Original Article

A New Mutation within the Porphobilinogen Deaminase Gene Leading to a Truncated Protein as a Cause of Acute Intermittent Porphyria in an Extended Indian Family

(acute intermittent porphyria / OMIM 176000 / porphobilinogen deaminase / exon 15 / 973insG mutation)

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Abstract. Based on Internet search, we were contacted by a 50-year-old man suffering from severe abdominal pain. Acute hepatic porphyria was considered from positive Watson-Schwartz test. He, not being a health professional, searched for centres with ability to do molecular diagnosis and for information about therapeutic possibilities. He asked his physician for haem-arginate (Normosang, Orphan Europe, Paris) treatment, arranged sending his blood to our laboratory and mediated genetic counselling for him and his family. Molecular analyses of the *PBGD* gene revealed a novel mutation in exon 15, the 973insG. Subsequently, genetic analysis was performed in 18 members of the proband's extensive family. In 12 members of the family, the same mutation was found. The mutation, which consisted of one nucleotide insertion, resulted in addition of four different amino acids leading to a protein that is prematurely truncated by the stop codon. The effect of this mutation was investigated by expression of the wild-type and mutated PBGD in a prokaryotic expression system. The mutation resulted in instability of the protein and loss of enzymatic function. The increasing access to a number of disease- and symptom-oriented web pages presents a new and unusual venue for gaining knowledge and enabling self-diagnosis and self-help. It is, therefore, important that disease-oriented Internet pages for public use should be designed with clarity and accurate current knowledge-based background.

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Abbreviations: AHP – acute hepatic porphyria, AIP – acute intermittent porphyria, ALA – δ-aminolevulinic acid, DP – Doss porphyria, GST – glutathione synthetase, HC – hereditary coproporphyria, PBG – porphobilinogen, PBGD – porphobilinogen deaminase, VP – variegate porphyria.

Introduction

Haem, an iron-containing tetrapyrrole, is critical for life. It is utilized by numerous proteins involved in basic cellular processes such as respiration (cytochrome oxidase), oxygen transport (haemoglobin), detoxification of foreign compounds (cytochromes P450), vascular homeostasis (nitric oxide synthases), cellular signalling (guanylate cyclase), cell death (cytochrome *c*), and a whole host of other functions (Granick and Beale, 1978; Ponka, 1999; Tang et al., 2003). Haem synthesis is a well-coupled complex of cellular machinery comprising eight enzymes (Meyer and Schmidt, 1978; Anderson et al., 2001; Kauppinen, 2005). The pathway is evolutionarily conserved from bacteria to humans. In mammals, the pathway is localized in cytosolic as well as in mitochondrial compartments (Fig. 1). Haem and porphyrins in general have unique structural features (Kral et al., 2006). The biological functions of haem are ensured by its metallocomplex with iron. It is, therefore, evident that a pathway of such biological significance will be regulated at many levels.

Porphyrias are caused by decreased activities of the enzymes in the haem biosynthetic pathway, with the exception of the first one in which a defect causes anaemia (Meyer and Schmidt, 1978; Anderson et al., 2001)

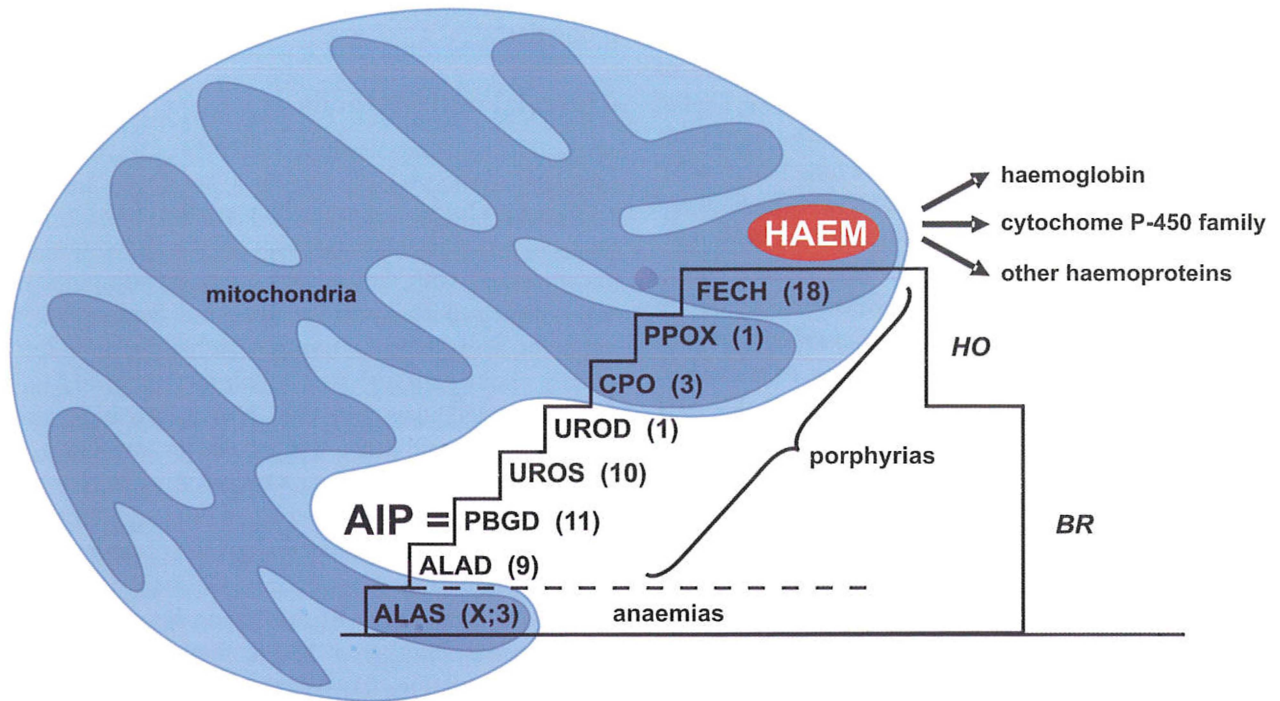

Fig. 1. Haem synthetic and degradation metabolic pathway. Enzymes involved are in Abbreviations and parenthetical numbers indicate chromosomal localization of the gene involved. ALAS: δ-aminolevulinic acid synthase; ALAD: δ-aminolevulinic acid dehydratase; PBGD: porphobilinogen deaminase; UROS: uroporphyrinogen III cosynthetase; UROD: uroporphyrinogen decarboxylase; CPO: coproporphyrinogen oxidase; PPOX: protoporphyrinogen oxidase; FECH: ferrochelatase; *Haem degradation enzymes:* HO – haem oxygenase; BR – biliverdin reductase. AIP: acute intermittent porphyria (defect in PBGD).

(Fig. 1). Each porphyria is characterized by a typical spectrum of accumulated and excreted porphyrins and their precursors, porphobilinogen (PBG) and δ-aminolevulinic acid (ALA). Acute porphyrias (acute intermittent porphyria, AIP; variegate porphyria, VP; hereditary coproporphyria, HC; Doss porphyria, DP) are all considered to be hepatic (Grandchamp, 1998; Martasek, 1998; Kauppinen, 2005). They all share possible precipitation of acute attacks. This might be a life-threatening medical emergency with neurovisceral symptomatology, believed to be caused by the administration of drugs (archetypal – barbiturates), which are metabolized by cytochrome P450 monooxygenases (Meyer and Schmidt, 1978; Yeung et al., 1987; Anderson et al., 2001; Kauppinen, 2005). This increased need in haem production may trigger a porphyric attack by a mechanism which is not yet understood. Other triggers for porphyric attack include a profound decrease in caloric intake, infections, and hormonal influences. The main clinical symptoms of an acute porphyric attack are (from the more frequent to the less frequent): abdominal pain, tachycardia, peripheral motor neuropathy, constipation, nausea, vomiting, mental changes, hypertension, sensory neuropathy, convulsion (Meyer and Schmidt, 1978; Yeung et al., 1987; Anderson et al., 2001). In case of VP and HC, skin symptoms may be present as well. Diagnosis in clinically manifested cases is based on typical biochemical findings (dark urine in all acute attacks, especially if exposed to light; AIP: increased urinary PBG and ALA, no elevation of faecal porphyrins; VP: increased faecal proto- and coproporphyrins, fluorescence plasma emission maximum 627 nm (exc. 400 nm); HC: high increase of faecal and urinary coproporphyrin, red fluorescence of faeces in UV light; DP: extremely rare, increased ALA urinary excretion) (Meyer and Schmidt, 1978; Grandchamp et al., 1996; Kauppinen, 2005). Once the mutation is found in responsible genes, a genetic-based diagnosis can be offered to all family members. Detection of the mutation will identify members with latent porphyria, and unaffected family members can be distinguished with assurance. An acute attack of porphyria in most cases requires hospital admission. High glucose intake (oral/intravenous) and haem-arginate are the principal therapeutic remedies (Kauppinen, 2005). An important aspect of therapy of acute hepatic porphyria (AHP) is prevention. Drugs known to provoke acute attacks should be avoided (for a complete list see web sites: http://www.porphyria-europe.com/03-drugs/how-to-use-info.asp) (Deybach et al., 2006)

The study of porphyria, as with other metabolic disorders, deals with gaining a deeper understanding of how defects in the enzymes that generate porphyrins

cause disease. Acute intermittent porphyria (AIP; OMIM 176000) is a low-penetrant autosomal dominant disorder caused by reduced activity (~50%) of porphobilinogen deaminase (PBGD; EC 4.3.1.8), the third enzyme of the haem synthetic pathway (Goldberg, 1959; Strand et al., 1970; Meyer et al., 1972; Meyer and Schmidt, 1978; Puy et al., 1997). This enzyme, also known as hydroxymethylbilane synthase, catalyses the head-to-tail condensation of four molecules of porphobilinogen to form hydroxymethylbilane (Jordan and Warren, 1987; Hart et al., 1988). The human *PBGD* gene has been cloned and his organization characterized. The *PBGD* gene is localized in 11q23.3 region (Wang et al., 1981; Namba, et al., 1991). It is split into 15 exons spread over 10 kb with a single open reading frame of 1038 bp (Raich et al., 1986; Yoo, et al., 1993). Two distinct promoters are located in the 5' flanking region and in intron 1, respectively, and generate housekeeping (exon 1 and 3–15) and erythroid-specific (exon 2–15) transcripts by alternative splicing of exon 1 and 2 (Grandchamp et al., 1987; Chretien et al., 1988; Chen et al., 1994).

AIP is the most frequent acute hepatic porphyria (Puy et al., 1997; Grandchamp, 1998). The disease is characterized by intermittent acute porphyric attacks with abdominal pain, hypertension, tachycardia, neurologic and psychiatric manifestation as previously mentioned. In difference to other hepatic porphyrias, skin photosensitivity is not present (Grandchamp, 1998; Kauppinen, 2005). To date, more than 300 mutations within the *PBGD* gene have been identified (Hrdinka et al., 2006).

In this study, we identify a new, previously non-characterized mutation, the 973insG, found in exon 15 of the *PBGD* gene in an extended Indian family. To establish the effect of this mutation on the protein function, we expressed the mutant protein in the prokaryotic expression system and analysed its biochemical and enzymatic properties.

Material and Methods

Patients

Based on Internet search, we were contacted by a 50-year-old proband suffering from severe abdominal pain accompanied with dark urine; acute hepatic porphyria was considered from positive Watson-Schwartz test. He, not being a health professional, searched for more information about porphyrias and centres with ability to do molecular diagnosis and give him information about therapeutic possibilities. He asked his physician for haem-arginate (Normosang, Orphan Europe, Paris), a treatment which achieved excellent clinical effects with prompt relief of abdominal pain. He subsequently arranged sending blood or genomic DNA via clinical geneticist (I.V., co-author of this paper, he also provided genetic counselling) from 18 members of his family to our laboratory (Fig. 2). We, therefore, analysed DNA from this extensive Indian family from Nepal.

DNA analysis

Genomic DNA was extracted from peripheral blood samples anticoagulated with EDTA according to a standard protocol. All 15 exons of the *PBGD* gene with surrounding exon/intron boundaries (more than 60 bp of their flanking regions) were amplified by PCR using specific primers (Puy et al., 1997; Genbank accession numbers, *HMBS* gene, M95623; HMBS cDNA, NM000190). Consequently, fragments of amplified DNA were analysed by denaturing gradient gel electrophoresis (Myers et al., 1987). Sequence analysis was performed in automatic sequencer ABI PRISM 3100/3100-Avant Genetic analyser (Applied Biosystems, Foster City, CA).

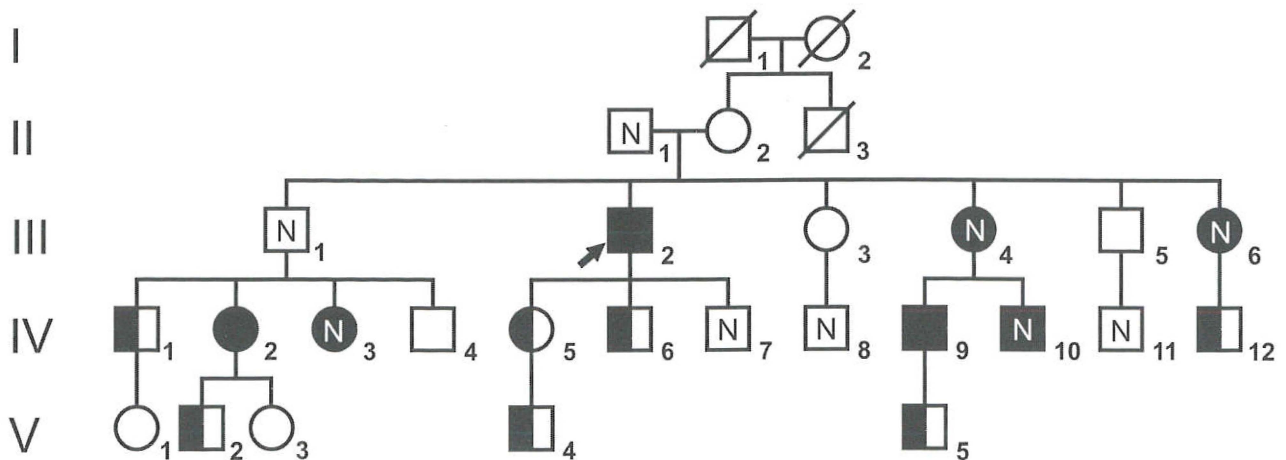

Fig. 2. Pedigree of the Indian family with acute intermittent porphyria (AIP). Proband is indicated by an arrow. Solid symbol represents a patient with clinically manifested disease. Half-solid symbols represent members of the family who carry the mutation but are asymptomatic. N – not tested

Plasmid construction and mutagenesis

Total RNA was extracted from fresh leukocytes isolated from EDTA-anticoagulated whole venous blood. One microgram of total RNA was reverse transcribed using 200 U M-MLV SuperScript III (Invitrogen, Carlsbad, CA) in the presence of oligo(dT) (Invitrogen) as a primer in the first step. The complementary cDNA spanning exons 1 and 3–15 was PCR-amplified using primers with specific restriction sites in the second step (cDNA *Bam*HI Fw: 5´ata tgg atc cat gtc tgg taa cgg 3´, cDNA *Xho*I Rev: 5´tat act cga gtt aat ggg cat cgt taa 3´). The human PBGD cDNA was ligated into the pGEX-4T-1 expression vector (Amersham Pharmacia Biotech, Piscataway, NJ) and then transformed into *E. coli* BL21 (DE3) (Stratagene). Plasmid DNA was amplified and isolated using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Site-directed mutagenesis of the 973insG mutation was performed with the following mutagenic primers: Fw: 5´cac tgc tcg taa cat tcc agc gag ggc ccc 3´, Rev 5´ggg gcc ctc gct gga atg tta cga gca gtg 3´ using QuikChange® Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). The PCR products were subsequently submitted to direct sequencing to confirm the result of mutagenesis.

Protein expression and purification

Mutated PBGD and wild-type PBGD were expressed as glutathione synthase (GST)-fusion proteins in BL21 cells using the optimal growing conditions (TB medium with ampicillin (100 μg/ml), IPTG (0.5 mM), 4 h at 30°C, aerobic conditions). All purification steps were carried out at 4°C. Bacterial cells were lysed using lysis buffer (PBS, pH 7.3) containing lysozyme (1 mg/ml) and Triton X-100 (0.5% v/v, Sigma, St. Louis, MO). The lysate was subjected to sonication thereafter. Cell debris was centrifuged at 4°C, 33,000 *g* for 30 min. Proteins were purified using affinity chromatography on the Glutathione Sepharose 4B column (Amersham Biosciences). GST-tag was cleaved by thrombin (20 U thrombin/1 mg of the protein, ICN Biomedicals, Irvine, CA) overnight at 20°C. All steps of the protein purification and digestion were confirmed by the SDS-PAGE (Laemmli et al., 1970).

PBGD enzymatic assay

The PBGD activity assay was carried out according to previously described methods (Erlandsen et al., 2000; Brons-Poulsen et al., 2005; Ulbrichova et al., 2006). The standard incubation system contained 1 μg of PBGD enzyme, incubation buffer (50 mM Tris-HCl, pH 8.2), and substrate PBG (100 μM, ICN Biomedicals) in a final volume of 400 μl. After pre-incubation at 37°C for 3 min, the reaction was started by substrate addition and incubation was carried out in the dark at 37°C for exactly 1 h. The reaction was stopped by adding TCA (trichloracetic acid, final concentration 12.5%). The uroporphyrinogens formed were photooxidized by exposing them to daylight for 60 min. The precipitated protein was discarded by centrifugation and total fluorescence intensity was measured using a Perkin Elmer LS 55 spectrofluorometer immediately thereafter. Uroporphyrin I (URO I, ICN Biomedicals) was used as the standard and 12.5% TCA as a blank. Protein concentration was determined by the method of Lowry (Lowry et al., 1951) employing bovine serum albumin (BSA) as standard.

Sequence and structure analysis

Sequence alignment was produced with T-coffee software on the server tcoffee@igs (Poirot et al., 2003). The structure was displayed and examined with The Molecular Biology Toolkit, Moreland et al. (2005).

Results and Discussion

The fragments of amplified DNA were analysed in the proband's DNA by denaturing gradient gel electrophoresis. An abnormal pattern in exon 15 was found. Subsequent sequencing analysis showed the insertion of one extra G in position 9205 on genomic DNA, resulting in a shift of the reading frame (Fig. 3). Analysis of the protein sequence indicated that following the mutation, four amino acids were different, leading to a prematurely truncated protein due to a stop codon in which 44 amino acids of the C-terminal of PBGD were missing. This mutation was subsequently found in 12 members of the family in addition to the proband. It is known that clinical expression of AIP is highly variable and a high percentage of AIP heterozygotes remain asymptomatic throughout life (Petrides, 1998). However, knowledge of their heteroallelic nature of gene abnormality might be an extremely important way to prevent devas-

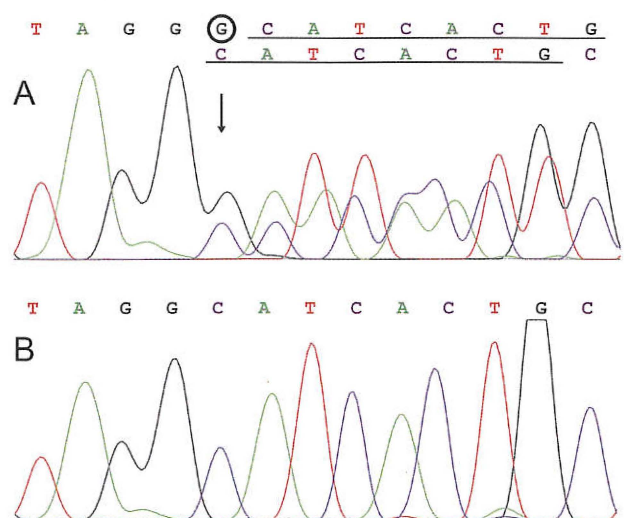

Fig. 3. DNA sequencing analysis in the *PBGD* gene of the proband (A) and (B) control. The mutation 973insG was localized in exon 15. This single-base insertion resulted in a shift in the open reading frame leading to a truncated and inactive protein.

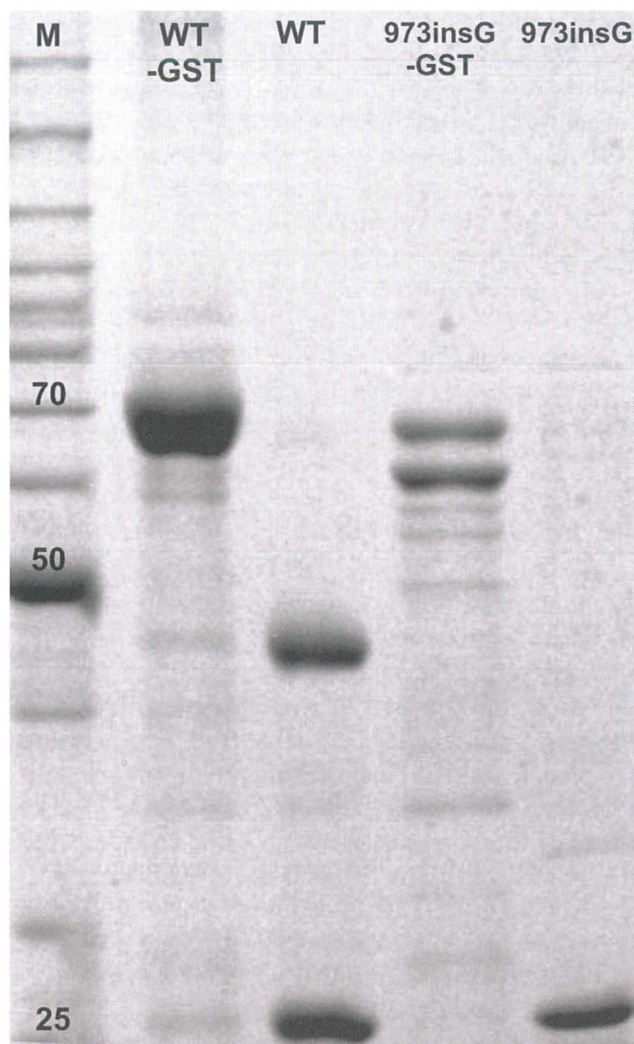

Fig. 4. SDS/PAGE analysis of wild-type and mutated porphobilinogen deaminase (PBGD). From the left: Marker; GST-fusion purified wild-type protein; thrombin-digested purified wild-type protein; GST-fusion purified mutated protein; Thrombin-digested mutated GST-fusion mutated products. Wild-type protein showed approximately Mr 68 kDa with GST-tag and Mr 42 kDa without GST-tag. The amount of protein loaded in each lane of the SDS-PAGE gel was adjusted to be identical (the same volume of affinity beads as well as elution buffer) for wild-type and mutant enzymes.

tating acute porphyria attack by carefully avoiding its precipitating factors. Additionally, two known polymorphisms within the *PBGD* gene were found in the proband: 3581A/G heteroallelic in ivs3 (RFLP *Bsm*AI) and 7064C/A heteroallelic in ivs10 (RFLP *Hinf*I).

In vitro expression remains a very useful tool in studying mutations in the *PBGD* gene (Delfau et al., 1990; Chen, et al., 1994; De Siervi, et al., 1999 & 2000; Solis, et al., 1999; Ulbrichova et al., 2006). The wild-type PBGD and the truncated mutated PBGD were simultaneously expressed as GST-fusion proteins (Fig. 4). The resulting SDS-PAGE gel showed distinct band(s) corresponding to the GST-PBGD of wild type and its cleaved products, PBGD and GST with the appropriate size. However, expression of the mutated protein was only possible, to a certain extent, as a GST-fusion protein. Attempts to purify the mutated PBGD, free of the GST, failed. We speculated at this point that this is due to instability of the truncated mutated protein. Results of the corresponding activity measurements are depicted in Table 1. Due to extreme instability of the mutated protein as described above, measurement of the activity was performed in GST-PBGD fusion protein of the mutated form and compared with the GST-PBGD fusion protein of the wild-type form expressed simultaneously under identical conditions. The activity of the mutated form was very low and amounted to only 0.5% of the activity of the control wild-type protein.

Table 1. Measurement of the activity was performed in GST-PBGD fusion protein of the mutated form and compared with the GST-PBGD fusion protein of the wild-type form expressed simultaneously under identical conditions

Activity	WT	973insG
Specific Activity (nmol URO I / h / μg of protein)	615	32
Relative Activity (%)	100	0.52

The availability of recent powerful sequence alignment algorithms and the increase of the number of PBGD homologues in the databases allowed us to improve the original (Louie et al., 1992; Lambert et al., 1994) *E. coli* and human PBGD sequence alignment. The sequences share 41% identity and 57% similarity in our alignment. The crystal structure of *E. coli* PBGD (Brownlie et al., 1994; Lambert et al., 1994; Louie et al., 1998) can thus be used for assessing the molecular and functional consequences of the missense mutation in the human enzyme.

Figure 5 shows the amino acid alignment of the N-terminal sequence for several bacterial and vertebrate PBGDs. It can be seen that the regular secondary structure elements are conserved among bacteria and vertebrates (β-sheet and two helices). Higher eukaryotes contain an extra segment (29 amino acids in humans) inserted between the β-sheet and the penultimate helix forming a large surface linker.

The effect of the deletion and few mutations is visualized in Fig. 6. The enzyme is formed by three domains in close interaction. Their interface forms the active site (Fig. 6a). The truncation described in the present report (2007) is located in the N-terminal domain (Fig. 6b). The domain consists of a three-stranded antiparallel β-sheet meander and three α-helices. Whereas the first two helices form a tightly packed unity with the β-sheet, the terminal helix is much more independent of the rest

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HEM3_HUMAN     290 SIQETMQATIHVPAQHEDGPEDDP--QLVGITARNIPRGPQLAAQNLGISLANLLLSKGAKNILDVARQLND-AH 361
HEM3_RAT       290 SMQETMQATIQVPVQQEDGPEDDP--QLVGITARNIPRGAQLAAENLGISLASLLLNKGAKNILDVARQLND-VR 361
HEM3_MOUSE     290 SMQETMQATIQVPVQQEDGPEDDP--QLVGITARNIPRGAQLAAENLGISLASLLLNKGAKNILDVARQLND-VR 361
HEM3_BOVIN     290 TMQDTMQTTIHVPVQHEDGPEDDP--QLVGITARNIPRQPQLAAENLGISLATLLLNKGAKNILDVARQLNE-AH 361
A2BFN6_DANRE   288 CLKDTMQTCVELDNKVNESTQRSA--N-VGVTACNISSSALEAAEKLGLDLANVLLNKGAKDILTTARKLND-AR 358
Q3KQ72_XENLA   280 SLKETMQSCINFPQQEVEGPNDDV--QHVGITALGVSHQALESAECLGTGLADLLLSKGAKEILTVARQLND-SR 351
Q503D2_DANRE   287 SLKETMQTSFNPDTQAEEQEKVDEKVQRVGITALKVAEAAQDAAMKLGVDLGNLLLSKGAKEILTVARQLND-AR 360
HEM3_ECOLI     271 IIRGERRGA-------------------------------PQDAEQMGISLAEELLNNGAREILAEVYNGDAP-A 313
HEM3_PROM5     274 LIKDESSGN-------------------------------VKYPEEVGKKLAEKLKLQGADKILSEIFEQFRD-K 316
HEM3_PROMP     274 LIKNESIGN-------------------------------IKYPEEVGKKLAEKLKLQGADKILSEIFEQFRD-K 316
HEM3_PROM0     274 LIKDQHIGN-------------------------------INDPEEIGKELAKKLKQQGAEEILSEIFEKFRE-K 316
HEM3_SYNS3     274 LIRDEQAGP-------------------------------LADPEAVGRDLAHKLKDQGAGEILQEIFEMERG-Q 316
HEM3_SYNPX     274 LIRDEASGS-------------------------------AADPESIGIELAGKLKHQGAGAILKEIFDEVRPEA 317
HEM3_PSEE4     271 LLVADARAP-------------------------------RASAEALGVQVAEDLLSQGAEAILKEVYGEAG-HP 313
HEM3_PSEPF     271 LLNAQARAP-------------------------------RAAAETLGVQVAEDLLSQGADDILKAVYGEAG-HE 313
HEM3_THICR     270 ILRSEARGS-------------------------------QKDPETLGVYVAEQLLEQGAGEILKEVYDQA---- 309
HEM3_BACHD     271 IFKEILRG---------------------------------KDPVQLGETMAQALMDQGAKEVLEQVKKGLE-SQ 311
HEM3_CARHZ     270 VIKGEKEGS-------------------------------IEEPEKVGISLAEELLTKGAQKILEEIRRDAN--E 311
SS                 EEEEEEEEC-------------------------------HHHHHHHHHHHHHHHHHCHHHHHHHCCCCCCCCCC 313
AIP                                                         ddd   x       x
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Fig. 5. Sequence alignment of the C-terminal part of PBGD. Selected vertebrate and bacterial sequences are compared (UniProt IDs and sequence numbering). The degree of conservation is expressed in three grades of background shade. The segment found to be deleted in the PBGD sequence of the AIP patient is highlighted in yellow in the human sequence. SS – secondary structure in the *E.coli* protein: E – β-sheet, H – α-helix (highlighted in deep blue and yellow, respectively, here and in the 3D structure in Fig. 6), C – coil (invisible from position 306 to the end in the crystal structure). AIP – acute intermittent porphyria caused by variants reported in Swiss-Prot database: d – deletion, m – missense mutations G335S, G335D, L343P (highlighted in red here in the secondary structure and 3D structure in Fig. 6b). Note a large, conserved segment of about 30 amino acids between the C-terminal β-sheet strand and C-terminal helix that is present in the vertebrate sequences but not in the bacterial ones (cf. Fig. 6, the segment would be inserted between the deep blue and yellow secondary structure elements in the 3D structure of the vertebrate proteins).

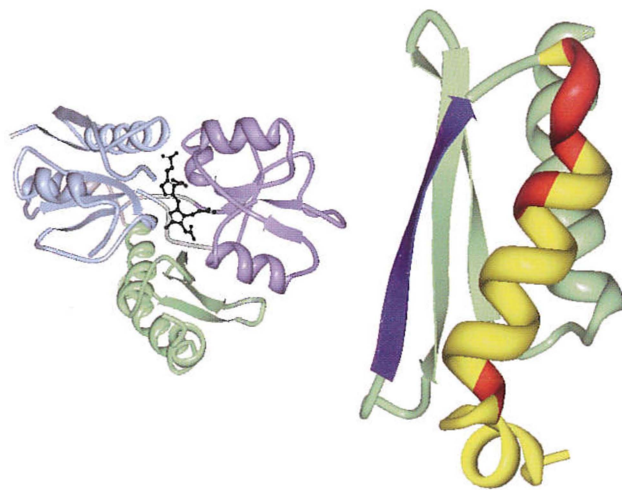

Fig. 6. Three-dimensional structure of *E. coli* porphobilinogen deaminase (PDB ID 1pda).
a) Left panel: Overall structure with N-terminal, central and C-terminal domains coloured in light blue, medium blue and green, respectively, connecting loops in grey and the dipyrromethane cofactor in the active site in black, b) Right panel: C-terminal domain, colour-coding as in Fig. 5.

of the structure and points towards the solvent. In agreement, the mutations in the penultimate helix or its removal destabilize the whole C-terminal domain. If the penultimate helix is missing, the N-terminal domain cannot fold in a stable unity. This will destabilize the whole protein. Even point mutations in the penultimate helix lead to AIP (Fig. 6b), putting in evidence the importance of this helix for the enzyme stability. On the other hand, no mutations have been reported in the C-terminal helix or in the vertebrate-only insert. Not surprisingly, the protein without the penultimate helix is unstable and if the GST in the fusion protein is removed, the protein probably precipitates and cannot be isolated.

The approach taken in this study is novel at several levels. First, it was initiated by a concerned patient who searched for knowledge on web pages. Second, it involved the full scale of finding the relationship between sequence and function. Hence, sequencing of genomic DNA was followed by protein analysis, expression of the mutated protein and measurement of functional activity. This full-scale analysis brought a yet unidentified mutation in the *PBGD* gene. This mutation yields a truncated and inactive protein and provides a mechanistic explanation for the symptoms this patient had. Third, the discovery of this mutation allowed genetic screening of the patient's family, 12 members of which had this mutation and thereby are at risk for AIP acute attack.

In summary, the increasing access to a number of disease- and symptom-oriented web pages presents a new and unusual venue for gaining knowledge and enabling self-diagnosis and self-help. It is, therefore, important that disease-oriented Internet pages for public use should

be designed with clarity and accurate current knowledge-based background. The current study together with the increasing global access to information over the Internet formed the impetus to our initiative of establishing a web page in conjunction with the activity of the European Porphyria Initiative – http://www.porphyria-europe.com (Deybach et al., 2006). We do believe it will add to the knowledge of the professional health-care providers as well as patients.

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