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INVITED PAPER, SPECIAL SECTION IN HONOR OF MAX PERUTZ

The three-dimensional structures of mutants of porphobilinogen deaminase: Toward an understanding of the structural basis of acute intermittent porphyria

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Abstract

Mutations in the human gene for the enzyme porphobilinogen deaminase give rise to an inherited disease of heme biosynthesis, acute intermittent porphyria. Knowledge of the 3-dimensional structure of human porphobilinogen deaminase, based on the structure of the bacterial enzyme, allows correlation of structure with gene organization and leads to an understanding of the relationship between mutations in the gene, structural and functional changes of the enzyme, and the symptoms of the disease. Most mutations occur in exons 10 and 12, often changing amino acids in the active site. Several of these are shown to be involved in binding the primer or substrate; none modifies Asp 84, which is essential for catalytic activity.

Keywords: acute intermittent porphyria; enzyme; gene; modeling; mutants; porphobilinogen deaminase; X-ray structure

Acute intermittent porphyria (AIP), an inherited autosomal dominant disorder in humans, results from a deficiency in the activity of the enzyme porphobilinogen deaminase (PBGD) (Kappas et al., 1989). This enzyme catalyzes an early step in the biosynthesis of heme that involves the sequential condensation of 4 molecules of porphobilinogen yielding the tetrapyrrole, preuroporphyrinogen. In this paper we discuss the impact of the most recent development, the determination of the X-ray structure of a PBGD (Louie et al., 1992) and its implications for understanding the molecular basis of AIP. The 3-dimensional structure of PBGD from *Escherichia coli* indicates strong conservation of structurally and functionally important amino acids in human PBGD and allows analysis of the reported mutations for likely effects on the structure and catalytic properties of the human enzyme. In many ways our analysis is inspired by and follows the approach developed by Max Perutz in analyzing mutants of human hemoglobin. This approach is further aided by the characterization of site-directed mutants of the *E. coli* enzyme, designed to probe the enzyme mechanism, and some of which have direct counterparts in AIP-associated human mutants (Jordan & Woodcock, 1991; Lander et al., 1991).

AIP is one of the most common of the porphyrias, with a frequency estimated at 1 per 10,000 persons. The disease manifests typically in acute attacks with symptoms that may include acute abdominal pain, vomiting, psychiatric involvement, constipation, peripheral neuritis, and paralysis. The urine contains elevated amounts of the heme precursors 5-aminolevulinic acid and porphobilinogen and has a diagnostic port-wine color on air oxidation. The neurological symptoms are almost certainly caused by 5-aminolevulinic acid, which bears a close structural similarity to the inhibitory neurotransmitter 4-aminobutyric acid (GABA) (Brennan et al., 1980; Bagust et al., 1985). In psychi-

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atric patient populations, the frequency of AIP can be as high as 1 in 500. Medical historians have assembled a strong but controversial case that the bouts of "madness" that afflicted King George III of England on 5 occasions were due to acute intermittent porphyria (Macalpine & Hunter, 1966). The King's illness, aptly named "Royal Malady," had a profound influence on the relationship between parliament and the crown in England and may well have contributed to the problems of the colonial administration in North America.

The human enzyme exists in 2 forms, encoded by a single structural gene made up of 15 exons extending over 10 kb of DNA on the long arm of chromosome 11 (Wang et al., 1981; Yoo et al., 1993). Expression is controlled by 2 promoters, an ubiquitous promoter, active in all tissues, upstream from an erythropoietic promoter, active only in erythroid cells (Raich et al., 1986; Grandchamp et al., 1987; Chretien et al., 1988). The ubiquitous enzyme has 361 amino acid residues, whereas the erythroid enzyme is derived from a transcript lacking exon 1 and is 17 residues shorter.

Rapid progress has been made recently in defining the genetic lesions responsible for AIP. PCR methods have been employed to amplify mutant DNA (either directly or derived from mRNA) for sequence analysis (Delfau et al., 1990; Scobie et al., 1990; Picat et al., 1991; Llewellyn et al., 1992a; Mgone et al., 1992) and detection of mutations. In some cases, the impact of a mutation on the properties of the enzyme has been evaluated by expression of the mutant protein in *E. coli* (Delfau et al., 1991). When combined with enzyme activity measurements, estimates of crossreacting immunoreactive material (CRIM) have provided a useful phenotypic characterization of AIP carriers (Desnick et al., 1985; Nordmann et al., 1990). In many cases, the level of enzymic activity is 50% of normal, correlating with the amount of CRIM arising from the normal allele. However, in some cases, the CRIM/activity ratio may be higher than normal, suggesting the presence of distinct classes of mutant type.

The crystal structure of *E. coli* PBGD reveals that the polypeptide chain of 313 amino acids is folded into 3 domains, each comprising β-sheet and α-helical secondary structure and a hydrophobic core (see Figs. 1, 3). The dipyrromethane cofactor, derived from 2 molecules of the substrate porphobilinogen (rings C1 and C2) (Hart et al., 1987; Jordan & Warren, 1987), is covalently bound to an invariant cysteine (C242) of domain 3 through ring C1 and lies in an extensive active-site cavity formed between the structurally related domains 1 and 2. An invariant aspartate (D84) hydrogen bonds the pyrrole nitrogens of the cofactor and is identified as a crucial catalytic residue (Louie et al., 1992). The acetate and propionate side groups of rings C1 and C2 form extensive salt bridge and hydrogen bond interactions with the protein, involving particularly the basic side chains of R131, R132, R149, R155, and K83, but also S81, T127, S129, and several main-chain functions (see Fig. 2A). The substrate is thought to interact with the positively charged side chains of R11, R149, and R155 at a binding site approximating the position of ring C2 of the cofactor in its oxidized form (Lambert et al., 1994). The structure possesses a large number of other ion pairs that may contribute to the considerable heat stability of the enzyme. Enzyme–intermediate complexes, generated as the 4 molecules of substrate are added sequentially to the cofactor primer, are stable to various degrees and may be isolated (Lander et al., 1991; Warren & Jordan, 1988). The escalating negative charge that develops as the pyrrole chain is elongated has focused attention on the role of the highly conserved basic residues in the protein. Site-directed mutagenesis of several of these residues results in the accumulation of distinct patterns of intermediates in the polymerization reaction, with some mutations (at R131 and R132) preventing the formation of the co-

Fig. 1. Schematic representation of the secondary structural elements in PBGD. The residues marking the ends of these elements in the human protein are labeled. The polypeptide chain has been colored to indicate the boundaries of the 15 exons in the human gene.

factor (Lander et al., 1991; Jordan & Woodcock, 1991). There is evidence that relative movement of the domains may be important during the catalytic cycle to accommodate the intermediates (Warren & Jordan, 1988).

Results

Modeling of the human PBGD

Amino acid sequences from a wide range of organisms have been determined, and the 58 invariant residues (18%) are shown in Figure 4. There is 43% identity between the *E. coli* and human sequences, rising to 60% for conservative changes, and this similarity forms the basis of the current analysis. All subsequent discussions will specify the human numbering system with the *E. coli* sequence numbering in brackets.

Conserved amino acids are clustered in the hydrophobic core of the molecule, in other conformationally important locations, and at the active site, suggesting that the deaminases have a similar fold. Both the ubiquitous and erythroid enzymes contain a 29-residue insertion in domain 3 compared to the *E. coli* protein. This can be placed either before, or after, strand $\beta 2_3$ of the *E. coli* structure. Secondary structure prediction methods (Eliopoulos et al., 1982) suggest a helix-turn-sheet-turn potential, which can be used to build plausible extensions to the domain 3 architecture.

Comparison of the aligned sequences with the enzyme fold allows structural features to be related to the organization of the human gene (see Fig. 1). All the exon/intron boundaries map to loops connecting secondary structural elements or to the extremities of these elements. Introns 7 and 11 occur in the coding region for the crossover strands linking domains 1 and 2, whereas the position of introns 8 and 12 demark approximately the boundaries of the motif whose repetition defines the symmetrical relation of the domains. No intron/exon boundaries are found in core regions of the domains, possibly reflecting the evolutionary origin of the enzyme's structural modules.

In many cases, the effect of a particular point mutation can be predicted by examination of the structurally encoded representation of the amino acid sequence of the *E. coli* enzyme provided by the JOY program (Overington et al., 1990) as shown

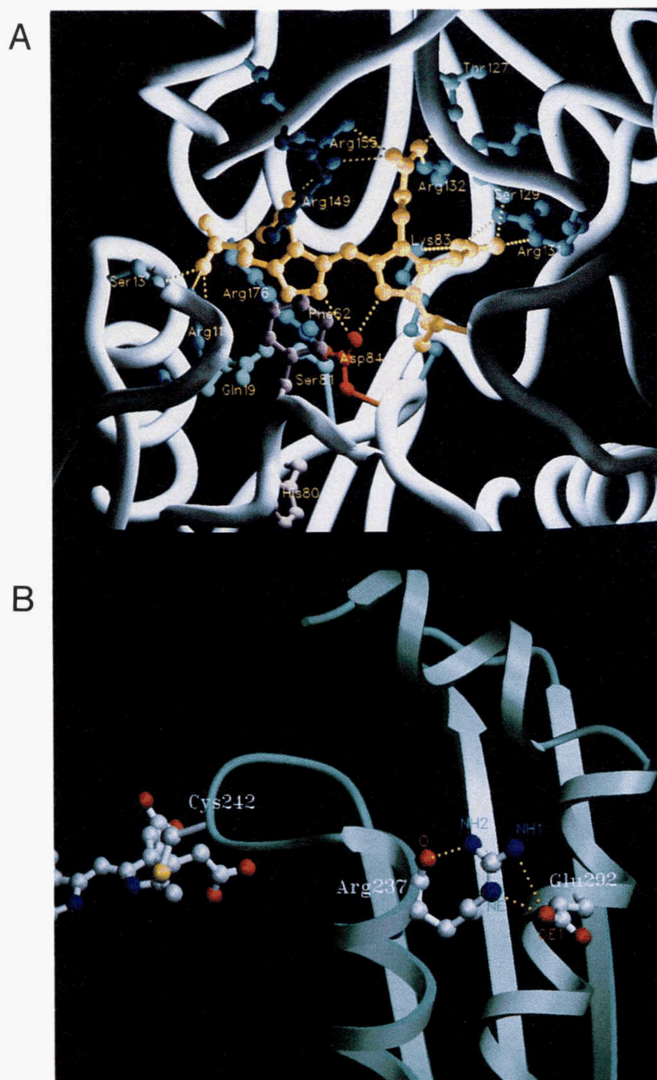

Fig. 2. A: Close-up view of the active-site cleft in *E. coli* PBGD. The polypeptide-chain backbone is represented as a white cylindrical ribbon. Direct hydrogen bond and salt bridge interactions between the cofactor dipyrrole (orange) and protein side chains are shown as yellow dotted lines. **B:** Hydrogen bond and salt bridge interactions formed in *E. coli* PBGD by Arg 237, at the end of helix $\alpha 1_3$, and Glu 292, from the adjacent helix $\alpha 2_3$. The polypeptide-chain backbone of domain 3 is represented as a green ribbon. The location of the cofactor dipyrrole (covalently attached to Cys 242) is shown for reference.

Fig. 3. Location of AIP mutations that cause single amino acid substitutions in human PBGD. The polypeptide-chain backbone is drawn in blue (β-strands: wide arrows; α-helices: ribbons; loops: thin rope), and the cofactor dipyrrole in orange. Mutation sites are highlighted in red.

in Figure 4. However, some mutations must be analyzed in terms of the local 3-dimensional structure of the human model to determine their likely influence.

Analysis of mutants

Where mutations in the PBGD gene lead to simple amino acid substitutions in the putative expression product, a straightforward assessment of the impact on the structure and functioning of the enzyme can usually be made. Mutations and their structural environment at each site are recorded in Table 1. It is important to note that all mutations have been found in patients independently diagnosed as suffering from AIP. For mutant alleles giving rise to a CRIM− class, the absence of detectable protein may be due to a variety of reasons, including transcriptional or translational disturbances, the formation of an unstable protein that is rapidly degraded by the cell, protein that is perturbed in conformation, or a polypeptide composition that is unrecognizable to the antibody. For instance, when the base change produces a synonymous codon (Mgone et al., 1992) we must assume that the CRIM− status derives from some defect at the nucleic acid level, involving perhaps promotor mutations outside the region sequenced. For CRIM+ mutants, where protein is clearly expressed, we can more reliably predict the consequences of sequence changes on the properties of the enzyme.

Many of the substitutions occur at sites that are generally well-conserved as polar residues close to the active site, and the structure shows clearly why they may be detrimental. Residues R149, R150, R167, and R173 [131, 132, 149, and 155 in *E. coli*] all provide stabilizing interactions with the acetate and propionate side groups of the cofactor in the active site as shown in Figure 2A. Residue R149 [131] interacts with side chains from ring C1 of the cofactor, and its change by site-directed mutagenesis to H131 or L131 in the *E. coli* enzyme prevents cofactor assembly and results in an inactive apo-enzyme (Jordan & Woodcock, 1991; Lander et al., 1991). The *E. coli* apo-enzyme is unstable (Scott et al., 1989) and the human enzyme is probably rapidly degraded, thus explaining the CRIM− status of the 149 [131] mutants. Residue R173 [155] interacts with side chains of rings C1 and C2 of the cofactor and, similarly, its substitution by His or Leu in the *E. coli* enzyme leads to severe impairment of the initial substrate binding, the final stages of pyrrole chain extension, and the release of the tetrapyrrolic product. Amino acid R167 [149] interacts with a ring C2 side chain when this cofactor pyrrole ring occupies the putative substrate binding site (see Fig. 2A), and its modification to H167 in the *E. coli* enzyme leads to an accumulation of the ES intermediate (enzyme with a single substrate attached) in the elongation reaction and a 5-fold rise in K_m (Jordan & Woodcock, 1991). A similar perturbation might also be expected for the W167 and Q167 [149] mutations. In accordance with this, a compound heterozygote R167W/Q (Llewellyn et al., 1992b) showed less than half the normal eryth-

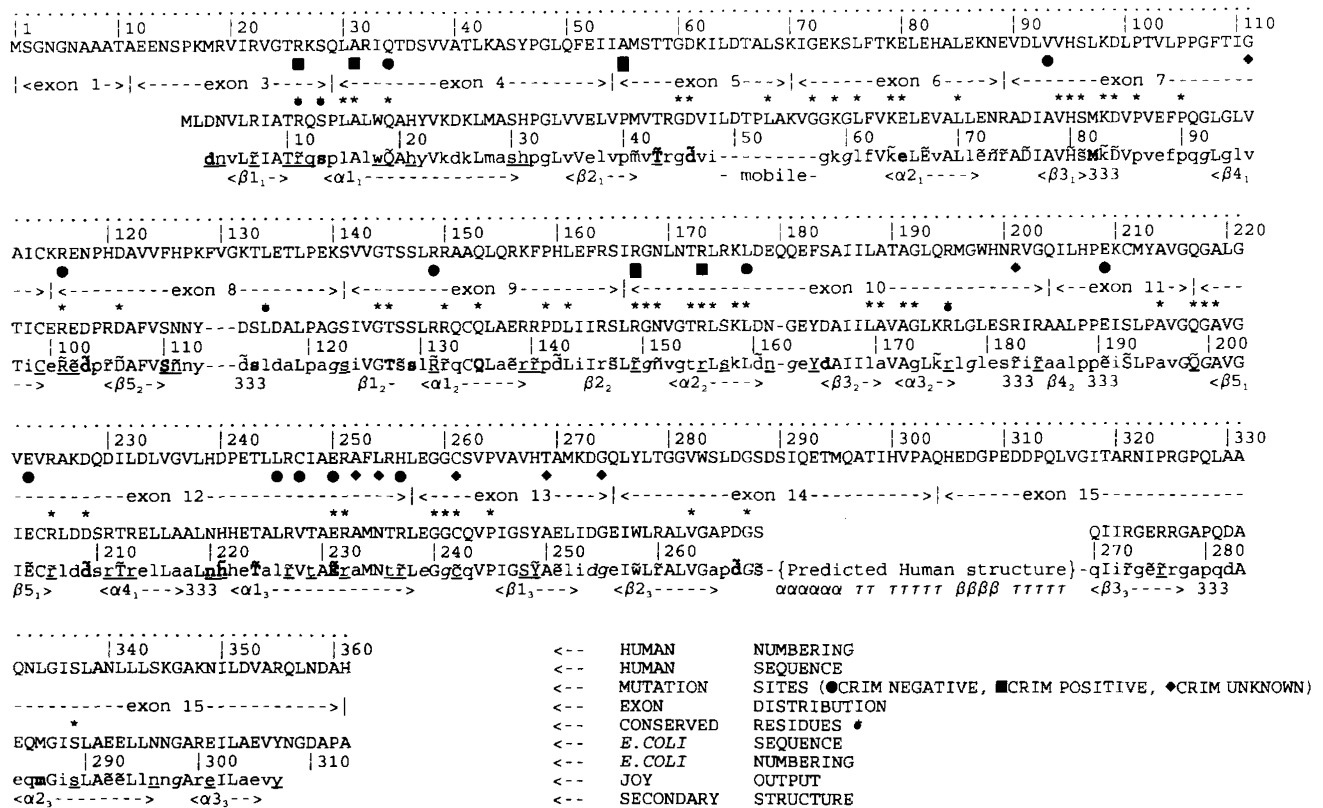

Fig. 4. Human exon distribution with *E. coli* residue environment (JOY). X, solvent inaccessible (upper case); x, solvent accessible (lower case); x̃, hydrogen bond to other side chain (tilde); **x**, hydrogen bond to main-chain amide (bold); x̲, hydrogen bond to main-chain carbonyl (underline); 3, 3_{10}-helix.

Table 1. *AIP associated mutations in the human PBGD gene*[a]

Exon	Mutation	Environment	Effect	Reference
1	Splice	Hydrophobic core adjacent L81, L85, and H80	Ubiquitous enzyme only deleted	Grandchamp et al., 1989b
1	Splice	Intron 1	Ubiquitous enzyme only deleted	Grandchamp et al., 1989c
3	Splice −		Truncated protein	Llewellyn et al., 1992a
3	R26H +	Salt bridge with propionate side chain of cofactor ring C2 at putative substrate site	Loss of interactions to substrate	Elder et al., unpubl.
4	A31T +	Packed against R11, insufficient space beyond $C\beta$	Steric disruption	Grandchamp et al., unpubl.
4	Q34K	Base of active site cleft, H bonds to S96 and R195	H bonding break, +ve charges close	Mgone et al., 1992
5	A55S +	Protein surface	Unknown	Grandchamp et al., unpubl.
5	Deletion −	Frameshift (at residue 58)	Truncated protein	Grandchamp et al., unpubl.
5	Insertion −	Frameshift (at residue 61)	Truncated protein	Grandchamp et al., unpubl.
5	Splice −	Intron 5	Truncated protein	Grandchamp et al., unpubl.
7	V93F	Hydrophobic core adjacent L81, L85, and H80	Steric disruption	Chen et al., 1992
7	G111R	Protein surface	Unknown	Grandchamp et al., unpubl.
8	R116T\			Mgone et al., 1992
8	R116W −	Interdomain salt bridge to E250, H bond to 198CO	Loss of stabilizing interactions	Chen et al., 1992 Lee et al., 1990
8	R116Q/			Gu et al., 1992
8	A122G	Hydrophobic core	Destabilizing cavity	Mgone et al., unpubl.
8	Deletion	704 bases missing	Truncated protein	Mgone et al., unpubl.
9	R149L\ −	Salt bridge interaction with cofactor ring	Cofactor binding interactions disrupted	Grandchamp et al., unpubl.
9	R149Q/ −	C1 acetate side chain		Delfau et al., 1991
9	Q155Stop −		Truncated protein	Scobie et al., 1990
10	Splice		Truncated protein	Lundin et al., 1993
10	R167W\ +	Salt bridge interaction with cofactor ring C2 acetate side group in putative substrate site	Substrate binding interactions disrupted	Llewellyn et al., 1992b
10	R167Q/ +			Delfau et al., 1990
10	R173W\ +	Salt bridge interactions to propionate of cofactor ring C1 and acetate of C2 in putative substrate site	Loss of binding interactions to substrate and cofactor	Gu et al., 1992;
10	R173Q/ +			Delfau et al., 1990
10	L177R	Hydrophobic core	Destabilizing buried charge	Mgone et al., 1992
10	W198Stop −		Truncated protein	Lee and Anvret, 1991
10	R201W	Salt bridge to D178	Loss of stabilizing interaction	Chen et al., 1992
10	Q204Stop		Truncated protein	Mgone et al., 1994
10	E209K −	Protein surface	Unknown	Grandchamp et al., unpubl.
10	Splice −	Cryptic splice site activated	Deletes residues 203–205	Delfau et al., 1991
11	E223K −	Salt bridge with H95 at base of active site cleft	Disruption due to close +ve charges	Grandchamp et al., unpubl.
12	Splice −	Intron 11	Truncated protein	Llewellyn et al., 1992a
12	T244STOP		Truncated protein	Mgone et al., 1992
12	Deletion −	Frameshift (R244) generating stop	Truncated protein	Grandchamp et al., unpubl.
12	Insertion−	Frameshift (R248) generating stop	Truncated protein	Grandchamp et al., unpubl.
12	C247F\	Buried in the hydrophobic core	Steric disruption	Chen et al., 1992
12	C247R/			Mgone et al., 1993
12	L245R −	Protein surface	Unknown	Delfau et al., 1991
12	E250K −	Buried interdomain salt bridge to R116	Disruption due to close +ve charges	Grandchamp et al., unpubl.
12	A252T\	Packed at interface of helices $\alpha 1_3$ & $\alpha 2_3$	Steric disruption	Mgone et al., 1993
12	A252V/			Mgone et al., 1993
12	L254P	Near end of $\alpha 1_3$ and cofactor linkage site	Conformation disrupted near cofactor	Mgone et al., unpubl.
12	H256N −	$\alpha 1_3$ helix cap and H bonds N340	Loss of stabilizing interactions	Mgone et al., 1992
12	C261Y	Cofactor linkage site	Cofactor bonding?	Mgone et al., unpubl.
12	Splice +		Exon 12 only deleted	Grandchamp et al., 1989a
13	T269I	Hydrophobic core	Steric disruption	Mgone et al., 1994
13	G274R	Loop $\beta 1_3/\beta 2_3$, positive phi	Folding defect?	Mgone et al., 1994
13	Splice −	Intron 13	Truncated protein	Llewellyn et al., 1992a
14	Deletion −	Frameshift (His 305) generating stop	Truncated protein	Delfau et al., 1991
14	W283stop			Mgone et al., 1994
14	Splice −	Intron 14	Truncated protein	Llewellyn et al., unpubl.

[a] Where known, the CRIM type is shown as + or −. Deletion and insertion refer to bases in the DNA. "Splice" indicates that the mutation has occurred in the consensus sequence for RNA processing; when such mutations occur within the intron, they are grouped with the adjacent exon.

rocyte enzyme activity but enhanced levels of CRIM. In some CRIM+ phenotypes, a range of enzyme intermediate complexes persists in the red cell and this could explain CRIM/activity ratios as high as 5.7 (Desnick et al., 1985; Nordmann et al., 1990). Mutations at R167 [149] and R173 [155] would show this result.

The mutations R116W and R116T [101] are expected to be detrimental. This invariant arginine is located on 1 of the 2 short strands linking domains 1 and 2, with the charged side chain participating in an ion pair with E250 [231]. Surprisingly, the equivalent site-directed mutations at this position in the *E. coli* enzyme [R101H or R101L] are not unduly detrimental and, although additional steric factors are likely with R116W, this cannot be the case with the R116T human mutant. The effect of disrupting this R116–E250 salt bridge would also seem to be confirmed by the CRIM− status of the human E250K [231] mutant.

Some mutations occur at conserved residues in the hydrophobic core and are expected to be deleterious to the protein structure, either because they introduce a buried charged group or lead to a large volume change and associated steric clashes, e.g., L177R [159] and A31T [16].

Other mutations occur at sites that are variable in the enzyme family. The human enzyme model is particularly instructive in such cases, especially where compensatory variations of interacting residues are present. The H256N mutation [R237 in *E. coli*] may fall into this class. In the *E. coli* protein, this basic residue is located at the C-terminus of $\alpha 1_3$, where it both hydrogen bonds with a terminal carbonyl of the α-helix and forms an ion pair with [E292] on $\alpha 2_3$ (Fig. 2B). Although a hydrogen bond is probably maintained between H256 and N340 in the normal human protein, the charge is lost and the equivalent hydrogen bond may be less stable in the mutant. The CRIM+ status of this mutation implies that the interaction is indeed important for activity, possibly due to the proximity of the cofactor attachment site to the C-terminus of $\alpha 1_3$ (see Fig. 2B). The modeled structure, however, fails to explain the CRIM− status of L245R [226], a mutation at the surface of the protein where natural variation includes the observed amino acid substitution.

Other mutations in the human gene result in frameshifts and premature stop codons, intron splice site disruptions, and extended deletions. These can usually explain satisfactorily the abnormalities in the protein and/or its expression. These mutations are summarized in Table 1 and the effect on the putative protein product can be judged from Figure 1. Surprisingly, complete deletion of exon 12, corresponding to the 40 residues 217-257 [198-238] making up $\beta 5_1$, $\alpha 4_1$, and $\alpha 1_3$, yields a protein product, albeit inactive, that persists and crossreacts with antibody raised against the native enzyme. On the other hand, the cryptic splice site activated by mutation of the last base of exon 10, which deletes only residues 203-205 ($\beta 4_2$) [185-187], yields a CRIM− phenotype.

Discussion

The largest number of mutations have so far been recorded in exons 10 (domain 2) and 12 (domain 1 + 3). The mutations leading to a simple change in amino acid sequence often seem to occur close to exon boundaries, and there are signs of a concentration of mutations in the code for $\alpha 1_3$ (see Fig. 3). This helix carries a number of polar groups that are involved in contacts between domains 1 and 3, and, even though quite distant from the active site, a mutation such as R232H in the *E. coli* protein (not known in human) is severely detrimental to enzyme activity. This is possibly due to perturbation of important conformational changes mediated through domain interfaces.

The large volume of the active-site cavity and the multipoint interaction envisaged between the enzyme and the growing polypyrrole chain during catalysis implies that, potentially, a large number of different mutants could be detrimental for activity but not affect the structure, giving rise to the CRIM+ phenotype. The high CRIM+/activity ratios of some mutants can be satisfactorily explained by the presence of stable enzyme intermediate complexes that are degraded more slowly than the wild-type enzyme. It seems likely, however, that if the mutational events are random, a spectrum of phenotypes may be defined. The delineation of the active-site pocket, potential catalytic residues, and stabilizing interactions for the protein fold in the human model 3-dimensional structure may be of use in the design of probes for more rapid and extensive molecular pathology testing.

Fortunately, only about 10% of known AIP heterozygotes are symptomatic. Early detection of carriers in affected lineages is, however, very important so that they can avoid precipitating factors. Poor diet, alcohol, certain drugs, and hormones that stimulate heme synthesis can precipitate acute and sometimes fatal exacerbations of neurologic dysfunction of the peripheral, autonomic, or central nervous system. The disease continues to be invoked to explain the recorded malaise of historically prominent individuals, including most recently, the artist Vincent van Gogh, whose appetite for the toxic liquor, absynthe, could have precipitated acute attacks of AIP (Loftus & Arnold, 1991).

Methods

A model of the human PBGD was constructed using the program COMPOSER (Srinivasan & Blundell, 1993) from the SYBYL suite (Tripos Assoc., California) and the refined coordinates of the *E. coli* enzyme (1PDA). The model was energy minimized.

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