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Porphobilinogen Deaminase and Uroporphyrinogen III Synthase: Structure, Molecular Biology, and Mechanism

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Porphobilinogen deaminase (hydroxymethylbilane synthase) and uroporphyrinogen III synthase (uroporphyrinogen III cosynthase) catalyze the transformation of four molecules of porphobilinogen, via the 1-hydroxymethylbilane, preuroporphyrinogen, into uroporphyrinogen III. A combination of studies involving protein chemistry, molecular biology, site-directed mutagenesis, and the use of chemically synthesized substrate analogs and inhibitors is helping to unravel the complex mechanisms by which the two enzymes function. The determination of the X-ray structure of *E. coli* porphobilinogen deaminase at 1.76 Å resolution has provided the springboard for the design of further experiments to elucidate the precise mechanism for the assembly of both the dipyrromethane cofactor and the tetrapyrrole chain. The human deaminase structure has been modeled from the *E. coli* structure and has led to a molecular explanation for the disease acute intermittent porphyria. Molecular modeling has also been employed to simulate the spiro-mechanism of uroporphyrinogen III synthase.

KEY WORDS: Porphobilinogen deaminase; uroporphyrinogen synthase; dipyrromethane cofactor; enzyme–intermediate complexes; uroporphyrinogen III; uroporphyrinogen I; preuroporphyrinogen; *hem* genes; acute intermittent porphyria; enzyme mechanism; polymerization; spiro-intermediate.

INTRODUCTION

The macrocyclic tetrapyrrole ring system has been adopted by living systems as a versatile chelating agent for the metal ions Fe^{2+}, Mg^{2+}, Co^{2+}, and Ni^{2+}. The metallotetrapyrroles cover an impressive spectrum of versatility and are employed for many of the most vital roles in bioenergetics. These include trapping of light energy (chlorophylls), transfer of electrons (cytochromes), and binding and reduction of oxygen and its derivatives (globins and oxidases). In addition, metallotetrapyrroles play key roles in methanogenesis (coenzyme F430) and in a variety of molecular rearrangements (vitamin B12 coenzyme). Recent volumes on the biosynthesis of tetrapyrroles are available [see Dailey (1990); Jordan (1991a), and Chadwick and Ackrill (1994)].

The progenitor tetrapyrrole, uroporphyrinogen III, is assembled in two enzymic reactions from the trisubstituted pyrrole, porphobilinogen (Scheme 1). Firstly, porphobilinogen deaminase catalyzes the polymerization of four molecules of porphobilinogen to furnish a highly unstable 1-hydroxymethylbilane intermediate called preuroporphyrinogen (for a review see Jordan, 1991b). The second enzyme, uroporphyrinogen III synthase (cosynthase), then catalyzes the rearrangement of the terminal "d" ring of preuroporphyrinogen followed by ring closure to form uroporphyrinogen III (Jordan, 1991b; Leeper, 1994). In the absence of uroporphyrinogen III synthase, or if the level of this enzyme is insufficient, preuroporphyrinogen undergoes spontaneous chemical cyclization to give the physiologically redundant uroporphyrinogen I isomer (Jordan, 1991b). The metal chelating properties of the tetrapyrrole macrocycle are only manifested after uroporphyrinogen III is selectively decarboxylated and oxidized to protoporphyrin IX (Dolphin, 1976) or, alternatively, after modification to related

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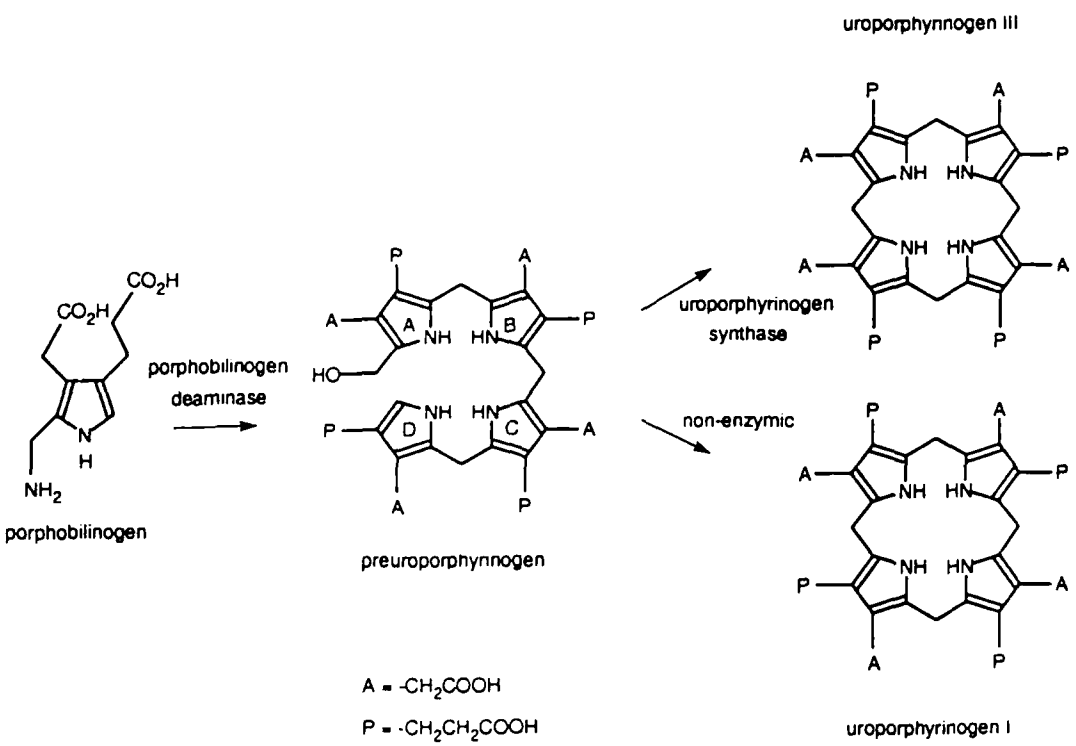
structures in which the pyrrole ring nitrogens are optimally arranged for specific metal chelation (Chadwick and Ackrill, 1994). This chapter is confined only to the two reactions in which the basic tetrapyrrole nucleus, uroporphyrinogen III, is assembled from the monopyrrole building unit of porphobilinogen.

ENZYMIC PROPERTIES OF PORPHOBILINOGEN DEAMINASE AND UROPORPHYRINOGEN III SYNTHASE

The two enzymes responsible for transforming porphobilinogen into uroporphyrinogen III were first described by Bogorad (1958). Porphobilinogen deaminase has since been isolated from a variety of sources but principally from *Rhodobacter spheroides* (Jordan and Shemin, 1973), spinach (Higuchi and Bogorad, 1975), human erythrocytes (Anderson and Desnick, 1980), *Euglena gracilis* (Williams *et al.*, 1981), and more recently from recombinant strains of *Escherichia coli* (Jordan *et al.*, 1988a) and *Arabidopsis thaliana* (Jones and Jordan, 1994). All deaminases exist as monomeric enzymes with Mr's from 34,000 to 44,000, isoelectric points between pH 4 and 5, and optimal enzyme activities in the region of pH 8. The K_m for porphobilinogen is in the 10–50 μM range with turnover numbers of about 0.5 mol $sec^{-1} \cdot mol^{-1}$. The deaminases from most sources show exceptional heat stability, which has greatly aided their purification and study.

In sharp contrast, uroporphyrinogen III synthases have proved extremely difficult to isolate because of their intrinsic temperature instability and low abundance. Furthermore the existence of the substrate, preuroporphyrinogen, became known relatively recently (Burton *et al.*, 1979; Jordan *et al.*, 1979; Battersby *et al.*, 1979a; Jordan and Berry, 1980) and only then was it possible to design an assay for the enzyme (Jordan, 1982). Despite these difficulties, uroporphyrinogen synthases (cosynthases) have been purified from spinach (Higuchi and Bogorad, 1975), rat liver (Kohashi *et al.*, 1984; Smythe and Williams, 1988), *Euglena gracilis* (Hart and Battersby, 1985), human erythrocytes (Tsai *et al.*, 1987), and from a recombinant strain of *Escherichia coliu* (Alwan *et al.*, 1989). The synthases are all monomeric enzymes with Mr's in the region 26,000 to 31,000, with isoelectric points around pH 5 and K_m values in the low micromolar range. They have



Scheme 1. Transformation of porphobilinogen into preuroporphyrinogen and uroporphyrinogens I and III.

turnover numbers in the order of 250 mol sec^{-1} · mol^{-1}, some 500 times those of the deaminases.

The levels of the deaminase and synthase are regulated closely *in vivo* since little porphobilinogen or uroporphyrinogen I accumulate under normal conditions. In humans, if imbalances between the levels of the enzymes exist, due to inherited defects, serious clinical conditions arise (for a review see Nordmann and Deybach, 1990). Deficiency of the deaminase leads to acute intermittent porphyria (AIP), a dominant inherited disease, in which there is a dramatic accumulation of porphobilinogen and 5-aminolevulinic acid, both of which are excreted in the urine. The molecular basis of AIP is covered later in this review. Deficiency of uroporphyrinogen III synthase causes the rare recessive human disease, congenital erythropoietic porphyria (CEP) (Nordmann and Deybach, 1990). In CEP there is insufficient uroporphyrinogen III synthase to ensure that all preuroporphyrinogen produced by the deaminase is transformed into uroporphyrinogen III. Consequently, chemical cyclization of this highly unstable 1-hydroxymethylbilane occurs, leading to the formation of uroporphyrinogen I, large amounts of which accumulate in erthrocytes and spleen. On oxidation to the highly photoreactive uroporphyrin I, acute photosensitivy results with extensive skin blistering and scarring.

The possibility that deaminase and synthase form a functional enzyme complex, first proposed by Higuchi and Bogorad (1975), has met with some support (Batlle and Rossetti, 1977), largely from the fact that the two enzymes appear to copurify through several stages during their isolation. It has been considered that the synthase may either accept a deaminase-bound bilane directly or, through substrate chaneling, may gain access to preuroporphyrinogen by enzyme–enzyme association. The availability of three purified deaminases and three purified synthases from *Rhodobacter spheroides, Escherichia coli*, and human erythrocytes allowed these possibilities to be tested (Jordan and Dailey, 1990). Since no significant differences were found in the rate of formation of uroporphyrinogen III with various permutations of deaminases and synthases, it is considered unlikely that the two enzymes have any specific interaction with one another in the form of a complex, or that substrate chaneling occurs. Had specific complex formation existed, it is expected that the rate of uroporphyrinogen III formation with enzymes from the *same* source would have been appreciably increased. The deaminase and synthase therefore appear to function independently and sequentially with preuroporphyrinogen as a free intermediate (Jordan *et al.*, 1979). The most likely reason for the enzymes' copurification is the fact that they are both monomeric proteins of similar sizes with similar isoelectric points.

THE FORMATION OF PORPHOBILINOGEN DEAMINASE ENZYME INTERMEDIATE COMPLEXES AND THE DISCOVERY OF THE DIPYRROMETHANE COFACTOR

The observation that porphobilinogen deaminase, when treated with tritiated porphobilinogen, yielded several labeled enzyme species (Anderson and Desnick, 1980) suggested that the deaminase reaction proceeds through enzyme intermediate ES, ES_2, ES_3, and ES_4. Similar complexes formed by the *R. spheroides* deaminase were isolated and characterized and shown to be covalently linked to the deaminase (Jordan and Berry, 1981; Berry *et al.*, 1981). Extensive studies with ^{14}C-labeled intermediate complexes of *E. coli* deaminase (Warren and Jordan, 1988) have revealed that the terminal α-free pyrrole unit of each complex is exchangeable and that the complexes are interconvertible. Of all the complexes, ES_4 is the most labile; however, it may be detected by electrospray mass spectrometry (Alpin *et al.*, 1991).

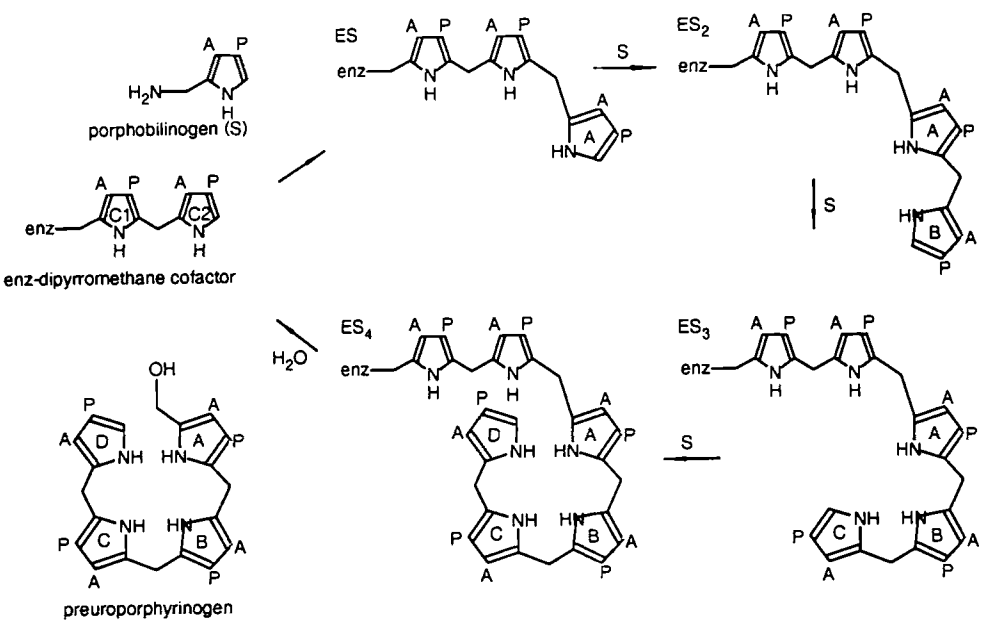
The order of addition of the four porphobilinogen units to the deaminase was shown by single turnover experiments to occur with ring "A" binding first followed by ring "B" and ring "C" and finally ring "D" (Battersby *et al.*, 1979b; Jordan and Seehra, 1979; Seehra and Jordan, 1980).

The finding that all four substrate molecules are attached covalently to the deaminase prompted a search for the enzymic residue that forms the covalent linkage. Results from nmr studies (Battersby *et al.*, 1983) and enzyme modification (Hart *et al.*, 1984) pointed to lysine as being the group involved in substrate covalent binding. However, proteolytic digestion of a deaminase ES_2 complex of the *R. spheroides* enzyme previously labeled with ^{14}C-porphobilinogen yielded a peptide bearing a pink *unlabeled* dipyrrole chromophore (Jordan and Berry, 1983, unpublished). This indicated that pyrrole units were already present in the enzyme *before* the addition of substrate. The availability of milligram amounts of the *E. coli* deaminase from a recombinant *hemC*-containing strain

(Thomas and Jordan, 1986) allowed further key labeling experiments to be carried out that established that the enzymic group forming the pink chromophore was involved in binding the substrate, and it was named the dipyrromethane cofactor (Jordan and Warren, 1987). This novel prosthetic group could be labeled specifically by growing a recombinant *hemA*$^-$ strain of *E. coli*, harboring the *hemC* gene, on 5-amino[^{14}C]levulinic acid (Warren and Jordan, 1988). Incubation of the resulting labeled deaminase with substrate did not release the [^{14}C] label, establishing that the dipyrromethane cofactor was not subject to turnover during the catalytic reaction cycle (Jordan and Warren, 1987; Warren and Jordan, 1988). These studies together with ^{13}C-nmr experiments (Hart *et al.*, 1987; Jordan *et al.*, 1988b; Beifuss *et al.*, 1988) confirmed the structure of the cofactor and established that it was covalently linked to the deaminase through a thioether linkage. The cysteine residue forming the covalent linkage with the cofactor was identified as cysteine-242 in the *E. coli* sequence (Jordan *et al.*, 1988b; Hart *et al.*, 1988; Miller *et al.*, 1988). The conclusion from these studies was that the four porphobilinogen units required for the assembly of preuroporphyrinogen are added in a stepwise fashion to the dipyrromethane cofactor that acts as a reaction primer. The resulting enzyme-bound "hexapyrrole" is then cleaved by reaction with water to yield preuroporphyrinogen and to regenerate the dipyrromethane cofactor, still linked to the deaminase. This reaction cycle is shown in Scheme 2. Most remarkably, no other enzyme seems to be necessary for the assembly of the cofactor. Thus the deaminase apoenzyme possesses the capacity to "self-assemble" its own cofactor from two molecules of porphobilinogen to form the holoenzyme (Warren and Jordan, 1988; Miller *et al.*, 1988; Scott *et al.*, 1989).

SUBSTRATE REQUIREMENTS AND INHIBITORS OF PORPHOBILINOGEN DEAMINASE

The substrate requirements for porphobilinogen deaminase are extremely rigid, as would be expected for a reaction cycle of such complexity. Only the physiological substrate, porphobilinogen [NH_2–CH_2–(AP)], and the nonphysiological hydroxy-analog [HO–CH_2–(AP)] (Battersby *et al.*, 1979c) act as substrates. Neither the chemically synthesized aminomethyldipyrromethane NH_2–CH_2–(APAP) nor the aminomethyltripyrrane NH_2–CH_2–(APAPAP) are accepted as substrates (Frydman *et al.*, 1976, 1978; Sburlati, 1983; Battersby and McDonald, 1976), despite the evidence for their existence in the form of the enzyme



Scheme 2. The stepwise assembly of preuroporphyrinogen attached to the dipyrromethane cofactor of porphobilinogen deaminase. A = –CH_2–CO_2H; P = –CH_2–CH_2–CO_2H; C1 and C2 are the two pyrrole rings of the cofactor; the preuroporphyrinogen structure is abbreviated to HO–CH_2–(APAPAPAP) in the text.

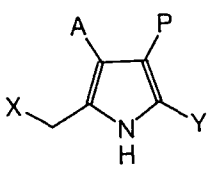
intermediate complexes ES_2 and ES_3. The deaminase thus prefers to assemble the tetrapyrrole from a monomeric pyrrole precursor. The enzyme does, however, accept the 1-aminomethylbilane [NH_2–CH_2–(APAPAPAP)] (Battersby *et al.*, 1977) and slowly catalyzes its deamination to preuroporphyrinogen [HO–CH_2–(APAPAPAP)]. Opsopyrrole, a porphobilinogen analog lacking the aminomethyl group, is a competitive inhibitor, indicating that the acetic (A) and propionic acid (P) side chains are essential for recognition and binding by the enzyme (Jordan, Cheung, and Leadbeater, 1995).

The porphobilinogen analog 2-bromoporphobilinogen inactivates porphobilinogen deaminase in a novel way by acting as a suicide inhibitor. This inhibitor is recognized as a substrate and is deaminated normally before being coupled to the enzyme. However, the presence of the bromine atom, at what is normally a free α-position in an ES complex, blocks any further reaction of substrate. After treatment of deaminase with 2-bromoporphobilinogen (I), in the presence of the substrate, porphobilinogen (S), a series of chain-termination complexes may be isolated (i.e., EI, ESI, ES_2I, and ES_3I) although the last is liberated from the enzyme as bromopreuroporphyrinogen (Warren and Jordan, 1988). The 2-fluoro analog of hydroxyporphobilinogen also acts as a chain terminator by a similar mechanism (Wang and Scott, 1994).

A comprehensive study to determine the specificity of the *E. coli* porphobilinogen deaminase has been carried out with a range of substrate analogs (Clemens *et al.*, 1994) the structures of which are shown in Table I. Whilst none of the analogs were able to turn over to yield a tetrapyrrole (bilane) product, important information was obtained about the nature of their interaction with the deaminase by examining their ability to form enzyme intermediate (inhibitor) complexes. Analogs in which the porphobilinogen acetic acid (A) side-chain had been replaced by a hydrogen atom were unable to bind to the enzyme; however, substitution by propionic acid allowed a single molecule of the analog to bind to form an EI complex. In contrast, when the propionic acid (P) side-chain was substituted by either acetic acid, butyric acid (B), or hydrogen, two or three molecules of the inhibitor became incorporated. Interestingly, isoporphobilinogen in which the A and P side chains are reversed is unable to form a product but can interact to form an EI complex (Clemens *et al.*, 1994). As expected, porphobilinogen analogs based on furan (Danso-Danquah and Scott, 1993) are unable to interact with the enzyme since the crucial NH of the pyrrole ring is absent.

Ammonia, the by-product of the deaminase reaction, inhibits the enzyme at high concentrations by reacting with the enzyme–intermediate complexes, or their equivalent, and liberating the 1-aminomethylpyrroles,

Table I. Reaction of Porphobilinogen Analogs with Porphobilinogen Deaminase



X	A	P	Y	Product of the reaction
NH_2	A	P	H	ES, ES_2, ES_3, ES_4, preuroporphyrinogen
HO	A	P	H	ES, ES_2, ES_3, ES_4, preuroporphyrinogen (Battersby *et al.*, 1979c)
NH_2	A	P	Br	EI, ESI, ES_2I, ES_3I, bromopreuroporphyrinogen (Warren and Jordan, 1988)
HO	A	P	F	EI, ESI, ES_2I, ES_3I, fluoropreuroporphyrinogen (Wang and Scott, 1994)
NH_2	A	A	H	EI, EI_2 (Clemens *et al.*, 1994)
NH_2	A	B	H	EI, EI_2, EI_3 "
NH_2	P	P	H	EI "
NH_2	P	A	H	EI "
NH_2	A	H	H	EI, EI_2 "
NH_2	H	P	H	No Complex "
NH_2	H	A	H	No complex "
NH_2	P	M	H	EI "
NH_2	H	H	H	No complex "

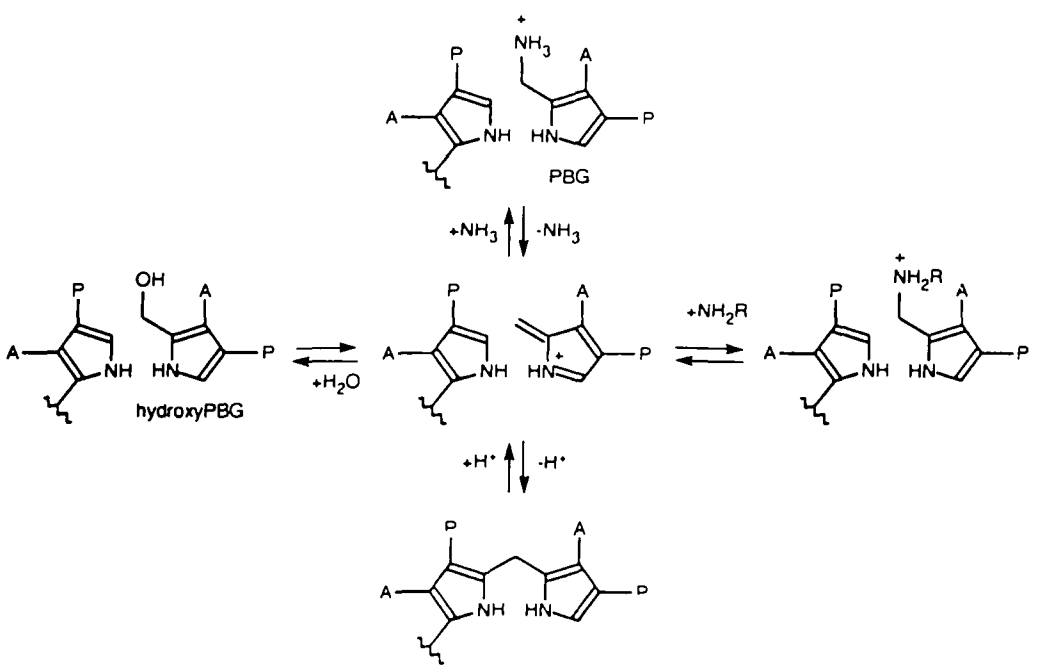
including the aminomethyl analog of preuroporphyrinogen (Radmer and Bogorad, 1972). Ammonia analogs such as hydroxylamine and methoxyamine have similar effects, the inhibitory base also being incorporated into the liberated intermediate (Davies and Neuberger, 1973) (see Scheme 3).

The overall conclusion from these studies is that porphobilinogen deaminase is able to catalyze deamination and dehydration reactions but that the enzyme is also able to catalyze the reverse reactions, namely, amination and hydration. The ability to catalyze hydration is essential for the ultimate release of the product from the ES_4 complex (Scheme 3).

X-RAY STRUCTURE OF PORPHOBILINOGEN DEAMINASE FROM *E. COLI*

The availability of milligram amounts of porphobilinogen deaminase from recombinant strains of *E. coli* (Thomas and Jordan, 1986) allowed the crystallization of the enzyme and the determination of the three-dimensional structure at 1.76 Å resolution (Louie *et al.*, 1992; Lambert *et al.*, 1994). The molecule has approximate dimensions of 57 Å × 43 Å × 32 Å and is composed of three domains of almost equal size linked together by flexible hinge regions as shown in Fig. 1.

The large catalytic cleft between domains 1 and 2 harbors the dipyrromethane cofactor which is covalently linked to cysteine-242, located on a loop from domain 3. X-Ray analysis of the reduced and oxidized deaminase structures has revealed three binding sites for pyrrole rings (Lambert *et al.*, 1994). Two of these sites are involved in binding the dipyrromethane cofactor (rings C1 and C2 in Scheme 2) and the third is proposed as the substrate binding site (S). The four carboxyl groups from the A and P side chains of the cofactor interact predominantly through the side chains of arginines 131, 132, 149, and 155 and lysine 83 while aspartate 84 forms hydrogen bonds with both pyrrole ring NH groups of the cofactor. The extensive number of interactions between the cofactor and the enzyme protein are likely to contribute significantly to the stability of the holoenzyme when compared with the apoenzyme (Scott *et al.*, 1989). The substrate binding site (Louie *et al.*, 1992; Lambert *et al.*, 1994) contains arginines 11, 149, and 155, aspartate 84, and phenylalanine 62 that makes a stacking interaction with the porphobilinogen pyrrole ring. The X-ray structural studies support the biochemical findings (Warren and Jordan, 1988) in which it was concluded that the deaminase possesses a single active site that is used for each porphobilinogen condensation in the catalytic cycle. Fig. 1 shows



Scheme 3. Reactions catalyzed by porphobilinogen deaminase through the intermediacy of a putative azafulvene intermediate.

the location of the catalytic cleft; fig. 2 shows detail of the amino acid residues in the catalytic cleft.

A detailed analysis of the porphobilinogen deaminase structure reveals that the three domains have relatively few interactions with one another and that they are connected by hinge regions that allow substantial flexibility between the domains. The connectivity of domains 1 and 2 in porphobilinogen deaminase has the same overall topology found in the transferrins and periplasmic binding proteins (Louie, 1993). These proteins are well known to undergo extensive conformational changes on ligand binding (Baker *et al.*, 1987). It is envisaged that the binding and assembly of the two cofactor rings in the deaminase catalytic cleft may be analogous to the ligand binding process of the two-domain transferrins. Whether the substrate binding also involves major changes between domains 1 and 2 is not known. Substrate binding certainly seems to result in a conformational change at the interface between domains 2 and 3 the magnitude of which is dependent upon the number of substrate molecules bound (Warren and Jordan, 1988). These changes have been studied in detail following the modification of cysteine-134 (Warren *et al.*, 1995).

THE MOLECULAR BIOLOGY OF PORPHOBILINOGEN DEAMINASE AND UROPORPHYRINOGEN III SYNTHASE

Since the first porphobilinogen deaminase gene (*hemC*) was identified, cloned, and sequenced in *Escherichia coli* (Thomas and Jordan, 1986), over a

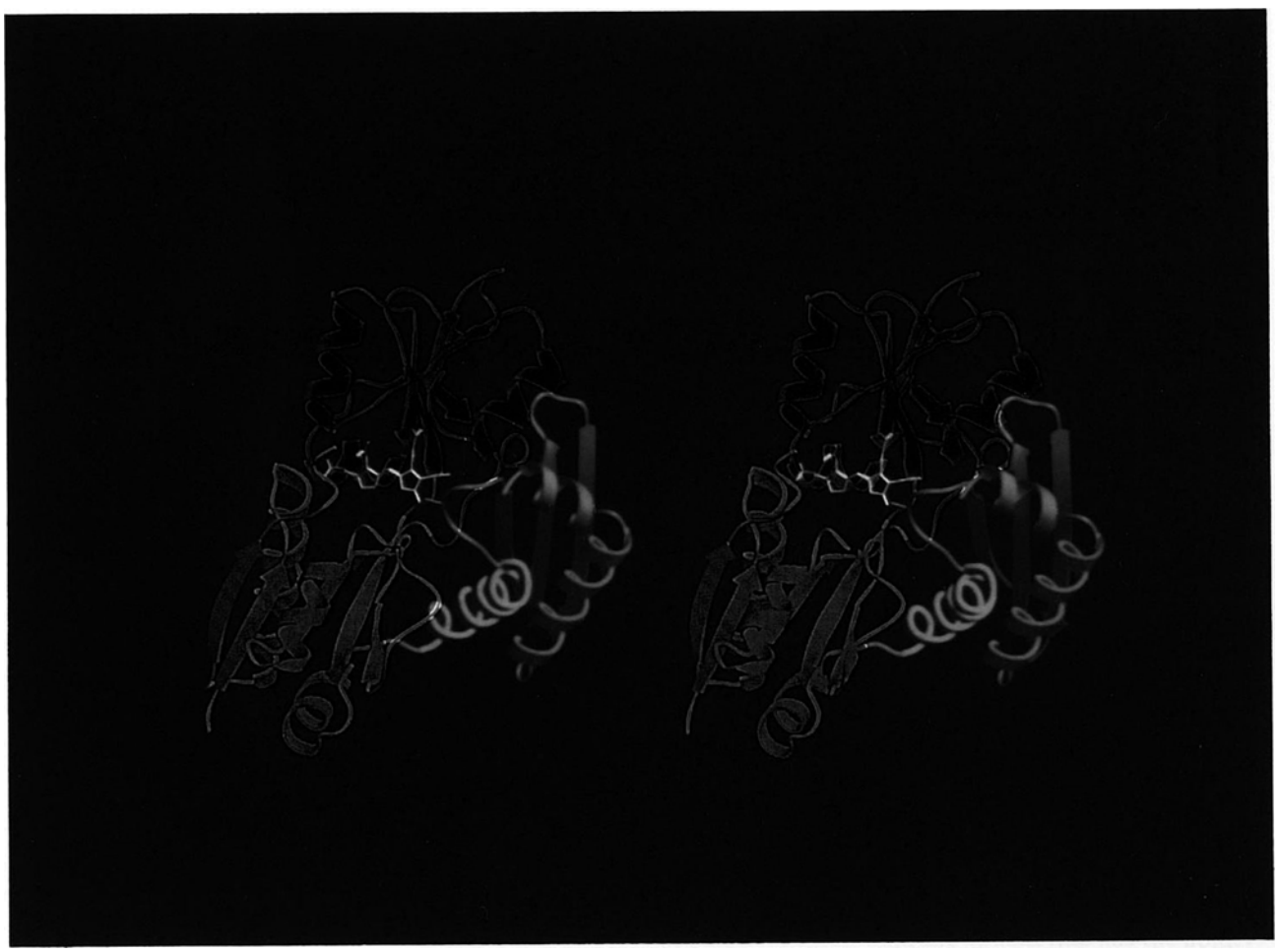


Fig. 1. Three-dimensional representation of *E. coli* porphobilinogen deaminase. Domain 1 is shown in blue, domain 2 is shown in green, and domain 3 is shown in purple. The dipyrromethane cofactor (oxidized form) is shown in orange in the catalytic cleft between domains 1 and 2. Residues 46–56 that form a loop over the catalytic cleft have been omitted. This figure was originally published in Chadwick and Ackrill (1994).

dozen genes or cDNAs have been isolated in less than a decade from a range of animals, plants, and micro-organisms. These sources include human cDNA (Raich *et al.*, 1986), rat cDNA (Stubnicer *et al.*, 1988), mouse (Beaumont *et al.*, 1989), *Euglena gracilis* (Sharif *et al.*, 1989), *Bacillus subtilis* (Petricek *et al.*, 1990). *Chlorobium vibrioforme* (Majumdar and Wyche, Accession number M96364), *Saccharomyces cerevisiae* (Keng *et al.*, 1992), *Pseudomonas aeruginosa* (Mohr *et al.*, 1994), *Pisum savitum* (Witty *et al.*, Accession number X73418), *Arabidopsis thaliana* (Witty *et al.*, Accession number X73535), *Clostridium josui* (Fujino *et al.*, Accession number D28503), and *Microbacterium leprae* (Robison and Smith, Accession number U00018).

Examination of the primary protein structures deduced from these nucleotide sequences reveals that 34 amino acids are invariant in all deaminases although many similarities are also present. Particularly notable are the invariant and highly conserved arginines at positions 11, 131, 132, 149, 155, 176 (*E. coli* numbering) that interact with the acetic acid and propionic acid side chains of the cofactor, substrate, and intermediate complexes. In addition, the cysteine to which the dipyrromethane cofactor is attached (cysteine 242), the catalytic aspartate 84, and its neighbour lysine 83 are also invariant, as are several alanine and leucine core residues.

Fewer genes/cDNAs specifying uroporphyrinogen III synthase have been identified. The first *hemD* gene to be characterized was from *Escherichia coli* (Jordan *et al.*, 1987; Sasarman *et al.*, 1987; Jordan *et al.*, 1988c) immediately following and overlapping with the *hemC* gene in a small *hem* operon. The *hemD* gene from *Bacillus subtilis* has been sequenced (Hansson *et al.*, 1991) and is also present in a *hemAXCDBL* gene cluster. Human uroporphyrinogen III synthase cDNA has been isolated and characterized (Tsai *et al.*, 1988), and gene defects have been identified in patients suffering from congenital erythropoietic porphyria (Boulechfar *et al.*, 1992; Xu *et al.*, 1993). Nucleotide sequences specifying uroporphyrinogen III synthases from several other sources are available: *Pseudomonas aeruginosa* (Mohr *et al.*, 1994), mouse (Bensidhoum *et al.*, Accession number U04439), *Chlorobium vibrioforme* (Majumdar and Wyche, Accession number M96364), *Anicystis nidulans* (Jones *et al.*, Accession number X70966), and *Clostridium josui* (Fujino *et al.*, Accession number D28503).

Comparison of the derived primary protein structure from these sequences shows minimal similarity with only five invariant residues. This is surprising in view of the high degree of similarity found in deaminases and other enzymes of the heme pathway. Analysis of the hydropathy indices of the synthases, however, shows a high degree of similarity, suggesting that the enzymes are related structurally. The N-terminal sequence of the *E. coli* synthase, NH_2-S-I-L-V-T-R-, isolated from an overproducing strain

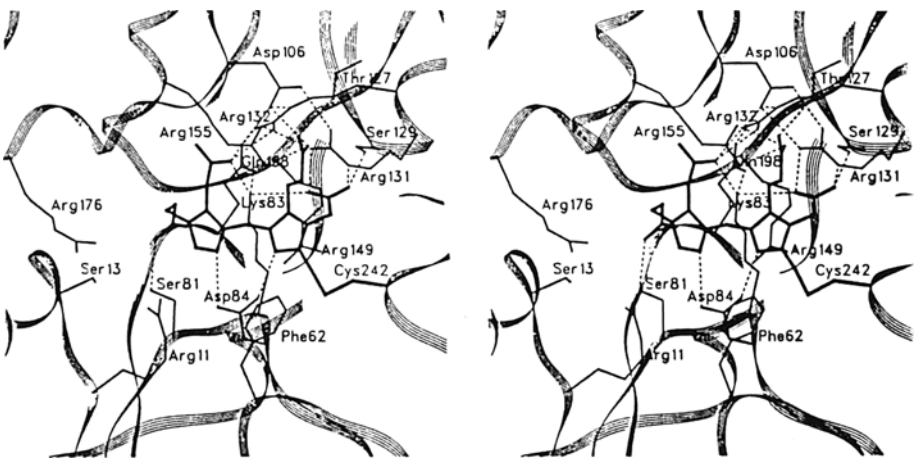


Fig. 2. The catalytic site of porphobilinogen deaminase. Stereo-diagram to show the interactions of the dipyrromethane cofactor, in its native form, with arginines 131, 132, and 155, lysine 83 and aspartate 106. Aspartate 84 hydrogen bonds with the two pyrrole rings through the NH groups. The vacant substrate binding site comprises arginines 11, 149, and 155. Aspartate 84 can hydrogen bond with the pyrrole ring –NH– and the amino group of the substrate. Arginine 176 is at the back of the catalytic cleft and may interact with enzyme intermediate complexes.

(Alwan *et al.*, 1989) indicates that the terminal methionine has been cleaved post-translationally.

SITE-DIRECTED MUTAGENESIS STUDIES ON *E. COLI* PORPHOBILINOGEN DEAMINASE

Site-directed mutagenesis of invariant residues has proved a powerful technique for investigating the structure and mechanism of porphobilinogen deaminase. A number of mutants have been generated in the *E. coli hemC* gene that have provided essential information about cofactor assembly (Scott *et al.*, 1988; Jordan and Woodcock, 1991), substrate binding (Hadener *et al.*, 1990; Jordan and Woodcock, 1991; Lander *et al.*, 1991), and catalysis (Woodcock and Jordan, 1994). Mutagenesis of arginines 131 and 132 to histidine or leucine prevented cofactor assembly and resulted in apodeaminases lacking catalytic activity. Mutagenesis of arginines 11 and 155 resulted in the inability of the enzyme to bind substrate, while substitution of arginines 149, 176, and 232 affected the polymerization mechanism. As expected, mutation of cysteine 242 to serine had a dramatic effect on cofactor assembly (Jordan *et al.*, 1988b; Scott *et al.*, 1988). The availability of the X-ray structure has permitted the construction of additional mutants to investigate further structure/function relationships of the enzyme. One such knowledge-based study has involved the substitution of the proposed catalytic amino acid aspartate-84. The glutamate-84 mutant possesses less than 100th of the wild type activity with a cofactor that is sensitive to oxidation. The reasons for this are evident from the X-ray structure determination of this mutant that shows the loss of a hydrogen bond, normally found between aspartate 84 and the C2 pyrrole ring NH of the cofactor (Lambert *et al.*, 1994). Alanine and asparagine substitutions for aspartate 84 are completely inactive with respect to the tetrapolymerization reaction but, nevertheless, contain the dipyrromethane cofactor, suggesting that aspartate 84 may not be obligatory for cofactor assembly.

STRUCTURAL BASIS FOR ACUTE INTERMITTENT PORPHYRIA FROM THE STUDY OF HUMAN MUTANTS FROM PATIENTS WITH THE DISEASE

Nature has provided a wealth of site-directed mutants of human porphobilinogen deaminase that have been identified in patients with the disease acute intermittent porphyria (for a survey see Brownlie *et al.*, 1994 and references therein). Most of these mutations involve residues that are invariant in all deaminases. Because of the close similarity between the *E. coli* and human deaminase primary structures (43% identity and 60% conservation), the *E. coli* structure has been used to model the three-dimensional structure of the human enzyme (Brownlie *et al.*, 1994). This model, shown in Fig. 3, is of great importance for mapping the mutations which give rise to acute intermittent porphyria (AIP) and has provided logical explanations for the majority of the properties of the mutants.

Arginine mutants are the most common and are found at positions 26, 116, 149, 167, 173, and 201 (equivalent to *E. coli.* positions 11, 101, 131, 149, 155, and 183 respectively). Arginines 26, 149, 167, and 173 interact with the cofactor or substrate, whereas arginines 116 and 201 form salt bridges that stabilize the protein structure (Brownlie *et al.*, 1994). Those mutants involving the substrate binding groups are CRIM + ve, indicating that the gross protein structure is little affected. Mutations involving key cofactor binding groups such as arginine 149 are CRIM-ve, reemphasizing the importance of the dipyrromethane cofactor in maintaining protein stability. This mutation correlates with the analogous *E. coli* arginine 131 mutants which lack the cofactor and exist in unstable apo-forms (Jordan and Woodcock, 1991; Lander *et al.*, 1991). Mutations of residues that play a structural role, such as arginine 116 and glutamate 250, which form a salt link between domains 1 and 3, also lead to a CRIM-ve status. Mutations in core hydrophobic residues (alanines 31, 122, and 252, valine 93, and leucine 177) are expected to be unstable and are generally CRIM-ve.

MECHANISM OF ACTION OF PORPHOBILINOGEN DEAMINASE

Porphobilinogen deaminase is a remarkable protein since it not only catalyzes the repetitive condensation of porphobilinogen units with an acceptor pyrrole chain but can "count" precisely and terminate the reaction when the tetrapyrrole chain has been assembled. In addition, the apoenzyme has the ability to install its own dipyrromethane cofactor to form the holoenzyme. Considering the small size of the protein, to perform so many reactions is an astonishing feat. A

clue to how the enzyme executes these many roles comes from considering the chemistry of the substrate porphobilinogen. α-Aminomethylpyrroles of this type are highly reactive compounds and are capable of polymerizing nonenzymically in dilute acid to form porphyrinogens (Mauzerall, 1960). Thus, if the properties of the enzyme can enable the intrinsic chemistry of porphobilinogen to express, then many of the mechanistic problems can be explained.

The formation of the holoenzyme may not be the same type of enzyme-catalyzed process as the tetrapolymerization reaction. By analogy with the two domain-binding proteins (Louie, 1993) it is envisaged that the porphobilinogen molecule that becomes the C1 ring of the cofactor may bind to the apoenzyme in such a way that the reactive aminomethyl carbon atom is placed adjacent to cysteine 242 so that C-alkylation can occur, presumably through the intermediacy of an azafulvene species. Such a mechanism is supported by the observations that arginine 131 and 132 mutants do not assemble the cofactor and that mutations of the catalytic aspartate 84 do not prevent cofactor assembly. Furthermore, the rate of cofactor assembly is a rather slow process compared with the catalytic cycle. The addition of the C2 ring of the cofactor may also be aspartate 84 independent. The assembly of the cofactor by a "nonenzymic" rather than an enzyme-catalyzed process has the attraction that once bound, the cofactor would not be susceptible to catalytic degradation.

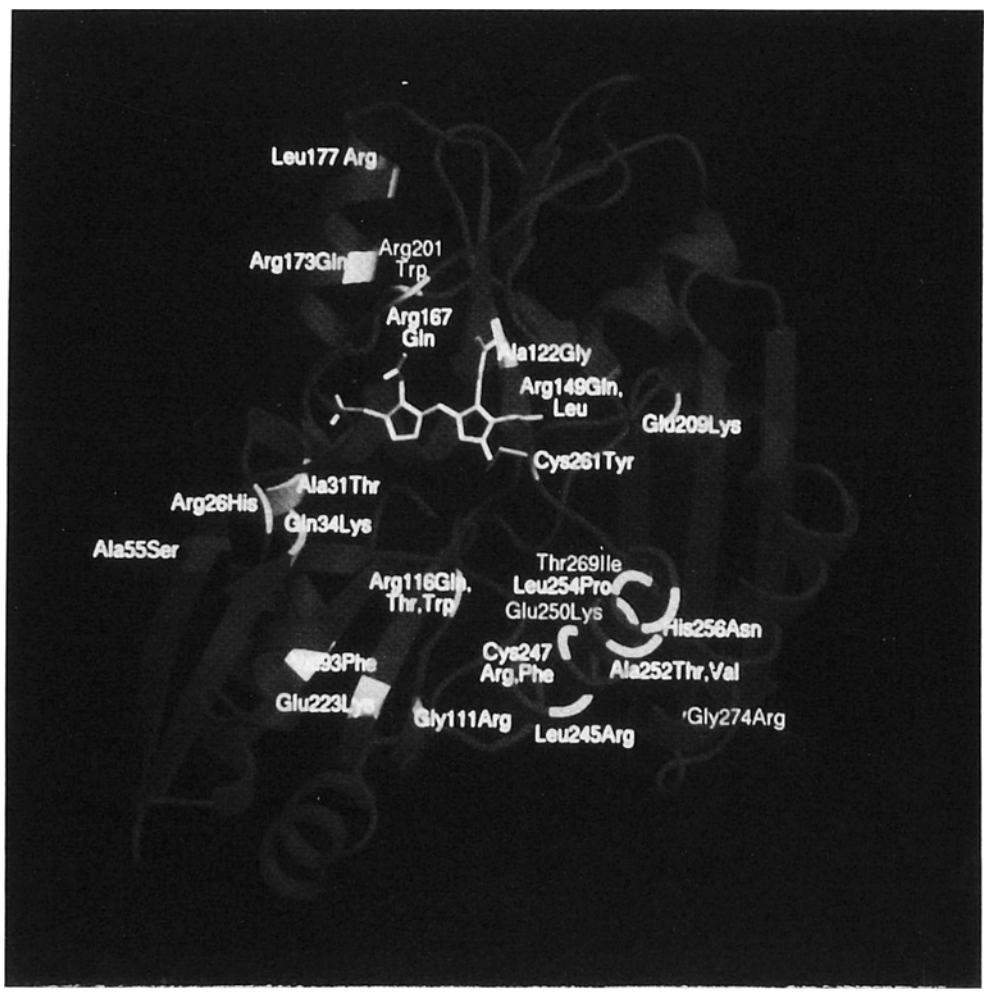
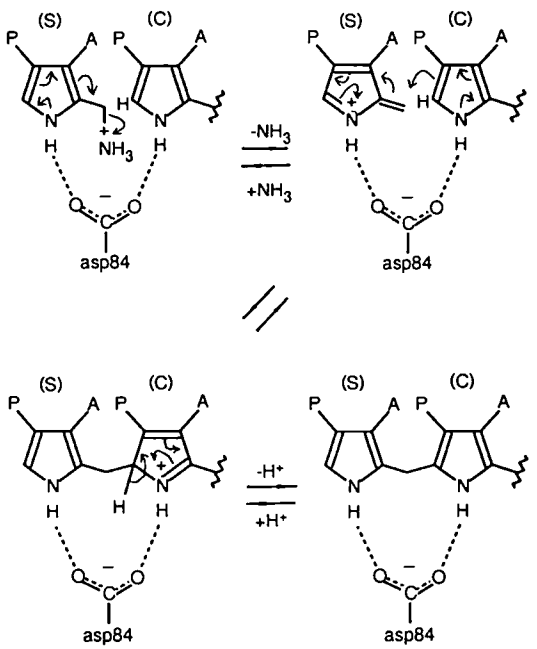


Fig. 3. Model of human porphobilinogen deaminase to show the site of mutations that cause acute intermittent porphyria. The numbering is that of the human enzyme. This figure was originally published in Brownlie *et al.* (1994).

There is direct evidence from nmr studies that a conformational change accompanies the formation of the holoenzyme (Scott *et al.*, 1989) and it is possible that this structural change prevents the reverse reaction, possibly through stereoelectronic control, so that the cofactor remains permanently bound. Treatment of the enzyme with acid is the only way of releasing the dipyrromethane cofactor (Jordan and Warren, 1987; Miller *et al.*, 1988).

Studies with the substrate analog hydroxyporphobilinogen (Battersby *et al.*, 1979c) and inhibitory bases (Radmer and Bogorad, 1972; Davies and Neuberger, 1973) and with enzyme–intermediate complexes (Warren and Jordan, 1988) have established that the enzyme can catalyze deamination and dehydration reactions in addition to the reverse of these reactions, amination and hydration, as shown in Scheme 3.

The mechanism of each stage of tetrapyrrole assembly also requires binding of the substrate and deamination to generate an azafulvene or an equivalent electrophilic species (Pichon *et al.*, 1992) that reacts with the nucleophilic free α-position of the dipyrromethane cofactor (or the free α-position in ES, or ES_2, or ES_3). This process requires aspartate-84 since alanine and asparagine 84 mutants do not support catalysis (Woodcock and Jordan, 1994). Aspartate 84 may have several roles including protonation of the leaving group ammonia, stabilization of the developing charges on the pyrrole rings during the condensation, and abstraction of the α-proton (Jordan, 1994; Woodcock and Jordan, 1994). A possible mechanism is shown in Scheme 4. Studies with *N*-methylporphobilinogen, 11-methylporphobilinogen, and 11-trifluoromethylporphobilinogen have been employed to investigate the detailed mechanistic course of the reaction (Pichon *et al.*, 1992). The results have been interpreted in terms of an E1 type displacement rather than an E2 mechanism, although the actual structure of the electrophile is still uncertain at the present time.



Scheme 4. Possible mechanism involving aspartate-84 as a catalytic residue in the condensation reaction catalyzed by porphobilinogen deaminase. Porphobilinogen binds to the S (substrate) site and is deaminated to the azafulvene. Bond formation occurs in which the positive charges are stabilized by aspartate 84. Loss of the α-hydrogen completes the reaction. If aspartate 84 is protonated initially, it could act as an acid to protonate ammonia and as a base to remove the α-proton. The site C (cofactor) can be occupied by the C2 ring of the cofactor or by the terminal ring of ES, ES_2, or ES_3.

Detailed studies with the enzyme intermediate complexes have established that there is a conformational change in the deaminase accompanying the reaction of each porphobilinogen unit, as judged by the increased reactivity of cysteine 134 to *N*-ethylmaleimide (Warren and Jordan, 1988; Warren *et al.*, 1995). Thus, cysteine 134 in ES_3 is modified far more rapidly than in ES_2 or in ES compared to the free enzyme. The X-ray structure shows that cysteine 134 is located on domain 2 at the interface with domain 3 supporting the involvement of this domain, to which the dipyrromethane cofactor is bound, in the regulation of polymer chain length.

A clue to the mechanism of chain termination, after four molecules of substrate have been added, has come from a detailed analysis of the X-ray structure. The size of the catalytic cleft is large enough to be able to accommodate up to four pyrrole units but not five (Louie *et al.*, 1992). It is thus attractive to propose that the pyrrole chain length is regulated predominantly by steric effects. Whatever process operates, the ES_4 complex would need to adopt a conformation in which the C2 ring of the cofactor and the "a" ring of the bound product are adjacent to the catalytic machinery so that cleavage and the addition of water can occur.

Studies with chirally labeled porphobilinogen have shown that the combined reactions catalyzed by the deaminase and synthase both proceed with rigid stereochemical control (Jones *et al.*, 1984; Jackson *et al.*, 1987). When chiral porphobilinogen is used to synthesize ES_4 in ammonia to give the

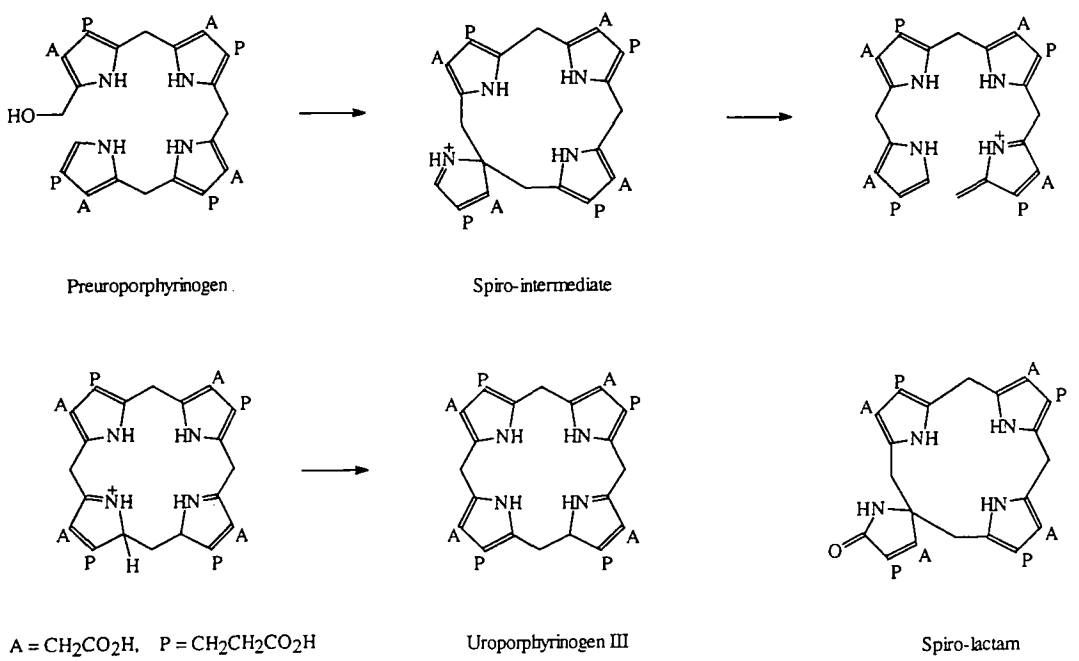
1-aminomethylbilane analog of preuroporphyrinogen, or in water to give the 1-hydroxmethylbilane, preuroporphyrinogen, the overall reaction proceeds with retention of configuration (Neidhart *et al.*, 1985; Schauder *et al.*, 1987).

MECHANISM OF ACTION OF UROPORPHYRINOGEN III SYNTHASE

The uroporphyrinogen III synthase reaction involves the rearrangement of the "D" ring of preuroporphyrinogen and cyclization to the uroporphyrinogen III macrocycle (Scheme 1). The inversion of the "D" ring during the mechanism was established by novel ^{13}C-nmr experiments using the 1-aminomethylbilane analog of preuroporphyrinogen, NH_2CH_2(APAPAPAP), that was thought to be the synthase substrate at that time (Battersby *et al.*, 1978a, b). The reaction mechanism has become an enigma with countless schemes having been proposed for the formation of uroporphyrinogen III, most of which were untenable after the discovery of preuroporphyrinogen. One of the most enduring mechanisms by which the synthase enzyme could catalyze the rearrangement involves the spiro-hypothesis of Mathewson and Corwin (1961). In this mechanism, the 1-hydroxymethylbilane substrate, preuroporphyrinogen, initially yields a spirocyclic pyrrolenine. This intermediate is then cleaved to generate an isomeric azafuvene that finally cyclizes to uroporphyrinogen III (Scheme 5).

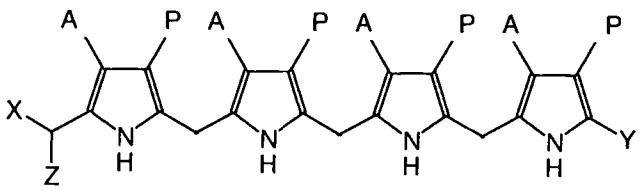
Although the spiro-intermediate has defied chemical synthesis to date, analogs closely resembling it have been synthesized (Stark *et al.*, 1986, 1993). One such analog is a spirolactam (Scheme 5) in which the "D" ring structure caries from the spiro-intermediate only by the presence of an amide rather than an imine. This spirolactam is a powerful inhibitor of the synthase with a K_i of 1–2 μM. One of the enantiomers of this compound is some 20 times better as an inhibitor (Leeper, 1994). Molecular mechanics studies have been employed to simulate the spiro-mechanism (Leeper, 1994). Recently, using high pH and low temperature and following the synthase reaction by nmr, the unrearranged azafulvene intermediate has been observed for the first time, suggesting that the formation of the spiro-intermediate, if it occurs, does not proceed through an initial concerted nucleophilic attack with loss of water (Pichon *et al.*, 1994a).

Another approach to the study of the uroporphyrinogen III synthase mechanism has been the synthesis of preuroporphyrinogen analogs. Several 1-hydroxymethylbilanes have been prepared, the



Scheme 5. The spirocyclic mechanism for the synthesis of uroporphyrinogen III and the structure of the spirolactam inhibitor.

Table II. Preuroporphyrinogen Analogs as Substrates and Inhibitors of Uroporphyrinogen III Synthase



X	Z	A	P	A	P	A	P	A	P	Y	
HO	H	A	P	A	P	A	P	A	P	H	Uroporphyrinogen III (Battersby *et al.*, 1979a)
HO	H	A	P	A	P	A	P	P	A	H	Uroporphyrinogen I (Battersby *et al.*, 1981)
HO	H	A	P	A	P	P	A	A	P	H	Uroporphyrinogen IV (Battersby *et al.*, 1981)
HO	H	A	P	P	A	A	P	A	P	H	No reaction (Battersby *et al.*, 1981)
HO	H	P	A	A	P	A	P	A	P	H	No reaction (Battersby *et al.*, 1981)
HO	H	P	A	P	A	P	A	A	P	H	No reaction (Battersby *et al.*, 1981)
HO	MeA		P	A	P	A	P	A	P	H	Inhibitor (Pichon *et al.*, 1994c)
OH	H	A	P	A	P	A	P	A	B	H	Uroporphyrinogen II analog (Pichon *et al.*, 1994c)
OH	H	A	P	A	P	A	P	Me	P	H	Poor substrate (Battersby *et al.*, 1983)
OH	H	A	P	A	P	A	P	A	Et	H	Poor substrate (Battersby *et al.*, 1983)
HO	H	A	P	A	P	A	P	A	P	Br	Inhibitor (Pichon *et al.*, 1994b)

structures of which are shown in Table II, to investigate the ability of the synthase to invert the "D" ring.

The findings shown in Table II highlight the importance of the "A" and "B" ring substutuents for recognition by the synthase. It is notable that the analog $HOCH_2$(APAPAPPA), in which the "D" ring is already inverted, is recognized by the enzyme and is partially rearranged to the uroporphyrinogen I isomer. The lack of total fidelity in the reaction of this analog, when compared with the natural substrate, is no doubt due to the imprecise binding if the "D" ring to the active site.The "D" ring of the natural substrate must bind initially in the "uroporphyrinogen III" conformation to ensure that the correct isomer is formed ultimately (Jordan, 1991b). A similar conclusion has been deduced from molecular dynamics studies (Leeper, 1994).

A possible criticism of the spiro-mechanism has been raised from these studies because the 1-hydroxymethylbilane $HOCH_2$(PAPAPAAP) is not a substrate for the synthase. This analog is related to the isomeric azafulvene proposed as the immediate precursor of uroporphyrinogen III in the spirocyclic mechanism. However, the reason this analog is not recognized may be because the "A" and "B" rings are in the wrong orientation for initial interaction with the synthase so that the azafulvene is never generated at the active site. A review on the deaminase and synthase (Battersby and Leeper, 1990) covers other possible mechanisms for the rearrangement.

The approach using preuroporphyrinogen analogs has recently been extended by the synthesis of the 19-bromo-1-hydroxymethylbilane related to preuroporphyrinogen (Pichon *et al.*, 1994b) and the related 1-hydroxyethlbilane (HEB) that acted as competitive inhibitors. An interesting analog in which butyrate was substituted for the propionate side chain on ring "D" $HOCH_2$(APAPAPAB) acted as a good substrate (Pichon *et al.*, 1994c) and produced a rearranged, type III product.

Whereas the mechanism of the deaminase reaction is beginning to become more clearly understood, the synthase reaction still remains rather a black box. This is largely because of the instability of the substrate and the enzyme alike, the apparent lack of any covalent intermediates, and the absence of structural information about the synthase protein., A combination of further studies with substrate analogs and site-directed mutants is required to probe the interactions between the enzyme and substrate.

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Chadwick and Ackrill (1994) and Fig. 3 in Brownlie *et al.* (1994). The BBSRC (previously the SERC and AFRC) provided funding for this work.

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