Two new mutations in the dihydropteridine reducase gene in patients with tetrahydrobiopterin deficiency

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Abstract

Two new mutations have been identified within the dihydropteridine reducase (DHPR) gene in two patients with DHPR deficiency. The total coding sequence of the cDNA has been screened by chemical cleavage of mismatch in both patients and selected portions of the cDNA have been sequenced. The first mutation identified causes a glycine to aspartic acid substitution at codon 23 and seems particularly frequent in Mediterranean patients. Its occurrence within a glycine string common to the amino-terminal region in NADH dependent enzymes suggests a possible causal mechanism for the defect. The second change involves a tryptophan to glycine substitution at codon 108 and is carried by both alleles in the second patient. It occurs in a motif which shows similarities with a region of dihydrofolate reducase (DHFR) and is highly conserved within different animal species.


Dihydropteridine reducase (DHPR, EC 1.6.99.7) is an essential enzyme in the hydroxylation system of the aromatic amino acids, since it catalyses the regeneration of tetrahydrobiopterin (BH₄), the natural cofactor of phenylalanine, tyrosine, and tryptophan hydroxylases, from the quinonoid-dihydrobiopterin produced in these coupled reactions.\(^\text{1}\) Inherited DHPR deficiency in humans causes hyperphenylalaninaemia as well as impaired production of monoamine neurotransmitters derived from tyrosine and tryptophan, dopamine, norepinephrine, and serotonin.\(^\text{2}\) This metabolic derangement leads patients to develop progressive and irreversible brain deterioration with severe neurological symptoms including psychomotor retardation, hypotonia of the trunk with limb hypertonia, and myoclonic epilepsy. A similar phenotype is shared by two additional inborn errors of BH₄ synthesis, 6-pyruvoyl tetrahydropterin synthase and GTP cyclohydrolase deficiencies, responsible for impaired cofactor availability. On the basis of worldwide series, an overall frequency of such cases of 1 to 3% has been estimated among all types of hyperphenylalaninaemia detected by newborn mass screening.\(^\text{3}\) However, higher incidences have been reported in Mediterranean and non-Caucasian populations.\(^\text{4}\)

All these disorders, at present called BH₄ deficiency, are clinically unresponsive to a restricted phenylalanine diet, unlike phenylketonuria, and so they have been previously described as 'malignant' or 'atypical' hyperphenylalaninaemia.\(^\text{5,6}\) The unfavourable outcome can be largely prevented by the early administration of synthetic BH₄, alone or in combination with diet and substitutive neurotransmitter therapy with hydroxylated precursors.\(^\text{7}\) The choice of the best treatment, and the individual adjustments required to obtain a good clinical response, are difficult to determine. Some DHPR deficient patients respond fully to BH₄ administration, thus avoiding both low phenylalanine diets and neurotransmitter therapy, whereas others need neurotransmitter precursor supplementation.\(^\text{8}\) The clinical heterogeneity of DHPR deficiency has been confirmed by enzyme studies, leading to the identification of patients carrying a mutated protein devoid of catalytic activity (DHPR "CRM"), and of others lacking any enzyme protein (DHPR "CRM"), or with different tissue distribution of residual activity.\(^\text{9-15}\)

So far, our understanding of how DHPR functions is limited. Protein chemistry has shown that the native NADH dependent enzyme exists as a homodimer with a subunit molecular weight of between 25 and 27 kDa.\(^\text{16}\) Four cysteine residues are found in each subunit of the human and rat enzyme.\(^\text{17-20}\) The role of these cysteines is unclear; however, studies with bovine DHPR suggest that these residues do not form disulphide bridges.\(^\text{21}\) An alternate role for at least one sulphhydr group might be found in NADH binding since preincubation with NADH protects the enzyme against inhibition by a wide range of thiol modifying agents.\(^\text{16,17,22}\)

Further information and possible genotype-phenotype correlations may arise from the identification of the underlying causal genetic lesions in patients with different clinical presentations.

The cDNA sequence (and by inference the amino acid sequence) of both human and rat DHPR have been determined\(^\text{18-21}\) and the rat DHPR has been shown to have a region of similarity with other NADH dependent enzymes.\(^\text{22}\)

So far it seems that most cases of DHPR deficiency result from point mutations, since analysis of the restriction map excludes any large deletion occurring within the gene. Molecular heterogeneity is, however, expected for this disease, as inferred from the clinical presentation and Northern analysis.\(^\text{23}\)
The identification of the molecular lesions responsible may be of great clinical value: identification of an association between phenotype and genotype may assist in a correct choice of therapy from birth. Furthermore, the identification of the molecular basis of DHPR deficiency may help to define the structure and organisation of the enzyme better. Finally, mutation detection may allow earlier prenatal diagnosis, now feasible only in the second trimester of pregnancy by evaluation of the enzyme activity in fetal erythrocytes.

Recently, the first causal mutation has been identified in a Turkish patient, the insertion of the triplet ACT (threonine) after alanine 122.24 We report here the characterisation of two new mutations within the DHPR gene in two patients with DHPR deficiency.

Materials and methods

Patients

DHPR deficiency was diagnosed by measurement of urinary pterins and dried blood spot assay of DHPR in two patients. Case 1 was born to consanguineous parents of Portuguese origin.25 Hyperphenylalaninaemia was detected on neonatal screening and DHPR deficiency was diagnosed at 3 months of age. Cultured fibroblasts were DHPR “CRM+”. Case 2 was diagnosed as having DHPR deficiency in the first month of life.2 He was born to a mother from southern Italy who had a heterozygous level of DHPR activity. However, the father showed normal DHPR activity. Paternity testing was not performed.

Mutation detection

Mutation detection was achieved by the chemical cleavage of mismatch (CCM) method.24,26 The DNA change was subsequently characterised by M13 subcloning27,28 and dideoxy chain termination sequencing.29 Total or messenger RNA from each patient was isolated from approximately $4 \times 10^7$ cultured skin fibroblasts, using a guanidinium/phenol/chloroform extraction technique, preceded by oligo(dT) cellulose to obtain poly(A)+ RNA. RNA was resuspended in $H_2O$.

First strand cDNA was synthesised from 1 µg of poly(A)+ RNA or 20 µg of total RNA using a cDNA synthesis kit (Amersham International plc). RNA secondary structure was minimised by heating to 65°C before cDNA synthesis.

The coding portion of the cDNA was either amplified in two overlapping sections using the primer pairs A-D and I-F or in one piece using the primers R114 and R113 (fig 1). Amplified cDNA from the patients was then mixed with similarly amplified and radio-labelled control DNA to generate a heteroduplex. Mismatch sites were cleaved using the chemical cleavage method. DHPR cDNA from two normal subjects was used as controls (KD4, S116); a further control corresponded to the human DHPR cDNA clone described by Dahl et al.28

For dideoxy chain termination sequencing the PCR products were purified by electrophoresis on a 3% Nusieve agarose gel and then subcloned into M13. Sequencing analysis was performed on several clones for each patient by means of the Sequenase sequencing kit (United States Biochemical Corporation).

Results

Chemical cleavage of a heteroduplex of case 2 and an internally labelled control probe derived from a human DHPR clone28 (PCR products R114–R113, corresponding to the full length coding sequence) showed three cytosines that reacted with hydroxylamine. The first at approximately 90 bases from the end of the probe (fig 2A) corresponded to a G to A conversion at base 92 (fig 2B). This results in the change of the codon for Gly23 (GGT) to an aspartic acid codon (GAT). The second faint band below the 92 bp mismatch (fig 2A)

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**Figure 1** Diagrammatic representation of the coding sequence of the human DHPR cDNA showing pairs of oligonucleotide primers used for PCR amplification. The numbering (according to Dahl et al28) indicates the base in the cDNA which is complementary to the 5' residue of the oligonucleotide. PCR was performed using the Cetus Perkin Elmer PCR protocol. For the primers A–D and I–F the final MgCl₂ concentrations were 2.5 mmol/l and 1.25 mmol/l, respectively. Thirty five cycles of denaturation (two minutes at 95°C), annealing (two minutes at 65°C), and extension (three minutes at 72°C) were performed. For the primers R114 and R113, 35 cycles of denaturation (two minutes at 98°C), annealing (two minutes, 30 seconds at 65°C), and extension (three minutes at 72°C) were performed at a final MgCl₂ concentration of 1.25 mmol/l.
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Discussion

In this paper we report two new mutations affecting the DHPR gene in two patients with DHPR deficiency. The mutation in case 2 is a conversion of glycine to aspartate at codon 23. This glycine is located within a highly conserved 'glycine fingerprint' found in human DHPR (fig 4) and common to most NADH binding enzymes at their amino-terminal. Since all the five sequenced M13 clones carried this change at codon 108 and since case 1 was born to consanguineous parents, he is homozygous for this mutation.
this regard to note that a role in NADH binding has been invoked for one of the four cysteines included in DHPR, since preincubation with NADH appeared to protect the enzyme against inactivation by thiol modifying agents. The cysteine at codon 26 might be the residue involved in this process.

Further studies are needed to confirm this hypothesis. However, enzymology shows that the Gly 23 Asp mutant protein expressed in Escherichia coli has no DHPR activity (Smooker et al, submitted).

Case 2 responds so well to BH₄ monotherapy that he is now on a free phenylalanine intake. This might be because of the presence of residual activity of the mutated enzyme which could be forced by a high concentration of substrate. Further studies are needed to elucidate the relevance of each of the mutations in case 2 to this effect.

Codon 108 lies in a sequence bearing similarities to a small region of DHFR, which contains six amino acids that are invariant in DHFR from a wide range of vertebrates suggesting functional importance (fig 5). Mutation of one of these conserved residues, Trp 108 to glycine, was identified in case 1 and, interestingly, the threonine insertion immediately after Ala 122, the first mutation recognised in a DHPR deficient gene, also lies in this region (fig 5).

In DHFR substitution of phenylalanine for Trp 24, corresponding to Trp 108 in DHPR, results in a more flexible conformation that shows general reduction in affinity for its substrates, suggesting that this residue plays a role in maintaining the structural integrity of DHFR. On the other hand, in rat DHPR substitution of phenylalanine for Trp 104 (the residue equivalent to human DHPR, Trp 108) increased the Km for NADH but did not significantly reduce Kcat. However, it is important to observe that in both these cases the mutation is conservative, since both phenylalanine and tryptophan are aromatic amino acids. Expression studies fit well with the former suggestion, since the DHPR protein with Trp 108 to Gly substitution shows an increased in vitro susceptibility to digestion by proteolytic enzymes (Smooker et al, submitted). Thus, this mutation probably inactivates DHPR by causing aberrant folding of the protein and consequent disruption of a region of functional importance. The hypothesis of an encoded non-functional protein is in agreement with the finding of CRM+ material within fibroblasts from this patient, homozgyous for the Trp to Gly substitution.

Finally, our study identified the change of the CTG codon for Leu 132 to CTA, an alternate leucine codon, in case 1 and in control S116 (fig 2). This change has been reported previously in another child with DHPR deficiency indicating that this is a common polymorphism. However, even harmless polymorphisms may be of clinical value in populations (or for diseases) where most of the causal mutations are unknown, since they allow the mutant allele to be traced in family studies.

In conclusion, two new mutations have been characterised in two patients with DHPR deficiency. The localisation of the mutations within highly conserved amino acid motifs within the protein and the preliminary results of expression studies suggest that they are most probably causal. Identification of the Gly 23 to Asp substitution in several patients of Mediterranean origin suggests that this mutation may be the most frequent molecular
cause of DHPR deficiency in Mediterranean populations (Smooker et al, submitted).

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