

De Novo Mutation Found in the Porphobilinogen Deaminase Gene in Slovak Acute Intermittent Porphyria Patient: Molecular Biochemical Study

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Summary

The porphyrias are group of mostly inherited disorders in which a specific spectrum of accumulated and excreted porphyrins and heme precursors are associated with characteristic clinical features. There are eight enzymes involved in the heme synthesis and defects in seven of them cause porphyria. Four of them are described as acute hepatic porphyrias, which share possible precipitation of acute attacks with symptoms engaging the nervous system. Acute intermittent porphyria (AIP), caused by partial deficiency of the porphobilinogen deaminase (PBGD), is the most frequent among hepatic porphyrias. Clinical expression is highly variable and ~ 90 % of AIP heterozygotes remain asymptomatic throughout life. During systematic genetic analysis of the AIP patients diagnosed in the Czech and Slovak Republics, we found a special case of AIP. In a 15-year-old boy with abdominal and subsequent neurological symptomatology, we identified *de novo* mutation 966insA within the PBGD gene leading to a stop codon after 36 completely different amino acids compared to the wt-sequence. To establish the effects of this mutation on the protein structure, we expressed mutant constructs with described mutation in *E. coli* and analyzed their biochemical and enzymatic properties. Moreover, computer-assisted protein structure prediction was performed.

Key words

Heme • Acute intermittent porphyria • Porphobilinogen deaminase • Porphyria

Introduction

Heme biosynthesis is one of the most conserved pathways in all living cells through many species (Mauzerall 1998). There are eight enzymes involved and defects in seven of them lead to porphyria.

The porphyrias are group of mostly inherited disorders in which specific spectrum of accumulated and

excreted porphyrins and heme precursors are associated with characteristic clinical features. Porphyrias can be divided into either hepatic or erythropoietic forms with respect to their anatomical origin and into acute or cutaneous forms with respect to their clinical presentation. Four of them are described as acute hepatic porphyrias, which share possible precipitation of the acute attacks. Acute attacks are manifested by a wide variety of clinical

features, including autonomous, central, motor or sensory symptoms, but the most common clinical presentation is the abdominal pain caused by the neurovisceral crises. An acute attack can be provoked by porphyrinogenic drugs such as barbiturates and sulfonamides, determining acute porphyria to be pharmacogenetic (Tschudy *et al.* 1975). Other known precipitants are various factors, including hormonal changes, alcohol, stress, infection and caloric deprivation; attacks are more common in women. Acute attacks can be potentially life-threatening and death may result from the respiratory paralysis (Goldberg 1959).

Acute intermittent porphyria (AIP) is an autosomal dominant inborn disorder (Meyer and Schmid 1978) and is the most frequent among hepatic porphyrias (Grandchamp 1998). The prevalence of symptomatic disease is only 1-2 per 100 000 (Badminton and Elder 2002). Clinical expression is highly variable and ~90 % of AIP heterozygotes remain asymptomatic throughout life (Petrides 1998). Individual gene carriers differ from each other in biochemical and clinical manner. Despite increased understanding of AIP, the mechanism causing symptoms of this disease remains unclear. There are many theories; two of them are on the hot spot of the current investigation – porphyrin precursor neurotoxicity (Brennan *et al.* 1980, Solis *et al.* 2004) and heme deficiency (Yeung Laiwah *et al.* 1987, Kupferschmidt *et al.* 1995, Litman and Correia 1985). Existence of the PBGD knock-out mouse, exhibiting many of the AIP symptoms (Lindberg *et al.* 1996), can greatly improve understanding of the mechanism of the pathophysiology of this disorder.

AIP results from the half-normal activity of the porphobilinogen deaminase enzyme (PBGD, EC 4.3.1.8) (Strand 1970, Meyer *et al.* 1972, Strand *et al.* 1972). PBGD is determined by a single gene located on the chromosome 11 (Meisler *et al.* 1980); assigned the locus to the long arm in the segment 11q23-qter (Wang *et al.* 1981). PBGD gene contains 15 exons and 14 introns and spans approximately 10 kb of DNA (Chretien *et al.* 1988). It has been shown that two distinct promoters generate the housekeeping and erythroid-specific transcripts by alternative splicing (Grandchamp *et al.* 1987, Chretien *et al.* 1988). The housekeeping HMBS transcript contains exons 1 and 3-15, the erythroid HMBS transcript is encoded by exons 2-15 (Chen *et al.* 1994). PBGD is the third enzyme of the heme biosynthetic pathway that catalyzes the step-wise head to tail condensation of four porphobilinogen molecules into the linear tetrapyrrole hydroxymethylbilane. Two PBGD

protein isoforms differ approximately by 2 kDa (40 and 42 kDa) (Grandchamp *et al.* 1987), the housekeeping form consists of 361 amino acids and the erythroid variant of 344 amino acids. The housekeeping isoform contains additional 17 amino acid residues at the N-terminus, but the function of these extra amino acids is not yet known (Grandchamp *et al.* 1987). The three-dimensional structure of PBGD has been defined in *E. coli* by X-ray analysis (Louie *et al.* 1992). The monomeric protein is organized in three domains, approximately equal in size (Louie *et al.* 1996). In the active site, there is dipyrromethane co-factor (Jordan and Warren 1987). The three-dimensional structure of the PBGD from *E. coli* indicates strong conservation of the protein and many amino acid similarities with the human PBGD (Brownlie *et al.* 1994).

To date, around 300 mutations in the PBGD gene have been identified. In this study, we report *de novo* mutation 966insA found within the PBGD gene in a Slovak AIP patient. To establish the effect of this mutation on the protein structure and its function, we expressed mutant protein with the desired mutation in *E. coli* and analyzed its biochemical and enzymatic properties. Moreover, computer-assisted protein structure prediction was performed.

Methods

Patient

A 15-year-old boy was hospitalized and diagnosed as having AIP after his first attack. The diagnosis of AIP was made on the basis of clinical features typical for AIP, including acute abdominal pain, cognition failure, hypertension, tachycardia and subsequent neurological symptomatology. Symptoms were in combination with highly increased urinary ALA and PBG and other porphyrin precursors and hyponatremia (coproporphyrins 1240.5 $\mu\text{mol/l}$ (N: 38-153), uroporphyrins 3298.3 $\mu\text{mol/l}$ (N: 6-24), ALA 1178.2 $\mu\text{mol/l}$ (N :< 42), PBG 331.2 $\mu\text{mol/l}$ (N :< 9). Five other family members out of two generations were without any clinical symptoms. Urinary porphyrin measurements were performed in the family, all subjects except the proband were within the normal concentration range.

Isolation and amplification of DNA

Genomic DNA was extracted from the peripheral blood leucocytes anticoagulated with EDTA

according to the standard protocol. Coding sequences of all exons 1-15 with flanking exon/intron boundaries were amplified. The PCR/DGGE primers were designed as previously reported (Puy *et al.* 1997). The PCR reactions of exon 1-15 were amplified in the total volume of 50 μ l including 1x Plain PP Master Mix (150mM Tris-HCl, pH 8.8, 40mM $(\text{NH}_4)_2\text{SO}_4$, 0.02 % Tween 20, 5mM MgCl_2 , 400 μ M dATP, 400 μ M dCTP, 400 μ M dGTP, 400 μ M dTTP, 100 U/ml Taq DNA polymerase; Top-Bio, Ltd.) and 0.4mM of each primer. Thermal cycling conditions: initial denaturation was performed at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55.8 or 59.3 or 62.9 °C for 30 sec (62.9 °C for exons 1 and 11, 59.3 °C for exons 3 and 5/6, 55.8 °C for the rest of the exons) and elongation at 72 °C for 40 sec, followed by final step 72 °C for 5 min, 95 °C for 5 min and 72 °C for 5 min.

DGGE analysis

Fourteen different PCR products were designed to cover the whole encoding sequence, including ~ 50 bp upstream and downstream of each exon/intron boundary of the PBGD gene (Puy *et al.* 1997). Complete DGGE setup was optimized according to previously reported method (Myers *et al.* 1987). DGGE was performed on linearly increasing denaturing gradient polyacrylamide gels of 35 – 90 % of denaturant (7M urea and 40 % deionized formamide, Bio-Rad). PCR products were analyzed using DCode™ (Bio-Rad). Gels had been run at 60 °C, 150 V for 3.5 hours in 1 x TAE buffer (40mM Tris-HCl, 20mM acetic acid, 1mM EDTA).

DNA sequencing and RFLP

The PCR-amplified double-stranded DNA products were purified from agarose gel using a QIAquick gel extraction kit (Qiagen). Exon 15 was sequenced in both directions on automatic sequencer ABI PRISM 3100/3100-Avant Genetic analyzer (Applied Biosystems) using the ABI PRISM BigDye terminator v 3.1 (Applied Biosystems). Identified mutation in exon 15 caused loss of the restriction site, as confirmed by RFLP. Restriction enzyme digestion was performed using *Mae III* (Roche Diagnostic) according to the manufacturer's instructions.

Plasmid construction and mutagenesis

Total RNA was extracted from the peripheral leukocytes isolated from EDTA-anticoagulated whole venous blood. cDNA sequence was obtained by reverse

transcription (RT-PCR) of the total RNA using oligo(dT) (GE Healthcare) as primer in the first step. Specific cDNA for PBGD with restriction sites *BamH I* and *Xho I* was amplified using specific primers in the second step: cDNA *BamH I* Fw: ata tgg atc cat gtc tgg taa cgg, cDNA *Xho I* Rev: tat act cga gtt aat ggg cat cgt taa. Human cDNA for PBGD was ligated into the pGEX-4T-1 expression vector (GE Healthcare) and transformed into *E. coli BL21 (DE3)* (Stratagene). Plasmid DNA was amplified and isolated using the QIAprep Spin Miniprep Kit (Qiagen). Site-directed mutagenesis of the mutation 966insA was performed with the following mutagenic primers: Fw: 5'gca tca ctg etc gta aac att cca cga ggg c 3' and Rev: 5'gcc ctc gtg gaa tgt tta cga gca gtg atg c 3', using QuikChange® Site-directed Mutagenesis Kit (Stratagene). The results of the mutagenesis were confirmed by sequencing.

Protein expression

All the proteins were expressed as GST-fusion proteins. *BL21* cells were grown at 37 °C in TB medium containing ampicillin (100 μ g/ml, Sigma). 0.05 % of the volume of the overnight-shaked culture was used to inoculate the growth medium. The cells were induced by IPTG (the final concentration of 0.5mM, Sigma) when reached OD at 600 nm between 0.4-0.6. Bacterial culture was grown under aerobic conditions for 4 hours at 30 °C. Bacterial cells were harvested by centrifugation at 4 °C for 10 min at 6000g.

Protein purification

All purification steps were carried out at 4 °C. Washed cells were resuspended in the lysis buffer: PBS (140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 , 1.8mM KH_2PO_4 , pH 7.3), protease inhibitor cocktail (Sigma) and Triton X-100 (0.5 % v/v, Sigma). The cells were lysed by lysozyme (1 mg/ml, ICN Biomedicals) on ice with gentle shaking for 1 hour. The lysate was sonicated 5 x 3 minutes with a 3 min pause in each cycle. Sonicated cells were centrifuged at 4 °C, 33.000g for 30 min. The supernatant was loaded onto the Glutathione Sepharose 4B column (GE Healthcare) and washed three times using the wash buffer (20mM Tris-HCl, 100mM NaCl, 1mM EDTA, 0.5 % Nonidet P-40, pH 8.2). Proteins were eluted in the freshly prepared 50mM Tris-HCl, pH 8.5 buffer with 20 mM glutathione (Sigma). Thrombin digest was performed by gentle shaking of protein mixed with thrombin (ICN Biomedicals) in concentration of 20 U thrombin / 1mg of the protein overnight at 20 °C.

Glycerol was added to the final concentration of 20 % and the aliquots were frozen and stored at -80°C . All results of the protein purification and digestion were confirmed by the SDS-PAGE (Laemmli 1970).

PBGD enzymatic assay

The PBGD activity assay was optimized according to the previously described methods (Erlandsen *et al.* 2000, Brons-Poulsen *et al.* 2005). 1 and 2.5 μg of the protein were diluted by the incubation buffer (50mmol/l Tris, 0.1 % BSA, 0.1 % Triton, pH 8.2) to the final volume of 360 μl . After pre-incubation at 37°C for 3 min, 40 μl of 1mM PBG (ICN Biomedicals) was added and samples were incubated in the dark at 37°C for exactly 1 hour. The reaction was stopped by adding 400 μl of 25 % TCA, (Fluka). Samples were exposed to fotooxidation for 30 min under UV light (350 nm) and then centrifuged for 10 min at 1.500g. To determine enzymatic activity, the fluorescence intensity was measured using 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. The exact concentration was determined at 22°C by measuring absorbance at 405 nm and calculated as A_{405}/ϵ ($\epsilon = 505 \times 10^3 \text{ l cm}^{-1} \text{ mol}^{-1}$). Standard curve in the linear range of fluorescence emission intensity and the concentration of URO I in 12.5 % TCA were created. All measurements were performed in triplicates and the negative control was included. The spectrofluorometer wavelength settings were set up at the maximum excitation of 405 nm and the maximum emission of 599 nm for URO I.

Computer-based structural modeling

For the prediction of the possible impact of the mutation on the protein structure and function, we used the correlations between a three-dimensional structure of human and bacterial *E. coli* (Brownlie *et al.* 1994, Louie *et al.* 1996) and human protein. The amino acid sequence of PBGD corresponding to proband was used to construct a homology model with *E. coli* PBGD as template. We also constructed a homology model with wild-type human PBGD. The resulting models were refined using CNS program to remove bad contacts and inspected using the program O.

Results

The proband was diagnosed at the age of nearly

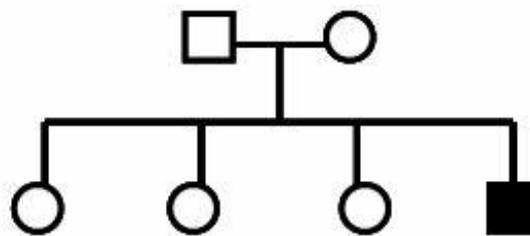


Fig. 1. Pedigree of the Slovak acute intermittent porphyria patient. Affected individual is indicated by solid symbol.

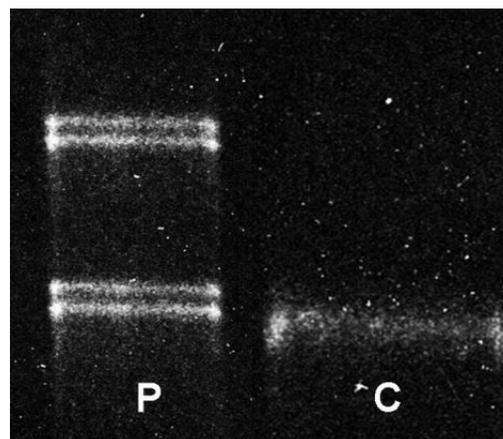


Fig. 2. Denaturing gradient gel electrophoresis (DGGE) based mutation screening of the porphobilinogen deaminase gene. Lane P – patient, lane C – control. The 35 – 90 % DGGE gels had been run at 60°C , 150V for 3.5 hours in 1 x TAE buffer. In case of the heterozygous mutated carrier, there occurs a specific exhibition of three- or four-band pattern. Two lower bands represent the normal and mutated homoduplexes and, the upper ones corresponding to the two types of the normal/mutated heteroduplexes. Only in one fragment of exon 15 did we detect an abnormal four-band pattern suggesting a DNA variation.

15 after he was hospitalized with an acute attack (abdominal and severe neurological symptomatology) after surgery for appendicitis. The patient was the only member of the family known to have AIP symptoms (Fig. 1). No clinical and molecular patterns were found after biochemical and molecular screening of his family. Nonpaternity was excluded by DNA microsatellite analysis. Based on these results, we suggested that 966insA was a *de novo* mutation.

All encoding sequences and exon/intron boundaries of the proband PBGD gene were screened for mutations. The PCR products were subjected to DGGE analysis. The sample with abnormal pattern suggesting certain sequence variation was detected (Fig. 2). This sequence variation was subsequently confirmed by direct sequencing and was further characterized as an insertional point mutation (Fig. 3). Mutation 966insA was located in the exon 15 and leading to formation of STOP

codon after 36 completely different amino acids compared to the original sequence. To further confirm the mutation, we proceeded to RFLP. Pursuant to which the identified mutation caused loss of the restriction endonuclease recognition site. The 317-kb fragment of genomic DNA containing exon 15 and flanking sequence was digested with the restriction endonuclease *Mae III*. In case of the normal homozygous allele control, there were two bands of 80 bp and 197 bp. In case of the mutant allele, three bands of 80 bp and 197 bp and, further, undigested 317 bp fragments appeared thus confirming the existence of the mutation.

To investigate the impact of the mutation on the protein structure and to study further functional consequences, we decided to purify protein expressed from mutated gene in the prokaryotic system. We isolated mRNA from the peripheral leukocytes, produced cDNA of PBGD by reverse transcription and amplified a fragment containing the complete encoding region. This fragment was inserted into prokaryotic expression vector. The cells of recombinant strain *E. coli* BL21 were transformed by this construct. By site-directed mutagenesis we introduced desired mutation into the construct. The accuracy and absence of additional base changes were confirmed by direct DNA sequencing.

To determine optimal growth conditions for high-level production of mutant PBGD, we proposed three different temperatures levels and expression durations. The decrease of the temperature during protein expression did not show any impact on the protein stability or solubility. As expected, in the case of the normal enzyme the purified protein product showed single homogeneous band on SDS-PAGE. In the case of mutant protein several bands appeared on SDS-PAGE suggesting low protein stability (Fig. 4). The amounts of proteins displayed in each lane of the SDS-PAGE gel were adjusted to be identical (the same volume of affinity beads as well as elution buffer) for the normal and mutant enzyme to reflect the potential of mutant protein expression and its solubility. PBGD enzymes of the normal as well as of the mutant were similar in size having approximately Mr 68 kDa with GST-tag and Mr 42 kDa without GST-tag.

Specific activities were estimated to be 1677.9 ± 75 U/mg for the normal PBGD (U/mg = nmol URO I/hr/mg of protein), 1320.1 ± 55 U/mg for the normal PBGD expressed in fusion with GST, 2.763 ± 0.136 U/mg for the mutant PBGD and 2.334 ± 0.129 U/mg for the mutant PBGD expressed in fusion with GST, when

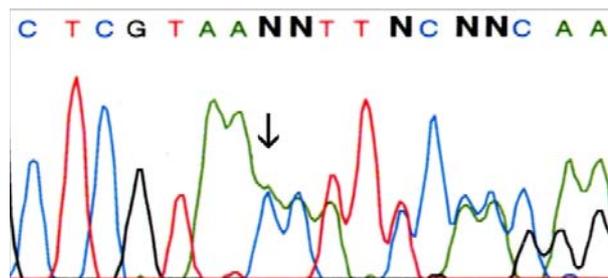


Fig. 3. Mutation detected in the porphobilinogen deaminase gene of a Slovak acute intermittent porphyria patient by sequencing analysis. The mutation 966insA was localized in the exon 15 and this single-base insertion was further identified as the causative mutation.

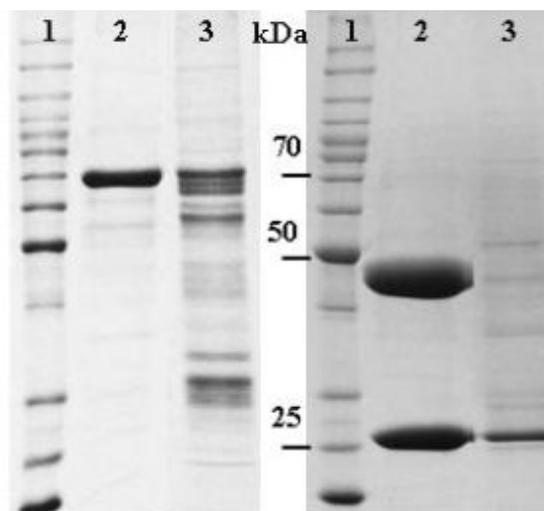


Fig. 4. SDS/PAGE analysis of the porphobilinogen deaminase. Left: GST-fusion purified enzymes. Right: Thrombin digested purified enzymes. Lane 1 – Marker, lane 2 – wild-type protein, lane 3 – mutated protein. PBGD enzymes of the normal as well as of the mutant were similar in size having approximately Mr 68 kDa with GST-tag and Mr 42 kDa without GST-tag. The amount of protein displayed 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 the normal and mutant enzyme to reflect the potential of mutant protein expression and its solubility.

measured at pH 8.2. The purified mutant enzyme had a relative activity 0.18 % of the average level expressed by the normal allele. We concluded that there was a significant difference in the specific and relative activity between the thrombin digested protein and the fusion protein. Fusion protein exhibited approximately 22 % higher activity than the thrombin digested enzyme.

Using the computer-assisted structure prediction, we designed the mutant protein structure. Due to the truncation, two helices α_2_3 and α_3_3 of the domain 3 were missing at the mutant protein in comparison with the normal protein (Fig. 5).

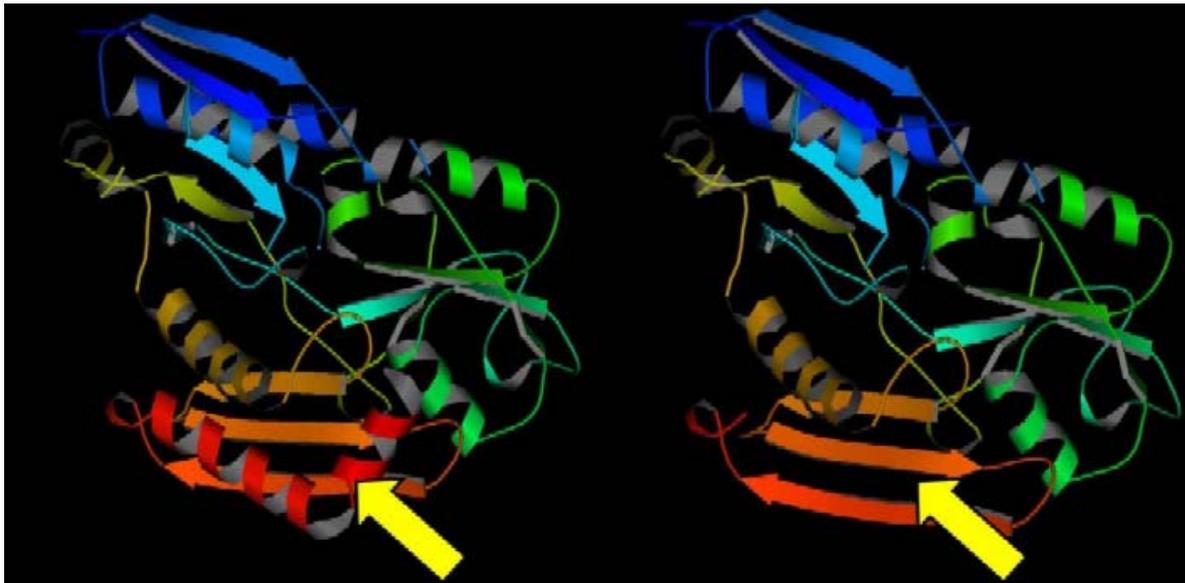


Fig. 5. Computer-based con-structured homology model with wild-type human PBGD (wild-type PBGD on the left side, mutant PBGD on the right side). This structural model shows small insertion 966insA, which resulted in the frameshift leading to the premature truncation. The truncated mutant protein consists of 357 amino acids as opposed to the normal 361 amino acids. The mutant protein includes 36 amino acids different from the normal, lacks 4 amino acids from the C-terminus and part of the third enzyme domain takes up a different shape formation. The premature termination of the mutant protein leads to the loss of protective helices as indicated by yellow arrows.

Discussion

The identification and characterization of the novel mutations within PBGD gene of the newly diagnosed AIP patients emphasize insight into the molecular heterogeneity of AIP. Accurate molecular diagnosis and detection of asymptomatic carriers in affected families helps them prevent potential precipitating factors. Investigation of the effects of the present mutation on the protein structure and its function provide further understanding of the molecular basis and cooperation of the molecules in the system.

PBGD is the third enzyme of the heme biosynthetic pathway and catalyzes the step-wise head to tail condensation of four porphobilinogen molecules into the linear tetrapyrrole hydroxymethylbilane. In AIP patients, the half-normal activity of PBGD results in the accumulation of high amount of porphyrin precursors as ALA and PBG (Strand 1970, Meyer *et al.* 1972, Strand *et al.* 1972). The accumulation leads to the potentially life-threatening state of acute attacks.

A great step forward was the isolation and characterization of the human PBGD cDNA and genomic sequence (Grandchamp 1987, Yoo *et al.* 1993). This resulted in a rapid progress in molecular diagnosis enabling us to employ several DNA variation screening methods for the accurate identification of mutation AIP

carriers. These methods include denaturing gradient gel electrophoresis DGGE and direct sequencing. On the DGGE gels, migration of the homozygous normal subject alleles gives a single-band specific for the normal homoduplex. In case of the heterozygous mutated carrier, there occurs a specific exhibition of three- or four-band pattern. DGGE detected sequence variations might be either disease causing the mutation or a neutral polymorphism. DGGE of the entire coding sequence including exons and exon/intron boundaries was performed. Only in one fragment of exon 15, we detected an abnormal four-band pattern suggesting a DNA variation. We proceeded to sequencing this fragment in both directions and we identified a single-base insertion 966insA as the causative mutation. In exon 15, together with exons 10 and 12, high mutation frequency within PBGD gene in AIP patients was detected (Gu *et al.* 1992, Gu *et al.* 1993a, Mgone *et al.* 1993, Grandchamp *et al.* 1996).

The deciphering of the structure of the PBGD enzyme had the same impact on the protein level. The three-dimensional structure of PBGD has been defined in *E. coli* by X-ray analysis (Louie *et al.* 1992). It has been shown that this monomeric protein is organized in three domains, approximately equal in size (Louie *et al.* 1996). The x-ray structure and results from the site-directed mutagenesis provided evidence for a single catalytic site

(Louie *et al.* 1992). In the active site, there is a dipyrromethane co-factor (Jordan and Warren 1987). The active site is located between domains 1 and 2 and, the dipyrromethane cofactor is covalently linked to a flexible loop of side-chain of cysteine 242 residue (Hart *et al.* 1988, Jordan and Warren 1987). Other important interactions in the process of tetrapyrrol formation are hydrogen-bonds and salt-bridges that are formed between its acetate and propionate side-groups and the polypeptide chain, which are supposed to anchor the cofactor within the active site cleft (Louie *et al.* 1996). Secondary structure of domains 1 and 2 is a modified doubly wound parallel beta-sheet which has a similar overall topology. Domain 3 is an open-faced three-stranded antiparallel beta-sheet, with one face covered by three alpha-helices (Louie *et al.* 1996, Lambert *et al.* 1994). The three-dimensional structure of the PBGD from *E. coli* shows approximately 45 % amino acid sequence identity and nearly 80 % of the amino acids are structurally related to human PBGD (Brownlie *et al.* 1994). Small insertion 966insA resulted in the frameshift leading to the incorporation of 36 completely different residues and to premature truncation. Usually, the truncation would lead to an unstable and inactive protein. Truncated proteins are likely to be rapidly degraded by proteasome. The truncated mutant protein consists of 357 amino acids as opposed to the normal 361 amino acids. The mutant protein includes 36 amino acids different from the normal, lacks 4 amino acids from the C-terminus and, part of the third enzyme domain takes up a different shape formation. The mutation is located in the β_3 sheet; two helices α_2 and α_3 of the domain 3 are completely missing compared to the normal protein. In the wild-type protein, the C-terminal helices protect the beta-strands from being exposed to solvent. This would be expected to expose the beta-strand core that could make the mutant protein prone to aggregation. In agreement with this prediction, stability of the mutant PBGD was severely impaired as we detected from SDS-PAGE results. During protein expression, high precipitation occurred, which decreased the amount of the soluble recombinant protein. We presumed that the premature truncation would cause a loss of enzymatic activity. We found that there was a

significant difference in the specific and relative activity between the thrombin digested and the fusion protein. Fusion protein exhibited approximately 22 % higher activity than the thrombin digested enzyme. This observation was in agreement with another study (Brons-Poulsen *et al.* 2005) and suggests that due to GST tag presence in this case, there is no correlation between steric barrier and lower substrate accessibility. The residual activity was extremely low, about 0.18 % of the normal protein activity, corresponding to the massive breakdown of the protein; therefore, kinetic studies of this mutant were not suitable.

Currently, around 300 mutations in the PBGD gene are known. Such known mutations include one promoter region mutation (Whatley *et al.* 2000), missense, nonsense, splicing and frame-shift mutations and in-frame deletion and insertion as well. Mutations are equally distributed among the PBGD gene and no prevalent site for mutation has been identified. In Czech and Slovak patients, 7 different mutations: G24S, R26C, R26H, 158insA, G111R, IVS 12 771+1 and V267M has been found (Rosipal *et al.* 1997, Kauppinen *et al.* 1995, Gu *et al.* 1993b, Llewellyn *et al.* 1993, Puy *et al.* 1997). 966insA is the first mutation found in the exon 15 in this population and indicates that AIP is a heterogeneous disease at the molecular level.

In summary, one *de novo* mutation was found in the Slavic population. Due to truncated protein sequence with an abnormal C-terminus, this small insertion mutation leads to an almost complete loss of the enzymatic function and decreases the stability of the protein structure. The probability of the life-threatening porphyric attack in AIP is a significant personal burden and poses a challenge for counseling and medical management. Introduction of molecular biology techniques to diagnostics increases our chances of more effective treatment of AIP.

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References

- BADMINTON MN, ELDER GH: Management of acute and cutaneous porphyrias. *Int J Clin Pract* **56**: 272-278, 2002.
BRENNAN MJW, CANTRILL RC, KRAMER S: Effect of δ -aminolevulinic acid on GABA receptor binding in synaptic plasma membranes. *Int J Biochem.* **12**: 833-835, 1980.

- BRONS-POULSEN J, CHRISTIANSEN L, PETERSEN NE, HORDER M, KRISTIANSEN K: Characterization of two isoalleles and three mutations in both isoforms of purified recombinant human porphobilinogen deaminase. *Scand J Clin Lab Invest* **65**: 93-105, 2005.
- BROWNLIE PD, LAMBERT R, LOUIE GV, JORDAN PM, BLUNDELL TL, WARREN MJ, COOPER JB, WOOD SP: The three-dimensional structures of mutants of porphobilinogen deaminase: Towards an understanding of structural basis of acute intermittent porphyria. *Prot Sci* **3**: 1644-1650, 1994.
- CHEN CH, ASTRIN KH, LEE G, ANDERSON KE, DESNICK RJ: Acute intermittent porphyria: identification and expression of exonic mutations in the hydroxymethylbilane synthase gene: an initiation codon missense mutation in the housekeeping transcript causes 'variant acute intermittent porphyria' with normal expression of the erythroid-specific enzyme. *J Clin Invest* **94**: 1927-1937, 1994.
- CHRETIEN S, DUBART A, BEAUPAIN D, RAICH N, GRANDCHAMP B, ROSA J, GOOSSENS M, ROMEO P-H: Alternative transcription and splicing of the human porphobilinogen deaminase gene result either in tissue-specific or in housekeeping expression. *Proc Nat Acad Sci* **85**: 6-10, 1988.
- ERLANDSEN EJ, JORGENSEN PE, MARKUSSEN S, BROCK A: Determination of porphobilinogen deaminase activity in human erythrocytes: pertinent factors in obtaining optimal conditions for measurements. *Scand J Clin Lab Invest* **60**: 627-634, 2000.
- GOLDBERG A: Acute intermittent porphyria. *Quart J Med* **28**: 183-209, 1959.
- GRANDCHAMP B, DE VERNEUIL H, BEAUMONT C, CHRETIEN S, WALTER O, NORDMANN Y: Tissue-specific expression of porphobilinogen deaminase: two isoenzymes from a single gene. *Europ J Biochem* **162**: 105-110, 1987.
- GRANDCHAMP B, PUY H, LAMORIL J, DEYBACH JC, NORDMANN Y: Review: molecular pathogenesis of hepatic acute porphyrias. *J Gastroenterol Hepatol* **11**: 1046-5210, 1996.
- GRANDCHAMP B: Acute intermittent porphyria. *Semin Liver Dis* **18**: 17-24, 1998.
- GU XF, DE ROOIJ F, VOORTMAN G, TE VELDE K, NORDMANN Y, GRANDCHAMP B: High frequency of mutations in exon 10 of the porphobilinogen deaminase gene in patients with a CRIM-positive subtype of acute intermittent porphyria. *Am J Hum Genet* **51**: 660-665, 1992.
- GU XF, DE ROOIJ JS, LEE F, TE VELDE K, DEYBACH JC, NORDMANN Y, GRANDCHAMP B: High prevalence of a point mutation in the porphobilinogen deaminase gene in Dutch patients with acute intermittent porphyria. *Hum Genet* **91**: 128-130, 1993a.
- GU XF, DE ROOIJ F, DE BAAR E, BRUYLAND M, LISSENS W, NORMANN Y, GRANDCHAMP B: Two novel mutations of the porphobilinogen deaminase gene in acute intermittent porphyria. *Hum Mol Gen* **2**: 1735-1736, 1993b.
- HART GJ, MILLER AD, BATTERSBY AR: Evidence that the pyrromethane cofactor of hydroxymethylbilane synthase (porphobilinogen deaminase) is bound through the sulphur atom of a cysteine residue. *Biochem J* **252**: 909-912, 1988.
- JORDAN PM AND WARREN MJ: Evidence for a dipyrromethane cofactor at the catalytic site of E. coli porphobilinogen deaminase. *FEBS Lett* **225**: 87-92, 1987.
- KAUPPINEN R, MUSTAJOKI S, PIHLAJA H, PELTONEN L, MUSTAJOKI P: Acute intermittent porphyria in Finland: 19 mutations in the porphobilinogen deaminase gene. *Hum Molec Genet* **4**: 215-222, 1995.
- KUPFERSCHMIDT H, BONT A, SCHNORF H, LANDIS T, WALTER E, PETER J, KRÄHENBÜHL S, MEIER PJ: Transient cortical blindness and bioccipital brain lesions in two patients with acute intermittent porphyria. *Ann. Intern. Med* **123**: 598-600, 1995.
- LAEMMLI UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685, 1970.
- LAMBERT R, BROWNLIE PD, WOODCOCK SC, LOUIE GV, COOPER JC, WARREN MJ, JORDAN PM, BLUNDELL TL, WOOD SP: Structural studies on porphobilinogen deaminase. *Ciba Foundation Symp* **180**: 97-110, 1994.

- LINDBERG RLP, PORCHER C, GRANDCHAMP B, LEDERMANN B, BURKI K, BRANDNER S, AGUZZI A, MEYER UA: Porphobilinogen deaminase deficiency in mice causes a neuropathy resembling that of human hepatic porphyria. *Nature Genet* **12**: 195-199, 1996.
- LITMAN DA, CORREIA MA: Elevated brain tryptophan and enhanced 5-hydroxytryptamine turnover in acute hepatic heme deficiency: Clinical implication. *J Pharm Exp Ther* **232**: 337-345, 1985.
- LLEWELLYN DH, WHATLEY S, ELDER GH: Acute intermittent porphyria caused by an arginine to histidine substitution (R26H) in the cofactor binding cleft of porphobilinogen deaminase. *Hum Mol Genet* **2**: 1315-1316, 1993.
- LOUIE GV, BROWNLIE PD, LAMBERT R, COOPER JB, BLUNDELL TL, WOOD SP, WARREN MJ, WOODCOCK SC, JORDAN PM: Structure of porphobilinogen deaminase reveals a flexible multidomain polymerase with a single catalytic site. *Nature* **359**: 33-39, 1992.
- LOUIE GV, BROWNLIE PD, LAMBERT R, COOPER JB, BLUNDELL TL, WOOD SP, MALASHEVICH VN, HADENER A, WARREN MJ, SCHOOLINGIN-JORDAN PM: The three-dimensional structure of Escherichia coli porphobilinogen deaminase at 1.76-A resolution. *Proteins* **25**: 48-78, 1996.
- MAUZERALL DC: Evolution of porphyrins. *Clin Dermatol* **16**: 195-201, 1998.
- MEISLER MH, WANNER LA, EDDY RE, SHOWS TH: Uroporphyrinogen I synthase: chromosomal linkage and isozyme expression in human-mouse hybrid cells. (Abstract) *Am J Hum Genet* **32**: 47A only, 1980.
- MEYER UA, STRAND LJ, DOSS M, REES AC, MARVER HS: Intermittent acute porphyria--demonstration of a genetic defect in porphobilinogen metabolism. *New Eng J Med* **286**: 1277-1282, 1972.
- MEYER UA, SCHMID R: The porphyrias. In: STANBURY JB, WYNGAARDEN JB, FREDRICKSON DS: *The Metabolic Basis of Inherited Disease*. McGraw-Hill, New York, pp 1166-1220, 1978.
- MGONE CS, LANYON WG, MOORE MR, LOUIE GV, CONNOR JM: Detection of a high mutation frequency in exon 12 of the porphobilinogen deaminase gene in patients with acute intermittent porphyria. *Hum Genet* **92**: 619-622, 1993.
- MYERS RM, MANIATIS T, LERMAN LS: detection and localization of single base changes by denaturing gradient gel electrophoresis. *Methods Enzymol* **155**: 501-527, 1987.
- PETRIDES PE: Acute intermittent porphyria: mutation analysis and identification of gene carriers in a German kindred by PCR-DGGE analysis. *Skin Pharmacol Appl Skin Physiol* **11**: 374-380, 1998.
- PUY H, DEYBACH JC, LAMORIL J, ROBREAU AM, DA SILVA V, GOUYA L, GRANDCHAMP B, NORDMANN Y: Molecular epidemiology and diagnosis of PBG deaminase gene defects in acute intermittent porphyria. *Am J Hum Genet* **60**: 1373-1383, 1997.
- ROSIPAL R, PUY H, LAMORIL J, MARTASEK P, NORDMANN Y, DEYBACH JC: Molecular analysis of porphobilinogen (PBG) deaminase gene mutations in acute intermittent porphyria: first study in patients of Slavic origin. *Scand J Lab Invest* **57**: 217-224, 1997.
- SOLIS C, MARTINEZ-BERMEJO A, NAIDICH TP, KAUFMANN WE, ASTRIN KH, BISHOP DF, DESNICK RJ: Acute intermittent porphyria: studies of the severe homozygous dominant disease provide insights into the neurologic attacks in acute porphyrias. *Arch Neurol* **61**: 1764-1770, 2004.
- STRAND LJ, FELSHER BF, REDEKER AG, MARVER HS: Heme biosynthesis in intermittent acute porphyria: decreased hepatic conversion of porphobilinogen to porphyrins and increased delta aminolevulinic acid synthetase activity. *Proc Nat Acad Sci* **67**: 1315-1320, 1970.
- STRAND LJ, MEYER UA, FELSHER BF, REDEKER AG, MARVER HS: Decreased red cell uroporphyrinogen I synthetase activity in intermittent acute porphyria. *J Clin Invest* **51**: 2530-2536, 1972.
- TSCHUDY DP, VALSAMIS M, MAGNUSSEN CR: Acute intermittent porphyria: clinical and selected research aspects. *Ann Intern Med* **83**: 851-864, 1975.
- WANG A-L, ARREDONDO-VEGA FX, GIAMPIETRO PF, SMITH M, ANDERSON WF, DESNICK RJ: Regional gene assignment of human porphobilinogen deaminase and esterase A(4) to chromosome 11q23-11qter. *Proc Nat Acad Sci* **78**: 5734-5738, 1981.

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- WHATLEY SD, ROBERTS AG, LLEWELLYN DH, BENNETT CP, GARRETT C, ELDER GH: Non-erythroid form of acute intermittent porphyria caused by promoter and frameshift mutations distant from the coding sequence of exon 1 of the HMBS gene. *Hum Genet* **107**: 243-248, 2000.
- YEUNG LAIWAH AC, MOORE MR, GOLDBERG A: Pathogenesis of acute porphyria. *Quart J Med* **63**: 377-392, 1987.
- YOO HW, WARNER CA, CHEN CHH, DESNICK RJ: Hydroxymethylbilane synthase: complete genomic sequence and amplifiable polymorphisms in the human gene. *Genomics* **15**: 21-29, 1993.
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