RESEARCH COMMUNICATION

Discovery that the assembly of the dipyrromethane cofactor of porphobilinogen deaminase holoenzyme proceeds initially by the reaction of preuroporphyrinogen with the apoenzyme

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The assembly process of the dipyrromethane cofactor of Escherichia coli porphobilinogen deaminase holoenzyme is initiated by the reaction of the porphobilinogen deaminase apoenzyme with preuroporphyrinogen. The resulting enzyme-bound tetrapyrrole (bilane) is equivalent to the holoenzyme intermediate complex ES₉ and yields the dipyrromethane cofactor by reactions of the normal catalytic cycle. These observations indicate that preuroporphyrinogen, rather than porphobilinogen, is the preferred precursor for the dipyrromethane cofactor and explain the existence of the D84A and D84N deaminase mutants as catalytically inactive ES₉ complexes.

INTRODUCTION

Porphobilinogen deaminase catalyses an early step in the tetrapyrole biosynthesis pathway involving the formation of the 1-hydroxymethylbilane, preuroporphyrinogen, from four molecules of porphobilinogen (Scheme 1). Porphobilinogen deaminases have been isolated from a number of sources, and their properties have been well established (see [1] for a review). In all organisms, deaminases have been found to exist as monomeric species with $M_r$ values between 33000 and 45000. The X-ray structure of the Escherichia coli deaminase [2,3] has revealed a protein with three domains attached to one another by flexible hinge regions. The nucleotide sequences of genes/cDNAs specifying the deaminases from bacterial, plant and animal sources show considerable conservation in the deduced protein sequences [4], suggesting that all deaminases are likely to be structurally related to the E. coli enzyme. The similarity between the human and E. coli enzymes has allowed the construction of a three-dimensional model of the human deaminase from the E. coli structure [5]. This model has given detailed insight into the molecular basis of the human inherited disease acute intermittent porphyria [6].

Extensive investigations with porphobilinogen deaminases from E. coli identified a novel prosthetic group named the dipyrromethane cofactor [7,8], made up of two porphobilinogen-derived units linked together (Scheme 1). Porphobilinogen deaminase lacking the cofactor, termed the apoenzyme, has been isolated from genetically engineered bacterial strains [9,10] or generated from the holoenzyme by acid treatment [11]. Although attempts to regenerate the holoenzyme from the apoenzyme using porphobilinogen have proved possible [9–11], the yields have tended to be disappointingly low, with recoveries between 10% and 40%. Furthermore, the regeneration of holoenzyme was observed to require an extended period of incubation with porphobilinogen before any reasonable activity was restored. Typically, incubations of apoenzyme with porphobilinogen for 2–4 h at 4 °C with a 4 mol excess of porphobilinogen were used [10,11]. No explanations have been put forward to account for the surprisingly slow rate of cofactor assembly.

The X-ray structure of the E. coli deaminase indicates that the cofactor resides in a large cleft between domains 1 and 2 [2,3] attached covalently to cysteine-242 of domain 3 through a thioether linkage. The cofactor acts as a primer for the synthesis of the linear tetrapyrole (bilane) chain that is built on to the free $\alpha$-position of the cofactor. This occurs by the sequential condensation of four porphobilinogen molecules through enzyme intermediate complexes, termed ES, ES$_9$, ES$_{10}$ and ES$_{11}$ (Scheme 1) [9,12–14]. The four carboxylic acid groups of the dipyrromethane cofactor interact with highly conserved arginine residues at positions 131, 132, 149 and 155 in the E. coli enzyme. Alteration of either arginine 131 or 132 by site-directed mutagenesis gives rise to inactive mutant proteins unable to assemble the dipyrromethane cofactor [15,16].

Scheme 1  The stepwise assembly of preuroporphyrinogen attached to the dipyrromethane cofactor of porphobilinogen deaminase

Abbreviations: A, CH$_2$CO$_2$H; P, CH$_2$CH$_2$CO$_2$H; enz, enzyme.

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Aspartate-84 has been identified from the X-ray structure as a key catalytic group [17]. Site-directed mutagenesis of aspartate-84 to glutamate (D84E) generates an enzyme with less than 0.5% of the wild-type catalytic activity, whereas mutations to alanine (D84A) or asparagine (D84N) yield completely inactive enzymes. The somewhat surprising observation that both the D84A and D84N mutants exist not as apoenzymes, but as inactive holoenzyme intermediate complexes, indicated that aspartate-84 is not essential for cofactor assembly [17]. This pointed to a mechanism for cofactor assembly from apoenzyme different from that employed by the holoenzyme for the normal catalytic cycle to form preuroporphyrinogen. This paper presents data that strongly implicate preuroporphyrinogen, rather than porphobilinogen, as the preferred precursor of the dipyrromethane cofactor.

MATERIALS AND METHODS

Porphobilinogen was obtained by enzymic synthesis from 5-aminolaevulic acid using purified 5-aminolaevulic acid dehydratase [18]. Preuroporphyrinogen (0.1 mM) was generated in a final volume of 1 ml of degassed Tris/HCl buffer, pH 9.1, from 0.5 µmol of porphobilinogen, using 100 µg of purified porphobilinogen deaminase. The porphobilinogen deaminase was separated from the deaminase by ultrafiltration through an Amicon PM10 membrane fitted to a 5 ml concentration cell. The preuroporphyrinogen was used immediately or frozen in liquid nitrogen until required. All routine chemicals were obtained from Sigma or Merck. Chromatographic materials were supplied by Pharmacia.

Porphobilinogen deaminase was expressed as BM3, constructed from E. coli strain TB1 [JM83 hsdR(Tg m-c)] [19] harbouring a plasmid (pBM3) constructed by cloning a 1.68 kb BamHI–SalI DNA fragment containing the hemC gene from pST48 [20] into pUC18 by standard methods [19]. The enzyme was isolated and assayed as previously described [21]. Mutant E. coli porphobilinogen deaminase, with aspartate-84 substituted by asparagine (D84N), was expressed and purified by published methods [17] and was completely separated from trace amounts of the wild-type enzyme during the ion-exchange stage. Other bacterial strains and methods used were as previously described [9].

Wild-type porphobilinogen deaminase apoenzyme was prepared from E. coli hemB strain RP523 [22] grown on Luria–Bertani broth supplemented with 7.8 µM haemin and harbouring the plasmid pBM3. The apoenzyme was prepared as follows: the cell paste from 2.0 l of culture was sonicated for 4 × 1 min periods in 30 ml of 20 mM Tris/HCl buffer, pH 8, containing 5 mM β-mercaptoethanol, using an MSE Ultrasonic Disintegrator at an amplitude of 10 µm. The extract was cooled by immersing the sonication vessel in an ice/water bath and by allowing 3 min for cooling between the periods of sonication. The resulting lysate was centrifuged at 10000 g for 10 min at 4°C, and the supernatant was then applied directly to a Mimetic Orange (Affinity Chromatography) column (2.5 cm × 14 cm) and eluted with a gradient of NaCl in the above buffer. The apoenzyme was judged to be in excess of 90% pure by SDS/PAGE [23].

After purification, all enzymes were desalted using a PD10 column (Pharmacia) and freeze-dried for storage at −20°C.

Non-denaturing polyacrylamide gel electrophoresis was performed according to Laemmli and Favre [23]. For separations using non-denaturing electrophoresis conditions, SDS and β-mercaptoethanol were omitted from the recipe. Electrophoresis using non-denaturing gels was conducted at 4°C in a cold room.

RESULTS AND DISCUSSION

Reconstitution of wild-type holo-porphobilinogen deaminase from wild-type apoenzyme with porphobilinogen

When wild-type porphobilinogen deaminase apoenzyme (1 mg/ml) was incubated with porphobilinogen (10 mol equivalents) at 4°C for various times and aliquots were analysed for active holoenzyme by assay with porphobilinogen, a pronounced lag phase in the production of active porphobilinogen deaminase was observed [Figure 1(□)]. If small amounts of porphobilinogen deaminase holoenzyme were included in the preincubation with porphobilinogen and apoenzyme (holoenzyme:apoenzyme ratio of 1:50), the lag period was reduced [Figure 1(○)]. The apo-enzyme → holoenzyme transformation was followed by electrophoresis, using 10% polyacrylamide non-denaturing gels. Holoenzyme migrates as a characteristic doublet with an Rf of 0.75 (Figure 2, lane a), whereas the apoenzyme appears as a broad smear running appreciably more slowly (Figure 2, lane b).

The amount of holoenzyme generated from the immediate addition of porphobilinogen to apoenzyme [Figure 1(□)] is barely detectable after 5 min when examined by electrophoresis (Figure 2, lane c).

The results in Figure 1 indicated that porphobilinogen was not able to form active holodeaminase from apodeaminase as rapidly as in experiments that already contained a small amount of active deaminase, suggesting that a cofactor precursor may be generated from porphobilinogen by the small amount of added holoenzyme. It was considered likely that the lag phase noted above [Figure 1(□)] was necessary to generate sufficient of this precursor from porphobilinogen to form the active holoenzyme. The most likely candidate for such a precursor is the reactive 1-hydroxymethylbilane, preuroporphyrinogen, the product of the deaminase reaction itself.

Figure 1 Formation of porphobilinogen deaminase holoenzyme from porphobilinogen apoenzyme under various conditions

Apoenzyme (1 mg/ml) was incubated in 20 mM Tris/HCl buffer, pH 8, containing 2 mM dithiothreitol with: (□) 10 mol equivalents of porphobilinogen; (○) as above but with added active holoenzyme (holoenzyme:apoenzyme ratio 1:50); or (○) preuroporphyrinogen filtered free of deaminase. The procedures are described in the Materials and methods section. Aliquots were removed and analysed for porphobilinogen deaminase activity. Values are corrected for the presence of holodeaminase (○) and the non-enzymic formation of uroporphyrinogen (measured as uroporphyrin) (○).
Figure 2  Non-denaturing PAGE of apo-porphobilinogen deaminase and its conversion into holo-porphobilinogen deaminase

Lane a, holo-porphobilinogen deaminase; lane b, apo-porphobilinogen deaminase; lane c, apoenzyme incubated with porphobilinogen [as in Figure 1 ()]; lane d, apoenzyme incubated with preuroporphyrinogen; lane e, heat-treated apoenzyme incubated with preuroporphyrinogen; and lane f, purified D84N deaminase mutant. Details of experiments are described in the Materials and methods section.

Reconstitution of porphobilinogen deaminase holoenzyme from apoenzyme with preuroporphyrinogen

In view of the above observations, preuroporphyrinogen was generated by reaction of holo-porphobilinogen deaminase with porphobilinogen in a separate incubation, under conditions that consumed all the porphobilinogen and generated preuroporphyrinogen in a yield approaching 80% [24]. The preuroporphyrinogen was separated from the holodeaminase by filtration at 4 °C under N₂, using an Amicon ultrafiltration unit. The deaminase-free preuroporphyrinogen was then incubated immediately with apodeaminase. The results shown in Figure 1 (○) indicate that an active holodeaminase was formed rapidly from a 10 mol excess of preuroporphyrinogen over apodeaminase, with no apparent lag phase. Furthermore, there was a four-fold increase in the amount of apoenzyme transformed in 5 min, compared with the incubations with porphobilinogen described above. Non-denaturing gel electrophoresis of the sample of apoenzyme that had been incubated with preuroporphyrinogen indicated the presence of the ES₂ intermediate complex (Figure 2, lane d), with only a small amount of free enzyme (E) having been produced. These observations strongly suggest that the apo-deaminase interacts with preuroporphyrinogen to yield directly the nascent holoenzyme in the form of the ES₂ intermediate complex.

E. coli porphobilinogen deaminase intermediate complexes, such as ES₂, have been characterized extensively and shown to undergo enzyme-catalysed hydrolysis to yield free enzyme (E) on heat treatment at 60 °C [9]. When the nascent holoenzyme (ES₂) regenerated from the incubation of apoenzyme and preuroporphyrinogen [Figures 1 (○) and 2 (lane d)] was subjected to heat treatment, two pyrrole units were lost, resulting in the conversion of most of the ES₂ complex into free holoenzyme (E) containing the dipyrromethane cofactor (Figure 2, lane e). It has been well established that the holoenzyme is particularly stable to heat treatment, compared with the apoenzyme, which is rapidly denatured. It is clear that the broad smear of the residual holoenzyme (Figure 2, lane e) is almost completely lost on heat treatment (Figure 2, lane e).

Formation of inactive holoenzyme and ES₂ complexes by porphobilinogen deaminase D84 mutants in vivo

Site-directed mutagenesis of the catalytic aspartate-84 in E. coli porphobilinogen deaminase to alanine or asparagine generated mutant enzymes that are catalytically inactive yet appear to exist as enzyme intermediate complexes [17]. To examine this further, the D84N mutant was purified and characterized by non-denaturing polyacrylamide gel electrophoresis for comparison with the wild-type nascent holoenzyme (ES₂ intermediate complex) generated from the incubation of apoenzyme and preuroporphyrinogen. Figure 2 (lane f) indicates that the D84N mutant does indeed exist as an ES₂ complex, despite the fact that it shows no catalytic activity. This confirms the observations first made on the D84 mutant deaminases [17].

Since the ES₂ complex contains a chain of four covalently bound porphobilinogen-derived units, two of which constitute the dipyrromethane cofactor [7,9], it is clear that the catalytically inactive D84N mutant apoenzyme must have reacted with preuroporphyrinogen directly to form the inactive mutant ES₂ complex. The presence of minute amounts of wild-type porphobilinogen deaminase, encoded by the chromosomal copy of the hemC gene, are able to generate sufficient preuroporphyrinogen, in vivo, for this purpose. The observation that the D84N exists as an ES₂ complex is therefore consistent with the direct coupling of preuroporphyrinogen to give ES₂. Since these mutants lack the catalytic aspartate, the inactive ES₂ complexes remain intact and cannot, unlike wild-type ES₂, complete the catalytic cycle with porphobilinogen to give the holoenzyme (Scheme 1). Nor can the ES₂ complexes of the mutants undergo enzyme-catalysed hydrolysis to ES or E [9].

In summary, the results described above show conclusively that the generation of E. coli wild-type holoenzyme from the apoenzyme occurs preferentially by the initial binding of preuroporphyrinogen to the apoenzyme followed by reaction with cysteine-242 to form a nascent holoenzyme (Scheme 2). The nascent holoenzyme is equivalent to the ES₂ intermediate complex and is able to complete the normal catalytic cycle to generate the holoenzyme containing the dipyrromethane cofactor (E) (Schemes 1 and 2). The dipyrromethane cofactor may also be generated artificially from the nascent holoenzyme by heat treatment (Figure 2, lanes d and e). Although porphobilinogen would also appear to be able to generate the holoenzyme, the rate of reaction is far slower and this route may play a relatively minor role in vivo.

Why should the apoenzyme use preuroporphyrinogen as the cofactor precursor rather than porphobilinogen? The answer undoubtedly lies in the fact that the 1-hydroxymethyl group of preuroporphyrinogen is more reactive than the aminomethyl group of porphobilinogen and more readily forms a reactive
azafulvene that can rapidly alkylate cysteine-242 to form the thioether link. The use of a preformed tetrapyrrole chain rather than a monopyrrole precursor also ensures that the catalytic machinery dedicated to the formation of the enzymic product cannot gain access to the two cofactor rings, explaining why, once formed, the cofactor C1 and C2 rings remain permanently attached to the holoenzyme whereas the substrate units in the ES, ES$_2$, ES$_3$, and ES$_4$ intermediate complexes are all catalytically accessible.

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REFERENCES


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