Human porphobilinogen deaminase mutations in the investigation of the mechanism of dipyrromethane cofactor assembly and tetrapyrrole formation

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Abstract
Porphobilinogen deaminase mutants that cause acute intermittent porphyria have been investigated as recombinant proteins expressed in Escherichia coli, yielding important insight into the mechanism of dipyrromethane cofactor assembly and tetrapyrrole chain polymerization. A mutation that affects a key catalytic residue, D99G, results in an inactive holo-protein that exists as a complex with two substrate molecules covalently bound to the dipyrromethane cofactor arising from the reaction between the apo-protein and pre-uroporphyrinogen. The R149Q mutant is also devoid of catalytic activity but the mutant protein is unable to assemble the dipyrromethane cofactor from pre-uroporphyrinogen and persists as an unstable, heat-labile apo-protein. The mutant, R173Q, has very low activity and, like R149Q, also exhibits largely as an apo-protein. The inability to reconstitute either R149Q or R173Q with exogenous pre-uroporphyrinogen confirms the importance of these two arginine residues for dipyrromethane cofactor assembly. In contrast, the mutant R167Q exists as a holo-enzyme but the catalytic cycle is severely compromised, leading to the accumulation of stable enzyme-substrate intermediates from the catalytic cycle.

Introduction
Porphobilinogen deaminase (also named hydroxymethylbilane synthase; EC 4.3.1.8), the third enzyme of the haem-biosynthesis pathway, catalyses a novel transformation in which four molecules of the pyrrole, porphobilinogen, are polymerized to give a linear tetrapyrrole called pre-uroporphyrinogen, as shown in Scheme 1 (for a review see [1]). Pre-uroporphyrinogen is subsequently cyclized, with rearrangement, to form uroporphyrinogen III in a reaction catalysed by uroporphyrinogen III synthase. As a 1-hydroxymethylbilane, pre-uroporphyrinogen is extremely unstable, cyclizing in a non-enzyme reaction to give uroporphyrinogen I with a half-life of 4 min at pH 8 and 37°C (see Scheme 1). In humans, under normal circumstances, because the activity of porphobilinogen deaminase is far lower than that of uroporphyrinogen synthase, pre-uroporphyrinogen never accumulates in vivo and porphobilinogen is converted by the two enzymes exclusively into uroporphyrinogen III.

The X-ray structure of porphobilinogen deaminase was solved in our laboratories over a decade ago [2] and this has provided extensive insight into how the enzyme catalyses what is a highly complex polymerization reaction. The key to the reaction is the presence, at the active site, of the dipyrromethane cofactor [3], an active-site group unique to all porphobilinogen deaminases. The dipyrromethane is elongated by the stepwise addition of four molecules of porphobilinogen through intermediates ES, ES2 and ES3 to give an enzyme-bound 'hexapyrrole', ES4. The enzymic product, pre-uroporphyrinogen, is released from ES4 by a hydrolytic reaction that regenerates the holo-deaminase with the cofactor still resident at the active site (Scheme 2). The dipyrromethane cofactor is not assembled from porphobilinogen but arises from pre-uroporphyrinogen in a fascinating reaction involving C-alkylation of Cys-261 of the apo-deaminase by reaction with the 1-hydroxymethyl group of pre-uroporphyrinogen. This generates ES2 directly [4].

A large number of human mutations have been identified in porphobilinogen deaminase that lead to the inherited disease acute intermittent porphyria (AIP). AIP is characterized by acute attacks, typically with severe abdominal pain and often accompanied by neurological symptoms including muscular weakness and psychotic episodes [5]. In all cases, acute attacks are accompanied by raised urinary levels of the haem-biosynthesis intermediates 5-aminolaevulinic acid and porphobilinogen. Despite extensive research, no direct link between the raised levels of 5-aminolaevulinic acid and porphobilinogen and the acute symptoms has been convincingly made, although there is little doubt that such a link exists. One of the most perplexing aspects of AIP
Scheme 1 | The formation of uroporphyrinogen I and III from porphobilinogen

A, –CH₂CO₂H; P, –CH₂CH₂CO₂H; PBG, porphobilinogen; PBGD, porphobilinogen deaminase.

Scheme 2 | The catalytic cycle of porphobilinogen deaminase

The reaction involves the elongation of the enzyme-bound dipyrromethane cofactor by the stepwise addition of four molecules of the substrate porphobilinogen through ES, ES₂ and ES₃ to give ES₄, an enzyme-bound ‘hexapyrrole’. The hydroxymethylbilane product pre-uroporphyrinogen is released from ES₄ by hydrolysis, regenerating the unchanged dipyrromethane cofactor. The dipyrromethane cofactor arises initially from the reaction of Cys-261 of the deaminase apo-enzyme with pre-uroporphyrinogen, and the resulting ES₂ complex then enters the catalytic cycle. The Figure 1 legend gives the structures of A and P.
is the low penetrance of the disease and the lack of any obvious relationship between genotype and phenotype. Thus heterozygotes, despite having only 50% of the normal level of porphobilinogen deaminase, may live their entire life without suffering an acute attack. Other carriers of the defect, however, may suffer debilitating and even life-threatening acute attacks at frequent intervals.

Mutations in porphobilinogen deaminase come under several categories with defects in mRNA splicing and exon deletions causing particularly catastrophic effects on the formation of the porphobilinogen deaminase protein. However, it is the investigation of the missense mutations and their molecular consequences that has provided the most valuable insight into structure/function relationships of the enzyme [6].

Mutations may be placed into several broad classes. There are those that lead to a truncated protein, such as W198X, a mutation exceptionally common in Sweden [7]. There are a number of mutations in the hydrophobic core of the enzyme that are likely to compromise normal protein folding or that result in an unstable protein [8]. Some mutations, like R149Q and R173Q [10], are thought to result in apo-proteins. Other mutations, at the active site, are expected to prevent normal substrate binding, such as R26H [11]. Mutations, like R167Q [9], are thought to compromise the polypyrole elongation mechanism to form the tetapyrpolyrole, resulting in the accumulation of stable enzyme–intermediate complexes. One mutation [12] involves a key catalytic group, D99G, and leads to the formation of an enzyme–intermediate complex that, nevertheless, has no catalytic activity. Some of the mutants result in a cross-reacting immunogenic material (CRIM)-negative status due to the instability of the protein.

This paper deals with four natural human mutant porphobilinogen deaminases, D99G, R149Q, R167Q and R173Q. All four mutants have been expressed in vitro using a recombinant *Escherichia coli* expression system. The study of these mutants has provided important information about apo-deaminase stability, dipyrromethane cofactor assembly, cofactor binding groups and the mechanism of pyrrole elongation during the catalytic cycle.

**Materials and methods**

Human ubiquitous porphobilinogen deaminase cDNA was generously provided by Professor B. Grandchamp (Hôpital Bichat, Paris, France) and, using PCR with the appropriate primers, was modified for ligation as an NdeI/BamHI fragment into pT7-7 [13]. Transformation of recombinant plasmids into *E. coli* BL21 was achieved using standard procedures. Recombinant human ubiquitous porphobilinogen deaminase expression was induced with isopropyl β-D-thiogalactoside after the bacterial culture had reached an absorbance of 0.8 and growth was extended for a further 4 h before harvesting the cells.

The human porphobilinogen deaminase was purified as follows. Bacterial pellets were initially sonicated under nitrogen gas and extracts were subjected to heat treatment at 60°C for 3 min followed by ultracentrifugation to remove cell debris. The enzyme was chromatographed on DEAE Sephacel in 20 mM Tris/HCl buffer, pH 8.2, and eluted with a gradient of KCl extending from 0 to 100 mM. The purified wild-type enzyme had a specific activity of 2.1 μmol of uroporphyrin/h per mg. Purifications of unstable mutant apo-deaminase proteins were carried out as described previously for the *E. coli* deaminase [4].

Human porphobilinogen deaminase was assayed by incubating enzyme (0.1 units) with 2 mM porphobilinogen in 50 mM Tris/HCl buffer, pH 8.2, containing 1 mM 2-mercaptoethanol in a final volume of 250 μl. The reaction was terminated by adding 1 M HCl and the uroporphyrinogen I formed was determined after oxidation to uroporphyrin with benzoquinone at 405 nm (ε405 = 548 000 in 0.1 M HCl). The dipyrromethane cofactor was identified by reaction with Ehrlich’s reagent as described previously [3,14].

**Results and discussion**

**Human porphobilinogen deaminase D99G mutant**

In human porphobilinogen deaminase, Asp-99 is a key catalytic residue, being involved in each of the four deamination and condensation reactions to form ES4, as well as in the hydrolytic reaction to release the product pre-uroporphyrinogen [1]. This mutant deaminase expressed normally and was stable to the heat-treatment stage used in the purification procedure. Assay of the purified protein with porphobilinogen indicated that the D99G mutant was totally devoid of any deaminase activity and SDS/PAGE revealed that the protein had the expected molecular mass of 44 kDa and migrated as a single band. Non-denaturing gel electrophoresis, however, showed that the mutant protein migrated substantially faster than native deaminase with a mobility similar to that expected for an ES2 enzyme–intermediate complex (see Scheme 2). Whereas reaction of the normal porphobilinogen deaminase with Ehrlich’s reagent showed an initial purple colour that faded to an orange colour over a period of 10 min (Figure 1A), reaction of the D99G protein gave an immediate orange colour reaction reminiscent of a tetapyrpolyrole (bilane) with no further change over 10 min (Figure 1B).

These findings suggest that the human D99G mutation results in an inactive *holo*-protein that is equivalent to the native enzyme–intermediate complex, ES2. Thus, despite the fact the enzyme is inactive, due to the loss of the catalytic Asp-99, pre-uroporphyrinogen can still bind to the active site and alkylate Cys-261 in the normal way. Once pre-uroporphyrinogen has reacted, the absence of the catalytic aspartate prevents the addition of any further substrates and also prevents the *holo*-protein from participating in the well-established ‘reverse’ reactions with NH3 or hydroxylamine (or water). The mutant *holo*-protein is thus completely blocked from further transformation. This mutation highlights the fact that, despite being essential for
Ehrlich’s reaction of human recombinant ubiquitous porphobilinogen deaminase with Ehrlich’s reagent

(A) Reaction of native porphobilinogen deaminase showing the characteristic initial absorbance at 555 nm given by the dipyromethene cofactor changing over a period of 10 min to a new maximum at 495 nm. (B) Reaction of D99G mutant porphobilinogen deaminase showing the immediate formation of an absorption maximum at 495 nm, characteristic of the reaction of a tetrapyrrole (ES₂). Solid lines, reaction after mixing; ○, reaction after 10 min.

Figure 1: Ehrlich's reaction of human recombinant ubiquitous porphobilinogen deaminase with Ehrlich's reagent

catalysis, Asp-99 is not essential for the binding or reaction of pre-uroporphyrinogen with the deaminase apo-enzyme during cofactor assembly. A related mutation, D84A, in the porphobilinogen deaminase from E. coli shows similar properties to the D99G human mutant in that it also appears to exist as an inactive ES₂ complex [15].

Human porphobilinogen deaminase R149Q and R173Q mutants

Both mutations were initially characterized in patients with acute intermittent porphyria [9,10] and affect two of the invariant arginine residues in the active-site cleft. Arg-149 makes contacts with the dipyromethene cofactor where it interacts with the acetic acid side chains of the C-1 ring. It also contributes to a complex hydrogen-bonding network involving Asp-124 and Lys-98. Arg-173 also makes interactions with the cofactor through the propionic acid side chain of ring C-1 and is implicated in binding the propionic acid side chain of the substrate porphobilinogen [2].

Both recombinant human mutants expressed normally and SDS/PAGE of crude extracts showed a band at 44 kDa for each. Neither protein exhibited any catalytic activity in crude extracts and, during the heat treatment used in the purification, the deaminase band was lost, suggesting that the mutant proteins existed as unstable apo-enzymes. Further investigation of crude extracts revealed that the R149Q mutant is Ehrlich’s negative, but that R173Q was weakly Ehrlich’s positive. Both proteins were purified to homogeneity by a modified procedure using an affinity column containing Mimetic Orange [4]. This matrix binds to the apo-deaminase but not to the holo-deaminase. Following purification, attempts to reconstitute the R149Q and R173Q apo-proteins with the cofactor precursor, pre-uroporphyrinogen, were made. The R149Q mutant failed to form any holo-protein; however, the R173Q mutant showed a very weak Ehrlich’s reaction after treatment with pre-uroporphyrinogen. Non-denaturing PAGE (results not shown) revealed that both mutant proteins migrated as ‘smears’ on the protein gel, indicating their existence as incompletely folded apo-enzymes [4].

The conclusions from these investigations highlight the importance of Arg-149 and Arg-173 in the initial recognition and binding of pre-uroporphyrinogen by the apo-deaminase. For pre-uroporphyrinogen to react with Cys-261, the rings destined to be C-1 and C-2 in the dipyromethene cofactor would need to bind to the protein in such a way as to place the hydroxymethyl group of pre-uroporphyrinogen close to Cys-261. These mutations clearly prevent the pre-uroporphyrinogen from docking correctly and suggest that both residues are essential for the apo-protein → holo-protein transformation. Broadly comparable results were obtained with equivalent mutations of Arg-131 and Arg-155 in the E. coli porphobilinogen deaminase [16]. The former is an inactive, heat-labile, apo-enzyme whereas the latter is a severely crippled enzyme with less than 1% of the activity of wild-type deaminase. These results contrast strongly with those for D99G in which pre-uroporphyrinogen can dock and react with the apo-deaminase but the resulting holo-protein cannot participate in catalysis.

Human porphobilinogen deaminase R167Q mutant

The final investigation involved R167Q, one of the first human porphobilinogen deaminase mutations to be characterized [9]. Arg-167 is located in the active-site cleft and is ideally placed to interact with the acetic acid side chain of the substrate, porphobilinogen. The purified R167Q mutant deaminase appeared normal in all aspects except that it exhibited less than 10% of the activity of the native enzyme when incubated with porphobilinogen and migrated as a less basic pair of protein species on non-denaturing PAGE analysis (Figure 2). Incubation of the R167Q mutant with porphobilinogen, as with native enzyme, revealed the immediate formation of enzyme–intermediate complexes (see Figure 2, lanes 3 and 4). However, the complexes of the R167Q mutant were exceptionally stable and persisted for over 30 min (see Figure 2, lane 8) whereas those formed by the native enzyme rapidly turned over to product and reformed the original enzyme (Figure 2, lane 7).
Figure 2 | Non-denaturing PAGE of human recombinant ubiquitous porphobilinogen deaminase to show the properties of the native enzyme and the R167Q mutant
Lane 1, native deaminase showing the characteristic two bands; lane 2, R167Q mutant; lane 3, native enzyme + porphobilinogen (1:10) after 3 s; lane 4, R167Q mutant + porphobilinogen (1:10) after 3 s; lane 5, native enzyme + porphobilinogen after 3 min; lane 6, R167Q mutant + porphobilinogen after 3 min; lane 7, native enzyme + porphobilinogen after 30 min; lane 8, R167Q mutant + porphobilinogen after 30 min.

Conclusions
The study of four naturally occurring human mutations of recombinant ubiquitous porphobilinogen deaminase has revealed important aspects about the process by which the dipyrrmethane cofactor is assembled. One of the mutants, D99G, gives rise to a totally inactive enzyme that, despite the absence of an essential catalytic group, harbours the dipyrrmethane cofactor, albeit in the form of a stable enzyme–intermediate complex ES2. Two other mutants, R149Q and R173Q, result in unstable, inactive apo-proteins, despite the fact that both contain the catalytic Asp-99 and the residue essential for cofactor attachment, Cys-261. These experiments highlight the importance of Arg-149 and Arg-173 for the initial binding and positioning of pre- uroporphyrinogen within the apo-deaminase active site. The last mutant investigated, R167Q, is particularly interesting because it provides evidence that Arg-167 is important for controlling the elongation process at all stages of tetrapyrrole formation. Thus the R167Q mutant appears to be capable of catalysing the bond-forming and -breaking events but the mechanism of translocation of the growing polypyrrole chain within the active-site cleft is severely compromised.

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References

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