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Mutations in Acute Intermittent Porphyria Detected by ELISA Measurement of Porphobilinogen Deaminase

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Summary: To study the existence of different mutations in acute intermittent porphyria, erythrocyte porphobilinogen deaminase activity and enzyme protein concentration were investigated in 125 porphyria gene carriers from 31 families, and in 121 apparently healthy controls. Porphobilinogen deaminase concentration (μg/gHb) was quantified using a recently developed double-sandwich ELISA. The ratio of enzyme catalytic activity to the concentration of enzyme protein was expressed as the porphobilinogen specific activity (nkat/g). The controls had a mean porphobilinogen deaminase concentration of 160 ± 35 μg/gHb and a specific activity of 762 ± 127 nkat/g. Two different types of mutation causing acute intermittent porphyria were detected. The majority (91%) of gene carriers, from 25 families, had a diminished porphobilinogen deaminase concentration of 102 ± 18 μg/gHb, with a slightly lowered specific activity of 634 ± 105 nkat/g. In 9% of the gene carriers, representing six different families, an increase in porphobilinogen deaminase concentration to 269 ± 46 μg/gHb, and a highly significant reduction in specific activity to 234 ± 48 nkat/g, were found, which indicates the presence of a different mutation.

Introduction

Acute intermittent porphyria is a dominantly inherited (1) inborn error of haem biosynthesis characterized by abdominal and neuropsychiatric symptoms (2, 3). The gene carrier state is often asymptomatic. Certain drugs, hormones, steroids, infections and starvation may precipitate acute attacks of the disease. Hepatic 5-aminolaevulinic acid synthase activity is induced and an increased urinary excretion of 5-aminolaevulinic acid and porphobilinogen occur. Heterozygotes for the acute intermittent porphyria gene have a reduced activity of porphobilinogen deaminase[1]). Measurement of the erythrocyte activity of the enzyme has proved to be a valuable tool in the detection of latent carriers of the disease. However, there exists an overlap zone of enzyme activity, where both acute intermittent porphyria gene carriers and non-carriers are found (4–9).

The porphobilinogen deaminase gene has been cloned (10) and shown to code for two enzymes (11, 12). One form (M_r 42 000) is found exclusively in erythopoietic tissue, and an extended version (M_r 44 000) is present in non-erythropoietic cells. The cloned gene has been used for restriction fragment length polymorphism studies (13, 14), and in informative families

[1]) Enzyme
Porphobilinogen deaminase (syn: uroporphyrinogen-I-synthetase, hydroxymethylbilane synthetase) EC 4.3.1.8

this approach has been of diagnostic value. Porphobilinogen deaminase has been purified and monospecific polyclonal anitbodies against the enzyme have been produced in rabbits (15–19). Different classes of enzyme defect have been identified from the amount of immunologically cross-reacting enzyme material (CRIM) in erythrocyte lysates of acute intermittent porphyria heterozygotes. Four classes of mutation were identified with the use of rocket immunoelectrophoresis (15–17), two CRIM-negative and two CRIM-positive. The majority of CRIM-negative families, designated type 1, exhibited half-normal porphobilinogen deaminase activity, which was accounted for by a reduction of porphobilinogen deaminase protein. Some families had a normal erythrocyte enzyme activity (20) and were designated CRIM-negative type 2. Other families had CRIM-positive mutations. One type had moderately elevated concentrations of porphobilinogen deaminase protein in erythrocyte lysate and was designated CRIM-positive type 1. The other CRIM-positive mutation, type 2, had markedly more enzyme protein. In the present study a Swedish collective of acute intermittent porphyria heterozygotes and normal controls was investigated for erythrocyte porphobilinogen deaminase activity, porphobilinogen deaminase protein concentration measured by ELISA (21) and porphobilinogen deaminase specific activity, i.e. enzyme activity related to the concentration of enzyme protein.

Materials, Methods and Human Subjects

Materials

Microtitre plates and an Immunowash 8 were obtained from Nunc (Roskilde, Denmark). ELISA absorbances were read in a MR 700 Microplate reader, Dynatech Laboratories (Guernsey, Great Britain). Evacuated heparinized test tubes were purchased from Becton-Dickinson (Plymouth, Great Britain).

Porphobilinogen deaminase activity

The activity of porphobilinogen deaminase in erythrocyte lysate was measured according to the method of *Magnussen* et al. (22). The enzyme activity was expressed in two ways: picokatal per gram haemoglobin (pkat/g Hb) and nanokatal per litre red blood cells (nkat/l). The latter method of calculation was employed in the original publication, and it is used again here to facilitate comparison with earlier reports and earlier investigations in our laboratory.

The ELISA

A monospecific polyclonal antiserum was raised in rabbits against the erythropoietic form of porphobilinogen deaminase, purified from human red blood cells (23). For immunological quantification of porphobilinogen deaminase enzyme protein in human erythrocyte lysate, a double-sandwich enzyme-linked immunosorbent assay (ELISA) was developed (21). The IgG fraction used was shown to be monospecific by immunoblotting, rocket immunoelectrophoresis and immunotitration. A measuring range from 4 ng to 50 pg was demonstrated. Intra- and inter-assay coefficients of variation were 6% and 7% respectively.

Lysis of the erythrocytes was achieved by freezing at −20 °C and thawing. Prior to analysis, 25 µl of the lysate was diluted by adding 100 ml of 50 mmol/l Tris-HCl pH 7.5, 0.15 mol/l NaCl. One hundred µl of the 1:4000 dilution was added to each of the microtitre plate wells. Duplicate samples were used. Erythrocyte lysate from a patient with a high concentration of porphobilinogen deaminase, as determined by titration against the purified enzyme, was used as an internal standard and included in each assay. Haemoglobin concentration was measured in the diluted erythrocyte lysate samples in the MR 700 Microplate reader as the absorbance at 410 nm. Assay results were expressed as microgram porphobilinogen deaminase enzyme protein per gram haemoglobin (µg/g Hb).

Specific activity

The porphobilinogen deaminase specific activity was determined in erythrocyte lysates from controls and acute intermittent porphyria by dividing the porphobilinogen deaminase activity (pkat/g Hb) by the porphobilinogen deaminase protein concentration (µg/g Hb) as measured by ELISA. The specific activity was expressed as nanokatal per gram porphobilinogen deaminase enzyme protein (nkat/g).

Human subjects

Carriers of the acute intermittent porphyria gene, 125 individuals from 31 different families, were investigated. According to genealogical data, 68 of the carriers were descended from the large family from northern Sweden first described by *Waldenström* (1, 24). The diagnosis of acute intermittent porphyria was documented by a careful investigation of biochemical findings, family history of acute intermittent porphyria and clinical symptoms. Eighty nine of the 125 acute intermittent porphyria gene carriers were investigated for 5-aminolaevulinate and porphobilinogen in the urine. Excess excretion was found in 82 of them and in seven a normal amount of 5-aminolaevulinate and porphobilinogen was found. The other 36 acute intermittent porphyria gene carriers were diagnosed only by porphobilinogen deaminase activity. There was no statistically significant difference in porphobilinogen deaminase activity between these groups (unpaired t-test, $p < 0.001$). As controls, 121 apparently healthy individuals of matching age and sex were used.

Blood samples

Heparinized blood was collected by venous puncture and sent by post to the laboratory. After centrifugation at 2000 *g* for 10 minutes at room temperature, the plasma was carefully removed. The erythrocytes were stored at −20 °C up to six months before assay. Storage within this period of time did not affect the assay results.

Results

Erythrocyte porphobilinogen deaminase activity

Figures 1a und b show the erythrocyte porphobilinogen deaminase activity in control persons and in acute intermittent porphyria heterozygotes, expressed as pkat/g Hb and nkat/l. The erythrocyte porphobilinogen deaminase activity was 120 ± 26 pkat/g Hb

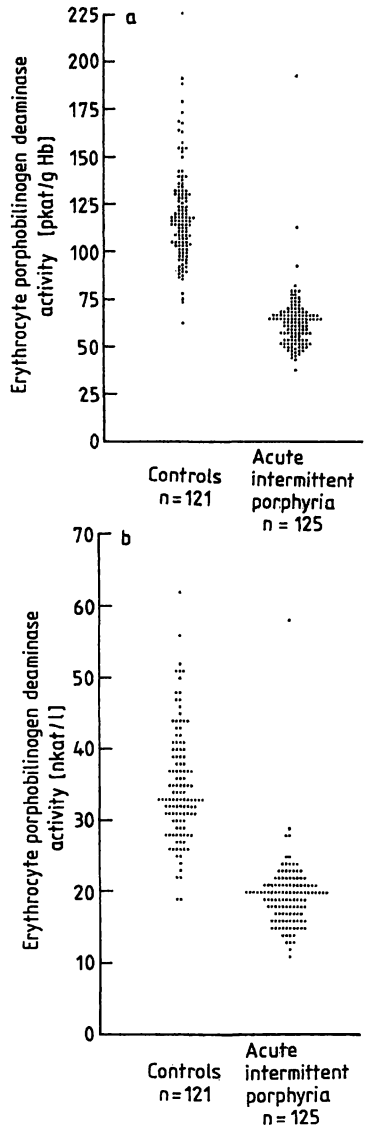

Fig. 1. Erythrocyte porphobilinogen deaminase enzyme activity in acute intermittent porphyria heterozygotes and in controls, enzyme activity expressed as pkat/g Hb (a) and nkat/l (b). One point represents one individual.

(36 ± 8 nkat/l) for the controls and 65 ± 16 pkat/g Hb (20 ± 5 nkat/l) for the acute intermittent porphyria gene carriers. A considerable overlap in activity between controls and patients is noted, in agreement with previous reports (4–9). At the time of the investigation one of the acute intermittent porphyria heterozygotes had a metastatic carcinoma of the prostate and severe anaemia with a haemoglobin concentration of 70 g/l. This patient exhibited a substantially increased porphobilinogen deaminase activity of 189 pkat/g Hb (58 nkat/l), (tab. 1), which is more than + 3 SD outside the mean for the remaining patients in the group. Three years earlier, when the patient had a normal haemoglobin concentration, his erythrocyte porphobilinogen deaminase activity was 20 nkat/l.

Erythrocyte porphobilinogen deaminase enzyme protein concentration

Figure 2 shows the porphobilinogen deaminase enzyme protein concentration in controls and acute intermittent porphyria heterozygotes. The controls had a porphobilinogen deaminase concentration of 160 ± 35 µg/gHb. The acute intermittent porphyria heterozygotes fell into two distinct groups. More than 90% of the patients (n = 113) from 25 different families, exhibited a lower concentration (102 ± 18 µg/g Hb), compared with the controls, which indicates a CRIM-negative mutation. However, 12 acute intermittent porphyria heterozygotes (9%) from six different families, had a markedly raised concentration of 277 ± 52 µg/g Hb, which signifies a CRIM-positive mutation.

Tab. 1. Erythrocyte porphobilinogen deaminase (PBGD) activity, enzyme protein concentration and specific activity in 121 controls and 125 acute intermittent porphyria heterozygotes.

	Controls n = 121			Acute intermittent porphyria n = 125			Acute intermittent porphyria CRIM negative, n = 113			Acute intermittent porphyria CRIM positive, n = 11[a])		
	Mean	S.D.	Range	Mean	S.D.	Range	Mean	S.D.	Range	Mean	S.D.	Range
PBGD activity (pkat/g Hb)	120	26	63– 225	65	16	38–189	64	11	38–114	61	6	52– 69
PBGD activity (nkat/l)	36	8	19– 70	20	5	11– 58	20	3	11– 30	19	2	16– 24
PBGD concentration (µg/g Hb)	160	35	102– 268	119	57	63–363	102	18	63–148	269	46	210–344
PBGD specific activity (nkat/g)	762	127	522–1189	600	149	170–950	634	105	430–950	234	48	171–320

[a]) One CRIM-positive patient was excluded (see Discussion), with a porphobilinogen deaminase activity of 189 pkat/gHb (58 nkat/l), a porphobilinogen deaminase concentration of 363 µg/g Hb and a porphobilinogen deaminase specific activity of 520 nkat/g.

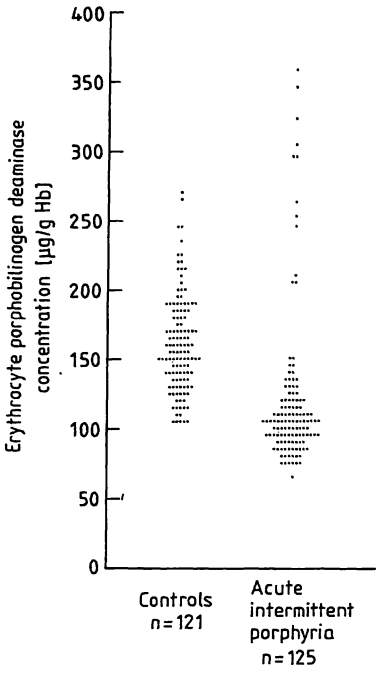

Fig. 2. Concentration of immunologically detectable porphobilinogen deaminase protein in erythrocyte lysates in acute intermittent porphyria heterozygotes and in controls, measured by ELISA.

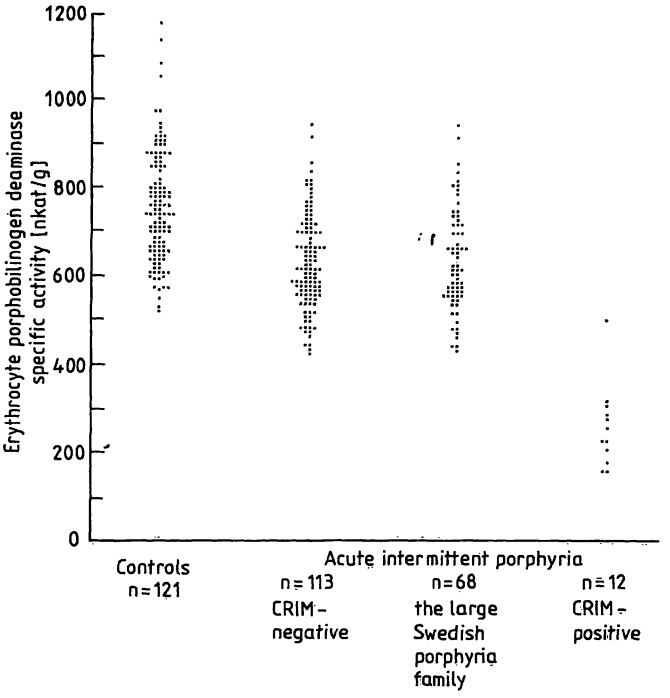

Fig. 3. Erythrocyte porphobilinogen deaminase specific activity in controls and in CRIM-negative and CRIM-positive acute intermittent porphyria heterozygotes. Individuals belonging to the same CRIM-negative family from northern Sweden are plotted separately.
CRIM = immunologically cross-reacting enzyme material

Erythrocyte porphobilinogen deaminase specific activity

The erythrocyte porphobilinogen deaminase specific activity for the controls was 762 ± 127 nkat/g. A significantly lower figure of 634 ± 105 nkat/g (unpaired t-test, $p < 0.001$) was obtained for the CRIM-negative acute intermittent porphyria heterozygotes. Sixty eight of the 113 acute intermittent porphyria heterozygotes were descended from one large Swedish family. They had the same distribution of values for porphobilinogen deaminase specific activity as the other individuals in the CRIM-negative group (fig. 3). The CRIM-positive gene carriers, with raised porphobilinogen deaminase concentration, exhibited a highly significant reduction in specific activity to 234 ± 48 nkat/g (n = 11) (tab. 1). One patient with raised specific activity, a proband in a CRIM-positive family (fig. 4, marked by arrow), was excluded (see Discussion). The inheritance of a CRIM-positive mutation is demonstrated in figure 4.

Discussion

An ELISA method for use in porphyria routine diagnostic work was developed previously (21) for quantitation of porphobilinogen deaminase enzyme protein concentration in erythrocyte lysates. In the present work it was applied in the study of a population of Swedish acute intermittent porphyria gene carriers. Two classes of mutations were revealed, one CRIM-negative with a low concentration of porphobilinogen deaminase enzyme protein and one CRIM-positive with a high porphobilinogen deaminase concentration, compared with controls. The CRIM-positive carriers exhibited a highly significant reduction in porphobilinogen deaminase specific activity, which indicates that immunoreactive non-catalytic enzyme protein encoded by the mutant allele was detected by the ELISA. The CRIM-positive carriers also had a considerable increase of catalytically inactive protein and are probably of a type similar to the CRIM-positive type 2 class, previously described (17), which may be caused by a mutation in the structural gene. However, the CRIM-negative acute intermittent porphyria heterozygotes also had a significantly lowered porphobilinogen deaminase specific activity, although not of the same magnitude as the CRIM-positive group. In a pure regulatory gene defect the mutant allele is not considered to produce an enzyme with diminished activity. The CRIM-negative mutation(s) may thus represent changes in the DNA, leading to altered enzyme stability, mRNA transcription or processing.

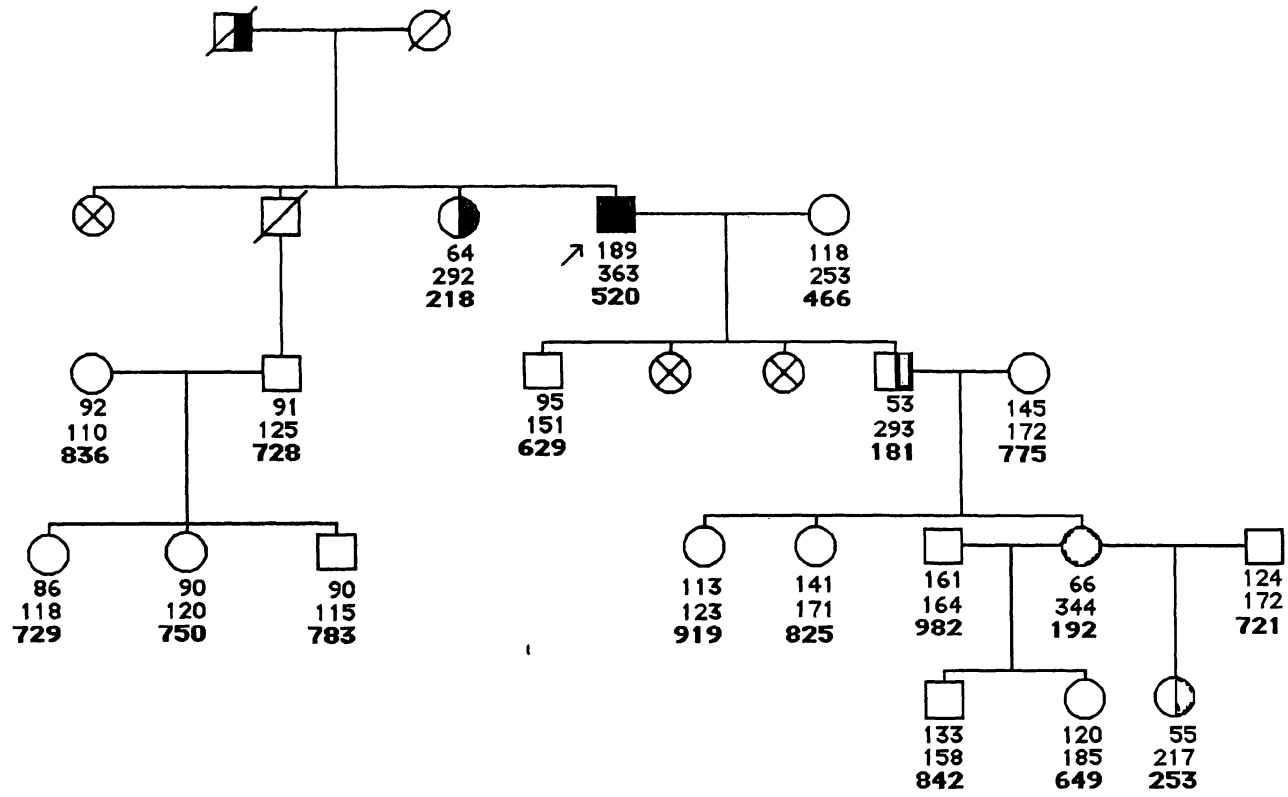

Fig. 4. A CRIM-positive acute intermittent porphyria family. Figures under symbols denote values obtained for porphobilinogen deaminase activity expressed as pkat/g Hb (upper), porphobilinogen deaminase concentration expressed as μg/g Hb (middle) and, in bold type, the porphobilinogen deaminase specific activity expressed as nkat/g (lower).

- ◧ deceased acute intermittent phorphyria heterozygote
- ○, □ unaffected
- ● acute intermittent porphyria heterozygote with clinically manifest disease
- ⊗ not investigated
- ◐, ◧ asymptomatic acute intermittent porphyria heterozygote
- ◘ proband with anaemia and metastatic cancer of the prostate

In the CRIM-positive family presented in figure 4, the inheritance of the mutation was clearly demonstrated, as all gene carriers showed an increased porphobilinogen deaminase concentration. All carriers also showed a decreased porphobilinogen deaminase specific activity, except the proband with anaemia and metastatic cancer of the prostate, who fell more than + 3 SD outside the group in this respect. He had a porphobilinogen deaminase concentration of the same magnitude as the other gene carriers in the family but considerably raised enzyme activity, which explains the increase in porphobilinogen deaminase specific activity to 520 nkat/g. Statistically he was not representative of the CRIM-positive group. It has been reported that the porphobilinogen deaminase enzyme activity in red blood cells is also subject to regulation by factors other than the major gene on chromosome 11 (25). It has further been shown that porphobilinogen deaminase activity may be increased in haemolytic disorders, in liver cirrhosis and other liver affections and in malignancies (26–28).

By measuring porphobilinogen deaminase protein concentration in addition to porphobilinogen deaminase activity, different mutations of acute intermittent porphyria can be distinguished. The information gained may be of diagnostic value, since in CRIM-positive families the raised porphobilinogen deaminase enzyme protein concentration and the decreased porphobilinogen deaminase specific activity distinguish between carriers and non-carriers of the gene defect. In CRIM-negative mutations, the porphobilinogen deaminase enzyme protein quantification yields additional information on the erythrocyte enzyme. The diagnostic potential of this information will be further evaluated. However, the ELISA method now available is less time-consuming than the traditional enzyme activity assay. It therefore offers a new means of rapidly investigating a large number of samples from populations at risk for the disease.

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