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Molecular Genetics of Variegate Porphyria in Finland

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Academic dissertation

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to my family

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Summary

Variegate porphyria (VP) is an inherited metabolic disease resulting from the partial deficiency of protoporphyrinogen oxidase (PPOX), the penultimate enzyme in the heme biosynthetic pathway. Biochemical abnormalities found in VP patients include overproduction and increased excretion of porphyrins and porphyrin precursors. Clinical manifestations include acute neurovisceral attacks and cutaneous photosensitivity resembling other acute porphyrias.

In the present study, eight mutations (I12T, 78insC, IVS2-2a→c, 338G→C, R152C, 470A→C, 609delG and L401F) were identified among 22 Finnish VP families by direct sequencing of the patients' genomic DNA. A founder mutation (R152C) was detected in 11 families. The outcome of seven mutations was studied by amplification and sequencing of the reverse-transcribed total RNA obtained from the patients' EBV-transformed lymphoblast cell lines. One of the mutations (338G→C) showed a decrease in the steady-state level of the mutant transcript in the patient's lymphocytes compared with that of the normal allele. For the other six mutations studied, no reduction of mRNA level could be demonstrated. The normal allele and six mutants were expressed in *E. coli* and in COS-1-cells. Enzyme activities of all mutated alleles I12T, 78insC, IVS2-2a→c, 338G→C, R152C and 470A→C were decreased to 0-5% of the wild-type activity both in *E. coli* and COS-1 cells, confirming the causality of the mutations and the enzymatic defects in this disease.

The mitochondrial targeting of the normal allele and seven PPOX mutants was investigated by constructing the corresponding green fluorescent protein (GFP) -fusion proteins and studying their intracellular localization in COS-1 cells. The 28 amino-terminal amino acids of PPOX contained an independently functioning mitochondrial targeting signal. The experiments with amino-terminally truncated GFP-fusion peptides revealed that amino acids 25-477 of PPOX contained at least one additional mitochondrial targeting signal. A structural model for the interaction between the amino-terminal end of PPOX and the putative mitochondrial receptor protein Tom20 was constructed. The model suggested that the leucine and isoleucine residues Leu8, Ile12 and Leu15 forming the hydrophobic motif L*XXX*I*XX*L were crucial for the recognition of the targeting signal. Mutants L8Q, I12T and L15Q, which disrupted the hydrophobic face of the L*XXX*I*XX*L helix, tested the validity of the model. Results from the *in vitro* expression studies and molecular modelling were in accordance, supporting the hypothesis that the recognition of mitochondrial targeting signal is dependent on hydrophobic interactions between the targeting peptide and the mitochondrial receptor.

The clinical and biochemical outcome of 103 Finnish VP patients diagnosed between 1966 and 2001 was evaluated. Fifty-two percent of patients had experienced clinical symptoms: 40% had photosensitivity, 27% acute attacks and 14% both manifestations. The proportion of patients with acute attacks had decreased

dramatically from 38% to 14% in patients diagnosed before and after 1980, while the prevalence of skin symptoms had decreased only from 45% to 34%. The correlation between *PPOX* genotype and clinical outcome was studied among 90 patients with the three most common Finnish mutations I12T, R152C and 338G→C. Patients with the I12T mutation experienced no photosensitivity and acute attacks were rare (8%). Therefore, the occurrence of photosensitivity was lower in the I12T group than in the R152C group (p=0.001), while no significant differences between the R152C and 338G→C groups could be observed. Biochemical abnormalities were significantly milder, suggesting a milder form of the disease in patients with the I12T mutation. In all VP patients, normal excretion of faecal protoporphyrin measured in adulthood predicted freedom from both skin symptoms and acute attacks. The most valuable test predicting an increased risk of symptoms was urinary coproporphyrin, but only a substantially increased excretion exceeding 1000 nmol/d was associated with an increased risk of skin symptoms and acute attacks. All patients with an excretion of more than 1000 nmol/d experienced skin symptoms, acute attacks, or both.

As a result of this study, DNA diagnostics of VP is now available for 20 of the 22 known Finnish VP families, where the mutation was detected. The sensitivity and specificity of the biochemical screening for VP were studied among 38 family members, for whom the results of both biochemical testing and mutation analysis were available. The sensitivity of urinary and faecal coproporphyrin analysis was 48% and 52%, respectively. The sensitivity of urinary uroporphyrin analysis was 71% and that for faecal protoporphyrin was 77%. Plasma fluorescence was sensitive in symptomatic patients even in remission but resulted in false-negatives in four symptom-free patients with normal excretion of porphyrins in urine. In our series of mutation screening, new symptom-free patients could be identified, demonstrating that DNA analysis is the only reliable method to screen symptom-free patients in remission.

Original publications

This thesis is based on the following original publications, which will be referred to in the text by the Roman numerals I to V.

I von und zu Fraunberg M, Kauppinen R. Diagnosis of variegate porphyria - hard to get? *Scandinavian Journal of Clinical and Laboratory Investigation* 2000; 60: 605-610.

II Kauppinen R, Timonen K, von und zu Fraunberg M, Laitinen E, Ahola H, Tenhunen R, Taketani S, Mustajoki P. Homozygous variegate porphyria: 20 y follow-up and characterization of molecular defect. *Journal of Investigative Dermatology* 2001; 116(4): 610-613.

III von und zu Fraunberg M, Tenhunen R, Kauppinen R. Expression and characterization of six mutations in the protoporphyrinogen oxidase gene among Finnish variegate porphyria patients. *Molecular Medicine* 2001; 7(5): 320-328.

IV von und zu Fraunberg M, Timonen K, Mustajoki P, Kauppinen R. Clinical and biochemical characteristics and genotype-phenotype correlation in Finnish variegate porphyria patients. *European Journal of Human Genetics* 2002; 10: 649-657.

V von und zu Fraunberg M, Nyrönen T, Kauppinen R. Mitochondrial targeting of normal and mutant protoporphyrinogen oxidase. *Journal of Biological Chemistry* (in press).

In addition, some unpublished data are presented.

Abbreviations

aa	amino acid
AIP	acute intermittent porphyria
ALA	5-aminolevulinic acid
ALAS	5-aminolevulinic acid synthase
ALAS1	housekeeping 5-aminolevulinic acid synthase
ALAS2	erythroid-specific 5-aminolevulinic acid synthase
bp	base pair
cDNA	complementary deoxyribonucleic acid
COS-1	simian virus 40-transformed monkey kidney cell line
CYP	cytochrome P-450
DNA	deoxyribonucleic acid
GFP	green fluorescent protein
kb	kilobase
kD	kilodalton
mRNA	messenger ribonucleic acid
PBG	porphobilinogen
PBGD	porphobilinogen deaminase
PCR	polymerase chain reaction
PPOX	protoporphyrinogen oxidase
RNA	ribonucleic acid
VP	variegate porphyria

Amino acids are abbreviated by either one or three letter codes and nucleotides by one letter code.

Introduction

The porphyrias are inherited disorders caused by a partial deficiency of one of the eight enzymes in the heme biosynthetic pathway. Heme is synthesised in most if not all cells and is an important constituent of the cellular hemoproteins responsible for such essential functions as the transport and storage of oxygen (e.g. hemoglobin, myoglobin), electron transport (e.g. respiratory cytochromes) and oxidation-reduction reactions (e.g. the cytochrome P450 enzymes). Heme is also utilized for other important cellular hemoproteins, such as catalase, peroxidase, tryptophan pyrrolase, prostaglandin endoperoxide synthase, indoleamine 2,3-dioxygenase and guanylate cyclase.

Variegate porphyria (VP) results from a defect in protoporphyrinogen oxidase (PPOX), the penultimate enzyme in heme biosynthesis, which catalyses the oxidation of protoporphyrinogen IX to protoporphyrin IX in the inner membrane of the mitochondrion. The biochemical abnormalities found in VP patients include overproduction and increased excretion of porphyrins and porphyrin precursors. VP can present with acute neurovisceral symptoms, photosensitivity, or both. The acute symptoms are indistinguishable from other acute porphyrias and include abdominal pain, vomiting, constipation, hypertension, tachycardia and peripheral neuropathy. Skin manifestations, which include increased skin fragility, vesicles, bullae, erosions, milia, hyperpigmentation and hypertrichosis in sun-exposed areas, manifest independently of acute attacks.

The clinical and biochemical picture of VP was first described in 1937. Despite intensive research in the field of porphyrias since the establishment of the heme biosynthetic pathway in the 1950s, the pathogenetic mechanisms of VP, together with other porphyrias, are poorly understood. The development of recombinant DNA techniques in the 1980s and the cloning of the *PPOX* gene in the 1990s have facilitated research of the disease mechanisms at the molecular level. The aim of this study was to identify the gene defects in Finnish VP families and develop methods for DNA diagnostics in these patients. The effects of the mutations on mitochondrial transport and enzymatic function of PPOX were studied. The clinical and biochemical outcome of patients was investigated based on follow-up from 1966 to 2001, and the correlation between *PPOX* genotype and VP phenotype was evaluated.

Review of the literature

1. Heme biosynthesis

The biosynthesis of one molecule of heme requires eight molecules of glycine and eight molecules of succinyl CoA. The first and last three enzymes in the heme biosynthetic pathway are located in the mitochondrion, whereas the other four are in the cytosol (Figure 1). The first enzyme, 5-aminolevulinic acid synthase (ALAS), catalyses the condensation of glycine, activated by pyridoxal phosphate and succinyl coenzyme A, to form aminolevulinic acid (ALA). In the second step, aminolevulinate dehydratase catalyses the condensation of two molecules of ALA to form porphobilinogen (PBG).

Porphobilinogen deaminase (PBGD, also known as hydroxymethylbilane synthase or uroporphyrinogen-I synthase) catalyses the head-to-tail condensation of four PBG molecules by a series of deaminations to form the linear tetrapyrrole hydroxymethylbilane (HMB). Uroporphyrinogen-III synthase catalyses the rearrangement and rapid cyclization of HMB to form the asymmetric octacarboxylate porphyrinogen uroporphyrinogen III. In the absence of uroporphyrinogen-III synthase, HMB may non-enzymatically close to form uroporphyrinogen I, which is normally present in only minute amounts and cannot convert to heme.

The fifth enzyme in the pathway, uroporphyrinogen decarboxylase catalyses the sequential removal of the four carboxylic groups of the acetic acid side chains in uroporphyrinogen to yield coproporphyrinogen. This compound then enters the mitochondrion, where coproporphyrinogen oxidase catalyses the decarboxylation of two of the four propionic groups in the presence of oxygen to form the two vinyl groups of protoporphyrinogen IX.

In the seventh step, protoporphyrinogen oxidase (PPOX, [E.C.1.3.3.4.]) oxidizes protoporphyrinogen IX to protoporphyrin IX by the removal of six hydrogen atoms. Protoporphyrinogen oxidation also occurs non-enzymatically at an appreciable rate. Finally, ferrous iron (Fe^{2+}) is inserted into protoporphyrin IX to form heme in a reaction catalysed by ferrochelatase (also known as heme synthase).

About 85% of the heme is synthesized in erythroid cells, where the majority is used for hemoglobin formation (Berk et al. 1976). Most of the remainder is produced in the liver, where 65% is utilized for the formation of the microsomal cytochrome P450 enzymes, 8% for the formation of cytochrome b_5, about 15% for the synthesis of peroxisomal catalase and 6% for other mitochondrial cytochromes (Sassa and Kappas 1981). The heme biosynthesis is regulated by housekeeping and erythroid-specific ALAS isozymes ALAS1 and ALAS2, respectively, which are encoded by separate genes (Bishop 1990). In the liver, heme represses the synthesis of ALAS1 mRNA at both transcriptional and translational levels (Hamilton et al. 1991) and inhibits its transfer from the cytosol into mitochondria (Ades and Harpe 1981). The

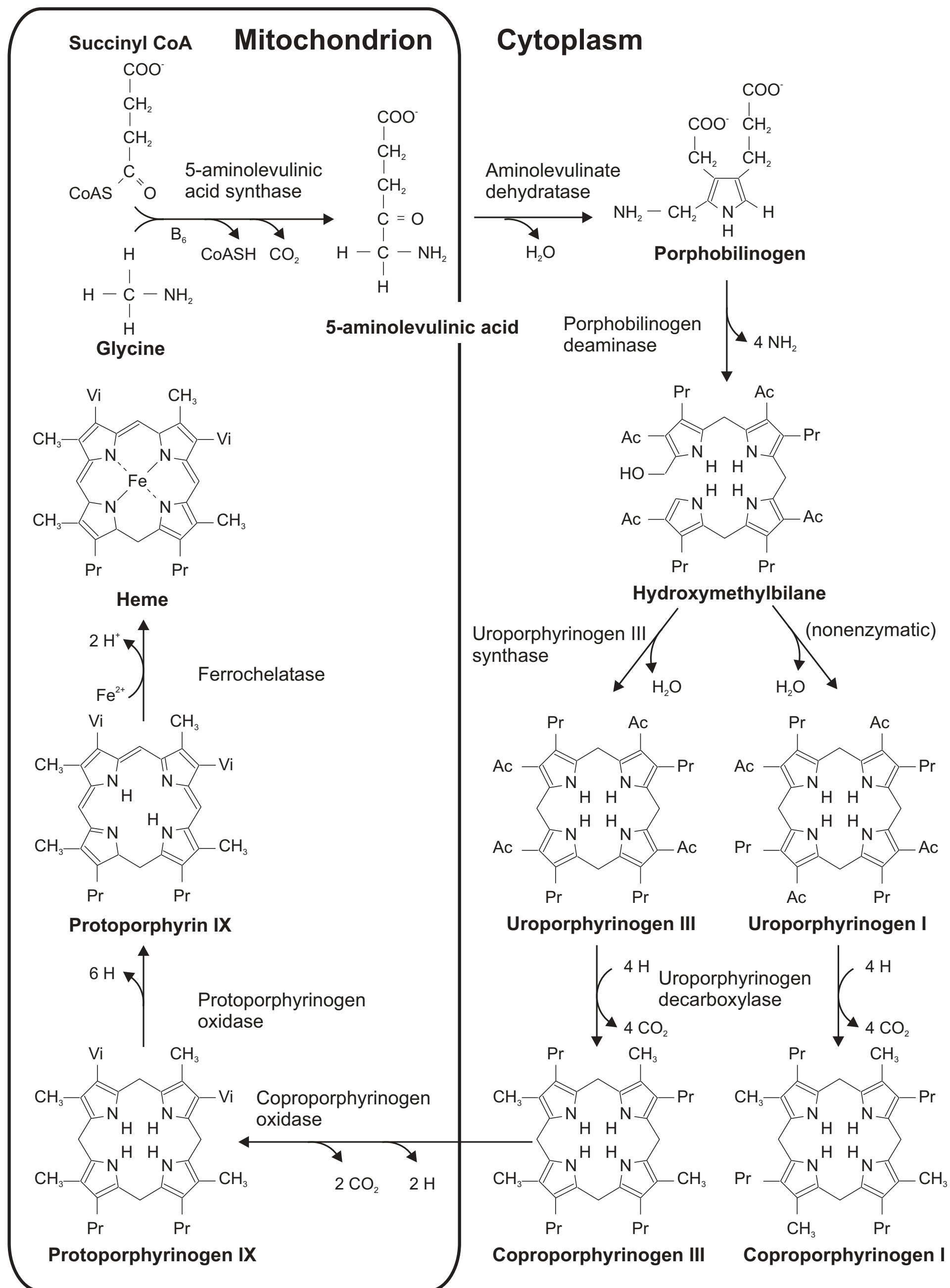


Figure 1. The human heme biosynthetic pathway. Ac=-CH_2COOH; Pr=-CH_2CH_2COOH; Vi=-$CH=CH_2$.

activity of this enzyme can be induced by various drugs, alcohol, steroids and many other chemicals, and reduced by e.g. glucose.

In erythroid cells, the regulation of heme synthesis differs from that in the hepatic cells. ALAS2 is not inducible by drugs that induce ALAS1 (Wada et al. 1967), and it is not regulated by feedback repression by heme (Sassa 1976; Fujita et al. 1991). Unlike in the liver, heme stimulates hemoglobin synthesis in erythroid cells (Ross and Sautner 1976; Dabney and Beaudet 1977) and increases the levels of ALAS2, aminolevulinate dehydratase, PBGD and Fe incorporation into heme (Granick and Sassa 1978). Ferrochelatase may also play a role in controlling the rate of heme formation in erythroid cells and become rate-limiting in these cells when the enzyme is partially defective (Anderson et al. 2001).

Heme degradation starts with the reductive breakdown of the heme macrocycle into carbon monoxide, Fe^{2+} and biliverdin in a reaction catalysed by heme oxygenase (Tenhunen et al. 1968). Heme oxygenase exists in two isoforms, heme oxygenase 1, which is inducible by stressors including cytokines, intake of heavy metals, hypoxia and oxygen-free radicals, and heme oxygenase 2, which is constitutive and non-inducible (Shibahara et al. 1985; Maines et al. 1986). Biliverdin reductase transforms biliverdin into the toxic and highly insoluble compound bilirubin in mammals but not in most non-mammalian invertebrates. Bilirubin is conjugated with glucuronic acid to form a more soluble bilirubin glucuronide, which is excreted in bile (O'Carra 1975). Since bilirubin is normally produced in humans at a rate of approximately 5 to 8 µmol bilirubin/kg body weight/day (Berk et al. 1974), it can be estimated that the daily ALA requirement for heme biosynthesis is 54 mg in hepatic and 304 mg in erythroid tissues (Anderson et al. 2001).

2. The porphyrias

The porphyrias are inherited or acquired disorders of specific enzymes in the heme biosynthetic pathway. Eight enzymes are involved in the synthesis of heme and all of these enzymes and their genes have been thoroughly characterized. A partial defect in seven of them is associated with a specific porphyria; a defect in ALAS2 results in X-chromosome-linked sideroblastic anaemia (Figure 2). Most of the porphyrias are inherited as an autosomal dominant trait, but ALA-dehydratase deficiency and congenital erythropoietic porphyria demonstrate an autosomal recessive inheritance. Porphyria cutanea tarda can be either sporadic (type I) or familial (types II and III). Hepatoerythropoietic porphyria is a rare homozygous form of the familial type of porphyria cutanea tarda. The porphyrias are classified as either hepatic or erythropoietic depending on the primary site of overproduction and accumulation of the porphyrin precursor or porphyrin (Table 1). The hepatic porphyrias manifest usually as neurovisceral disorders, including abdominal pain, neuropathy and mental disturbances, whereas the erythropoietic porphyrias characteristically cause photosensitivity and disrupted erythropoiesis.

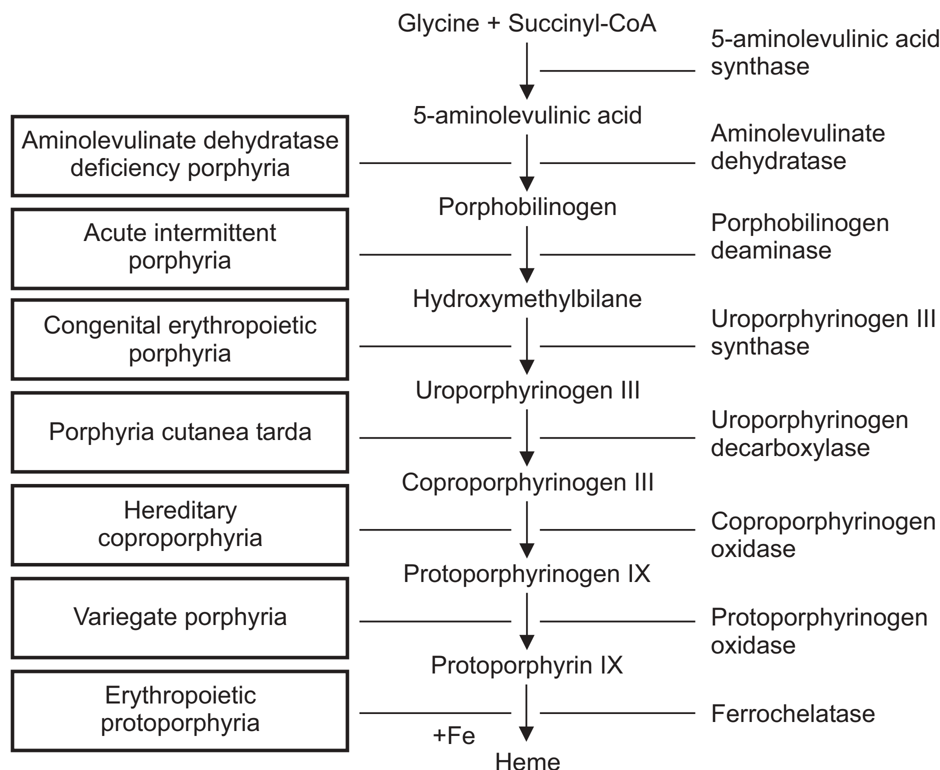


Figure 2. Heme biosynthesis and porphyrias. The enzymatic block is shown on the right panel and the associated disease on the left.

3. Variegate porphyria (VP)

3.1. History and prevalence

The clinical and biochemical picture of VP was first described by van der Bergh and Grotepass in 1937 (van der Bergh and Grotepass 1937). Following the first South African report in 1945 (Barnes 1945), case reports have been published with increasing frequency and a great amount of work was carried out to identify affected subjects and to classify the disease as a separate entity. The prevalence of VP is exceptionally high in the South African Afrikaans-speaking population (1:300) (Dean 1969). There, the history of VP can be traced back to the marriage of a Dutch couple in the Cape of Good Hope in 1688, and the R59W mutation identified from the family members has become enriched within the population of Dutch origin (Meissner et al. 1996; Warnich et al. 1996). As many as 20 000 South Africans may carry this trait due to the founder mutation. In Finland, the prevalence of VP has been estimated to be 1.3:100 000 (Mustajoki 1980), as compared with 0.5:100 000 reported in Great

Table 1. Classification of porphyrias.*

Classification	Principal symptomology
Erythropoietic	
Congenital erythropoietic porphyria	Photosensitivity
Erythropoietic protoporphyria	Photosensitivity
Hepatic	
Aminolevulinate dehydratase deficiency porphyria	Chronic neurological symptoms
Acute intermittent porphyria	Acute attacks
Hereditary coproporphyria	Acute attacks and/or photosensitivity
Variegate porphyria	Acute attacks and/or photosensitivity
Porphyria cutanea tarda	Photosensitivity
Hepatoerythropoietic porphyria	Photosensitivity

*Adapted from Anderson et al. (2001).

Britain (Elder et al. 1997) or 1:100 000 reported in Sweden (Wiman 2003). VP is the second most common porphyria in Finland after AIP, which has a prevalence of approximately 3:100 000 (Kauppinen and von und zu Fraunberg 2002). Porphyria cutanea tarda and erythropoietic protoporphyria are less common in Finland, with prevalences of 1:100 000 and 0.6:100 000, respectively (K. Timonen, personal communication), in contrast to most other countries, where porphyria cutanea tarda predominates (Anderson et al. 2001). VP has been termed the "Royal Malady" since some British royalty have been suggested to have had this condition (Macalpine et al. 1968). These suggestions are not very reliable, though, as they are based on retrospective analysis of rather unspecific symptoms without proper biochemical confirmation. Synonyms for VP include porphyria variegata, protocoproporphyria and South African genetic porphyria.

3.2. Clinical findings

3.2.1. Skin symptoms

VP can present with neurological manifestations, photosensitivity, or both – hence it was termed "variegate". Skin manifestations often occur apart from neurovisceral symptoms and are usually chronic in nature. They are often indistinguishable from those seen in porphyria cutanea tarda and hereditary coproporphyria, and include increased skin fragility on sun-exposed areas, most frequently on the backs of the hands (Day 1986). Even minor trauma results in the epidermis becoming detached from the dermis, followed by erosions and blisters (Figure 3A) (Timonen et al. 1990b). These lesions heal slowly, especially in the presence of secondary infection, and often result in scars, which may be pigmented. Hypertrichosis and hyperpigmentation are fairly common on facial skin (Day 1986).

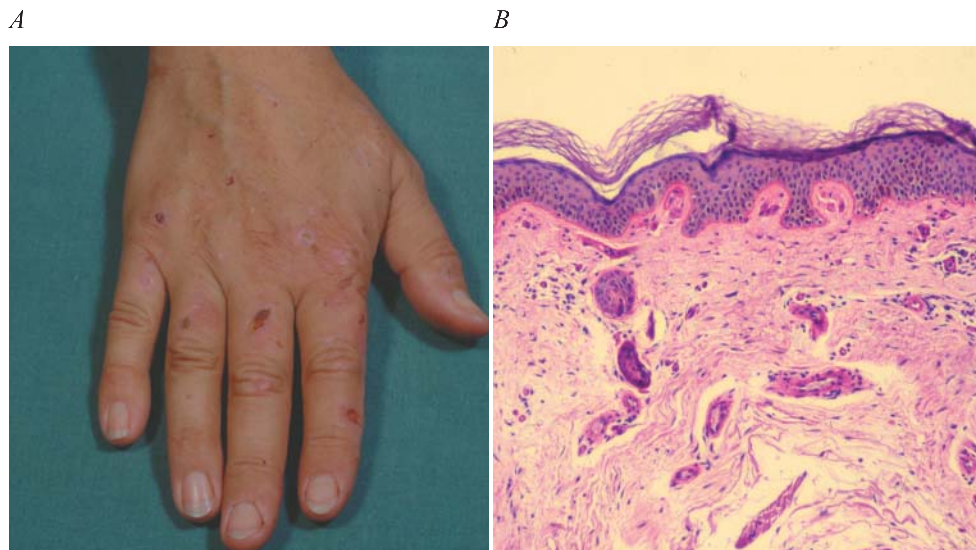


Figure 3. A Erosions and superficial scars on the skin of the hand of a VP patient. *B* Thickening of the superficial dermal vessel walls in the sun-exposed skin of a 80-year-old patient with skin symptoms (periodic acid-Schiff staining; x 120). (Courtesy of Dr. K.Timonen)

Histopathological changes in VP are identical to those of porphyria cutanea tarda and hereditary coproporphyria and include homogenous periodic acid-Schiff stain-positive thickening and immunoglobulin G deposition in superficial dermal vessel walls (Figure 3B) (Timonen et al. 1990b). The immunoglobulins apparently represent circulating proteins, and their deposition in the vessel wall is probably a result of phototoxic damage to the vessel walls (Cormane et al. 1971; Epstein et al. 1973). The changes are more prominent in sun-exposed areas, but slight changes can also be detected in sun-protected areas, indicating that porphyrins may induce microscopic changes in the vessels with only minimal light exposure (Timonen et al. 1990b).

Three decades ago, the prevalence of skin symptoms was 70-80% in South Africa, where the disease is common (Eales et al. 1980), while in Finland less than half of the patients experienced only mild skin fragility (Mustajoki and Koskelo 1976). This lower frequency of skin symptoms at northern latitudes probably reflects the absence of chronic exposure to strong sunlight (Muhlbauer et al. 1982), but recent South African studies also report a lower frequency (38%) of skin symptoms (R. Hift, personal communication).

3.2.2. Acute attacks

Acute attacks of VP are identical to those of other acute porphyrias. All symptoms during the attack are suggested to be due either to autonomic or peripheral neuropathy

Table 2. Clinical features of an acute attack of VP

	Mustajoki 1980 n=18 (%)	Eales 1980 n=100 (%)	Kirsch et al. 1996 n=23 (%)
Abdominal pain	95	98	95
Tachycardia	90	80	35
Dark or red urine	80		
Constipation	80	60	25
Muscle weakness	80	60*	10*
Psychiatric symptoms	80		
Abdominal tenderness	80		
Non abdominal pain	70		
Vomiting	65	75	60
Hypertension	60	60	55
Respiratory muscle paralysis	40		
Objective sensory disturbances	40		
Epileptic seizures	25	25	5
Cranial nerve abnormalities	20	20	5

Adapted from Eales et al. (1980); Mustajoki (1980); Kirsch et al. (1998)
* recorded as neuropathy without further definition.

or to CNS involvement (Crimlisk 1997). Symptoms of autonomic neuropathy include abdominal pain, which is the key symptom, hypertension, tachycardia, vomiting and constipation (Eales et al. 1980; Mustajoki 1980; Kirsch et al. 1998). The sensory neuropathies usually manifest as pain in the extremities or in the back, and motor neuropathy as variable pattern symmetrical weakness that may progress to paralysis and respiratory failure requiring prolonged mechanical ventilation (Ridley 1969). Peripheral neuropathy has become rare during the last decades due to early treatment of all acute attacks with heme arginate (Mustajoki and Nordmann 1993) and improved counselling (Kauppinen and Mustajoki 1992). The most common sign of CNS involvement is psycho-organic syndrome with anxiety, depression, hallucinations, delirium and dementia (Crimlisk 1997). Hyponatremia with evidence of sodium depletion or inappropriate ADH secretion can occur during acute attacks (Eales 1963) and is suggested to be due to hypothalamic involvement (Suarez et al. 1997). Epileptic seizures are known to be present in 5-20% of acute attacks and are often associated with hyponatremia (Stein and Tschudy 1970; Bylesjo et al. 1996). Urine can be dark in colour because PBG is auto-oxidized to porphobilin and porphyrins are formed non-enzymatically (Bissell 1982) (Table 2).

Peripheral nerve conduction studies show a primarily axonal degeneration rather than demyelinization (Flugel and Druschky 1977; Wochnik-Dyjas et al. 1978), and these findings are supported by histological studies (Cavanagh and Mellick 1965). Slight peripheral neuropathy may be demonstrable in the latent form of acute porphyria and even in patients who have never been symptomatic (Mustajoki and Seppäläinen 1975; Kochar et al. 2000).

Three decades ago, 17-38% of VP patients experienced acute attacks (Eales et al. 1980; Mustajoki 1980; Muhlbauer et al. 1982), but milder symptoms of porphyria are more common, occurring in 30-40% of patients (Kauppinen and Mustajoki 1992). Symptoms rarely occur before puberty and more than 50% of carriers of the affected gene probably remain symptom-free throughout their lives (Mustajoki 1980).

Acute attacks are often induced by various exogeneous and endogeneous precipitating factors such as drugs (for an extensive list of potentially safe and unsafe drugs, see http://www.uq.edu.au/porphyria), alcohol (Thunell et al. 1992), infection (Paxton et al. 1974), fasting (Perlroth et al. 1968; Quiroz-Kendall et al. 1983) or the menstrual cycle (Tschudy et al. 1975; Mustajoki and Koskelo 1976; Kauppinen and Mustajoki 1992). Most precipitating factors have the capacity to induce production of hepatic cytochrome P450 enzymes, which may cause heme depletion and secondary ALAS1 activation. Direct transcriptional activation of ALAS1 via drug-responsive enhancer sequences has also been reported (Fraser et al. 2002). ALAS1 induction leads to an increased synthesis and accumulation of heme biosynthetic pathway intermediates in the presence of the enzymatic blockade in VP. Individual susceptibility to clinical manifestations is likely determined by other genetic factors (Grandchamp 1998). Sulphonamide antibiotics exert their harmful effects by a different mechanism, probably via PBGD inhibition (Peters et al. 1980; Tishler 1999), since they apparently do not induce production of ALAS1 and cytochrome P450 enzymes.

VP patients occasionally present with chronic liver abnormalities including mild inflammation and fibrosis of the portal tracts and iron deposits (McGrath et al. 1984; Mascaro et al. 1985). Increased risk of hepatocellular carcinoma has been reported among patients with VP, as in AIP and porphyria cutanea tarda (Berman and Braun 1962; Kauppinen and Mustajoki 1988; Tidman et al. 1989; Germanaud et al. 1994; Andant et al. 1998).

3.2.3. Homozygous VP

The first cases of homozygous VP were described in 1984, and since then, 14 homozygous patients have been reported world-wide (Kordac et al. 1985; Murphy et al. 1986; Mustajoki et al. 1987; Coakley et al. 1990; Norris et al. 1990; Gandolfo et al. 1991; Hift et al. 1993; Roberts et al. 1998; Corrigall et al. 2000). Six of the patients were heteroallelic (Meissner et al. 1996; Frank et al. 1998d; Roberts et al. 1998; Palmer et al. 2001) and two homoallelic, with a varying amount of residual PPOX activity *in vitro* (9-25%) (Roberts et al. 1998).

In the homozygous form of VP, patients have markedly reduced PPOX activity in the lymphocytes or cultured fibroblasts (0-20% of normal) (Mustajoki et al. 1987; Norris et al. 1990) and severe clinical manifestations with onset in childhood (Hift et al. 1993). Severe photosensitivity is accompanied in 50-70% of patients by neurological symptoms, convulsions and developmental disturbances. Growth

Table 3. Comparison of the clinical features of homozygous and heterozygous VP.

	Homozygous VP (n / total)	(%)	Heterozygous VP (%)
Age at onset	<18 months		after puberty
Developmental delay	5 / 5	100	
Hand deformities	9 / 9	100	
Photosensitivity	11 / 12	92	38-70
Growth retardation	7 / 9	78	
Fits	8 / 11	73	
Retarded bone age	2 / 3	67	
Nystagmus	5 / 10	50	
Mental retardation	6 / 13	46	
Acute attacks	2 / 12	17	17-38
Death	1 / 9	11	

Adapted from Kordac et al. (1985); Murphy et al. (1986); Mustajoki et al. (1987); Coakley et al. (1990); Norris et al. (1990); Gandolfo et al. (1991); Hift et al. (1993); Roberts et al. (1998); Corrigall et al. (2000).

retardation in infancy or childhood has been reported in 80% and mental retardation in 50% of patients (Table 3).

3.3. Biochemical findings

Biochemical abnormalities found in VP patients include overproduction and increased excretion of porphyrins and porphyrin precursors. Routes of excretion are determined in part by the degree of porphyrin solubility in water, which is increased in the presence of carboxylic acid side chains. Uroporphyrin is the most water-soluble of the porphyrins derived from the heme biosynthetic pathway, and protoporphyrin the least soluble. Hence, uroporphyrin is excreted predominantly in urine and coproporphyrin mostly in urine but partly in bile, whereas protoporphyrin is so hydrophobic that it is excreted only in bile and faeces (Anderson et al. 2001). Much of the protoporphyrinogen IX that accumulates in VP undergoes auto-oxidation to protoporphyrin IX before excretion.

When VP is clinically active, faecal copro- and protoporphyrin and urinary uro- and coproporphyrin are elevated. Of the urinary coproporphyrin, 70-90% is type III isoform, as in AIP (Mustajoki 1980; R. Kauppinen, personal communication). The proportion of coproporphyrin III in faeces is 60-85% in VP (Mustajoki 1980), which is slightly less than the 30-60% encountered in AIP, but due to wide inter-individual variation, the fractionation should not be used to distinguish AIP and VP (R. Kauppinen, personal communication). The concerted excretion of proto- and coproporphyrin to bile could be explained by the close association between

PPOX in the inner mitochondrial membrane and coproporphyrinogen oxidase in the intermembrane space (Deybach et al. 1985). The plasma fluorescence spectrum shows a VP-specific emission maximum at 626 nm (Enriquez de Salamanca et al. 1993; Long et al. 1993; Da Silva et al. 1995). Biliary porphyrins are also increased (Logan et al. 1991), suggested to lead to the formation of gallstones, which contain protoporphyrin (Herrick et al. 1991).

Urinary PBG and ALA are increased during an acute attack but may be normal or only slightly increased during remission (Meissner et al. 1993). In heterozygous VP patients, half-normal activity of PPOX can be demonstrated in cultured skin fibroblasts, peripheral leucocytes and lymphocytes, cultured lymphoblasts and hepatocytes (Brenner and Bloomer 1980; Deybach et al. 1981; Viljoen et al. 1983; Meissner et al. 1986; Li et al. 1989). In the homozygous form of VP, patients have an 8- to 15-fold increase in erythrocyte protoporphyrin levels, 60-80% of which is zinc-bound (Kordac et al. 1985; Murphy et al. 1986; Mustajoki et al. 1987). A less marked 1.5–fold increase has been reported in heterozygous cases (Mustajoki 1980). A distinguishing feature in VP is the presence, especially in the bile and faeces, but also in the plasma and occasionally the urine, of hydrophilic porphyrin-peptide complexes termed X-porphyrins (Rimington et al. 1968; Smith et al. 1968, 1969; Elder et al. 1974).

3.4. Pathogenesis

3.4.1. Acute attacks

The acute symptoms in VP are identical to those in other acute porphyrias and result from neurological dysfunction. The direct or indirect induction of ALAS1, which is the rate-limiting enzyme of the pathway, plays a key role in the pathogenesis of acute attacks. If heme requirements in the cell are increased, for instance by increased turnover of certain hemoproteins, ALAS is induced (Meyer et al. 1998) and the normal activity of PBGD becomes the rate-limiting step (Elder et al. 1976), resulting in the accumulation of precursors ALA and PBG. Decreased PBGD activities (50-75% of normal) have also been reported in VP patients' lymphocytes (Meissner et al. 1993; Weinlich et al. 2001), and this inhibition is presumably consequent upon the accumulation of intracellular protoporphyrinogen (Meissner et al. 1993).

Neurotoxicity of porphyrin precursors

According to the leading hypothesis, neurotoxicity of ALA or PBG may cause neuropathy during the acute attacks, although the mechanism of neural damage is poorly understood (Bonkowsky and Schady 1982; Yeung Laiwah et al. 1987). The levels of ALA and PBG are virtually never normal during an acute attack. These

precursors most likely originate from the liver, where ALAS1 is induced by factors known to provoke acute porphyria, such as certain drugs and hormones (Anderson et al. 2001).

Current theories of the porphyric neuropathy implicate ALA as a potential neurotoxin. ALA can enter most tissues readily and undergo metal-catalysed oxidation, promote lipid peroxidation and mediate oxidative damage through release of iron from ferritin in the liver (Oteiza et al. 1995). ALA is also structurally similar to γ-aminobutyric acid (GABA) and can interact with GABA receptors, which could explain the CNS involvement but not the neuronal damage (Müller and Snyder 1977; Brennan and Cantrill 1979).

In vitro, ALA caused misfiring of electrically stimulated neurons of crayfish (Bonkowsky and Schady 1982) and death of cultured neuronal and glial cells of chick embryos (Percy and Lamm 1981). *In vivo*, acute administration of ALA did not provoke any symptoms in a volunteer (Mustajoki et al. 1992b) or AIP patients (Dowdle et al. 1968; Meyer et al. 1972; Shimizu et al. 1978), indicating that a more prolonged exposure is required to develop acute symptoms of porphyria. In human, the blood-brain barrier (BBB) is only weakly permeable to ALA, and the concentrations detected in the cerebrospinal fluid (CSF) are only 2-3% of blood levels (Bonkowsky et al. 1971; Percy and Shanley 1977; Gorchein and Webber 1987), which may be insufficient to explain the CNS symptoms (Meyer et al. 1998). However, the peripheral nerves are not protected by the blood-brain barrier and are exposed to levels of ALA comparable with those in blood (Meyer et al. 1998).

Heme deficiency in nerve cells

An alternative hypothesis suggests that depletion of heme in the nerve cells could impair the functions of several hemoproteins, such as microsomal cytochromes, which are required for oxidative phosphorylation or to catalyse mixed-function oxidation (Watson 1975). A further suggestion is that heme deficiency in the liver might dispose to unsaturation of hepatic tryptophan pyrrolase and lead to increased tryptophan delivery to the central nervous system (Litman and Correia 1985). Disturbance of the serotonergic system could explain the autonomic dysfunction during an acute attack (Meyer et al. 1998).

Depletion of essential substrates or cofactors

The increased activity of ALAS1 may also lead to the depletion of essential substrates or cofactors such as pyridoxal phosphate (PLP), zinc or glycine. Neuropathic manifestations of pyridoxal phosphate deficiency resemble acute porphyrias, and there is evidence of low pyridoxal phosphate status in some AIP patients (Cavanagh and Ridley 1967; Hamfelt and Wetterberg 1969).

Heme biosynthesis and degradation are closely interrelated to the function of cytochrome P450 enzymes. Several polymorphisms in cytochrome P450 enzymes exist (van der Weide and Steijns 1999), and these genes are good candidates for modifying porphyrin metabolism and phenotype. A diminished supply of heme may lead to insufficient function of P450-mediated reactions in VP patients: the metabolism of antipyrine and aminopyrine is prolonged (Birnie et al. 1987), which can be reversed using exogenous heme administration (Tokola et al. 1988; Mustajoki et al. 1992a). In addition, the activity of normally functioning P450 enzymes can be further induced by exogenous heme administration (Mustajoki et al. 1994). The effect is isozyme-specific in both VP patients and healthy subjects, and thus, exogenous heme can accelerate the reactions mediated by the cytochrome isozymes CYP2D6 and CYP3A4 but not by CYP1A2 and CYP2A6 (Mustajoki et al. 1994). In a rat porphyria cutanea tarda model, CYP3A induction was shown to attenuate the hepatic accumulation and urinary excretion of uro- and heptacarboxylporphyrins (Franklin et al. 2000).

The removal of excess porphyrins and their precursors from the liver could be at least partly due to ATP-binding cassette (ABC) efflux proteins, which can transport virtually any class of substrate, including drugs, steroids, polysaccharides, amino acids and peptides, across membranes (Kerb et al. 2001). MRP2 is an ABC efflux protein located at the apical surface of the hepatocytes and epithelial cells of the gastrointestinal tract and kidney (Figure 4) (Cole et al. 1992). A defect in the *MRP2* gene has been linked to the Dubin-Johnson syndrome, which is characterized by impaired secretion of anionic conjugates from hepatocytes into the bile and conjugated hyperbilirubinemia (Dubin and Johnson 1954; Kartenbeck et al. 1996; Wada et al. 1998). In Dubin-Johnson syndrome, the excretion of coproporphyrin in urine was increased 1.5- to 2-fold (Koskelo and Mustajoki 1980), and the proportion of isomer I in homozygotes and heterozygotes was 88.9% and 31.6%, respectively, whereas in normal individuals, it was 24.8% (Wolkoff et al. 1973). Hence, polymorphisms in the *MRP2* gene may well contribute to the accumulation of porphyrins and their precursors in hepatocytes and predispose to acute attacks.

P-glycoprotein (PGP) is another ABC efflux protein, which is capable of clearing cells of cytotoxic substances such as several anticancer drugs and other therapeutic agents (Gros et al. 1986; Fromm 2000). P-glycoprotein is coded by the multi-drug resistance gene 1 (*MDR1*) and expressed at the apical surface of hepatocytes, where it contributes to the drug efflux to bile. The repression of P-glycoprotein in *MDR1*(-/-) mice has been shown to increase CYP3A4-mediated drug metabolism in the liver 1.5- to 1.9-fold by increasing substrate availability (Lan et al. 2000). MDR1 deficiency could be postulated to lead to an increasing demand for CYP450 hemeoproteins and, consequently, heme depletion and ALAS1 induction. In addition, the removal of porphyrins and their precursors from hepatocytes could be impaired, leading to their accumulation in the liver. Several polymorphisms in the *MDR1* gene exist (Kerb et al. 2001), but only the 3435C→T polymorphism has been shown to be associated with

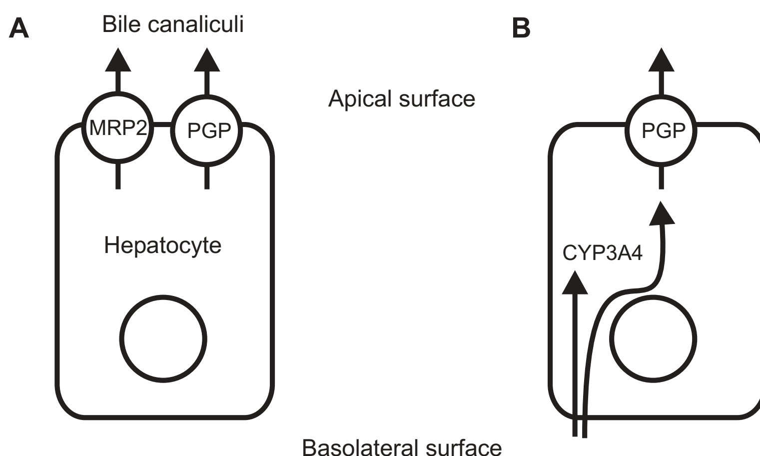


Figure 4. A Localization of drug transport proteins in liver cells. *B* Pathway of substrates common for P-glycoprotein and CYP3A4. MRP2, Multidrug resistance protein 2; PGP, P-glycoprotein. Adapted from Borst et al. (1999); Lan et al. (2000).

decreased P-glycoprotein activity (Hoffmeyer et al. 2000). Since it is located at a non-coding non-promoter region, it is unlikely to influence P-glycoprotein expression, but may be linked to other as yet unidentified modifier loci.

Mouse models of acute porphyrias

Recently, a mouse model of VP with the R59W mutations has been developed in C57/BL6 mice using targeted gene replacement (Medlock et al. 2002). Hepatic PPOX activity was reduced by approximately 50% in heterozygous mice. Urine and faecal samples from these mice demonstrated elevated concentrations of porphyrins and porphyrin precursors resembling the biochemical characteristics of human VP. ALA feeding of these mice resulted in an accentuated pattern of porphyrin excretion characteristic of VP, and urinary ALA and PBG were increased significantly. This suggests that the mice present a good model of the biochemical abnormalities in VP, but the clinical phenotypes have not yet been characterized (Medlock et al. 2002).

A PBGD-deficient mouse has been generated to gain insight into the pathophysiological mechanisms of porphyric neuropathy in AIP (Lindberg et al. 1996, 1999). Compound heterozygous mice T1/T2 (-/-) with a loss-of-function allele (T2) and a partial disruption of PBGD (T1) are viable and have 30% of normal PBGD activity. The PBGD-deficient T1/T2 (-/-) mice have normal levels of urinary ALA and PBG, but after phenobarbital treatment, the plasma levels and urinary excretion of ALA are massively increased, resembling levels found during an acute attack in man. Behavioural studies with the mice suggest that heme deficiency and consequent

dysfunction of hemoproteins cause chronic and progressive neuropathy, leading to impairment of motor co-ordination and muscle weakness (Lindberg et al. 1999). Hence, the mouse model resembles the chronic neuropathy occasionally seen in AIP and VP patients, but provides no information about pathogenetic mechanisms of an acute attack.

3.4.2. Photosensitivity

Most of the knowledge about the chronic type of cutaneous VP lesions comes from studies of the identical lesions in porphyria cutanea tarda. The proposed pathogenetic mechanism is that excess porphyrins in plasma and/or skin interact with light energy, inducing a phototoxic reaction and tissue damage (Day 1986). Absorption of a photon of near ultraviolet light (~400 nm) raises the porphyrin to its singlet-excited state, which may be converted to the metastable triplet-excited state. In the singlet state, the excited porphyrins may directly react with biomolecular targets, whereas the oxidative damage of the triplet-state porphyrin is mediated by the production of singlet-oxygen, destructive for most biological systems (Spikes 1975; Bodaness and Chan 1977). This event may be accompanied by photoactivation of the complement system, which acts synergistically to amplify the tissue damage (Lim et al. 1984; Meurer et al. 1985).

The prevailing hypothesis states that the excess porphyrins in the skin of patients with cutaneous porphyrias originate from the circulating plasma porphyrins (Day et al. 1978; Longas and Poh-Fitzpatrick 1982). In VP, the plasma porphyrins are mostly hydrophobic coproporphyrin and protoporphyrin (Poh-Fitzpatrick 1980; Long et al. 1993). Quantitative thin-layer chromatography did not reveal an association between circulating plasma porphyrin levels and cutaneous symptoms in a large series of patients with VP and porphyria cutanea tarda (Day et al. 1978). However, frequency of cutaneous symptoms has been shown to be positively correlated with urinary coproporphyrin levels (Timonen et al. 1990b).

3.5. Diagnosis

VP should be included whenever AIP or other acute porphyrias are considered as a potential cause of acute neurovisceral symptoms. During an acute attack measurement of urinary PBG and ALA is essential, and the plasma fluorescence emission spectrum and faecal protoporphyrin measurements can be used to distinguish VP from other acute porphyrias (AIP and hereditary coproporphyria). If a patient experiences photosensitivity, measurements of the plasma fluorescence emission spectrum, urinary copro- and uroporphyrins and faecal protoporphyrins are indicated and distinguish VP from other cutaneous porphyrias (porphyria cutanea tarda, hereditary coproporphyria, erythropoietic protoporphyria and congenital erythropoietic

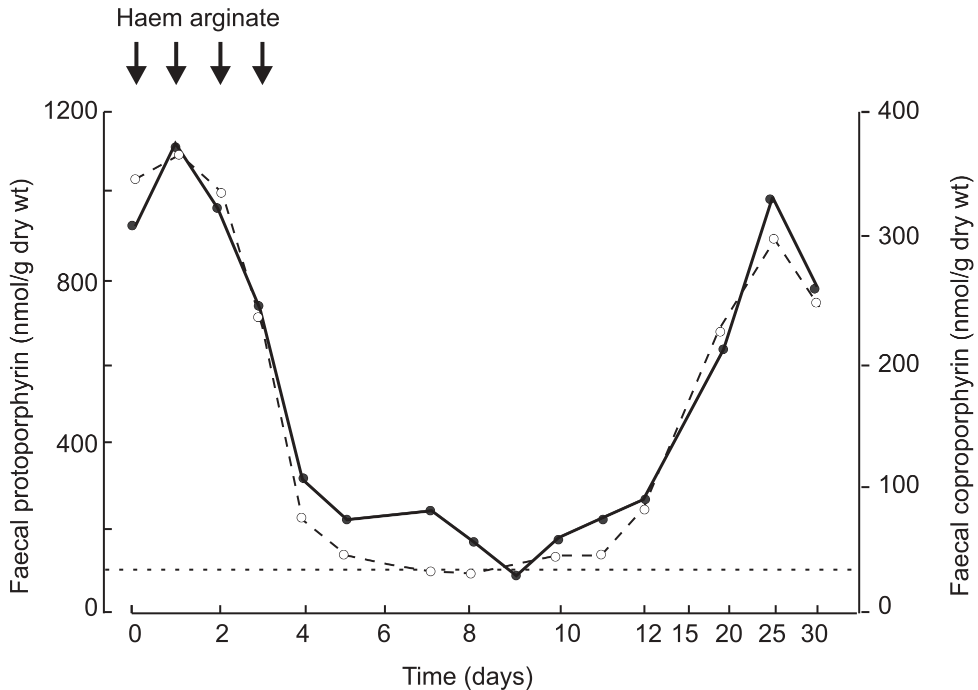


Figure 5. Faecal excretion of protoporphyrin (solid line) and coproporphyrin (dashed line) after administration of heme arginate in a patient with VP in remission. The dotted line gives the upper limit of the normal value. Adapted from Mustajoki et al. (1986).

porphyria) (Mustajoki 1980; Poh-Fitzpatrick 1980; Timonen et al. 1990b; Da Silva et al. 1995). The measurement of PPOX activity in cells that contain mitochondria, such as lymphocytes, may provide additional information if other biochemical analyses remain inconclusive. The assays for PPOX activity are laborious and not widely available.

3.6. Treatment

Specific treatment of acute attacks involves prompt initiation of intravenous heme (heme arginate 3 mg/kg daily for 2 to 4 days) and administration of glucose to avoid fasting (Watson et al. 1978; Mustajoki et al. 1986; Mustajoki and Nordmann 1993). Symptomatic treatment of pain, nausea and electrolyte imbalance is necessary. General and supportive measures are also important, including monitoring of nutrional status and withdrawal of precipitating factors (Mustajoki and Nordmann 1993). Heme therapy is the most effective treatment since it represses hepatic ALAS1

(Granick et al. 1975; Sassa et al. 1979) and reduces urinary PBG and ALA levels in AIP and faecal protoporphyrin and coproporphyrin levels in VP (Bonkowsky et al. 1971; Mustajoki et al. 1986) (Figure 5).

Heme treatment should be started as early as possible since the clinical response depends on the degree of neuronal damage that occurred before the initiation of therapy (Mustajoki et al. 1986; Mustajoki and Nordmann 1993). Currently available heme preparations for intravenous infusion include hematin (heme hydroxide) (Watson et al. 1978; Lamon et al. 1979) and heme arginate (Sievers et al. 1987; Tenhunen et al. 1987). Heme arginate is preferable as it is more stable and seldom associated with phlebitis and coagulopathy, which are common side effects of hematin (Tenhunen and Mustajoki 1998).

The prevention of cutaneous symptoms is difficult and therapies such as propranolol, d-penicillamine, hemodialysis, alkalization of urine, repeated venesections, cloroquine and β-carotene are of little or no benefit (Cramers and Jepsen 1980; Kirsch et al. 1998). Heme arginate therapy did not change the photoreactivity of the skin or improve skin lesions (Timonen et al. 1990a). Protection from sunlight using proper clothing, etc., is the only effective way to avoid cutaneous symptoms.

4. Protoporphyrinogen oxidase (PPOX)

4.1. The PPOX gene

The human PPOX-cDNA was isolated by complementation *in vivo* of a hemG mutant of *E. coli* (Nishimura et al. 1995), and subsequently, the *PPOX* gene was mapped to chromosome 1q23. The entire coding region for human PPOX was characterized (Roberts et al. 1995; Taketani et al. 1995); the gene is 5.5 kb including a 660 bp promoter region. The coding sequence is 1431 bp and encodes a 477 amino acid polypeptide with a M_r of 50.8 kDa (Puy et al. 1996; Dailey and Dailey 1997b) (Figure 6).

The human, mouse and bacterial PPOX amino acid sequences show a high degree of homology near the N-terminus of the polypeptide (Nishimura et al. 1995; Dailey and Dailey 1998). This area contains a characteristic βαβ dinucleotide-binding motif, which is often found within flavine adenine dinucleotide (FAD) -binding domains (Wierenga et al. 1986). Consistent with this finding, human PPOX has been shown to contain non-covalently bound FAD (Dailey et al. 1995). Northern blot analyses from various tissues indicate the presence of a single PPOX transcript of 1.8 kb in all tissues (Dailey et al. 1995; Nishimura et al. 1995; Dailey and Dailey 1996). Gel filtration of the purified protein under non-denaturing conditions suggests that the active enzyme functions as a homodimer (Dailey and Dailey 1996).

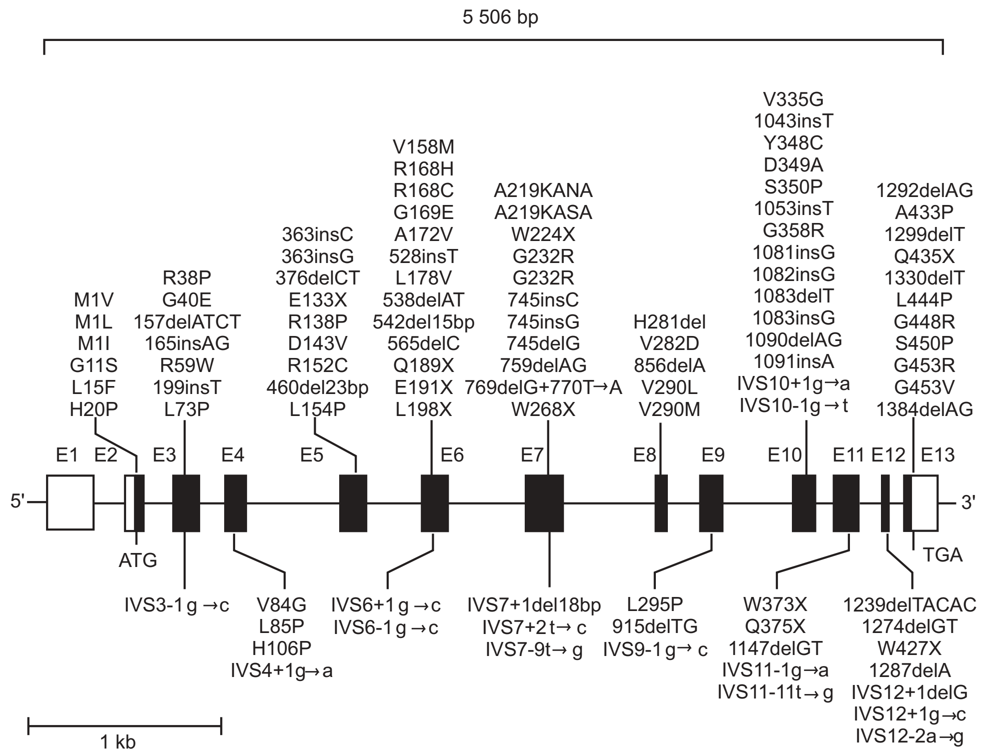


Figure 6. Exon/intron organization of the human protoporphyrinogen oxidase gene and reported mutations responsible for VP outside Finland (November 2002). Exons are indicated by boxes: closed and open boxes represent protein-coding and untranslated regions, respectively.

4.2. Function and localization of PPOX

PPOX catalyses the six-electron oxidation of protoporphyrinogen IX to the planar, fully conjugated macrocycle protoporphyrin IX in the inner membrane of the mitochondrion and requires oxygen for its activity (Dailey 1990). The oxidation occurs in three steps, consuming 3 mol of O_2 and producing 3 mol of H_2O_2. Dihydroporphyrin and tetrahydroporphyrin intermediates of the reaction are released and subsequently rebound by the enzyme for further oxidation to the end product, protoporphyrin (Figure 7).

PPOX acts specifically on protoporphyrinogen and does not catalyse the oxidation of coproporphyrinogen I, coproporphyrinogen III or uroporphyrinogen I. The activity of PPOX can be enhanced by sulfhydryl-reducing agents, like glutathione at low concentrations (Poulson 1976). Hemin at concentrations of 50 μM inhibits the

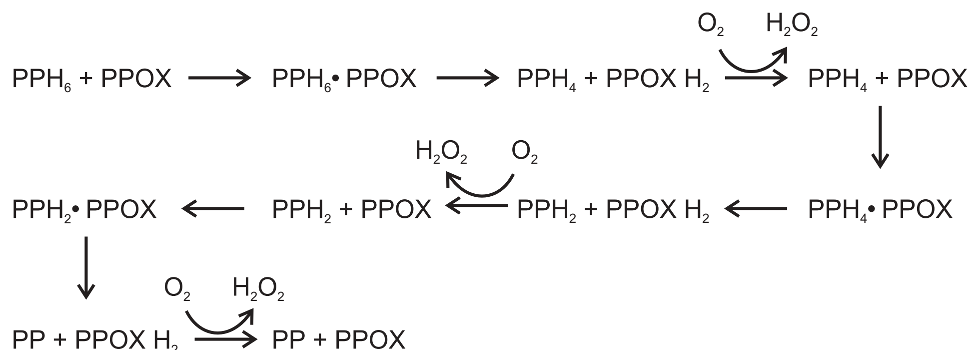


Figure 7. Proposed model for the action of PPOX. PPH_6, protoporphyrinogen IX; PPH_4, tetrahydroprotoporphyrin; PPH_2, dihydroprotoporphyrin; PPH, protoporphyrin. Adapted from Dailey and Dailey (1997b).

enzyme activity in a non-competitive and irreversible fashion (Poulson and Polglase 1975).

PPOX is associated with the lipid bilayer of the inner membrane of the mitochondria in eucaryotes (Deybach et al. 1985), with its active site facing the cytosolic side of this membrane (Ferreira et al. 1988). The anchoring of PPOX to the inner membrane may involve amphipathic helical domains that are responsible for insertion of the enzyme into the inner mitochondrial membrane. Another possibility is that PPOX is anchored to the inner mitochondrial membrane by a different mechanism, such as acylation (Arnould et al. 1999).

PPOX has been postulated to interact with ferrochelatase to channel protoporphyrin, and the terminal three enzymes to form an enzyme complex at the inner mitochondrial membrane (Ferreira et al. 1988) (Figure 8). The close association of the two enzymes would help explain the accumulation of both coproporphyrin and protoporphyrin in the excretions of VP patients. Studies with radiolabelled substrates and quantification of substrate utilization and product formation have demonstrated, however, that substrate channelling between the terminal three enzymes is not obligatory (Proulx et al. 1993).

4.3. Mitochondrial targeting of PPOX

The mitochondrial targeting mechanism of PPOX is currently unknown since the gene does not encode typical mitochondrial targeting and import sequences or membrane-spanning regions (Puy et al. 1996; Kirsch et al. 1998). The majority of proteins destined for import into mitochondria contain a signal sequence in their amino terminus that directs them into mitochondria (Pfanner 2000). Signal sequences can also be found in the carboxyl terminus of the protein mediating the import in a

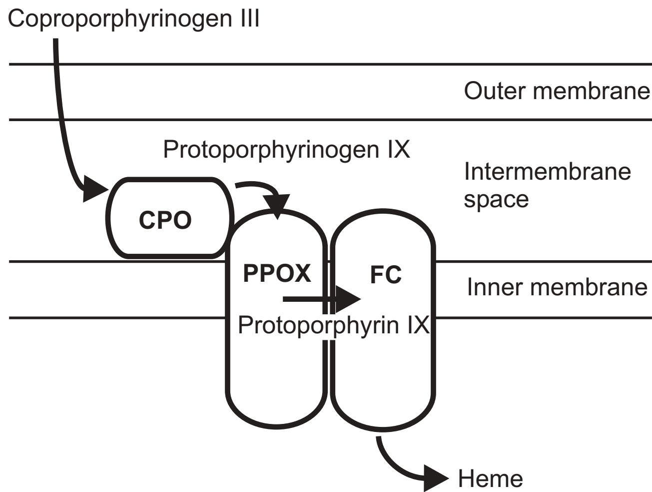


Figure 8. A proposed model for the terminal three membrane-associated enzymes of the heme biosynthetic pathway. CPO copropoprhyrinogen oxidase; PPOX protoporphyrinogen oxidase; FC ferrochelatase. Adapted from Ferreira et al. (1988).

reverse carboxy- to amino-terminal direction (Pfanner et al. 1987; Lee et al. 1999). These mitochondrial targeting signals comprise some 20 to 60 amino acid residues that have the potential to form amphiphilic α-helices with one hydrophobic and one positively charged side (von Heijne 1986; Roise et al. 1988). The presequence peptides of different mitochondrial proteins do not show amino acid sequence identity but do have characteristic physico-chemical properties. They are enriched in positively charged, hydroxylated and hydrophobic residues, have no acidic residues and usually are able to form an amphiphilic secondary structure (von Heijne 1986; Roise and Schatz 1988).

The amino terminus of PPOX contains a characteristic βαβ dinucleotide-binding motif often found within flavine adenine dinucleotide (FAD) -binding domains (Wierenga et al. 1986). The amino terminus is the putative mitochondrial targeting domain since it contains three basic residues and no acidic residues, which is characteristic for a mitochondrial targeting presequence, and is capable of forming an α-helix.

Protein import into mitochondria is mediated by protein assemblies, translocases of outer and inner mitochondrial membranes (TOM and TIM, respectively) (for review, see Voos et al. (1999); Herrmann and Neupert (2000)) (Figure 9). The N-terminal targeting signal initially interacts with the general import receptor Tom20, which is part of the TOM complex (Ramage et al. 1993; Brix et al. 1997). After binding, the polypeptide is allowed to cross the lipid bilayer through the general translocation pore formed by receptors Tom22, Tom40 and the intermembrane space domains of Tom22 and Tim23 (Schatz and Dobberstein 1996; Neupert 1997; Voos

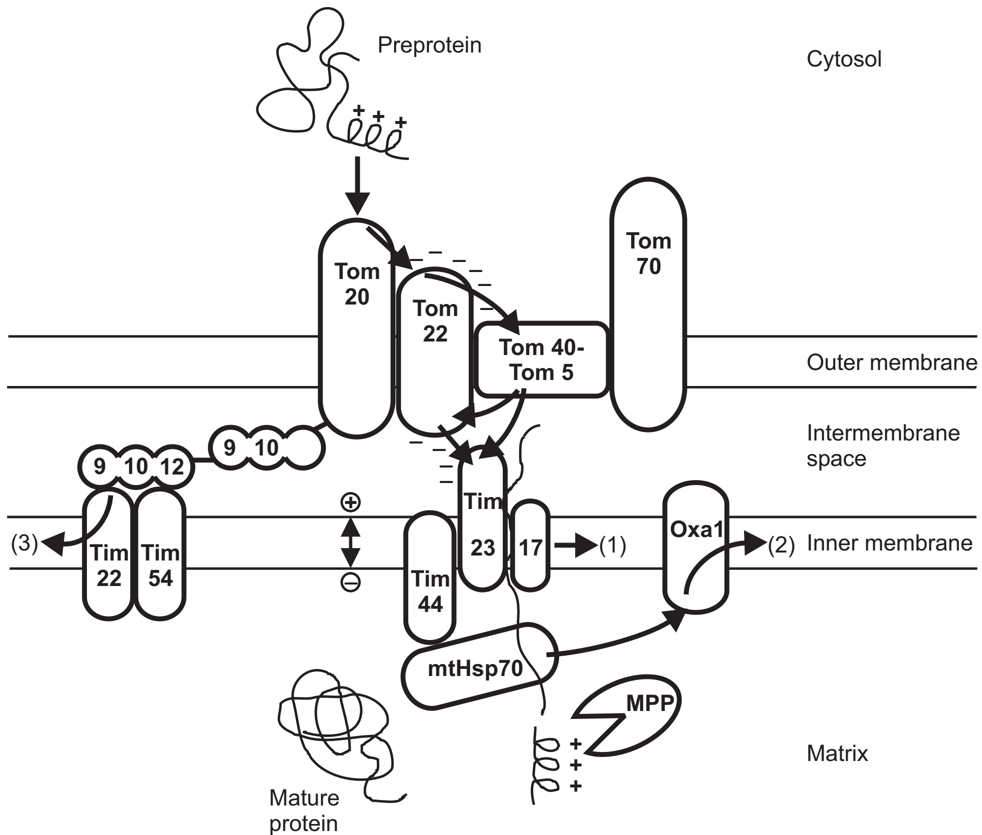


Figure 9. Transport pathway of an amino terminal presequence-carrying preprotein from the cytosol into mitochondria. Preproteins can be inserted into the inner membrane by several different pathways: (1) arrest at the level of the TIM23 complex and lateral insertion into the lipid bilayer (stop-transfer mechanism); (2) reinsertion into the inner membrane by the Oxa1 complex; or (3) transfer to the Tim22 complex by the soluble Tim9/Tim10 complex. Adapted from Pfanner (2000); Herrmann and Neupert (2000).

et al. 1999). The preprotein is driven across the inner membrane by the membrane potential and the heat shock protein mtHsp70 (Jensen and Johnson 1999). Eventually, the presequence is cleaved off by the mitochondrial processing peptidase (MPP) and the protein folds into its functional conformation.

Recently, the NMR structure of rat Tom20 in complex with a mitochondrial presequence peptide has been determined, revealing that an amphiphilic α-helical structure of presequences is critical for binding to the receptor. The hydrophilic residues, including the two positively charged arginines in the presequence, are

dispensable for binding to Tom20; however the hydrophobic residues are critical (Abe et al. 2000).

The proteins are incorporated into the inner membrane by several different pathways. Firstly, proteins can become arrested at the level of the TIM23 complex and then laterally inserted into the lipid bilayer (stop-transfer mechanism) (Kaput et al. 1982; van Loon and Schatz 1987). Secondly, proteins can be completely transported into the matrix from where they reinsert into the inner membrane by the Oxa1 complex (Hell et al. 1998). Thirdly, after translocation through the TOM complex, a Tim9/Tim10 soluble complex can bind preproteins and transfer them to the TIM22 complex, which mediates the insertion of the preproteins into the inner membrane (Sirrenberg et al. 1996; Koehler et al. 1998a, 1998b; Sirrenberg et al. 1998; Adam et al. 1999).

4.4. Mutations causing VP

By November 2002, a total of 102 mutations causing VP had been reported in the *PPOX* gene outside Finland. The mutations are distributed throughout the coding region of the *PPOX* gene, with no exon having >13 or <2 different mutations. No mutational hot spots have been identified (Figure 6, Table 4) (Deybach et al. 1996; Meissner et al. 1996; Warnich et al. 1996; Frank and Christiano 1997; Lam et al. 1997, 2001; Corrigall et al. 1998, 2000, 2001; Frank et al. 1998a, 1998b, 1998c, 1998d, 1998e, 2001a, 2001b; Roberts et al. 1998; Whatley et al. 1999; De Siervi et al. 2000a, 2000b; Maeda et al. 2000; Palmer et al. 2001; Donnelly et al. 2002). Of the mutations, 36 (35%) are small insertions or deletions, 40 (39%) are missense mutations, 15 (15%) change invariant nucleotides at splice sites, 1 (1%) is a gross deletion and 10 (10%) produce stop codons.

The effects of 27 *PPOX* mutations have been analysed in a prokaryotic expression system. The South African mutation R59W resulted in a loss of enzyme activity (Meissner et al. 1996), as did mutations G40E, L85P, G232R, H281del, V282D, L295P, V335G, S350P, L444P and G453V (Morgan et al. 2002). In mutations A219KANA, D349A, G358R and A433P found in homozygous VP patients, 9.5-25% of wild-type activity was preserved (Dailey and Dailey 1997a; Roberts et al. 1998). The enzyme activities of mutations L15F, R38P, L73P, V84G, D143V, R152C, L154P, V158M, R168H, A172V, V290L and G453R ranged from 1% to 9.2% of wild-type activity (Morgan et al. 2002).

In addition to the above-described mutations, several intragenic single nucleotide polymorphisms (SNP) have been found in the *PPOX* gene, both in the translated and untranslated regions (Kotze et al. 1998; Whatley et al. 1999). *In vitro* studies using a luciferase assay revealed that a common promoter region polymorphism, –1081G→A, resulted in a reduction in transcriptional activity but did not affect the clinical expression of the disease (Warnich et al. 2002).

Table 4. Reported mutations in the PPOX gene

Mutation	Outcome*	Reference	Mutation	Outcome	Reference
Exon 2:			Intron 6:		
1A→G	M1V	13	IVS6+1g→c	del exon 6	13
1A→C	M1L	13	IVS6-1g→c	del exon 7	13
3G→C	M1I	13	Exon 7:		
31G→A	G11S	18	657ins12bp	A219KANA**	12
45G→C	L15F	13,21	657ins12bp	A219KASA**	20
59A→C	H20P	2	672G→A	W224X	13
Exon 3:			694G→C	**G232R**	3,13
113G→C	R38P	13	695G→C	**G232R**	13
119G→A	**G40E**	13	745insC	stop +31	3,13
157delATCT	stop+13	13,23	745insG	stop +31	3
165insAG	stop +2	5	745delG	stop +23	13
175C→T	R59W	1,2	759delAG	stop +26	14
199insT	stop+10	13	769delG+770T→A	stop +16	21
218T→C	L73P	13	803G→A	W268X	13
Intron 3:			Intron 7:		
IVS3-1g→c	del exon 4	13	IVS7+1del 18bp	del exon 7**	12
Exon 4:			IVS7+2t→c	del exon 7	13
251T→G	V84G	13	IVS7-9t→g	8bp into cDNA	13
254T→C	**L85P**	13	Exon 8:		
317A→C	H106P	15	841delCAC	**H281del**	13
Intron 4:			845T→A	**V282D**	13
IVS4+1g→a	splicing defect	18	856delA	stop +20	13
Exon 5:			868G→C	V290L	13
363insC	stop +21	13	868G→A	V290M	21
363insG	stop +21	22	Exon 9:		
376delCT	stop +16	13	884T→C	L295P	13
397G→T	E133X	6	915delTG	stop +42	18
413G→C	R138P**	22	Intron 9:		
428A→T	D143V	13	IVS9-1g→c	del exon 10	13
454C→T	**R152C**	10,13	Exon 10:		
460del 23bp	stop +15	13	1004T→G	V335G	13
461T→C	L154P	13	1043insT	stop +1	16
Exon 6:			1043A→G	Y348C**	22
472G→A	V158M	13	1046A→C	D349A***	12
503G→A	**R168H**	7,13	1048T→C	**S350P**	13
502C→T	R168C	2	1053insT	stop +3	13
505G→A	G169E**	9,12	1072G→A	G358R**	9,12
515C→T	A172V	13	1081insG	stop +19	13
528insT	stop +1	13	1082insC	stop +18	13
532C→G	L178V	15	1083delT	stop +3	4,13,18
538delAT	stop +10	11,13	1083insG	stop +18	13
542del 15bp	stop +291	13	1090delAG	stop +15	13
565delC	stop +44	13	1091insA	stop +16	18
565C→T	Q189X	13,18	Intron 10:		
571G→T	E191X	18	IVS10+1g→a	splicing defect	19
593T→G	L198X	13	IVS10-1g→t	del exon 11	13

Table 4 continued. Reported mutations in the PPOX gene

Mutation	Outcome	Reference	Mutation	Outcome	Reference
Exon 11:			IVS12+1g→c	splicing defect	18
1119G→A	W373X	13	IVS12-2a→g	del exon 13	13,18
1123C→T	Q375X	21	Exon 13:		
1147delGT	stop +50	13,18	1292delAG	stop +2	13
Intron 11:			1297G→C	A433P***	12
IVS11-1g→a	splicing defect	20	1299delT	stop +4	14
IVS11-11t→g	mRNA <10%**	12	1303C→T	Q435X	13,18
Exon 12:			1330delT	stop	17
1239delTACAC	stop +19	17	1331T→C	L444P	13
1274delGT	stop +7	13	1340G→A	G448R	14
1281G→A	W427X	13	1348T→C	S450P	8
1287delA	stop	13	1357G→A	**G453R**	13
Intron 12:			1358G→T	**G453V**	13
IVS12+1delG	del exon 12	13	1384delAG	stop +13	13

1. Meissner et al. (1996); 2. Warnich et al. (1996); 3. Deybach et al. (1996); 4. Frank and Christiano (1997); 5. Lam et al. (1997); 6. Frank et al. (1998b); 7. Frank et al. (1998a); 8. Frank et al. (1998c); 9. Frank et al. (1998d); 10. Frank et al. (1998e); 11. Corrigall et al. (1998); 12. Roberts et al. (1998); 13. Whatley et al. (1999); 14. Maeda et al. (2000); 15. De Siervi et al. (2000a); 16. De Siervi et al. (2000b); 17. Frank et al. (2001a); 18. Frank et al. (2001b); 19. Lam et al. (2001); 20. Palmer et al. (2001); 21. Corrigall et al. (2001); 22. Corrigall et al. (2000); 23. Donnelly et al. (2002).

*mutations in bold have been expressed in *E. coli*

**homozygous VP, patient heteroallelic for the mutation

***homozygous VP, patient homoallelic for the mutation

Aims of the study

At the beginning of this study in 1998, two different mutations causing VP had been identified in 13 of the 22 biochemically and clinically well-characterized Finnish families (Mustajoki 1980; Timonen et al. 1990b). The pathogenetic mechanisms of VP at the molecular level were poorly known, as were the effects of mutations on PPOX activity.

The aims of this study were:

1. To identify the gene defects causing VP in Finland.

2. To characterize the outcome of the mutations at mRNA and polypeptide levels and to study the mitochondrial transport and localization of mutant and wild-type PPOX.

3. To investigate the clinical and biochemical outcome of patients and the genotype-phenotype correlation among Finnish VP patients.

4. To develop methods for DNA diagnostics of VP in Finnish families.

Materials and methods

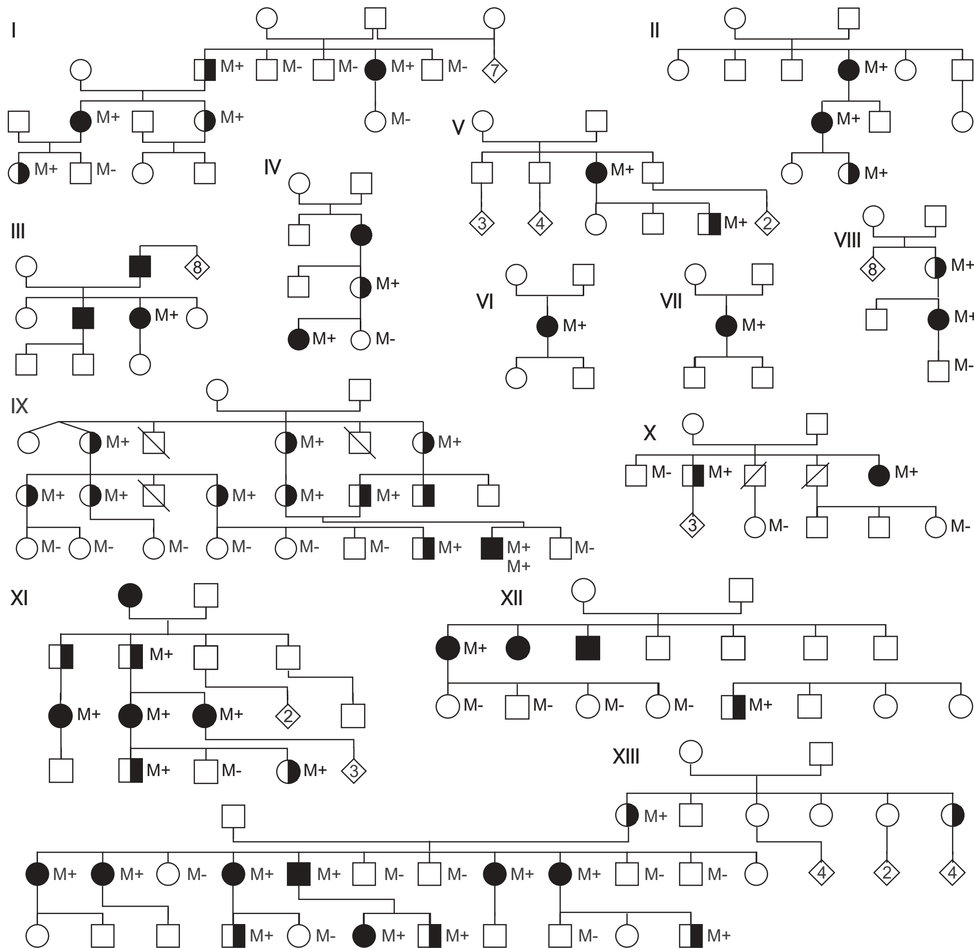
1. Patients and biochemical analyses

Since 1966, we have conducted a systematic follow-up of all Finnish patients known to have VP. To date, 143 VP patients belonging to 22 different families have been diagnosed, and in 20 of these families, ancestors were traced back to the 18th or 19th centuries using church registers (Mustajoki 1980) (Figure 10). Diagnosis of VP was based on mutation analysis (n=60), typical clinical symptoms with elevated faecal protoporphyrin excretion (n=68) (Li et al. 1986), characteristic plasma fluorescence emission spectrum (n=14) (Poh-Fitzpatrick 1980), low lymphocyte PPOX activity (n=25) (Deybach et al. 1981) and/or pedigree analysis (n=6).

PBG and ALA were measured using the ALA/PBG Column test (Bio-Rad, CA, USA) as described by Mauzerall and Granick (1956). Until 1988, urinary excretions of uro- and coproporphyrin and faecal excretions of copro- and protoporphyrin were measured according to Rimington (1958) and Holti et al (1958). Since 1988, all measurements have been obtained using high-pressure liquid chromatography (HPLC) (Lim and Peters 1984; Li et al. 1986). Biochemical measurements were taken in adolescence or adulthood (14 to 83 y). Porphyrins and porphyrin precursors were measured during remission of acute symptoms but in the presence or absence of skin disease. Informed consent was obtained for all DNA testing and the study protocol was approved by the Ethics Committee of the Department of Medicine, University Central Hospital of Helsinki.

2. Mutation analyses

For mutation analyses, a blood sample was available from at least one patient from each family. DNA was isolated from blood leucocytes using a QIAmp Blood Kit (Qiagen, Hilden, Germany) (Higuchi 1989) and amplified using polymerase chain reaction (PCR) (Mullis and Faloona 1987). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced using the dideoxynucleotide chain termination method (Sanger et al. 1977) with the Amplicycle Sequencing Kit (Perkin Elmer, Wellesley, MA, USA) according to the manufacturer's instructions. When the mutation was identified in a family, DNA samples of known family members were either analysed using restriction digestion, when a specific enzyme was available, or by direct sequencing. The analyses were repeated at least twice for each sample in the presence of negative and positive controls. The PCR conditions and primer sequences are given in Studies I-III. In addition, the DNA of 40 or more healthy unrelated controls were tested to exclude common polymorphisms.



To verify the outcome of the mutations at mRNA level, total RNA was extracted from Epstein-Barr virus transfected lymphoblastoid cell lines of patients using the guanidium isothiocyanate method (Chirgwin et al. 1979; Sambrook et al. 1989). Complementary DNA was synthesized from 5-10 µg of total RNA using Superscript II Rnase H$^-$ Reverse Transcriptase (Gibco BRL Life Technologies, Gaithersburg, MD, USA) and random hexamers or oligo(dT)s and directly sequenced as described above.

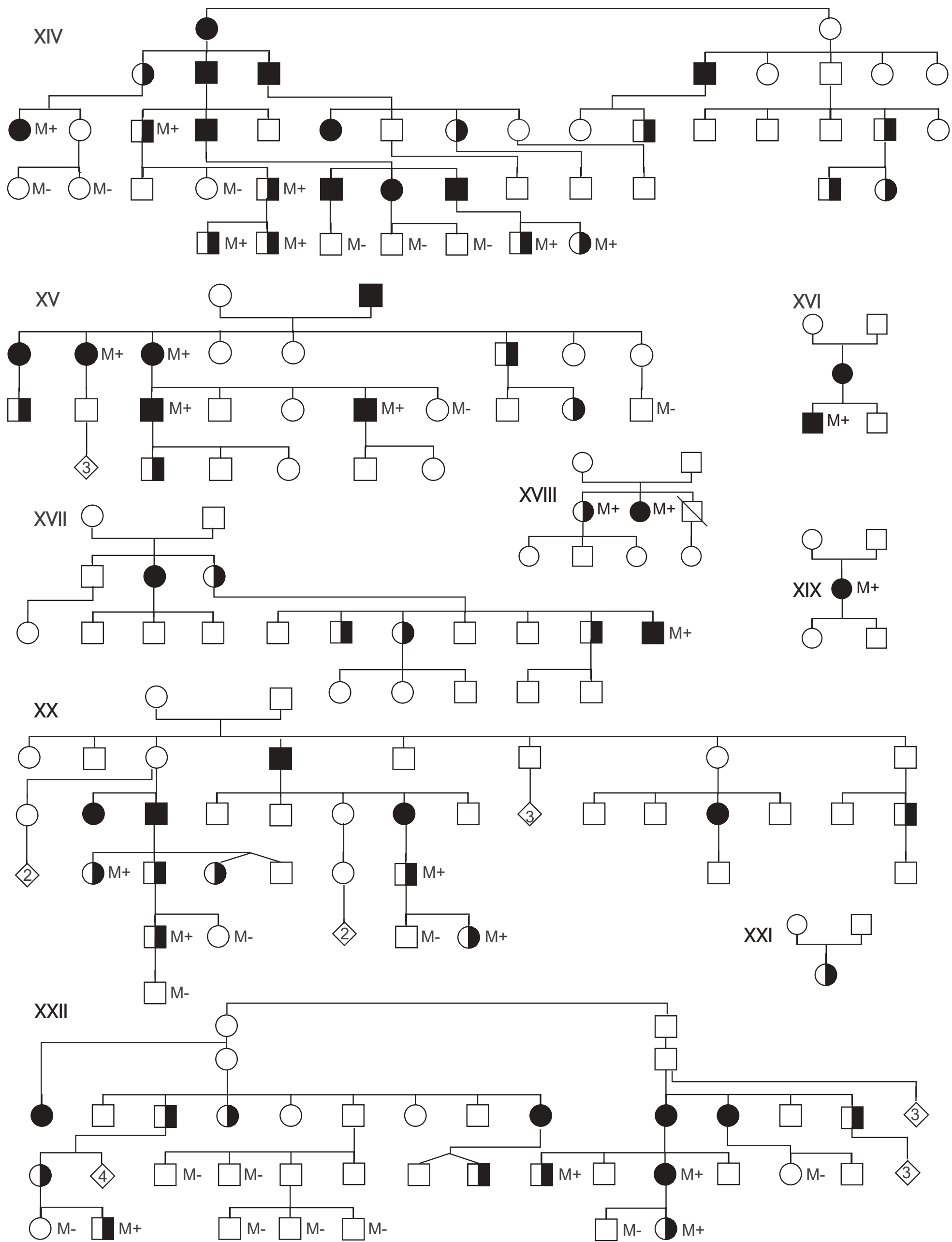


*Figure 10***.** VP pedigrees. Closed circle and square = affected female and male with clinically symptomatic disease; open circle and square = unaffected family member; half-filled circle = asymptomatic individual carrying the affected gene; M+ = mutation-positive; M- = mutation-negative.

3. Prokaryotic and eukaryotic expression of PPOX mutations

The normal and mutant PPOX alleles were expressed in *Escherichia coli* using the pUC18 vector (Pharmacia LKB Biotechnology Inc., Picataway, NJ, USA). The mutations were inserted into human PPOX-pUC18 (kindly provided by Prof. S. Taketani, Kansai Medical University, Osaka, Japan) using Chameleon double-strand site-directed mutagenesis kit (Stratagene Cloning Systems, La Jolla, CA, USA) or by ligating mutated RT-PCR fragments as a cassette into the corresponding sites of the PPOX-pUC18 using T4 DNA ligase (New England Biolabs, Beverly, MA, USA). The details of plasmid construction are given in Studies II-III. For eucaryotic expression, normal and mutant PPOX fragments were ligated to the corresponding sites of the modified pCMV1-vector, pCMV5 (Andersson et al. 1989). COS-1 cells were transfected using 100 µM chloroquine and $CaCl_2$ precipitation (Sambrook et al. 1989). The transformed COS-1 cells were incubated in Dulbecco's modified Eagle's medium (Gibco-BRL, NY, USA) supplemented with 10% foetal heat-inactivated bovine serum and antibiotics for 48 h before harvesting.

4. Assay of PPOX activity

Crude cell extracts of plasmid-containing cultures were assayed and the enzyme activities obtained were compared with those of background activity of PPOX in *E. coli* or COS-1 cells. Transformed cells were lysed with 300 µl of incubation buffer (150 mM Tris-HCl, 1mM EDTA, 1% (vol/vol) Tween 20) and sonicated (3 times 12 s), spun down for 10 min at 4^0C, and 50 µl of the supernatant was used for the PPOX activity assay (Deybach et al. 1981). Protoporphyrinogen was prepared from protoporphyrin IX (Porphyrin Products, Logan, UT, USA) by sodium amalgam reduction just before use. The protoporphyrinogen solution was diluted with 50 mM GSH incubation buffer 1:1 (vol/vol) to avoid auto-oxidation, and 10 µl was used as a substrate in the reaction. The assays were carried out at 37^0C in the dark. At 5 and 25 min, the samples were withdrawn and the reaction was stopped by mixing with 1 ml of 100 mM Tris-HCl, 1 mM EDTA, 0.1% Triton-X-100 (vol/vol), pH 8.7, and 2 mM GSH. Background fluorescence was controlled in each series by measuring the buffers only. The formation of protoporphyrin was determined by fluorescence using a Hitachi spectrofluorometer (wavelength of excitation 403 nm, emission 631 nm), and the protein concentration by the method of Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA, USA).

5. GFP fluorescence, mitochondrial staining and confocal laser scanning microscopy

For GFP fluorescence, the COS-1 cells were seeded on a 3-cm 6-well plate at 400 000 cells/well and grown overnight. The 50-70% confluent cells were transfected with 1.5 µg of human PPOX-pcDNA3.1/CT-GFP wild-type or mutant constructs by lipofection using FuGENE6 transfection reagent (Boehringer Mannheim, Germany). The details of the plasmid construction are given in Study V. For mitochondrial staining, 40 nM of MitoTracker Red CMXRos (Molecular Probes, Eugene, OR, USA) was added to the media for 45 min at 48 h post-transfection. Cells were immediately fixed with 3% paraformaldehyde for 30 min at room temperature. After fixation, the cells were washed three times in PBS and mounted in Mowiol 4-88 mounting medium (Calbiochem, Germany). The cells were observed on a Leica TCS confocal laser scanning microscope (Leica Microsystems GmBH, Heidelberg, Germany). Fluorescence of GFP was excited using a 488-mm argon/krypton laser, and emitted fluorescence was detected with a 500- to 530-nm band pass filter. For Mitotracker Red, a 543-nm helium-neon laser was used for excitation, and fluorescence was detected with a 565- to 699-nm band pass filter. For 3D imaging, a stack of 30 images with 0.20 µm distances was taken, and the 3D picture was constructed using Leica Confocal Software 2.00.

6. Statistical analyses

Fischer's exact test was used for comparison of categorical variables (number of symptomatic vs. non-symptomatic patients with different mutations). Continuous variables (biochemical excretions in patients with different mutations or different symptoms) were analysed using the Mann-Whitney U-test when two groups were compared or Kruskal-Wallis one-way ANOVA when more than two groups were compared simultaneously. Logistic regression with maximum likelihood estimation as an optimization criterion was employed to evaluate the association between the occurrence of skin symptoms and biochemical excretions. The model was adjusted for gender, age at time of diagnosis, year of diagnosis and mutation type. Statistical calculations were performed with SPSS version 10.04 and NCSS 2000.

7. Molecular modelling and polypeptide structure prediction

The model of the 18-residue peptide PPOX-(6-23) bound to the mitochondrial import receptor (Tom20) was built using a Bodil modelling environment (http://www.abo.fi/fak/mnf/bkf/research/johnson/bodil.html) and Sybyl 6.8 (Tripos Inc., St. Louis, MO, USA). The co-ordinates of the NMR structure of rat Tom20 in a complex with the recognition peptide of rat aldehyde dehydrogenase (pdb-entry: 1OM2) were used as a template for model building. Tertiary structure of the PPOX amino-terminus was predicted using program Swiss-Model (Peitsch 1996) (http://www.expasy.org/swissmod/SWISS-MODEL.html) with MAO-B structure (pdb-entry: 1GOS) as a template. Secondary structure calculation and figure drawing were carried out using PredictProt (Rost and Sander 1993; Rost and Sander 1994; Rost 1996) and MolMol (Koradi et al. 1996). The details of the model building are given in Study V.

Results

1. Mutations in the PPOX gene among Finnish VP patients (II,III,V)

1.1. Identification and characterization of the mutations

To identify the mutations in the PPOX gene, all exons and exon-intron boundary regions were directly sequenced after amplification of genomic DNA samples of 22 Finnish VP patients representing their families. In each case, the mutation was confirmed by repeating amplification and sequencing in both sense and antisense orientation, and by restriction digestion whenever available (Figure 11).

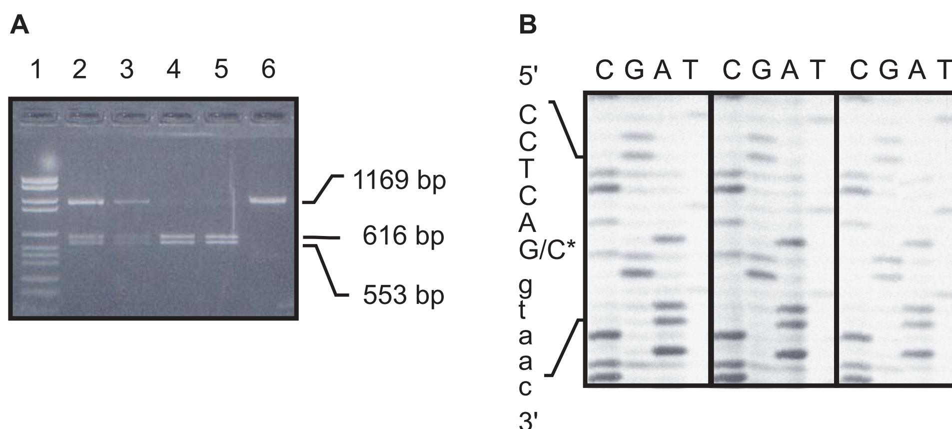


Figure 11. Identification of a mutation using two alternative methods. *A* Restriction site analysis of a patient's genomic DNA in the presence of negative and positive controls. A 1260-bp fragment spanning exons 2 to 5 was PCR-amplified and digested with Bsu36I. Since the mutation 338G→C abolishes a restriction site, an extra band of 1169 bp is visible in agarose gel electophoresis for a heterozygous patient. *Lane 1* DNA molecular weight marker VI (Boehringer Mannheim, Germany); *Lane 2* the patient's DNA sample; *Lane 3* mutation-positive control; *Lanes 4 and 5* mutation-negative controls; *Lane 6* undigested sample. *B* Direct sequencing of the genomic DNA of a patient (left) in the presence of mutation-positive (middle) and mutation-negative (right) controls. The mutation G→C is seen as an extra band in PAGE electrophoresis for a heterozygous patient.

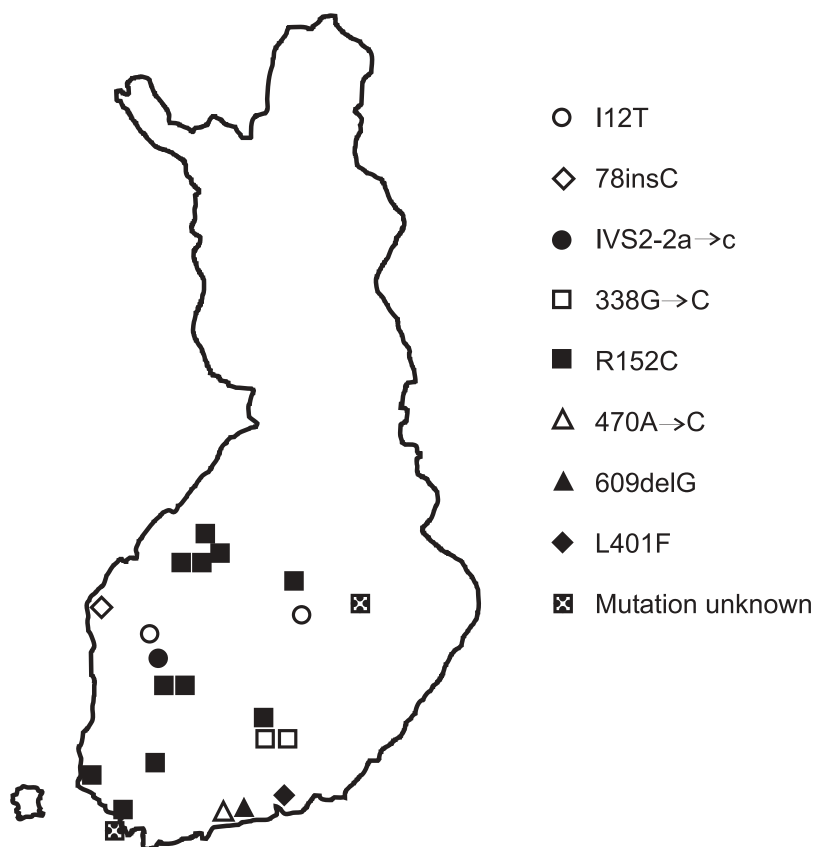


Figure 12. Geographical origin of the 22 VP families in Finland.

DNA samples of healthy unrelated Finnish controls (n≥40) were analysed to exclude common polymorphisms. Eight different mutations were identified in the PPOX gene among the 22 known Finnish VP families, and the ancestors were traced back to the 18th or 19th centuries using church registers (Figure 12).

The outcomes of the mutations were studied by amplification and sequencing of the reverse-transcribed total RNA obtained from patients' EBV-transformed lymphoblast cell lines. Seven of the mutations (I12T, 78insC, IVS2-2a→c, 338G→C, R152C, 470A→C and L401F) were identified in the direct sequencing of cDNA. This method gives in most cases a rough estimation of the steady-state transcript level of the mutant allele since the mutant allele is detected in cDNA if the level exceeds 30%

Table 5. Characterization of mutations in Finnish VP patients.

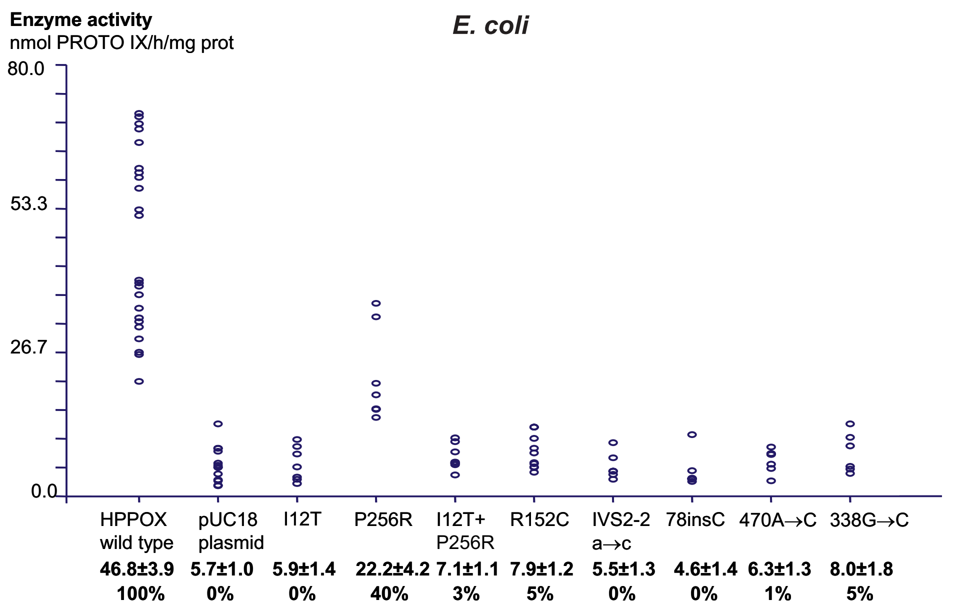
Mutation		Outcome	Residual activity *E. coli*	COS-1	cDNA[a]	mRNA level[b]	Mito-chondrial[c]	Patients/ families	Ref
35T$\rightarrow$C	Ex 2	I12T	3%	1%	+	n.d.	+	12/ 2	II
78insC	Ex 2	stop +8 amino acids	0	0	+	n.d.	+	5/ 1	III
IVS2-2a$\rightarrow$c	IVS 2	34 bp retention of intron 2	0	0	+	N	+	2/ 1	III
338G$\rightarrow$C	Ex 4	Deletion exon 4	5%	0	+	↓	+	11/ 2	III
454C$\rightarrow$T	Ex 5	R152C	5%	5%	+	n.d.	+	67/ 11	III[d]
470A$\rightarrow$C	Ex 5	Deletion exon 5 + 19 bp retention of intron 5	1%	0	+	N	+	1/ 1	III
609delG	Ex 6	Frameshift or splicing defect	n.d.	n.d.	n.d.	n.d.	n.d.	3/ 2	-[e]
1203A$\rightarrow$C	Ex 11	L401F	n.d.	n.d.	+	n.d.	+	2/ 1	III

[a] + mutation seen in direct sequencing of cDNA
[b]transcript level of the mutant allele compared to normal allele, ↓ reduced, N normal
[c]localization of the corresponding GFP-fusion protein, + localized in mitochondria
[d]mutation reported in Acta Haematologica (1997) 98 (suppl 1) 1-128 (Abstr.) and in Frank et al. (1998e) and Whatley et al. (1999).
[e]unpublished data
n.d. not done

of the normal allele. In addition, for three mutations, IVS2-2 a$\rightarrow$c, 338G$\rightarrow$C and 470A$\rightarrow$C, the relative amount of expressed mutant allele was estimated by comparing bands corresponding to the mutant and wild-type alleles in the agarose gel (Table 5).

To further characterize the PPOX mutations and to confirm the enzymatic defect, the normal and five mutant polypeptides were expressed both in prokaryotic and eukaryotic cell lines. PPOX activities of the normal transcripts and mutants were measured in *E. coli* and in COS-1 cells (Table 5, Figure 13). The normal allele showed high levels of PPOX activity in both expression systems; the increase was 8-fold in *E. coli* and 3-fold in COS-1 cells compared with background activity. This validated the use of both expression systems (Table 5).

Transport of the wild-type and seven mutated PPOX polypeptides to mitochondria was investigated by constructing the corresponding green fluorescent protein (GFP) fusion proteins, expressing them in COS-1 cells and studying their intracellular localization (Table 5).



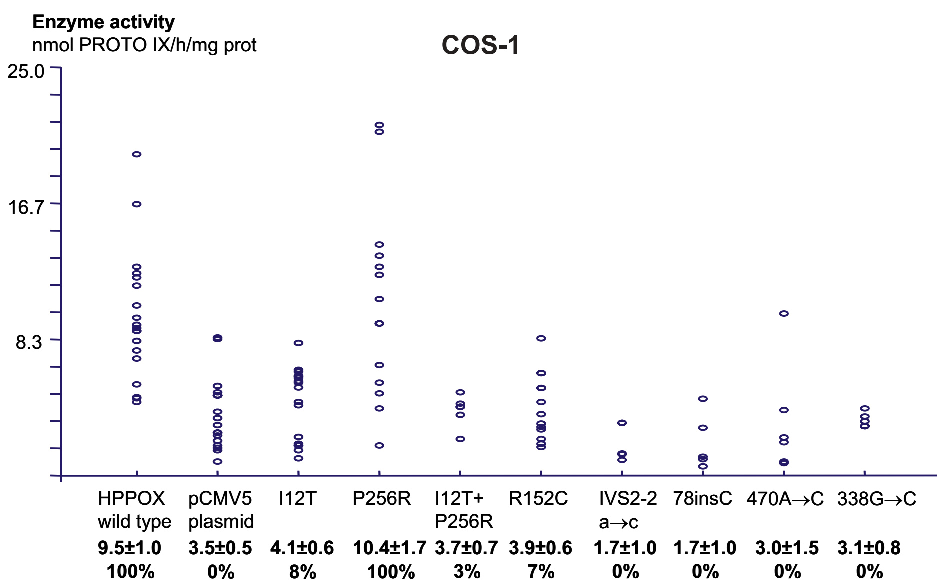


Figure 13. Enzyme activities of normal and mutant PPOX alleles measured in *E. coli* and COS-1 cells.

1.2. Outcomes of the mutations

I12T (35T→C) Substitution of the non-polar isoleucine with the polar threonine in exon 2 (Figure 14) resulted in the loss of enzymatic activity in both prokaryotic and eukaryotic expression systems (Table 5). The mutation could be detected with *Ban*I digestion since it abolished a restriction site. The full-length PPOX with the I12T mutation is transported into mitochondria, although the mutation is able to disrupt the N-terminal targeting signal in a truncated polypeptide with only the first 29 amino acids. In the two families studied, the mutation 35T→C co-segregated with a single nucleotide polymorphism 767C→G in all patients. The C to G transversion in exon 7 at position 767 converted proline to arginine (P256R) but demonstrated up to 50% residual activity in *E. coli* and normal activity in COS-1 cells. P256R polymorphism has also been reported in other western European populations (Whatley et al. 1999). This study demonstrated that this amino acid substitution did not affect the PPOX activity. Neither of these two amino acid changes was detected in any of the 40 unrelated healthy Finnish controls.

78insC Insertion of nucleotide C after nucleotide position 78 in exon 2 caused a frameshift and a premature stop codon 8 codons downstream in exon 2. As expected, the mutation abolished enzyme activity in both *E. coli* and COS-1 cells, but the GFP fusion protein studies showed that it nonetheless targeted into mitochondria (Table 5).

IVS2-2a→c –2a→c substitution at the 3' acceptor splice site of intron 2 altered the consensus sequence (ag/GT→cg/GT) and activated a cryptic splice (cag/gc) 35 bp upstream from the authentic splice site. Exons 1 and 2 were transcribed normally, but a frameshift and a premature stop codon occurred in exon 3. The predicted length of the translated polypeptide was 47 amino acids. The mutation abolished enzyme acticity in both *E. coli* and COS-1 cells, but mitochondrial transport was unaffected (Table 5). The mutation was confirmed by restriction cleavage since it abolished a *BstN*I cleaving site.

338G→C (–1G→C) Mutation at the 5' splice site of intron 4 altered the consensus sequence (AG/gt→AC/gt) and resulted in exon 4 skipping. Exons 1 to 3 were transcribed normally, but a frameshift and a premature stop codon occured in exon 5. In RT-PCR, the steady-state level of the mutant transcript in the patient's lymphocytes was markedly decreased compared with that of the normal allele. The mutation abolished enzyme acticity in both *E. coli* and COS-1 cells, but the mitochondrial transport was unaffected (Table 5). The predicted length of the translated polypeptide was 103 amino acids. The mutation was confirmed by restriction cleavage since it abolished a *Bsu36*I cleaving site.

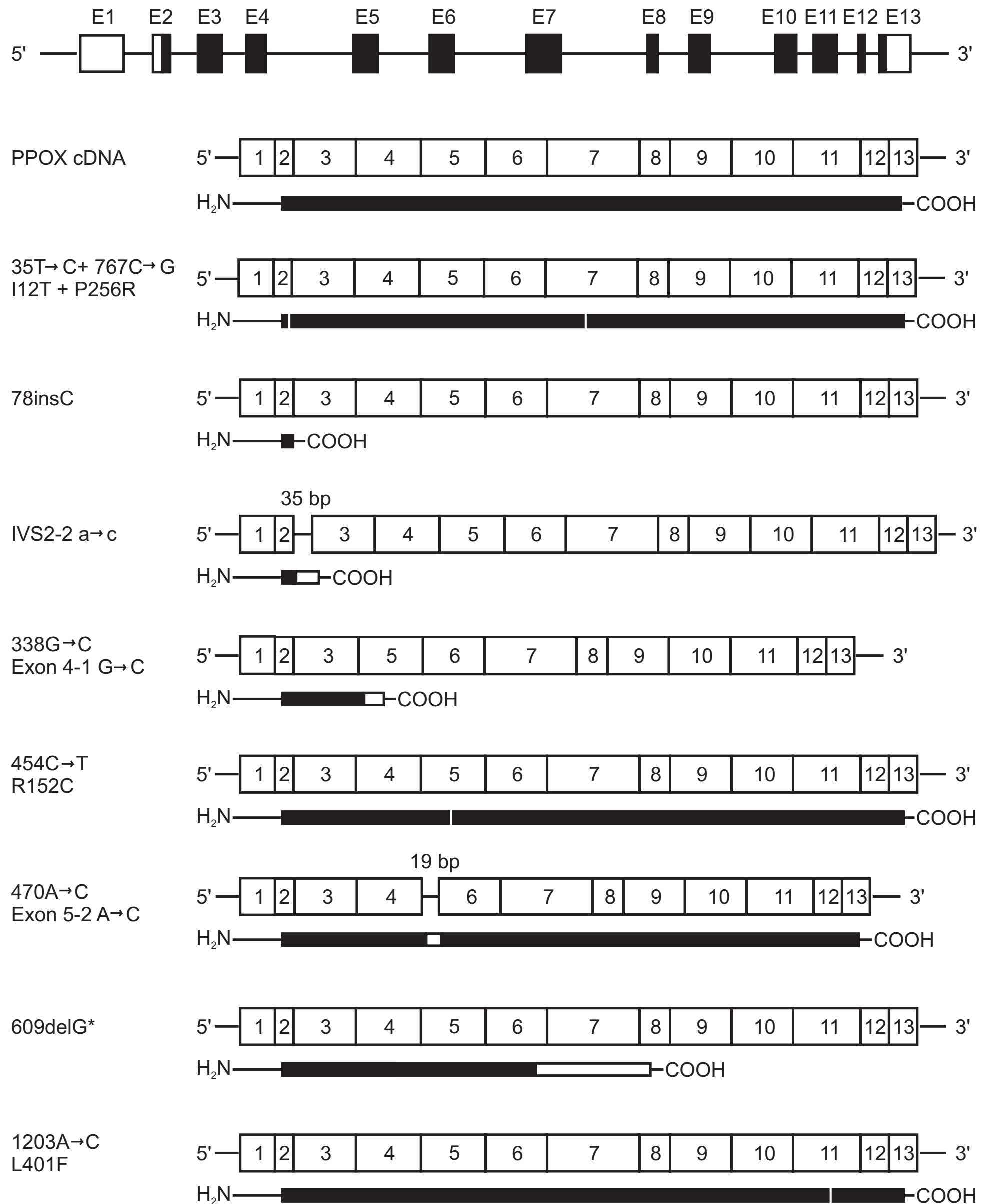


Figure 14. Schematic representation of outcomes of the mutations. Above exon/intron structure of human PPOX gene; solid and open boxes represent protein-coding and untranslated regions. Below the cDNA and protein translation are shown for each mutation; solid bars represent normally translated amino acids and open bars represent nonsense amino acids. * unpublished data; not confirmed at cDNA level.

R152C (454C→T) Substitution of basic arginine residue with neutral, sulphur-containing amino acid cysteine (R152C) resulted in a dramatic decrease of enzyme activity (5% of wild type activity) in both *E. coli* and COS-1 cells (Table 5). This arginine residue has been conserved in humans, mice, bacteria, yeast and plants, indicating its importance in PPOX function. The mutation had no effect on mitochondrial transport and was not detected in any of the 80 alleles of unrelated healthy controls, excluding a common polymorphism. The mutation could be detected with *HhaI* digestion since it abolished a restriction site.

470A→C (–2A→C) Mutation at the 5' splice site of intron 5 altered the consensus sequence (AG/gt→CG/gt) and resulted in exon 5 skipping and activation of a cryptic splice site (ag/tc) 19 bp upstream from the authentic 3' splice site of intron 5. The reading frame was unaffected and exons 1 to 4 and 6 to 13 were transcribed normally. The mutation abolished enzyme acticity in both *E. coli* and COS-1 cells, but mitochondrial transport was not affected (Table 5). The predicted polypeptide contained 112 translated amino acids encoded by exons 1 to 4, followed by 7 amino acids encoded by the inserted fragment of intron 5 instead of exon 5, and 320 normally translated amino acids encoded by exons 6 to 13. The mutation was confirmed by restriction cleavage since it abolished a *Bsu36*I cleaving site.

L401F (1203A→C) Mutation in exon 11 was predicted to result in a substitution of a non-polar amino acid phenylalanine for non-polar leucine (L401F). The effect of this apparently mild amino acid change on the function of the enzyme was difficult to predict since the three-dimensional structure of the PPOX has yet to be determined. Homology comparisons showed that this leucine residue was conserved in humans, mice and yeast but not in bacteria. The mutation had no effect on mitochondrial transport. The mutation segregated within the family and was not detected in any of the 80 alleles of unrelated healthy controls, excluding a common polymorphism. No other mutations were detected by direct sequencing of the exons and intron-exon boundaries of the proband's *PPOX* gene.

609delG Deletion of nucleotide G after nucleotide position 609 in exon 6 was detected in the direct sequencing of a patient's genomic DNA (unpublished data). The mutation resided at the 5' splice site of intron 6 and was predicted to cause either a frameshift with a premature stop codon at exon 8 or a splicing defect. This could not be confirmed at the mRNA level since cDNA analysis of the mutation is yet to be accomplished. The mutation segregated within the family and was not detected in any of the 80 alleles of unrelated healthy controls, excluding a common polymorphism. No other mutations were detected by direct sequencing of the exons and intron-exon boundaries of the proband's *PPOX* gene.

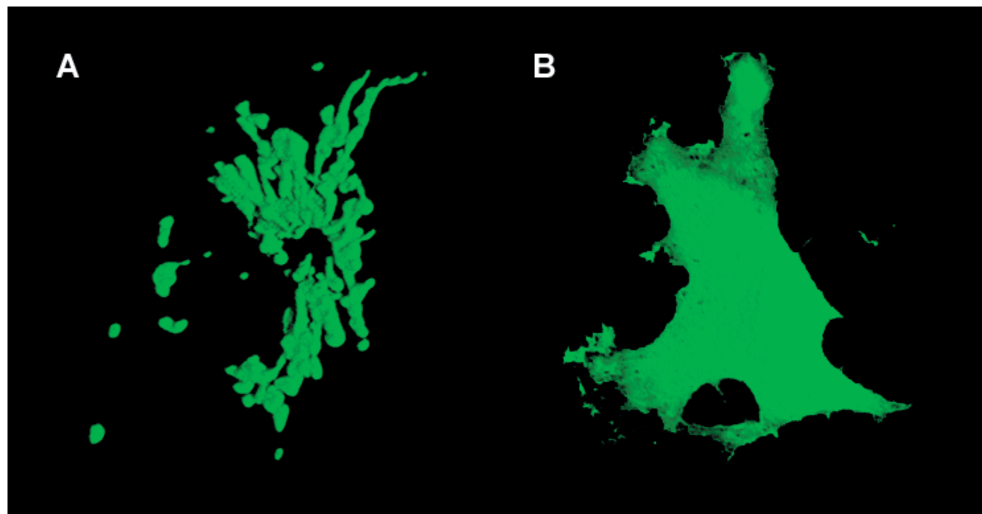


Figure 15. 3D image of a COS-1 cell transfected with the PPOX-GFP construct or GFP alone. After transfection, the cells were fixed and analysed by fluorescence confocal microscopy. *A* PPOX-GFP; *B* GFP alone.

2. Mitochondrial targeting of PPOX (V)

Transport of the wild-type and seven PPOX mutants (I12T, 78insC, IVS2-2a→c, 338G→C, R152C, 470A→C and L401F) to mitochondria was investigated by constructing green fluorescent protein (GFP) fusion proteins in which GFP was located in the carboxyl-terminal end of PPOX. COS-1 cells were transfected with the constructs and GFP fluorescence was analysed with confocal laser scanning microscopy. The cells expressing the wild-type PPOX-GFP demonstrated a typical mitochondrial pattern (Figure 15A) that was clearly distinguishable from the cytosolic pattern of GFP-constructs alone (Figure 15B). Each of the seven PPOX mutants showed a pattern similar to the PPOX-GFP, indicating that transport to mitochondria in all of them was unimpaired.

We showed that the 28 amino-terminal amino acids of PPOX contained an independently functioning mitochondrial targeting signal. In addition, experiments with a GFP-fusion protein where amino acids 5 to 24 were removed revealed that

Figure 16. Alignment and secondary structure prediction of PPOX peptide sequences using PredictProtein (Rost 1996). Residues are numbered according to the human sequence. Invariant residues are coloured in yellow and internal leucine-rich α-helical segments in blue (*next page*)

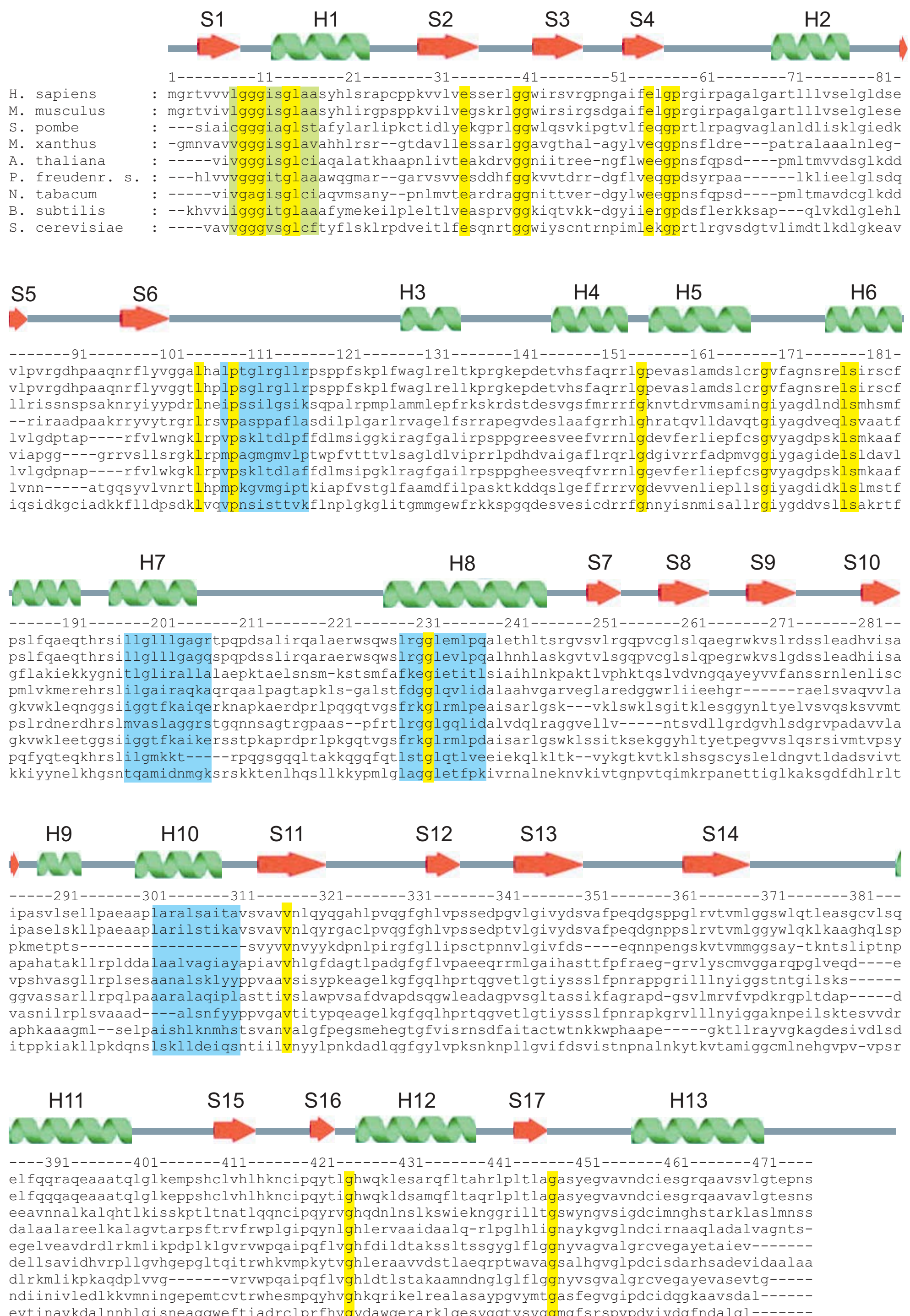


Figure 16 (see previous page)

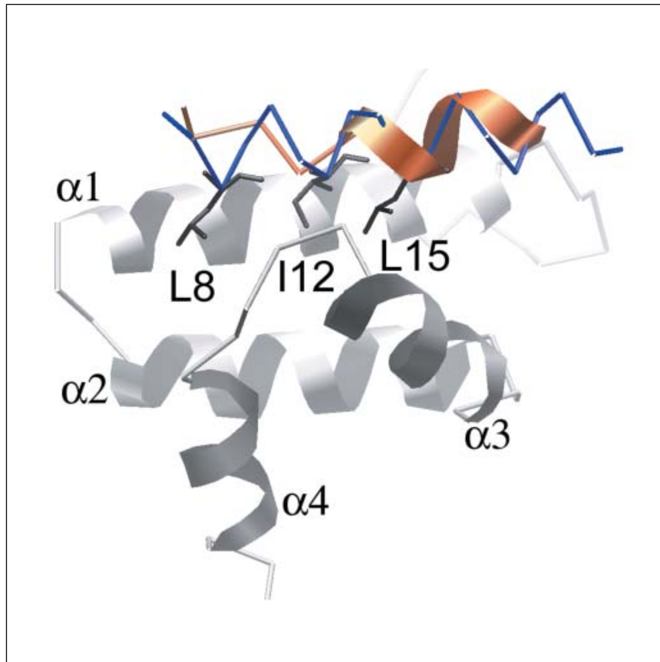


Figure 17. Model of the interaction between Tom20 (grey) and PPOX-(6-23) (blue). Rat aldehyde dehydrogenase presequence peptide pALDH-(12-22) in a complex with Tom20 (NMR structure, pdb-entry: 1OM2) is shown in orange. The hydrophobic Leu8, Ile12 and Leu15 residues are organized on one side of the helix in the model. The model locates the motif L*XXX*I*XX*L of PPOX-(6-23) in contact with the hydrophobic groove on the surface of Tom20. © The American Society for Biochemistry & Molecular Biology, reprinted with permission.

amino acids 25 to 477 of PPOX contained at least one additional mitochondrial targeting signal. Secondary structure prediction of PPOX revealed several internal leucine-rich a-helical segments with a net positive charge, which could act as internal mitochondrial targeting signals (Figure 16).

To create a structural hypothesis for the molecular recognition of the amino-terminal mitochondrial targeting signal of PPOX, we constructed an α-helical model of PPOX-(6-23) and a model of the interaction between PPOX-(6-23) and the mitochondrial import receptor Tom20. The model placed the L*XXX*I*XX*L (Leu8, Ile12 and Leu15) motif of PPOX-(6-23) in contact with the hydrophobic groove on the Tom20 surface (Figure 17).

We tested our structural model by mutating the PPOX-(6-23) residues Ile12 into threonine and residues Leu8 and Leu15 into glutamine. These amino acid substitutions disrupted the hydrophobic face of the L*XXX*I*XX*L helix model, which should affect the capability of interaction with Tom20. All three mutations caused a disruption of PPOX transport into mitochondria, confirming that each of these residues was essential for PPOX targeting. In the amino-terminus of PPOX, the L*XXX*I*XX*L motif, including residues Leu8, Ile12 and Leu15, appeared to be crucial for the putative interaction between PPOX and Tom20.

Table 6. Clinical manifestations and biochemical characteristics (mean±S.E.) in VP patients with the three most common Finnish mutations.

Mutation	Photo-sensitivity and acute attacks (%)	Photo-sensitivity only (%)	Acute attacks only (%)	Symptom-free (%)	Urinary copro-porphyrin (nmol/d)	Urinary uro-porphyrin (nmol/d)	Faecal copro-porphyrin (nmol/g)	Faecal proto-porphyrin (nmol/g)
I12T	0	0	8	92	164±12	30±11	67±38	156±67
R152C	15	34	10	40	743±124	105±15	212±28	554±50
338G→C	18	9	18	55	981±499	158±65	113±34	361±82

3. Homozygous variegate porphyria – molecular genetics and long-term follow-up (II)

The molecular defect was characterized from a homozygous VP patient, who was followed up in 1980-2000. The patient was homoallelic with amino acid substitutions I12T and P256R. He developed a severe skin disease post-partum with fragility, blister formation and scarring. The patient had never experienced acute attacks, although he had been exposed to potential predisposing agents such as infections. Minor verbal and visuospatial deficiencies as well as fine motor co-ordination disturbances were present, but mental status had remained normal. Increased serum creatinine levels, hematuria, proteinuria and increased blood pressure indicated renal failure. Heterozygous relatives with the I12T mutation experienced no cutaneous symptoms or acute attacks (Figure 10, pedigree IX). In another family with the mutation I12T (Figure 10, pedigree VIII), only one patient had experienced two acute attacks in her youth due to use of sulphonamides, suggesting a milder phenotype of this mutation in the heterozygous state.

4. Genotype-phenotype correlation in VP (IV)

The correlation between *PPOX* genotype and phenotype was investigated for 90 patients with the three most common Finnish mutations R152C, I12T and 338G→C. Twenty-two (24%) had experienced acute attacks, 36 (40%) photosensitivity and 12 (13%) both symptoms before or during the follow-up period from 1966 to 2001 (Table 6). None of the patients with the I12T mutation manifested photosensitivity, and only one out of 12 patients had experienced two acute attacks in her youth. The occurrence of photosensitivity was significantly lower in the I12T group compared with the R152C group (p=0.001), whereas no significant differences between the R152C and 338G→C groups could be observed.

Patients with the I12T mutation excreted significantly less uro- and coproporphyrins in urine than those with the R152C (p=0.001 for coproporphyrin and p=0.01 for uroporphyrin) or 338G→C (p=0.01 for coproporphyrin) mutation. The excretions of PBG and ALA in remission were also lowest in the I12T group. No differences in urinary excretions of uro- and coproporphyrins were observed between the R152C and 338G→C groups. Patients with the I12T mutation excreted significantly lower amounts of copro- and protoporphyrins in faeces than patients with the R152C (p=0.001 for coproporphyrin and p=0.0002 for protoporphyrin) or 338G→C (p=0.01 for protoporphyrin) mutation. The plasma fluorescence spectrum was normal in three patients tested in the I12T group, whereas in the R152C group, it was positive in eight out of nine patients and in the 338G→C group in two out of three patients.

5. Biochemical findings and prognosis of VP (IV)

To evaluate the prognostic value of biochemical tests in VP, results were compared between patients known to have experienced acute symptoms and/or skin symptoms and asymptomatic patients who had never been clinically active. Regardless of mutation type, urinary and faecal excretions of porphyrins were significantly higher in patients with prior symptoms, even during remission. The faecal protoporphyrin test was 92% accurate in predicting those individuals who would remain symptom-free with respect to acute attacks. Normal protoporphyrin excretion in the post-menopausal phase was demonstrated in one patient, who had previously experienced cyclical acute attacks. The negative predictive value for skin symptoms was 92% if the measurement was made during post-puberty. A 64-year-old patient, who had experienced mild skin fragility in his youth, had normal porphyrin excretion in faeces and urine but a positive plasma fluorescence spectrum for VP. In contrast, normal urinary excretion of porphyrins, PBG or ALA, or normal faecal excretion of coproporphyrin did not predict freedom from symptoms for VP patients; the negative predictive values varied from 61% to 82%. Urinary coproporphyrin levels were positively correlated with the risk of both skin symptoms and acute attacks, and virtually all patients with an excretion of more than 1000 nmol/d experienced skin symptoms, acute attacks, or both. Similarly, 90% of patients with faecal coproporphyrin excretion exceeding 200 nmol/g (twice the upper normal limit) were symptomatic.

6. Sensitivity and specificity of biochemical tests in the diagnosis of VP (I)

The sensitivity and specificity of biochemical screening for VP were studied among 38 family members for whom both DNA analysis and biochemical analysis were

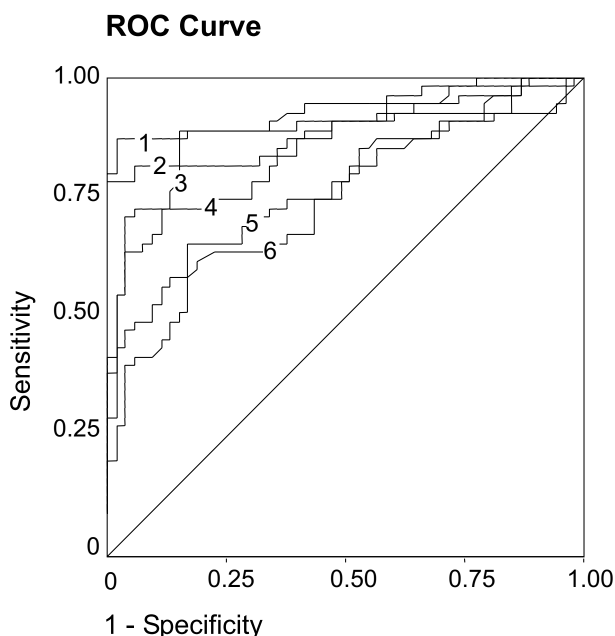


Figure 18. Comparison of sensitivity and specificity of different biochemical tests for VP in remission. (1) Faecal protoporphyrin; (2) Faecal coproporphyrin; (3) Urinary uroporphyrin; (4) Urinary coproporphyrin; (5) Urinary ALA; (6) Urinary PBG

available. These individuals were either asymptomatic or had experienced occasional skin symptoms, acute attacks, or both. The sensitivity of urinary and faecal coproporphyrin analysis was 48% and 52%, respectively. The sensitivity of urinary uroporphyrin analysis was 71% and of faecal protoporphyrin 77%. None of the biochemical measurements gave false-positive results, although urinary excretion of coproporphyrins may increase, for example, in many liver diseases and heavy metal intoxications (Moore et al. 1987). In two patients, the sensitivity of biochemical analysis was increased up to 83% by combining the results of faecal protoporphyrin and urinary coproporphyrin tests. The sensitivity of biochemical analysis could not be further improved by combining other tests. The plasma fluorescence emission spectrum, measured for 15 patients, was characteristic for VP in all symptomatic patients, even in remission, but failed to detect four asymptomatic patients who excreted normal amounts of porphyrins in urine. The overall sensitivity of plasma fluorescence analysis was 70%.

ROC-curve analysis was performed on 55 patients and 53 healthy relatives, including both individuals tested and not tested for mutation, for whom the results of all biochemical tests were available (unpublished data) (Figure 18). According to this analysis, the faecal protoporphyrin test was slightly better than the other screening tests in remission. Areas under curves were 0.93 [95% confidence interval (CI), 0.88-0.98] for fecal protoporphyrin, 0.89 (95% CI, 0.82-0.96) for fecal coproporphyrin, 0.90 (95% CI, 0.84-0.96) for urinary uroporphyrin, 0.86 (95% CI, 0.79-0.93) for urinary coproporphyrin, 0.78 (95% CI, 0.69-0.87) for urinary ALA and 0.74 (95% CI, 0.64-0.83) for urinary PBG.

Discussion

1. Mutations in the PPOX gene among Finnish VP patients

1.1. Genetic heterogeneity of VP in Finland

This series of investigations revealed eight different mutations in the PPOX gene among the 22 known Finnish VP families. This demonstrates the genetic heterogeneity of the disease in Finland, as in other countries. Seven of the mutations are family-specific and have thus far only been found in Finland, whereas the major mutation (R152C), which was identified in 11 (50%) of the 22 Finnish VP families, has also been reported in France and in USA (Frank et al. 1998e; Whatley et al. 1999).

A founder effect was observed among VP patients in Finland, in contrast to AIP, porphyria cutanea tarda and erythropoietic protoporphyria. The dual theory of inhabitation supported by recent analyses of Y chromosome haplotypes suggests that the early migration wave of eastern Uralic speakers took place some 4000 years ago (Kittles et al. 1998; Peltonen et al. 1999). The majority of the genes of the present Finnish population originated from later founder populations, which migrated from the south over the Gulf of Finland approximately 2000 years ago. Up to the 16th century, only the southwest and southeast corners and the coastal areas were regularly inhabited, with the population expansion in the north and east starting for the most part after 1500 (Norio et al. 1973).

The distribution of the major mutation R152C coincides with the early settlement of the Finnish population in the south-western part of the country, suggesting that the putative founder mutation emerged in the population more than 15 to 25 generations ago (Norio et al. 1973). Since the mutation has also been reported in France and in USA (Frank et al. 1998e; Whatley et al. 1999), it is possible that it has migrated from Central Europe during the late immigration ca. 2000 years ago. However, this has not been confirmed by haplotype studies. Another possible explanation would be that the mutation has propagated during the internal migration movement, which began in the 16th century from a small southeastern area in South Savo to the middle, western and finally northern and eastern parts of the country (Figure 19).

1.2. Effects of the mutations on PPOX function

Eight mutations, including three amino acid substitutions, an insertion and three splicing defects, were identified among Finnish VP families. The outcome of the mutation 609delG was not confirmed at the mRNA level, and since it resides in the exon 6-intron 6-boundary region, it is predicted to cause either a frameshift or a splicing defect. The consequences of the mutations were studied at the mRNA

Figure 19. Geographical origin of the Finnish VP families and the settlement of Finland. Closed circles the founder mutation R152C; Open circles other mutations. The grey line indicates the northern and eastern boundaries of the early settlement in the 16th century. Adapted from Nevanlinna (1972); Norio et al. (1973).

and polypeptide levels and mitochondrial transport and localization of mutant and wild-type PPOX were investigated to elucidate the pathogenetic mechanisms at a molecular level.

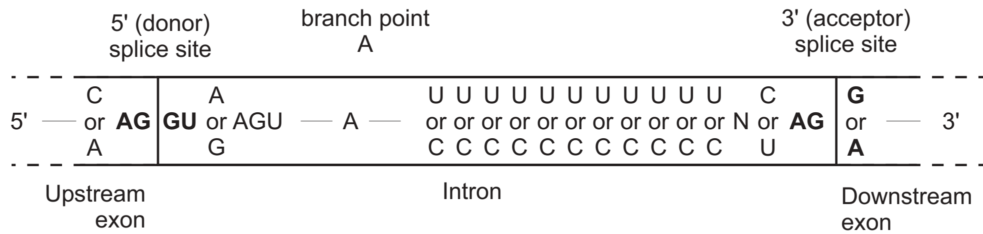


Figure 20. Consensus sequences for RNA splicing in higher eukaryotes. Adapted from Alberts et al. (1994).

1.2.1. Splicing defects

Three of the eight mutations were splicing defects. Two of the mutations were located at the 5' donor splice site and one at the 3' acceptor site. The consensus sequence for RNA splicing is known (Figure 20), but the splicing mechanisms have not been thoroughly clarified. Studies of different types of mutations at the splice sites have provided useful information about aberrant splicing patterns (Krawczak et al. 1992; Nakai and Sakamoto 1994).

Interestingly, in the case of mutation 338G→C (-1G→C), exon skipping was preferred, although a potential cryptic splice site (ACAG/GTTC) resembling the authentic splice site (TCAG/GTAA) existed 48 bp upstream of the authentic splice site in exon 4. This may support the "exon-definition hypothesis" derived from experimental analyses stating that each exon sequence in the pre-mRNA is defined and then the introns between the exons are recognized by splicing machinery (Niwa et al. 1992). According to the model, intron retention is permitted only when the intron is small enough to satisfy the exon length limitation. In this case, the total length of exon 4, intron 4 and exon 5 was 756 bp, which may violate the length limitation and thus prevent intron 4 retention. This kind of multiple aberrant splicing pattern with simultaneous exon skipping and cryptic splice site activation appears to be an infrequent phenomenon, as it was not documented in the database consisting of 209 point mutations leading to aberrant splicing (Nakai and Sakamoto 1994).

In the case of the mutation IVS2-2a→c, the –2a→c substitution at the 3' acceptor site of intron 2 causes a cryptic splice (cag/gc) activation 35 bp upstream from the authentic splice site. Cryptic splice site activation is a relatively common phenomenon, occurring in 30% of splice site mutations. The activated cryptic splice sites are usually located relatively near the authentic splice site within 100 nucleotides (Nakai and Sakamoto 1994). In the activation of 3' cryptic splice sites, the cryptic sites downstream from the authentic sites are selected more frequently (Nakai and Sakamoto 1994). In this case, however, the cryptic site was located upstream from the authentic site, indicating that splice site selection is not a simple one-way scanning process.

1.2.2. Decrease in enzymatic activity in prokaryotic and eukaryotic cells

The loss of enzymatic function of the truncated polypeptides 78insC, IVS2-2a→c and 338G→C was expected since they included less than 103 of the 477 amino acids (22%) of the normal polypeptide. The mutation 470A→C coded a polypeptide in which 45 amino acids encoded by exon 5 were missing. Since less than 1% enzyme activity was observed in both *E. coli* and COS-1 cells, the domain coded by exon 5 is essential for enzyme function. In the case of mutation R152C, the substitution of basic arginine residue with neutral, sulphur-containing amino acid cysteine resulted in a dramatic decrease of enzyme activity (5% of wild-type activity) in both *E. coli* and COS-1 cells, confirming the causality of the mutation and the disease. This arginine residue is evolutionarily conserved in humans, mice, bacteria, yeast and plants, indicating its importance in PPOX function (Figure 16).

Our findings are in line with other studies, where enzyme activities of 0-10% have been reported for VP-causing mutations in prokaryotic expression systems (Meissner et al. 1996; Dailey and Dailey 1997a; Roberts et al. 1998; Morgan et al. 2002). In many cases, the effect of a single nucleotide change on the enzyme function is difficult to predict, and the expression of the mutated allele *in vitro* is the only reliable way to exclude a rare polymorphism. The results must be interpreted with caution, however, since the accurate measurement of low tissue activities is technically complicated and the PPOX activity *in vivo* may be modified by its mitochondrial environment, enzyme stability or formation of mutant-normal heterodimers (Roberts et al. 1998).

The consequences of PPOX mutations have thus far been analysed only in prokaryotic expression systems (Meissner et al. 1996; Dailey and Dailey 1997a; Roberts et al. 1998). Expression studies performed in bacterial or mammalian cells may give controversial results due to the different localization and transport mechanisms. PPOX is bound to cytoplasmic membrane and coupled to the cell's respiratory chain in many prokaryotes (Dailey 1990), whereas the *Bacillus* oxidase overexpressed in *E. coli* is soluble and includes no membrane-spanning structure (Dailey et al. 1994). In our series, the results from prokaryotic and eukaryotic expression systems were mostly in accord with one another. Only in the case of amino acid substitution P256 did the prokaryotic expression system produce consistently lower activities relative to the normal allele (50%) than the eukaryotic system (100%). The transfection efficiency of COS-1 cells could be assessed only when the cells were transfected with PPOX-GFP fusion protein constructs and analysed by fluorescence microscopy. The efficiencies were constantly less than 10% with both chloroquine-$CaCl_2$ precipitation and Fugene6 lipofection techniques. Since the quantity of the expressed protein in the prokaryotic expression system is much higher than in the eukaryotic system, the enzyme activity assay is far more sensitive. Despite differences in cell organelles and transport mechanisms, we believe that the prokaryotic expression system is sufficiently reliable for analysing the effect of a single nucleotide change on enzyme function in humans.

1.2.3. Reduced levels of mRNA transcripts

The mutation 338G→C showed a dramatic decrease in the steady-state level of the mutant transcript in the patient's lymphocytes compared with that of the normal allele. This is consistent with previous findings in which several nonsense or frameshift mutations were associated with dramatic reductions in the steady-state level of cytoplasmic mRNA (Cooper 1993). However, the mutations 78insC, R152C, 470A→C and IVS2-2a→c did not demonstrate a reduction in the mRNA level at steady state. This was unexpected since the mutation IVS2-2a→c produces a termination codon near the 5' end of the gene, and normally, the closer a termination codon is to the 5' end of the gene, the lower the level of mRNA transcripts is likely to be (Cooper 1993). The mechanisms of how premature termination codons reduce mutant mRNA levels are not yet known. Because the rate of gene transcription is not decreased (Urlaub et al. 1989; Cheng and Maquat 1993), mRNA decay must take place in the translation phase. It was anticipated that the decay would occur in the cytoplasm, where the ribosomes would scan the transcript and recognize the termination codon, initiating the down-regulation of mutant mRNA (Urlaub et al. 1989; Carter et al. 1996). A model in which the mRNA decay is a nuclear event has also been proposed (Urlaub et al. 1989).

1.2.4. Mitochondrial transport of PPOX

The mitochondrial targeting signal was localized in the 28 amino acids in the amino-terminal end of PPOX. The predicted secondary structure of the PPOX amino-terminus consists of a βαβ-motif (Nishimura et al. 1995) that contains an α-helix, which is a common structure found in mitochondrial targeting signals. Our structural model of the interaction between the amino-terminus of PPOX and Tom20 and the experiments with amino-terminally mutated fusion proteins indicated that the critical residues for recognition of the PPOX targeting signal include leucine and isoleucine residues that form a hydrophobic motif L*XXX*I*XX*L. Our findings support the hypothesis that recognition of the mitochondrial targeting signal is dependent on hydrophobic interactions with the mitochondrial receptor. The NMR structure of rat Tom20 in a complex with the mitochondrial presequence peptide revealed that an amphiphilic α-helical structure of the presequences was important for binding to the receptor (Abe et al. 2000). Subsequent mutagenesis studies showed that the hydrophobic residues were essential for binding to Tom20, while the hydrophilic residues, including two positively charged arginines in the presequence, were dispensable (Abe et al. 2000). Replacing a single hydrophobic leucine or isoleucine in the amino-terminal recognition sequence of PPOX with a hydrophilic residue of the same size consistently prevented the mitochondrial transport.

The amino terminus of PPOX contains only three positively charged residues, which is less than in most presequences (Tzschoppe et al. 2000). It has been postulated that the positively charged residues could be involved in subsequent ionic interactions

between the targeting signal and Tom22, which is also known to bind amino-terminal recognition sequences, especially their carboxyl-terminal parts, in a salt-sensitive manner (Brix et al. 1997, 1999). In the case of PPOX, two positively charged residues Arg23 and Lys29 located in the carboxyl-terminal part of the recognition sequence, could facilitate this interaction.

Surprisingly, PPOX derivatives where the amino-terminal targeting signal was removed either totally or partially were still located in the mitochondria. This implies that the residual part of PPOX must contain an additional mitochondrial targeting signal(s) (Tzschoppe et al. 2000). PPOX is a further example of mitochondrial proteins whose import is not strictly dependent on the presence of an amino-terminal presequence (Michaelis et al. 1991; DeLabre et al. 1999; Krause-Buchholz et al. 2000; Tzschoppe et al. 2000). Secondary structure prediction of PPOX reveals several internal leucine-rich α-helical segments with a net positive charge (Figure 16). Such segments can putatively form hairpin-like structures that mimic a typical amphiphilic presequence and function as an internal mitochondrial targeting sequence (Folsch et al. 1998). Without knowledge of the tertiary structure of PPOX, it is, however, difficult to predict which of these segments is accessible to the receptor and serves as an effective targeting signal.

2. Clinical and biochemical features and pathogenetic mechanisms of VP

2.1. Acute attacks and photosensitivity – different pathogenetic mechanisms?

Our series includes both patients with symptoms (52%) and phenotypically normal carriers (48%), thus providing information about the clinical and biochemical outcome among VP patients in general. The proportion of patients with acute attacks (27%) in this series is somewhat lower than in some extensive family studies, in which up to 38% of patients had experienced acute attacks (Eales et al. 1980; Whatley et al. 1999), but higher than the 4-15% recently reported for a large South African kindred (R. Hift, personal communication). The frequency of photosensitivity in our series (40%) is lower than reported previously in South Africa and France (70%) (Eales et al. 1980; Whatley et al. 1999), but comparable with that of the recent South African study (39%) (R. Hift, personal communication). Extended mutation screening among symptom-free family members and improved counselling explain the differences in these numbers.

Since only 26% of symptomatic patients suffered both photosensitivity and acute attacks, the majority of the patients with each of these manifestations were in two distinct groups. This is in line with other studies, where 79% and 77% of symptomatic patients experienced either photosensitivity or acute attacks, but not both (Eales et al. 1980; Whatley et al. 1999). We have shown that the occurrence of acute attacks

has decreased markedly during the last two decades, whereas no such tendency has been observed for skin symptoms (Timonen et al. 1990b; Kauppinen and Mustajoki 1992). The decrease has been more prominent in males since they are not prone to cyclical factors. This indicates that different pathogenetic mechanisms may underlie the development of skin symptoms and acute attacks. The latter may be more readily prevented by avoiding precipitating factors.

The occurrence of skin symptoms was related to a more than 4-fold increase in urinary copro- and uroporphyrin excretion. In contrast, normal faecal protoporphyrin excretion as well as negative plasma fluorescence predicted freedom from skin symptoms. These findings support the theory that the severity of chronic skin symptoms is likely to depend on the permanent circulating levels of uro- and coproporphyrins (Day 1986; Timonen et al. 1990b). In the pathogenesis of acute attacks, the direct or indirect induction of ALAS, which is the rate-limiting enzyme of the pathway, plays a key role. ALAS induction leads to a transient increase of circulating porphyrin precursors ALA and PBG, which could be responsible for the neurological manifestations during an acute attack (Meyer et al. 1998).

2.2. Genotype-phenotype correlation and modifying genetic factors

Since patients with the I12T mutation experienced no photosensitivity and acute attacks were rare, a correlation could be made between the *PPOX* genotype and phenotype. In addition, biochemical abnormalities were milder, suggesting a less severe form of the disease in patients with the I12T mutation than in those with other mutations. No such correlation could be demonstrated in another study of 100 families, where the relationship between mode of presentation (skin lesions alone, acute attack alone, or both together) and type of mutation (nonsense or frameshift, missense or splice site) was studied (Whatley et al. 1999). Our results show that pooling of mutations should be avoided since the effect of the mutation on enzyme function is often difficult to predict and genotype-phenotype relationships within the subgroups can go undetected. Detailed phenotyping of all symptomatic and symptom-free patients in the families is essential and should involve the assessment of skin symptoms and acute attacks and relate these to the biochemical findings.

The milder phenotype of I12T could not be predicted by expression studies. The mutation lies in the highly conserved FAD-binding domain in the amino-terminal region of the *PPOX* gene (Nishimura et al. 1995; Dailey and Dailey 1996), whereas the R152C mutation causes an amino acid substitution in exon 5 and the 338G→C mutation results in exon 4 deletion and a truncated polypeptide. Prokaryotic and eukaryotic expression studies revealed a dramatic decrease in enzyme activity for the I12T mutation, which was comparable to the activities found in the R152C and 338G→C mutations. This suggests that in each mutation type post-translational factors and interaction between the mutant and normal polypeptide may vary and can modify enzyme activity *in vivo,* explaining the milder phenotype in patients with I12T.

The penetrance of the *PPOX* defect in our material was 52% (n=103), which is close to the 45% estimated previously by Mustajoki (1980) (n=48). The incomplete penetrance may be due to other modifying gene(s), such as cytochrome *P450*, *MDR2* or *MRP1*, which could modify porphyrin metabolism in the liver and the permanent level of circulating porphyrins predisposing to skin symptoms. Alternatively, environmental influences may modify the phenotype, especially in relation to acute attacks, which may be provoked by several exogenous factors.

2.3. Homozygous variegate porphyria

The clinical and biochemical findings in our patient are consistent with earlier reported cases of homozygous VP. The CNS involvement with minor motor co-ordination disturbances and slight verbal and visuospatial deficiencies seems to be weaker than in half of the cases where patients suffer from mental retardation. This implies that considerable variation may exist in the homozygous VP phenotype. Severe photosensitivity with early onset in childhood is common in all reported cases, as are mild developmental abnormalities, suggesting that certain early stages of development may be critically dependent on heme biosynthesis (Roberts et al. 1998). During the 20-year follow up no signs of acute attacks were observed in our patient. This is in accordance with previous reports, where only two of the 14 known homozygous patients had experienced such attacks (Coakley et al. 1990; Corrigall et al. 2000). This implies that factors other than the absolute level of PPOX may be important in the aetiology of acute attacks (Palmer et al. 2001).

In homozygous VP, at least one of the mutations in the PPOX must have substantial residual activity, otherwise heme biosynthesis would not be possible and the patient could not survive. This is probably the case with the South African mutation R59W since no homozygous patients have been encountered despite the high prevalence of the mutation (Meissner et al. 1996; Warnich et al. 1996). This is compatible with our findings that in heterozygous patients the I12T mutation causes a significantly milder clinical phenotype than in other patients. Without the homozygous patient in the family, all heterozygous patients would have remained undiagnosed.

In the previously reported mutations in homozygous VP patients, 10-25% residual activity was observed in prokaryotic expression systems (Dailey and Dailey 1997a; Roberts et al. 1998), which is slightly more than observed for the I12T mutation (0% in *E. coli* and 8% in COS-1 cells). Unlike these mutations, the I12T mutation resides in the conserved amino-terminal part of PPOX (Figure 16) and could cause the disease by a different mechanism, such as disruption of mitochondrial transport or FAD binding (Nishimura et al. 1995). However, our study showed that riboflavin treatment could not restore enzyme activity *in vitro* or alleviate clinical manifestations. While the mutation was able to disrupt the amino-terminal mitochondrial transport of the truncated PPOX, the corresponding full-length PPOX was nevertheless transported into mitochondria. It would be intriguing to hypothesize that in this patient the secondary mitochondrial targeting signal(s) could serve as a backup system, which

directs the peptide into mitochondria if the primary signal fails. This transport may, however, be less specific and efficient (Zara et al. 1992) and lead to non-optimal submitochondrial compartmentalization. This could explain the 10% residual activity *in vivo* measured from the patient's lymphocytes, which is sufficient for him to survive.

Non-optimal association of the I12T polypeptide with the mitochondrial membranes could disrupt the final steps of heme biosynthesis in the inner mitochondrial membrane, especially if substrate channelling between the last three enzymes of the pathway, coproporphyrinogen oxidase, PPOX and ferrochelatase, occurs through an enzyme complex, as suggested by Ferreira et al. (1988). Disruption of the enzyme complex could explain the low ferrochelatase activity measured from homozygous and heterozygous patients' erythrocytes, 10% and 30-40% of normal activity, respectively (R. Kauppinen, personal communication). Low ferrochelatase activity with elevated zinc protoporphyrin concentration in erythrocytes has also been reported in other cases of homozygous VP (Kordac et al. 1985; Murphy et al. 1986).

3. Diagnostics of VP

Conventional assays of urinary and faecal porphyrins detect only about 70% of VP patients; thus a large number of latent cases remain undetected. The identification of PPOX mutations in the Finnish VP families now facilitates reliable DNA-based diagnostics in each family. If a patient belongs to a known VP family, the mutation analysis is reliable and easy to perform. In families, where the mutation is unknown, the value of DNA analysis is reduced. Mutations can be detected using various DNA techniques, which can be chosen according to the experience of the laboratory personnel. In asymptomatic patients, DNA analysis alone is sufficient for the diagnosis of VP, whereas in symptomatic patients a proper clinical investigation, detailed family history and biochemical measurements should be conducted before the diagnosis is confirmed.

During an acute attack the measurement of urinary porphobilinogen and delta-aminolevulinic acid is the method of choice, and measurements of plasma fluorescence and faecal protoporphyrins are useful in distinguishing VP from other acute porphyrias (AIP and hepatic coproporphyria). If a patient experiences photosensitivity, plasma fluorescence, urinary copro- and uroporphyrin and faecal protoporphyrin measurements are essential and distinguish VP from other cutaneous porphyrias (porphyria cutanea tarda, hepatic coproporphyria, erythropoietic protoporphyria, congenital erythropoietic protoporphyria) (Mustajoki 1980; Poh-Fitzpatrick 1980; Timonen et al. 1990b). The assay for lymphocyte protoporphyrinogen oxidase is technically laborious but may provide additional information if other biochemical analyses remain inconclusive. In each case, the diagnosis of VP should be confirmed using DNA analysis whenever possible, and mutation screening is indicated for family members.

Conclusions

This work describes the molecular genetics of VP in Finland. The main results are summarized as follows:

- Eight mutations, including three amino acid substitutions, an insertion and three splicing defects, were identified among Finnish VP families. A founder mutation, R152C, was detected in 11 families.
- The outcomes of seven mutations were studied by amplification and sequencing of the reverse-transcribed total RNA obtained from patients' EBV transformed lymphoblast cell lines. One of the mutations (338G→C) showed a decrease of the steady-state level of the mutant transcript in the patient's lymphocytes compared with that of the normal allele. No reduction in mRNA level could be demonstrated for the other six mutations studied.
- The normal and five mutant polypeptides were expressed in *E. coli* and in COS-1 cells. Enzyme activities of all mutated alleles (78insC, IVS2-2a→c, 338G→C, R152C and 470A→C) were decreased to 0-5% of wild-type activity in both cell types, confirming the causality of the mutations and enzymatic defects in the disease.
- The mitochondrial targeting signal was localized in the 28 amino acids in the amino-terminal end of PPOX. Amino acids 25-477 of PPOX contain at least one mitochondrial targeting signal in addition to the amino-terminal signal.
- Of the 103 Finnish VP patients diagnosed between 1966 and 2001, 40% had photosensitivity, 27% acute attacks and 14% both manifestations. Acute attacks have decreased dramatically from 38% to 14% in patients diagnosed before and after 1980, whereas the prevalence of skin symptoms has decreased only subtly from 45% to 34%.
- Patients with the I12T mutation experienced no photosensitivity and acute attacks were rare (8%). The occurrence of photosensitivity was therefore lower in the I12T group than in the R152C group ($p=0.001$), whereas no significant differences between the R152C and 338G→C groups were observed. Biochemical abnormalities were also significantly milder in patients with the I12T mutation.
- Normal excretion of protoporphyrin in faeces in adulthood predicted freedom from both skin symptoms and acute attacks. All patients with urinary coproporphyrin excretion exceeding 1000 nmol/d experienced skin symptoms, acute attacks, or both.
- DNA diagnostics of VP was developed for 20 of the 22 known Finnish VP families in which the mutation was detected.

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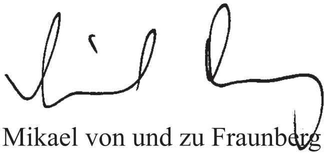

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Original publications