presence (□) of 5-aminolevulinic acid.

substrate for the porphobilinogen deaminase and is thus the main building block for all tetrapyrroles but that it is also the direct precursor for the dipyrromethane cofactor (Jordan & Warren, 1987). In an attempt to investigate this dual role of porphobilinogen, we studied the effects of a mutation in the *hemA* gene on the formation of catalytically active porphobilinogen deaminase compared to the level of heme (Smith et al., 1988). The *hemA* mutant will only grow fermentatively under strictly anaerobic conditions since there is little requirement for heme under these conditions. The data in Figure 1 indicate that the level of porphobilinogen deaminase is practically zero in extracts from the *hemA* mutant grown in the absence of 5-aminolevulinic acid. However, when this organism was grown anaerobically, but in the presence of 5-aminolevulinic acid, the level of the porphobilinogen deaminase was raised dramatically and was similar to that of wild type grown under similar anaerobic conditions (Figure 1). The amount of heme produced in the *hemA* mutant when grown on 5-aminolevulinic acid was also similar to that of wild-type *E. coli* (Smith et al., 1988). We conclude therefore that in the *hemA* mutant the inability to biosynthesize 5-aminolevulinic acid, and hence porphobilinogen, prevents the formation of active porphobilinogen deaminase due to the inability of the organism to form the dipyrromethane cofactor. It is also interesting that *E. coli hemB* mutants, in which 5-aminolevulinate dehydratase is absent and which also cannot biosynthesize porphobilinogen, produce an inactive porphobilinogen deaminase enzyme (Li et al., 1988). These observations may therefore be explained, in light of our findings, as a requirement for 5-aminolevulinate dehydratase in order to permit the biosynthesis of porphobilinogen essential for the formation of the dipyrromethane cofactor.

Since the *hemA* mutant did not produce active porphobilinogen deaminase holoenzyme, we investigated the possibility that, in the absence of the precursor for the dipyrromethane cofactor, inactive apoenzyme may still be present and susceptible to activation by porphobilinogen. In an attempt to activate any apoenzyme in the *hemA* mutant, grown in the absence of 5-aminolevulinate, the extract was incubated with prophobilinogen. Only a small amount of porphobilinogen deaminase was recovered although results were variable and

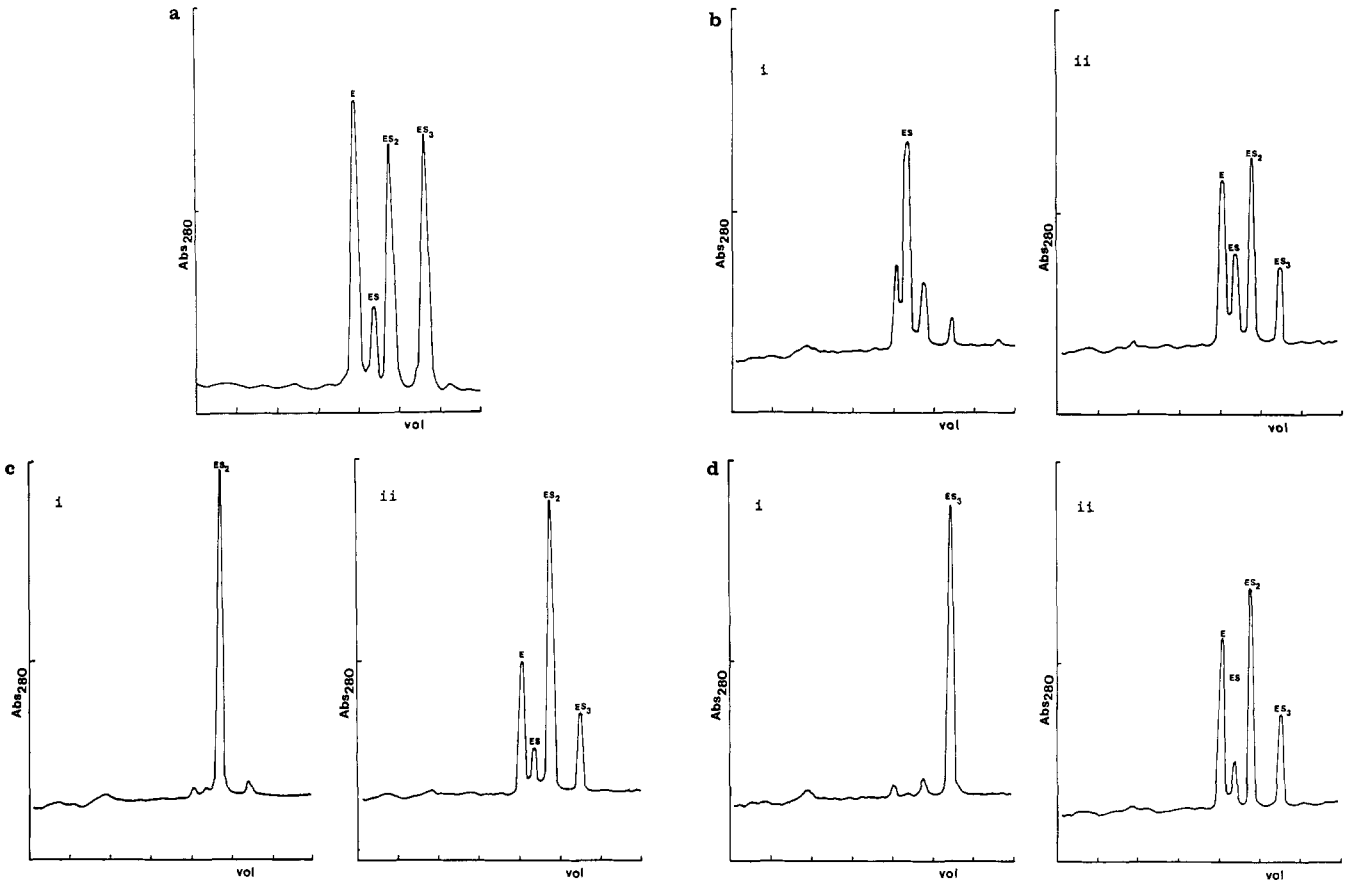



FIGURE 2: (a) FPLC profile resulting from the reaction of porphobilinogen deaminase with increasing amounts of substrate. Porphobilinogen deaminase (10 nmol) was mixed with porphobilinogen (100 nmol), and the enzyme–intermediate complexes formed (ES, $ES_2$, and $ES_3$) were separated from free enzyme (E) on a Pharmacia MonoQ column as described under Methods. (b) Isolated ES complex (from panel a) before (i) and after (ii) incubation at 37 °C for 15 min. (c) Isolated $ES_2$ complex (from panel a) before (i) and after (ii) incubation at 37 °C for 15 min. (d) Isolated $ES_3$ complex (from panel a) before (i) and after (ii) incubation at 37 °C for 15 min.

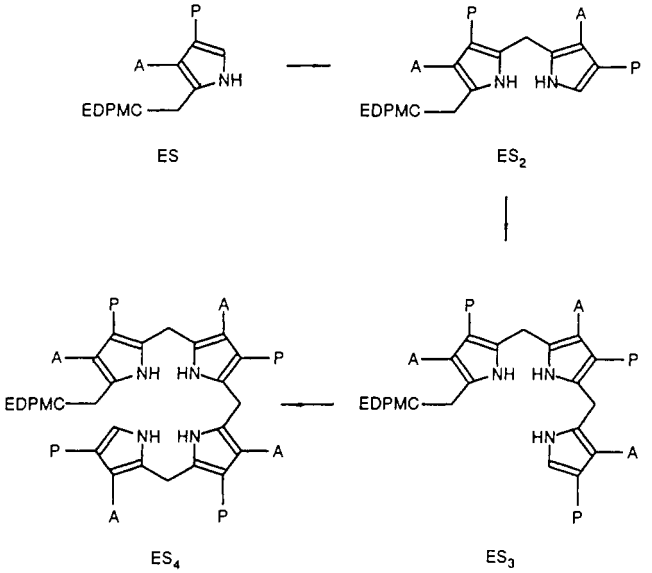
up to 15% of the activity could be recovered on prolonged incubation (data not shown). These observations suggest that the deaminase itself is responsible for the assembly of the dipyrromethane cofactor. This is not altogether unexpected since the formation of the cofactor involves the deamination of porphobilinogen in a reaction closely related to the normal catalytic reaction to form the tetrapyrrole product (see Scheme IV). Failure to recover maximum activity suggests that the apoenzyme may be unstable in vivo and in cell-free extracts. In this context it has been observed that the human porphobilinogen deaminase holoenzyme is somewhat unstable in the absence of added substrate (Beaumont et al., 1986), so it would not be surprising if the apoenzyme was highly susceptible to denaturation or to destruction by proteolytic enzymes. It was not possible to establish that the "apo" deaminase was indeed present in the extracts from *hemA* mutants grown in the absence of 5-aminolevulinic acid because of the very small amounts of protein produced in this strain. It was significant, however, that the enzyme 5-aminolevulinic acid dehydratase is present in the *hemA* mutant whether 5-aminolevulinic acid is present or absent. Since the level of deaminase produced by the wild type was similar in the presence or absence of added 5-aminolevulinic acid (Figure 1), we can only assume that the "apo" deaminase protein was produced in the mutant grown in the absence of 5-aminolevulinic acid but that it was unstable.

To increase the level of "apo" deaminase, the *hemA* mutant was transformed with pST48, a plasmid which when present in *E. coli* HB101 leads to the production of almost 100 times the wild-type level of porphobilinogen deaminase (Thomas & Jordan, 1986). The transformed strain was unable to produce active enzyme except when grown on medium supplemented with 5-aminolevulinic acid. Addition of porphobilingen to the cell-free extracts from the hemA:pST48 strain also led to the recovery of small amounts (10–15%) of deaminase activity. Overall, these findings suggest that the deaminase is able to catalyze the deamination of two porphobilinogen molecules which may then couple to cysteine-242 to give the dipyrromethane cofactor; however, this aspect requires further investigation.

*Properties of E. coli Porphobilinogen Deaminase–Intermediate Complexes.* Porphobilinogen deaminase catalyzes the stepwise synthesis of the (hydroxymethyl)bilane preuroporphyrinogen via stable enzyme–intermediate complexes, each of which is covalently linked to the dipyrromethane cofactor (DPMC) (Jordan & Warren, 1987), Scheme II. Previous reports in our laboratory have described the formation and isolation of enzyme–intermediate complexes from *R. spheroides* (Berry et al., 1981). In these studies the complexes, formed on incubation of the deaminase with [$^{14}$C]porphobilinogen, were isolated by gel electrophoresis at 4 °C and characterized by a lengthy procedure (Berry et al., 1981). The availability of FPLC and milligram quantities of the *E. coli* enzyme from overproducing strains (Jordan et al., 1988a) permits the rapid isolation of the enzyme–intermediate complexes and a more detailed study of their properties as follows.

Incubation of purified *E. coli* enzyme with increasing amounts of [$^{14}$C]porphobilinogen (1, 2, and 3 mol equiv) at 4 °C, as described under Methods, followed by FPLC afforded ES, $ES_2$, and $ES_3$ (Figure 2a).[1] The individual complexes

Scheme II: Porphobilinogen Deaminase–Intermediate Complexes[a]




[a] DPMC = dipyrromethane cofactor; Cys = cysteine-242 of *E. coli* porphobilinogen deaminase.

could be isolated and stored at 4 °C without undue decomposition. At higher temperatures the situation was very different. Figure 2b shows that the complexes varied greatly in their stability on incubation at 37°C for 15 min. ES was particularly labile and was rapidly converted into E, $ES_2$, and $ES_3$. The $ES_3$ complex was more stable and was partially converted into $ES_2$ at 37 °C. The most stable intermediate complex was $ES_2$, which remained substantially unaffected by treatment at 37 °C. Heating all the enzyme–intermediate complexes at 60 °C, however, caused a rapid loss of bound intermediates from all complexes, yielding fully active free enzyme and unstable pyrroles which finally yielded uroporphyrin (data not shown).

It may be concluded therefore that at 37 °C the linkage between the first substrate molecule and the enzyme-bound DPMC is the most labile but that on binding a second substrate molecule a far more stable complex is formed. Addition of the third substrate molecule yields $ES_3$, a more labile intermediate complex than $ES_2$, but which is more stable than ES. It is important to note that no $ES_4$ complex has been observed either with the *R. spheroides* enzyme or in the current studies with the *E. coli* deaminase. Presumably, the tetrapyrrole product must be so rapidly released from the enzyme that $ES_4$ never accumulates.

The studies of Radmer and Bogorad (1972) and Davies and Neuberger (1974) established that polypyrroles could be released from the deaminase enzyme by treatment with bases such as $NH_3$, $NH_2OH$, or $NH_2OCH_3$. When hydroxylamine was incubated with any of the *E. coli* porphobilinogen deaminase intermediate complexes discussed above, free enzyme was regenerated, and hydroxylamine adducts of mono-, di-, and tripyrroles were released into solution. These adducts were isolated and characterized by high-voltage electrophoresis as previously described (Pluscec & Bogorad, 1970) (data not shown).

[1] Enzyme–intermediate complexes are abbreviated to ES (enzyme with one substrate molecule bound), $ES_2$ (enzyme with two substrates bound), and $ES_3$ (enzyme with three substrate molecules bound). Since the substrate has been deaminated on binding to the enzyme, it is not *strictly* the substrate; however, the notation S has been adopted for simplicity.

Table I: Exchange of the Terminal $^{14}C$-Labeled Substrate in Enzyme–Intermediate Complexes[a]

| intermediate complex | % radioactivity found in complexes and product after heating to 37 °C | | | |
|---|---|---|---|---|
| | ES | $ES_2$ | $ES_3$ | released tetrapyrrole |
| ES* | 22 | 40 | 28 | 10 |
| ESS* | 9 | 54 | 22 | 15 |
| ESSS* | 6 | 31 | 20 | 43 |

| intermediate complex | molar ratio of $^{14}C$:protein in complexes after incubation at 37 °C | | |
|---|---|---|---|
| | ES | $ES_2$ | $ES_3$ |
| ES* | 1.00 | 2.00 | 3.00 |
| ESS* | 0.40 | 1.01 | 1.51 |
| ESSS* | 0.31 | 0.42 | 0.74 |

[a] Enzyme–intermediate complexes carrying a terminal $^{14}C$-labeled substrate were prepared as described under Methods. Complexes ES*, ESS*, and ESSS* were individually incubated at 37 °C for 15 min, after which each was analyzed by FPLC followed by radioactive determination of $^{14}C$ label in the resulting complexes. Molar ratios of $^{14}C$ to protein were determined for all complexes (initial $^{14}C$:protein ratios = 1:1).

*Exchange of the Pyrrole Rings in the Porphobilinogen Deaminase Intermediate Complexes Using Regiospecifically Labeled Complexes.* The aforegoing discussion highlights that the interconversion of the enzyme–intermediate complexes in the *absence* of added porphobilinogen must involve the release of pyrrole residues from one intermediate followed by their reaction either with free enzyme or with another intermediate complex. In order to establish the mechanistic course of these interconversions and to investigate the possibility of exchange, ES, $ES_2$, and $ES_3$ were prepared regiospecifically labeled at the *terminal* pyrrole position by titration of enzyme, ES, and $ES_2$ with limiting [$^{14}C$]porphobilinogen (S*) to yield ES*, ESS*, and ESSS*, respectively. This was carried out at 4 °C to prevent any unwanted exchange of label. The labeled complexes thus generated were each independently incubated at 37 °C for 15 min in the absence of porphobilinogen, the resulting enzyme–intermediate complexes were then reisolated by FPLC, and their radioactivity was determined. Table I shows that after incubation of ES* at 37 °C for 15 min the radioactivity was found in all three complexes. The ESSS* terminal label was also labile but not to the degree found for ES*. The most stable complex, ESS*, showed far less loss of label as compared to labeled ES* and ESSS*. The conclusion from these experiments is that at 37 °C all the complexes are able to release single pyrrole units as hydroxyporphobilinogen. This free pyrrole is then able to participate as a substrate in a reaction with other existing enzyme–intermediate complexes or free enzyme, ultimately generating a mixture in which $ES_2$ predominates followed by $ES_3$ and ES. Reaction of a liberated pyrrole moiety with $ES_3$ generates $ES_4$ which is rapidly released from the enzyme to generate the product—the (hydroxymethyl)bilane preuroporphyrinogen. This latter reaction pulls the equilibrium to the right, leading ultimately to the conversion of all enzyme-bound pyrroles to product and the regeneration of free enzyme. This is particularly rapid at 60 °C. Since ammonia is not present in the incubation, the back-reaction to re-form porphobilinogen is not possible, and the terminal pyrrole unit is therefore liberated by reaction with water to yield the hydroxy derivative of porphobilinogen. Since hydroxyporphobilinogen has been shown to act as an alternative substrate (Battersby et al., 1979b), the deaminase is able to carry out the dehydration–hydration reaction almost as well as the deamination–amination reaction. The exclusive formation of uroporphyrinogen I suggests that the reactions which occur are all mediated by

Table II: Effect of Inhibitors on Native Porphobilinogen Deaminase and on the Enzyme–Intermediate Complex ES[a]

| inhibitor | concn | % inhibition E | % inhibition $ES_2$ complex |
|---|---|---|---|
| *p*-(chloromercuri)benzoate | 5 μM | 17 | 35 |
| | 10 μM | 38 | 93 |
| | 100 μM | 100 | 100 |
| *N*-ethylmaleimide | 1 mM | 11 | 45 |
| | 5 mM | 47 | 89 |
| pyridoxal 5′-phosphate | 1 mM | 15 | 22 |
| | 5 mM | 33 | 35 |
| | 10 mM | 58 | 60 |
| pyridoxal 5′-phosphate (after reduction with sodium borohydride) | 1 mM | 77 | 45 |
| | 5 mM | 81 | 55 |
| | 10 mM | 83 | 60 |
| phenylglyoxal | 1 mM | 0 | 0 |
| | 5 mM | 5 | 7 |
| | 10 mM | 25 | 23 |
| butanedione | 1 mM | 0 | 0 |
| | 5 mM | 1 | 2 |
| | 10 mM | 5 | 4 |

[a] Inhibitors were incubated with the enzyme as described under Methods. Where necessary, the inhibitor was removed by gel filtration prior to assay.

the catalytic machinery of the deaminase enzyme, since the nonenzymic formation of uroporphyrinogens from porphobilinogen is accompanied by the formation of other uroporphyrin isomers (Mauzerall, 1960).

*Susceptibility of Enzyme–Intermediate Complexes to Reaction with Sulfhydryl Reagents.* Sulfhydryl reagents have been shown to inhibit porphobilinogen deaminase from several sources, implying that a cysteine residue may play an important role in the enzyme reaction (Russell & Rockwell, 1980). NMR studies have also pointed to the involvement of cysteine in the functioning of the enzyme from *R. spheroides* (Evans et al., 1986).

The native *E. coli* deaminase is remarkably unreactive to sulfhydryl reagents such as *p*-(chloromercuri)benzoate, *N*-ethylmaleimide, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and only high concentrations of these reagents caused enzyme inactivation (Table II). However, in marked contrast, when the reaction of these reagents with enzyme–intermediate complexes was investigated, the situation was completely different (Table II). For instance, when the ES complex was exposed to *N*-ethylmaleimide, a time-dependent inactivation of the enzyme was observed (Figure 3a). Addition of further substrate residues to give $ES_2$ and $ES_3$ resulted in an even more rapid inactivation of the enzyme with *N*-ethylmaleimide. DTNB also showed a greatly increased rate of reaction with the enzyme–intermediate complexes (Figure 3b).

These observations lead one to the tentative conclusion that the stepwise addition of substrate molecules causes a conformational change which leads to the exposure of a sulfhydryl group previously buried in the native enzyme. Since a considerable conformational change is envisaged during the stepwise addition of substrate molecules to the enzyme-bound cofactor, further study on this cysteine may provide valuable information about the mechanics of the polymerization process.

*Reaction of E. coli Porphobilinogen Deaminase with Pyridoxal Phosphate.* The nature of the active site group which is responsible for covalent binding of the substrate and intermediates has been a matter of some controversy. From $^{13}C$ NMR studies (Battersby et al., 1979), the *Euglena* enzyme appears to bind the substrate through a lysine residue. This work has been extended by studies with pyridoxal 5′-phosphate (Hart et al., 1984) which inactivates the deaminase enzyme

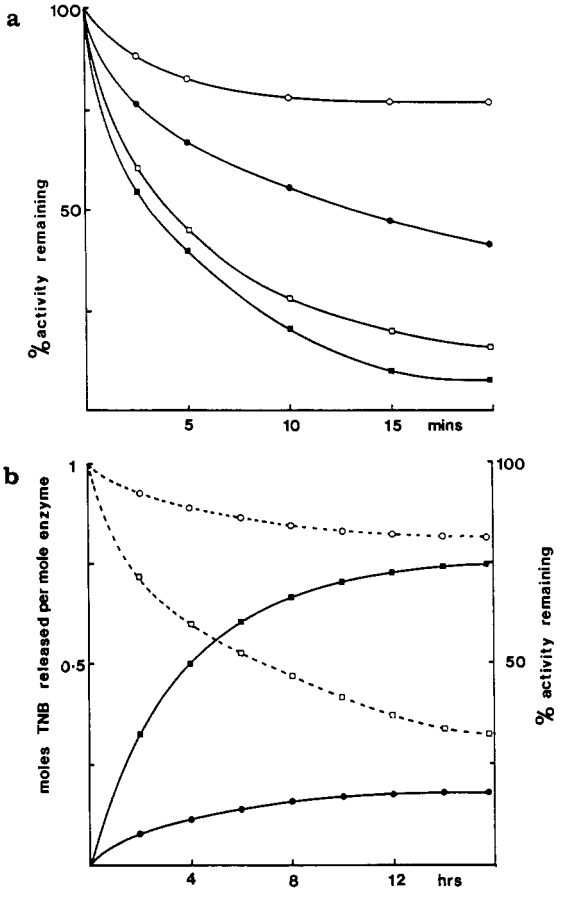



FIGURE 3: (a) Time course to show the effect of *N*-ethylmaleimide on the inactivation of native porphobilinogen deaminase and on the enzyme–intermediate complexes ES, $ES_2$, and $ES_3$. Reaction conditions are described under Methods. Native enzyme (○); enzyme–intermediate complexes ES (●), $ES_2$ (□), and $ES_3$ (■). (b) Reaction of DTNB with native porphobilinogen deaminase and enzyme–intermediate complex ES. Reaction conditions are described under methods. Inactivation of E (○) and $ES_2$ (□); moles of thionitrobenzoate (TNB) released by E (●) and $ES_2$ (■).

in the presence of sodium borohydride. When porphobilinogen is present, the enzyme is protected from inactivation. Together with the $^{13}C$ NMR studies, this has been interpreted to indicate the involvement of lysine in a covalent linkage with the substrate.

The discovery that the active site of the porphobilinogen deaminase, not only from *E. coli* (Jordan & Warren, 1987) but from all other deaminases investigated (Warren & Jordan, 1988), contains a dipyrromethane cofactor which is responsible for the binding of the substrate is thus completely different from the conclusions made from the earlier studies on the *Euglena* enzyme and prompted us to investigate further the possible involvement of lysine as an additional catalytically important group.

Accordingly, enzyme was reacted with pyridoxal 5′-phosphate in the presence of the substrate porphobilinogen—conditions under which only a marginal loss of enzyme activity occurs (Hart et al., 1984). The enzyme was next reacted with sodium borohydride, and the modified enzyme was treated with hydroxylamine to remove the protecting substrate. The enzyme was then reacted, in sequence, with [$^{14}C$]pyridoxal 5′-phosphate and sodium borohydride as described under Methods. A time-dependent loss of activity occurred accompanied by incorporation of $^{14}C$ label into the deaminase protein. However, the loss of activity did not parallel the incorporation of label into the enzyme protein as expected for a group involved in the covalent attachment of the substrate. In fact,

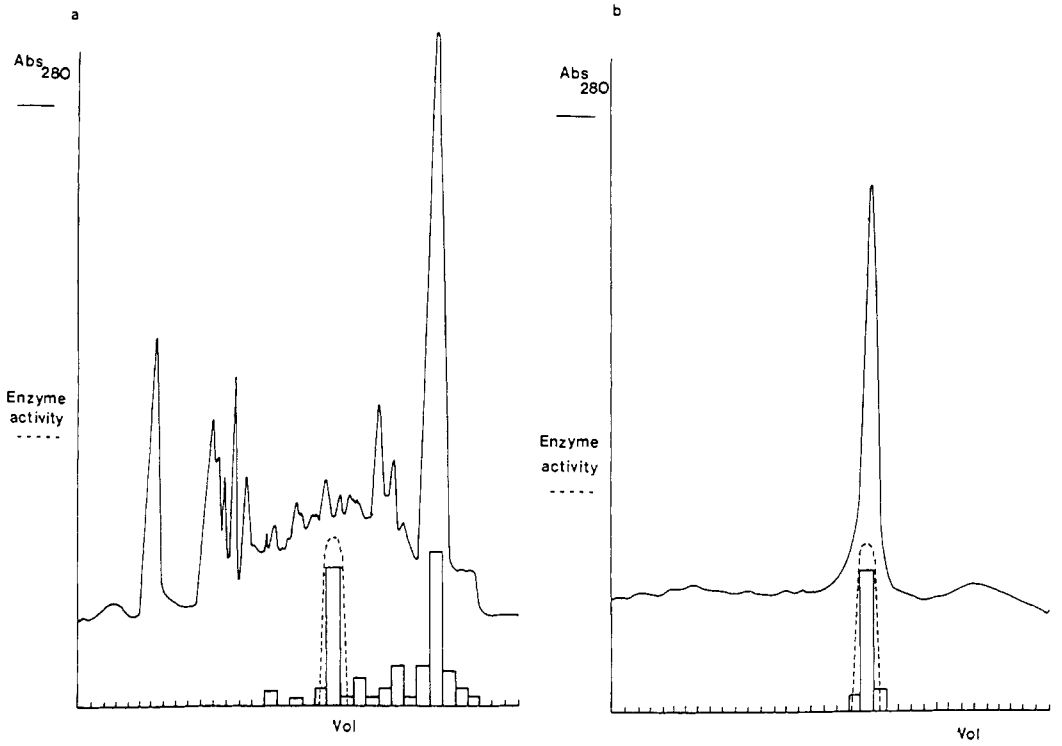



FIGURE 4: Biosynthesis of [$^{14}$C]porphobilinogen deaminase. *E. coli* strain ST1048 was grown on medium contaning 5-amino[5-$^{14}$C]levulinic acid. Cell-free extracts were analyzed for protein, enzyme activity, and radioactivity. (a) FPLC profile of protein from cell-free extracts of sonicated bacteria (—); (--) porphobilinogen deaminase activity; $^{14}$C radioactivity is shown in a bar chart form. (b) FPLC profile of purified porphobilinogen deaminase (symbols as in panel a).

incorporation of 1 mol of [$^{14}$C]pyridoxal 5′-phosphate led to a loss of only 60% of the enzyme activity. Had the modified lysine been involved in a covalent link with the substrate, it is unlikely that any enzyme activity would have remained. We suggest that if a lysine is involved its role is to permit noncovalent interaction between enzyme and substrate.

*Investigations with Porphobilinogen Deaminase Containing $^{14}$C-Labeled Dipyrromethane Cofactor.* Studies in our laboratory have established that the porphobilinogen deaminase from *E. coli* contains a dipyrromethane cofactor which is located at the catalytic site (Jordan & Warren, 1987). Since this cofactor appears to be made up of the same pyrrolic units as the substrate, it was important to differentiate unambiguously between the pyrrole rings of the cofactor and those of the enzyme–intermediate complexes. This was accomplished by two related approaches.

Growth of *E. coli* strain ST1048 with amino[5-$^{14}$C]levulinic acid resulted in the formation of porphobilinogen deaminase containing $^{14}$C label. When 50 $\mu$Ci of the labeled 5-aminolevulinic acid was used, approximately 7 $\mu$Ci of $^{14}$C label was taken up by the bacteria. Sonication of the bacterial cells followed by FPLC analysis of the centrifuged cell extract using a MonoQ column (see Methods) revealed two major radioactive peaks, one of which coincided with the porphobilinogen deaminase and the other which eluted with porphyrins at the end of the gradient (Figure 4a). No other proteins were labeled significantly. The porphobilinogen deaminase was further purified by the stages previously described (Jordan et al., 1988a), and at each stage the radioactivity was found to be exclusively associated with the porphobilinogen deaminase enzyme. The resulting homogeneous enzyme (10 mg) isolated by FPLC (Figure 4b) contained 1.5 $\mu$Ci representing an overall yield of 3% of the initial $^{14}$C label used. On the basis of the enzyme units originally in the extract and the total $^{14}$C label taken up by the bacteria, it was possible to calculate that some 40% of the $^{14}$C radioactivity taken up by the bacteria was associated with the porphobilinogen deaminase dipyrromethane cofactor.

The porphobilinogen deaminase thus labeled with $^{14}$C in the dipyrromethane cofactor (E*) was subjected to treatment with nonlabeled porphobilinogen, and the radioactive E*S, E*$S_2$, and E*$S_3$ intermediate complexes were isolated by FPLC as previously described. Exposure of any of the $^{14}$C-labeled enzyme–intermediate complexes to hydroxylamine, known to release bound substrate from the enzyme (Davis & Neuberger, 1974), led to the regeneration of free active enzyme which, most importantly, contained all the original $^{14}$C radioactivity as judged by the dpm per enzyme unit. For example, when E*S containing 3204 dpm/unit was treated with hydroxylamine, the resulting free enzyme contained 3174 dpm/unit. Alternatively, the complexes were incubated with 0.5 mM porphobilinogen to allow catalytic turnover to take place. Consistent with the above findings, the uroporphyrinogen I generated was completely unlabeled, and the $^{14}$C radioactivity remained in the enzyme-bound cofactor.

The converse experiments in which [$^{14}$C]porphobilinogen was incubated with nonradioactive enzyme to give enzyme–intermediate complexes (ES*, ES*S*, ES*S*S*) confirmed the above findings. In this case, incubation of these complexes with hydroxylamine yielded radioactive polypyrroles and enzyme totally devoid of radioactivity. For instance, when ES* containing 3001 dpm/unit was treated with hydroxylamine, the resulting free enzyme contained only 5 dpm/unit. Alternatively, incubation of these labeled enzyme–intermediate complexes with 0.5 mM porphobilinogen liberated all the $^{14}$C radioactivity from the enzyme, generating $^{14}$C uroporphyrinogen I and unlabeled enzyme (data not shown).

These experiments firmly establish that the pyrrole rings in the DPMC and those contributing to the enzyme–intermediate complexes although structurally related behave completely differently. The DPMC thus acts as a true cofactor rather than a substrate and is not subject to catalytic turnover.

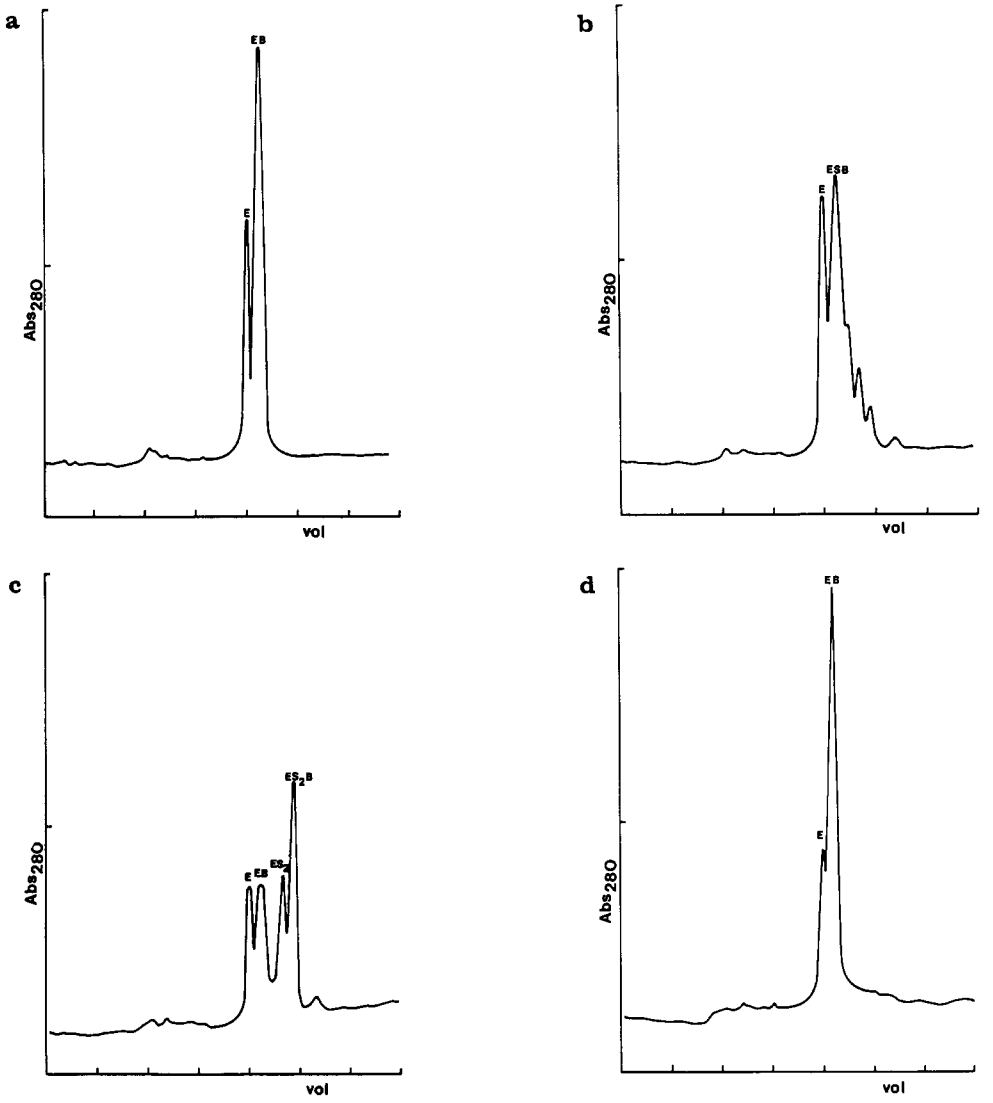



FIGURE 5: Reaction of 2-bromoporphobilinogen with porphobilinogen deaminase and enzyme–intermediate complexes. FPLC analysis of enzyme or enzyme–intermediate complexes after reaction with 2-bromoporphobilinogen. (a) Enzyme + bromoporphobilinogen; (b) ES + bromoporphobilinogen; (c) $ES_2$ + bromoporphobilinogen; (d) $ES_3$ + bromoporphobilinogen.

Release of the dipyrromethane from the $^{14}C$-labeled "holoenzyme" could only be achieved by prolonged treatment with formic acid. This yielded [$^{14}C$]uroporphyrin I and III (80:20) and nonradioactive, inactive enzyme protein. The findings are thus in complete agreement with our preliminary results (Jordan & Warren, 1987).

*Reaction of 2-Bromoporphobilinogen with Porphobilinogen Deaminase.* 2-Bromoporphobilinogen is closely related to the substrate and differs only in having bromine at the key reactive $\alpha$-position. Bromoporphobilinogen would be expected to bind to the deaminase and form a covalent complex similar to that of the substrate; however, the blocked $\alpha$-position would be expected to prevent the binding of further substrate molecules. As expected, incubation of enzyme with bromoporphobilinogen (25 $\mu$M) led to a rapid inactivation of the enzyme within 5 min (Table II). The substrate, porphobilinogen (0.3 mM), greatly reduced the rate of inactivation. The deaminase–bromoporphobilinogen complex (EB) could be isolated by FPLC (Figure 5) where it chromatographed in a similar but slightly different position to the ES complex. Bromoporphobilinogen (B) also reacted with ES and $ES_2$, yielding ESB and $ES_2B$ as judged by FPLC analysis (Figure 5b,c). All these complexes were enzymically inactive. Reaction of $ES_3$ with bromoporphobilinogen completed the turnover, yielding bromopreuroporphyrinogen and enzyme, the latter which then reacted with more bromoporphobilinogen to form the enzyme–bromoporphobilinogen complex (Figure 5d). The behavior of bromoporphobilinogen is thus as a suicide inhibitor which is recognized by the catalytic site as a substrate where it is first deaminated and then coupled either directly to the cofactor or to an intermediate complex. Once bound however, the presence of the Br atom at the $\alpha$-position blocks further reaction both with substrate and with additional bromoporphobilinogen residues, leaving the enzyme in an inactivated state. It is interesting that heating the inactivated species (EB, ESB, ESSB) or treatment with 0.2 M hydroxylamine causes the liberation of the bound bromo intermediates leading to complete restoration of enzyme activity.

The native porphobilinogen deaminase enzyme gives a characteristic Ehrlich's positive reaction due to the resident dipyrromethane cofactor. Since the bromoporphobilinogen reacts with the enzyme by a similar mechanism to that of the substrate, prior exposure of the enzyme, or enzyme–intermediate complexes, to bromoporphobilingen would be expected to block the reaction with Ehrlich's reagent due to absence of a free $\alpha$-position in EB, ESB, or $ES_2B$.

When Ehrlich's reagent was added to enzyme that had been treated with bromoporphobilinogen, the development of the

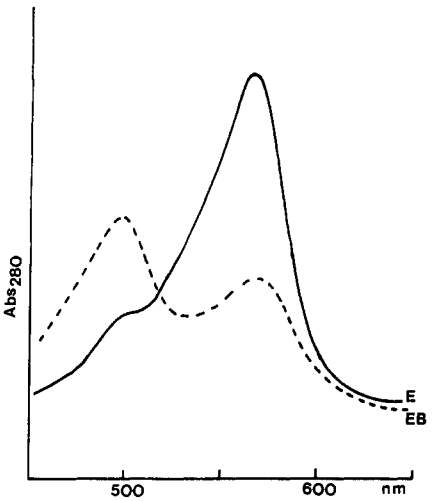



FIGURE 6: Reaction of Ehrlich's reagent with native enzyme (E) and with enzyme–bromoporphobilinogen complex (EB). The reactions and FPLC conditions are as described under Methods. The EB complex contained approximately 10% of free enzyme.

characteristic pink color was almost completely inhibited. The same result was obtained for ESB and $ES_2B$ where the Ehrlich's reaction was also totally inhibited (data not shown). The spectrum in Figure 6 shows the reaction of enzyme with Ehrlich's reagent compared to the reaction of enzyme–bromoporphobilinogen.

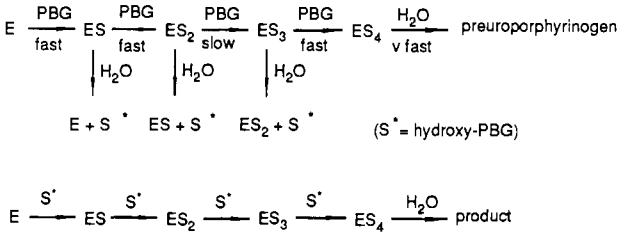
These observations provide unequivocal evidence that the native enzyme contains a reactive dipyrromethane cofactor and, more importantly, that this cofactor is involved *directly* in the sequential binding of the substrate molecules during the catalytic reaction.

## SUMMARY

In conclusion we have confirmed that the porphyrin precursor 5-aminolevulinic acid is essential for the formation of catalytically active porphobilinogen deaminase. Growth of a *hemA*⁻ *E. coli* mutant in the absence of 5-aminolevulinic acid resulted in inactive deaminase whereas addition of 5-aminolevulinc acid to the growth medium restored the deaminase to normal wild-type levels. The requirement for 5-aminolevulinic acid relates directly to its incorporation into porphobilinogen and then into the dipyrromethane cofactor at the catalytic site of the deaminase. This assertion was confirmed by the specific incorporation of $^{14}C$ label into the cofactor when the *E. coli* was grown on 5-amino[5-$^{14}C$]levulinic acid. Subjection of the $^{14}C$-labeled enzyme to catalytic turnover with the substrate porphobilinogen did not result in the loss of any radioactive label from the enzyme, thus confirming that the dipyrromethane was acting as a true cofactor.

Studies with purified enzyme–intermediate complexes with one, two, and three pyrrole units bound covalently to the enzyme have revealed that all three complexes, when incubated in the absence of substrate, are able to participate in enzyme-catalyzed exchange reactions involving the terminal pyrrole unit. The terminal pyrrole unit is removed as the hydroxy form of the substrate which, as a substrate itself, can react with any existing intermediate complex. The ultimate result is that the intermediate complexes are transformed into the (hydroxymethyl)bilane product preuroporphyrinogen. From investigations with the enzyme–intermediate complexes, we have concluded that the slowest reaction in the absence of porphobilinogen is that in which $ES_2$ is converted into $ES_3$. We believe that this may be due to a conformational adjustment in the enzyme structure in order to accommodate additional porphobilinogen units. The exposure of a previously unreactive cysteine residue on binding substrate provides preliminary experimental evidence for the occurrence of such a conformational change.

Scheme III: Catalytic Cycle for Porphobilinogen Deaminase in the Presence and Absence of Porphobilinogen[a]



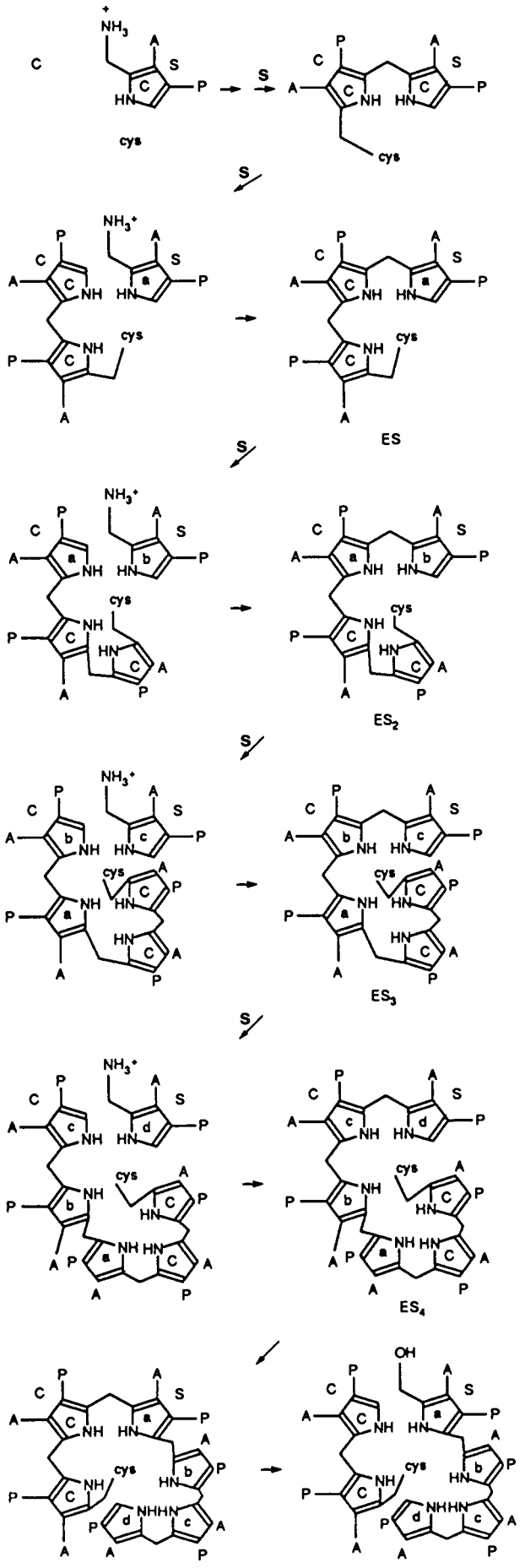

[a] In the absence of porphobilinogen each enzyme–intermediate complex (ES, $ES_2$, $ES_3$, or $ES_4$) may be cleaved by hydrolysis. The resulting hydroxyporphobilinogen, which also acts as a substrate, can be added to any intermediate complex yielding, ultimately, the product, preuroporphyrinogen.

Conclusive evidence for the direct covalent interaction of the substrate with the dipyrromethane cofactor is provided by the experiments with bromoporphobilinogen. This substrate analogue differs from the substrate only in having a bromine atom at the $\alpha$-position. It is therefore recognized as a substrate and forms stable enzyme–intermediate–inhibitor complexes. These, by virtue of a blocked $\alpha$-position, are unable to react with further substrate, and the enzyme is therefore no longer catalytically active. Most importantly, the reaction of the deaminase with bromoporphobilinogen abolishes the typical dipyrromethane cofactor reaction of the enzyme with Ehrlich's reagent, establishing that it is the cofactor which forms the covelent linkage with the substrate.

These cumulative results allow us to propose a mechanism for the reaction of the deaminase enzyme. This involves a single active site which is able to catalyze two basic reactions: first, the deamination of the substrate and the condensation of the deaminated intermediate with either the dipyrromethane cofactor or an enzyme–intermediate complex; second, the hydrolytic cleavage to yield a hydroxymethyl product. The latter activity is normally confined to the release of the tetrapyrrole product, but in the absence of substrate any of the enzyme–intermediate complexes can be similarly cleaved, albeit at a slow rate. These proposals are summarized in Scheme III.

The topography of the catalytic center may be envisaged as having two pyrrole recognition sites, one for the incoming substrate, site S, and a second which can accommodate a pyrrole ring with a free $\alpha$-position in readiness for the reaction with the substrate bound at site S. In the free holoenzyme this second site is occupied by the dipyrromethane cofactor and has been designated as site C. In the case of the apoenzyme the cofactor is first assembled (Scheme IV) by the deamination of one porphobilinogen molecule at the catalytic site followed by reaction with cysteine-242 (Cys). The second porphobilinogen unit of the dipyromethane cofactor is inserted similarly, and the deaminase assumes the configuration of the holoenzyme in which the cofactor is permanently bound to the deaminase. The subsequent stages in Scheme IV outline the stepwise addition of the four substrates in the assembly of the tetrapyrrole preuroporphyrinogen. The first substrate occupies the S site and reacts with the dipyrromethane cofactor, which acts as a "primer" for the reaction. The resulting cofactor enzyme–inermediate complex is then translocated at the active site so that the newly bound pyrrole unit occupies the C site. The translocation occurs as a result of the binding of porphobilinogen which has a higher affinity for the S site than the newly bound pyrrole unit. In the absence of substrate the

Scheme IV: Formation and Role of the Dipyrromethane Cofactor at the Catalytic Site of Porphobilinogen Deaminase




pyrrole ring bound in the ES complex remains in the S site and can be released by hydrolytic cleavage. In the presence of substrate, sequential deamination occurs at the S site followed by translocation resulting in the formation of $ES_2$ followed by $ES_3$ and finally $ES_4$. At this stage it is envisaged that steric considerations prevent the binding of a "fifth" substrate and that hydrolytic cleavage, which is normally far slower than the condensation reaction, becomes significant and results in the liberation of the (hydroxymethyl)bilane product preuroporphyrinogen. This would require that the a ring of $ES_4$ occupies the S site. It is well established that the enzyme-bound tetrapyrrole exists in this form since incubation of deaminase with substrate in the presence of ammonia leads to the production of (aminomethyl)bilanes of the type $NH_2APAPAPAP$ (Radmer & Bogorad, 1972). Furthermore, the enzyme is also able to deaminate the (aminomethyl)bilane $NH_2APAPAPAP$ to the hydroxymethyl equivalent. The function of the cofactor is thus 2-fold—first to act as a primer for the reaction and second to impose a steric restriction on the number of pyrrole rings which can be incorporated to four. This latter property is due to the fact that the cofactor is permanently linked to the deaminase protein. It is thought that this is the only example known in which the "primer" is covalently bound to the enzyme.

## Added in Proof

Independent work has also established that the dipyrromethane cofactor is attached to a cysteine residue (Hart et al., 1988).

**Registry No.** Porphobilinogen deaminase, 9074-91-3; 5-aminolevulinic acid, 106-60-5; porphobilinogen, 487-90-1; α-bromoporphobilinogen, 115828-91-6.

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# Interaction of Diacylglycerols with Phosphatidylcholine Vesicles As Studied by Differential Scanning Calorimetry and Fluorescence Probe Depolarization†

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ABSTRACT: Mixtures of 1,2-dipalmitoylglycerol (1,2-DPG), 1,2-dioleylglycerol (1,2-DOG), 1,2-dicapryloylglycerol (1,2-DCG), 1,3-dioleylglycerol (1,3-DOG), and 1,3-dicapryloylglycerol (1,3-DCG) with dipalmitoylphosphatidylcholine (DPPC) have been studied by means of differential scanning calorimetry (DSC) and fluorescence depolarization of the probe diphenylhexatriene (DPH). DSC measurements showed that the tested diacylglycerols (DG's) modified both the pretransition and the main transition of DPPC, but whereas increasing concentrations of 1,2-DPG tended to produce mixtures with transition temperatures higher than that of pure DPPC, all the other diacylglycerols tested tended to decrease this temperature. This is interpreted as a preferential partitioning of 1,2-DPG into rigid domains whereas all the other DG's preferentially partition into fluid domains. Lateral phase separation was detected in all the mixtures, so that the presence of diacylglycerols produced lipid immiscibilities. The phase diagrams constructed from the calorimetric data showed that 1,2-DPG induced solid-phase immiscibility from 0 to 12.5 mol %, whereas 1,2-DCG produced fluid-phase immiscibility at low concentrations, with an eutectic point at 0.64 mol %. 1,2-DOG also showed fluid-phase immiscibility. 1,3-DCG behaved differently than 1,2-DCG, but 1,3-DOG was rather similar in its effects to 1,2-DOG. Fluorescence depolarization of DPH included in these lipid mixtures was measured at different temperatures, so that phase transitions and the order of the bilayer were monitored. The phase transitions observed by the fluorescence technique were in general in agreement with those monitored by calorimetry. 1,2-DPG did not change the anisotropy value, as referenced to pure DPPC, neither above nor below the phase transition interval, but 1,2-DCG and 1,2-DOG decreased the anisotropy below the phase transition and increased it above this transition. 1,3-DCG decreased the anisotropy at all temperatures, and 1,3-DOG behaved similarly to 1,2-DOG. The physiological importance of the preferential partition of diacylglycerols into domains of different fluidity and their ability to produce lipid immiscibilities at relatively low concentrations are discussed. Since it has been described that only some isomers of diacylglycerol elicit biological responses, the distinct types of perturbation of the phospholipid bilayer produced by the different isomers of diacylglycerol tested here may be a significantly important phenomenon to be considered when studying the mechanism of action of these compounds.

Diacylglycerols (DG's)[1] are nonpolar molecules which are currently the focus of attention of many workers. DG's are generated in response to the stimulation of phosphoinositide breakdown by extracellular agents, acting then as a second messenger by activating protein kinase C (Nishizuka, 1984; Downes & Mitchell, 1985) together with calcium and phosphatidylserine. It has been also described that DG's enhance the activity of a variety of phospholipases when incorporated into their substrates (Dawson et al., 1983; Dawson et al., 1984). The generation of DG's has been observed in many different cells, eliciting a great variety of biological activities [see Kikkawa and Nishizuka (1986), Abdel-Latif (1986), and Berridge (1987) for recent reviews].

The crystalline structure of DG's has been studied, indicating that 1,3-DG's and 1,2-DG's have different structures. 1,3-DG molecules pack in a triclinic lattice with T parallel subcells and the chain extended on both sides of the glycerol (Larsson, 1963). 1,2-DG's, however, pack in a stable mono-

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[1] Abbreviations: $\Delta H$, enthalpy change of the phase transition; DG, diacylglycerol; DCG, dicapryloylglycerol; DOG, dioleylglycerol; DPG, dipalmitoylglycerol; DPH, diphenylhexatriene; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; $T_c$, onset temperature of the phase transition.

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