Identification and *in Vitro* Expression of Mutations Causing Dihydropteridine Reductase Deficiency

Peter M. Smooker,* David W. Howells, and Richard G. H. Cotton

The Murdoch Institute, Royal Children's Hospital, Flemington Road, Parkville 3052, Australia

Received January 5, 1993; Revised Manuscript Received March 29, 1993

ABSTRACT: Six mutations resulting in the recessive inherited disorder dihydropteridine reductase deficiency are reported, five of which are previously unknown. Two are nonsense mutations, resulting in premature termination of the protein, with the remaining four being missense mutations. The mutations found lie in the middle to 3′ end of the dihydropteridine reductase reading frame, with the exception of one mutation which lies at codon 23, which is the only mutation found in more than one patient. The mutation pattern can be described as heterogeneous. The wild type and several of the mutant DHPR cDNA's were expressed in *E. coli* and the proteins purified and examined by a variety of techniques, including calculation of kinetic constants. One mutation (Gly23→Asp) results in completely inactive protein, while a second (Trp108→Gly) has substantial activity but does not completely dimerize. Both this mutant and a third, His158→Tyr, are extremely susceptible to *in vitro* protease digestion, indicating that their three-dimensional structure has been altered. The protein studies underline the heterogeneous nature of DHPR mutations, in that the effects of different amino acid substitutions on the DHPR enzyme are varied.

Dihydropteridine reductase (DHPR, EC 1.6.99.7) is a key enzyme required for the hydroxylation of the aromatic amino acids phenylalanine, tyrosine, and tryptophan. Its role is to reduce quinonoid dihydrobiopterin and thus regenerate tetrahydrobiopterin (Craine et al., 1972), the cofactor that donates the electrons required to activate molecular oxygen prior to amino acid hydroxylation (Lazarus et al., 1983). In man, inherited deficiency of DHPR causes a variant form of phenylketonuria associated with a debilitating neurological disease characterized by hypokinesis and other features of basal ganglia disease. The neurological symptoms are associated with reduced hydroxylation of tyrosine and tryptophan to L-dopa and 5-hydroxytryptophan, precursors in the synthesis of the neurotransmitters dopamine and serotonin, respectively, and to a secondary folate deficiency (Cotton, 1986; Smith et al., 1986; Scriver et al., 1989).

We have previously described the characterization of the DHPR cDNA (Dahl et al., 1987) and five mutations within the protein coding regions of the cDNA which we propose to be causative for DHPR deficiency (Howells et al., 1990; Blau et al., 1992, Dianzani et al., 1992). DHPR has two substrates, the quinonoid dihydrobiopterin which it reduces to the tetrahydro form and the electron-donating cofactor NADH. A mutation previously identified in one patient (Gly23→Asp) alters a highly conserved residue in a region proposed to comprise part of the NADH binding domain (Dianzani et al., 1992). It is probable that the mutant enzyme fails to bind and/or oxidize the NADH cofactor. We have extended the study of DHPR mutations and here report the characterization of a number of further mutant alleles. All the previously unknown mutations found lie in the middle to 3′ end of the coding sequences of the DHPR cDNA. In fact, of the 10 DHPR mutations we have found that only one lies in the N-terminal region of the protein. A second mutation in this region (Trp36→Arg) was recently reported by Matsubara et al. (1992); however, no details other than the mutational change were given. To determine the consequences of the naturally occurring mutations for DHPR structure and function a program of *in vitro* expression of mutant enzymes has begun, and the results are reported.

Table I: DHPR Mutations Found in This Study (Mutations Found Are Listed, as Are the Inferred Amino Acid Changes)

name	CRM[a]	refs or source[b]	mutation	predicted AA change
MMZ	+ve	Danks et al., 1979; Firgaira et al., 1981b	G92A Het.	Gly23→Asp
FMZ	+ve		G92A Het.	Gly23→Asp
LR	ND	B. Steinmann, R. Gitzelmann, N. Blau	G92A Hom.	Gly23→Asp
DP[c]	38%	Firgaira et al., 1983; Dianzani et al., 1992	T346G Hom.	Trp108→Gly
PM	100%	Cotton et al., 1986	C496T Hom.	His158→Tyr
NP	ND	Young et al., 1983	G523A Hom.	Gly170→Thr
AA	ND	M. Bitzan, N. Blau	C685T Hom.	Arg221→Stop
NF	–ve	Smith et al., 1985; Firgaira et al., 1983	C458T Het.	Pro145→Leu
			T638G Het.	Leu205→Stop

[a] CRM = cross reacting material, determined in cultured fibroblasts. [b] Where there is previous publication this reference is given, otherwise the clinicians and co-workers supplying the fibroblasts are listed. [c] Mutation determined by Dianzani et al. (1992), but mutant protein expressed in this study. Het. and Hom. are heterozygous and homozygous, respectively. ND = not determined.

EXPERIMENTAL PROCEDURES

Patients and Cell Lines. DHPR deficiency was confirmed by either the tetrahydrobiopterin loading test (Danks et al., 1979) or enzyme assay (Firgaira et al., 1979). Several of the patients have been the subject of previous publications, and these references are given in Table I. Fibroblasts were cultured in basal eagle medium supplemented with 10% fetal calf serum, collected and washed in phosphate buffered saline (PBS). Approximately 5×10^7 fibroblasts were obtained, of which half were used for the isolation of RNA.

Nucleic Acid Manipulations. Whole cell RNA was isolated from approximately 2×10^7 fibroblasts using guanidine hydrochloride as a ribonuclease inhibitor as previously described (Ramus et al., 1992). Approximately 10–20 μg of RNA was used in a first-strand cDNA synthesis reaction, using oligo dT as a primer. The reaction was performed as in Ramus et al. (1992).

0006-2960/93/0432-6443$04.00/0

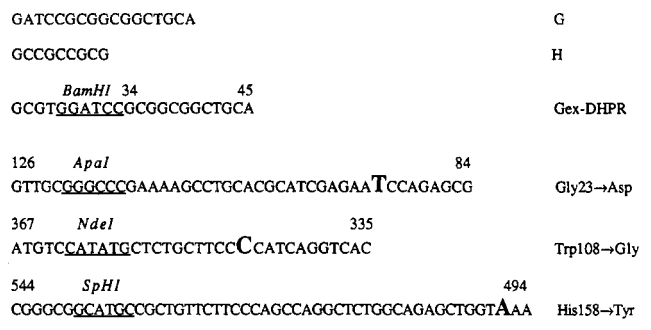


FIGURE 1: Synthetic oligonucleotides used in this study. G and H are complementary and form a *BamHI-PstI* linker. Other oligonucleotides were used as PCR primers. The three mutagenic primers are shown with the mutated nucleotide highlighted, and the restriction site used for cloning is listed.

The polymerase chain reaction (PCR) was performed using Taq polymerase, and the cDNA's were amplified using the primers Gex-DHPR (GD) and F (Figure 1). Primer F is complementary to the DHPR cDNA 3′ to the stop codon, from nucleotides 784 to 803 (numbering according to Dahl et al. (1987)). Primer GD is complementary to the DHPR cDNA from nucleotides 34 to 45, with a 10 bp tail containing a *BamHI* site. This primer was designed for use in the *in vitro* mutagenesis of the DHPR cDNA but was found to be very efficient in the PCR reaction while oligonucleotide primers in the 5′ untranslated region were not. For radioactive labeling, 0.3-0.5 μL of ^{32}PdCTP (or 5 μL of ^{35}SdATP) was added to the PCR. Each cycle consisted of two min of denaturation at 94 °C, 2 min of annealing at 60-65 °C, and 2 min of extension at 72 °C. The reaction proceeded for 35 cycles, after which the products were recovered by ethanol precipitation. If required, the PCR product was purified by electrophoresis in an agarose gel and collection on 3MM paper.

Chemical cleavage of mismatch (CCM) was performed as described by Cotton et al. (1988) and Howells (1990). A radioactively labeled probe (amplified from the wild type cDNA clone) was made, and heteroduplexes were formed by reaction with cDNA amplified from the fibroblast cell lines. After the chemical reactions with hydroxylamine and osmium tetraoxide, the modified strands were cleaved by reaction with piperidine, the products electrophoresed in 8% polyacrylamide, 7.5 M urea sequencing gels, and cleavage products detected by autoradiography.

DNA sequencing was performed using a Sequenase kit (United States Biochemicals). For sequencing PCR products, the modifications of Bachmann et al. (1990) were adopted. Synthetic oligonucleotides complementary to the DHPR cDNA were used as primers for sequencing (not shown).

Restriction endonuclease digestions and DNA ligation followed manufacturer's (Boehringer Mannheim) recommmendations. Plasmid DNA was amplified in *E. coli* strain JPA101 (Sambrook et al., 1989) and prepared using the Qiagon plasmid preparation kit.

Construction and Expression of DHPR Enzymes in E. coli. The DHPR cDNA clone was engineered to allow expression of the DHPR protein as a fusion with the glutathione transferase enzyme, using the pGEX-2T vector system (Smith and Johnson, 1988). The 1.2-kb DHPR cDNA (in a plasmid vector) was excised by digestion with *BamHI* and *PstI*. This was ligated into the pGEX-2T vector digested with *BamHI* by the use of *BamHI-PstI* linker constructed from oligonucleotides G and H (Figure 1). The assembled construct is depicted in Figure 5A. The N-terminus of recombinant DHPR will contain an additional two amino acids, glycine and serine, due to the sequence of the thrombin cleavage site. Thus, the N-terminal sequence will be GSAAAAGEAR, as compared to the wild-type sequence of MAAAAAAGEAR predicted from the cDNA sequence. This has been confirmed by protein sequence analysis (W. Armarego, personal communication). In the engineered version the three N-terminal amino acids predicted by the cDNA sequence were omitted, as amino acid analysis by DHPR peptide fragments indicated they were not present in enzyme purified from human liver (F. Morgan, W. Russell, & R. G. H. Cotton, unpublished results. The sequence could not be directly determined as the N-terminus of DHPR is blocked).

The ligation mixture was transformed into *E. coli*, and clones which expressed a 50-kDa fusion protein were selected. A plasmid clone was selected for further use and was termed pDHPR-WT. Mutant DHPR enzymes were constructed using PCR. Oligonucleotides were designed which spanned the mutation site and which contained a restriction site (Figure 1). PCR products were generated, digested with the appropriate restriction endonuclease, and ligated into the pDHPR-WT vector from which the corresponding DNA cassette had been removed. After transformation into *E. coli*, clones expressing the fusion protein were isolated. Selected clones were assayed for the presence of the desired mutation by nucleotide sequence determination. To ensure the absence of other mutations the sequence of the entire reading frame was determined. Three mutant enzymes were constructed: Gly23→Asp, Trp108→Gly (mutation reported in Dianzani et al. (1992)), and His158→Tyr. All three mutant enzymes were found to be expressed efficiently in *E. coli*.

Protein Analysis. Recombinant fusion protein (Glutathione transferase-DHPR) was isolated basically as described by Smith and Johnson (1988), with the modification that proteins were usually digested with thrombin without elution from the glutathione–agarose beads. Approximately 1 μg of plasmid DNA was transformed into *E. coli* and grown overnight at 37 °C in the presence of ampicillin. The culture was diluted 1:10, and after growth for a further hour, IPTG was added to a final concentration of 0.2 mM. After 3 h, cells were harvested and resuspended in 10 mM Tris–HCl, pH 8. Lysozyme was added to 1 mg/mL and the mixture incubated on ice for 30 min. The cells were lyzed by mild sonication (4 × 15 s), cell debris was removed by centrifugation, and the fusion protein was recovered from the supernatant by affinity chromatography to glutathione–agarose. After being washed in PBS to remove material nonspecifically bound, the fusion protein was either eluted by the addition of reduced glutathione to 5 mM, followed by thrombin digestion, or the beads were added to a thrombin digestion mix and the DHPR moiety cleaved from the glutathione transferase. The digestion mix contained PBS with 2.5 mM $CaCl_2$ and thrombin/fusion protein at a ratio of 1:100 (w/w). Digestion proceeded for 1–2 h at rt after which the proteins were stored at −20 °C until use.

Cleavage of the proteins with chymotrypsin was performed at 37 °C in a buffer containing 10 mM Tris–HCl, pH 7.4, 0.9% NaCl, with a chymotrypsin/DHPR ratio of 1:50 (w/w). Cleavage of protein was monitored by the withdrawl of samples from the digestion mix at varying times and addition to SDS-PAGE sample buffer. Proteins were electrophoresed using the Biorad SDS-PAGE minielectrophoresis system and visualized by staining with Coomassie blue.

Gel filtration was performed using a Biosil SEC-125 size exclusion column (Biorad). Proteins (20 μg) were applied in TBS at a flow rate of 0.5 mL/min. Elution was followed by

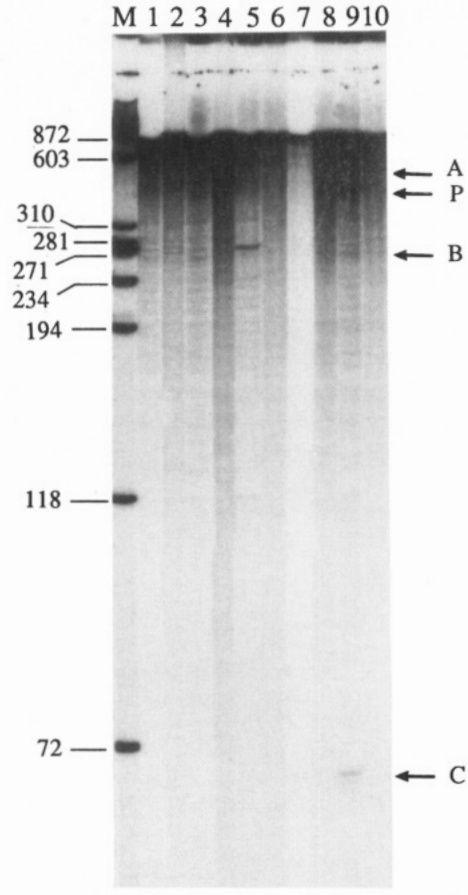


FIGURE 2: Mutation detection by chemical cleavage of mismatch (CCM). Each pair of lanes is a single heteroduplex, with odd-numbered lanes the result of hydroxylamine modification and even-numbered lanes the result of osmium tetraoxide modification. Lanes 1 and 2 are homoduplex control, 3 and 4 are a patient for which no mismatch was detected, and lanes 5 and 6, 7 and 8, and 9 and 10 are patient NP and parents MMZ and FMZ, respectively. Observed mismatches are arrowed. Note the doublet at approximately 400 bp in lane 9. This is the Leucine polymorphism (see text). The band at 69 bp in lane 7 is clearly visible on a longer esposure, but the polymorphism is not present. Lane M is a ϕX/*HaeIII* digest.

measurement of A_{280nm}. Size standards were used as followed: thyroglobulin (M_r 670 000), gamma globulin (158 000), ovalbumin (44 000), myoglobin (17 000), and vitamin B-12 (1350).

DHPR activity was measured as described in Firgaira et al. (1981a) by monitoring the oxidation of NADH in the presence of the synthetic cofactor 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine hydrochloride ($DMPH_4$; this is the product of the reaction and is oxidized to the unstable quinoid substrate by peroxidase). Initial enzyme activity was measured at 37 °C over 1 min in a Beckmann DU-50 spectrophotometer. All components except one were incubated for 5 min prior to the reaction being started by the addition of the final component, either the enzyme or $DMPH_4$. Each reaction mix contained 50 mM Tris–HCl, pH 7.2, 10 μg of horseradish peroxidase, 1 mM H_2O_2, 5–200 μM $DMPH_4$, 5–100 μM NADH, and 50 ng of DHPR. Kinetic constants were calculated from a plot of $[S]/[v]$ vs $[S]$, using the program MMPLOT. All points were the average of duplicate experiments. This gives a value for V_{max} and k_m and estimates the standard deviation.

RESULTS

Identification of Mutations. In order to search for mutations affecting the DHPR protein the cDNA derived from the RNA of affected patients was screened. For mutation detection, each of the PCR products derived from patient fibroblasts was hybridized to a radioactively labeled reference PCR product derived from the DHPR cDNA clone (Dahl et al., 1987). The heteroduplex DNA was subjected to standard chemical cleavage reactions (Cotton et al., 1988), and the cleavage products were subjected to electrophoresis and autoradiography. An example of the patterns obtained is shown in Figure 2. Included here are a negative control (homoduplex, lanes 1 and 2), a patient for which no mismatch was observed (lanes 3 and 4), and three individuals for which mismatches were detected (NP, MMZ, and FMZ, lanes 5 and 6, 7 and 8, and 9 and 10, respectively). The bands resulting from cleavage of mismatch are arrowed. Bands A and B (lane 5) are the result of a single mismatch in the cDNA of NP. Band C is present in both MMZ and FMZ. The second band for this pair is near the top of the gel and difficult to see. Band P is a doublet resulting from a common polymorphism seen in the DHPR cDNA (see below). The size of the bands resulting from cleavage of mismatches (arrowed) was estimated and the nucleotide sequence of the mutant PCR product around this point determined (Figure 3). As the PCR product will contain sequences from both DHPR alleles (assuming that both are expressed), the zygosity of the mutations could be determined. If both wild type and mutant sequences are present (half-intensity bands in two positions in the sequencing autoradiogram) the mutation is heterozygous (Figure 4). Homozygous mutations show only one sequence.

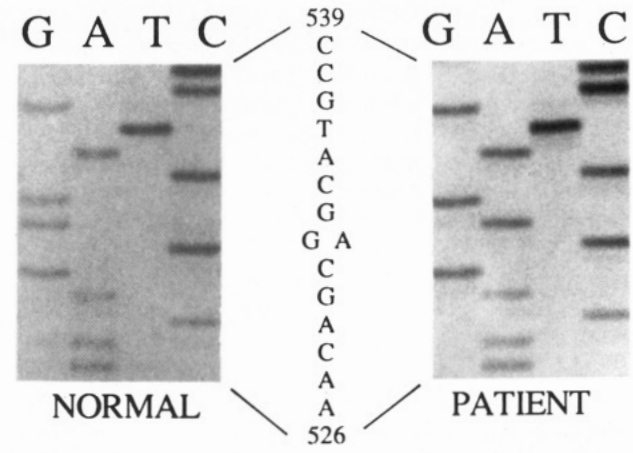


FIGURE 3: Nucleotide sequence across the point determined to contain a mutation in patient NP. A homozygous G to A mutation is apparent at nucleotide 532. This results in a Gly170→Thr substitution.

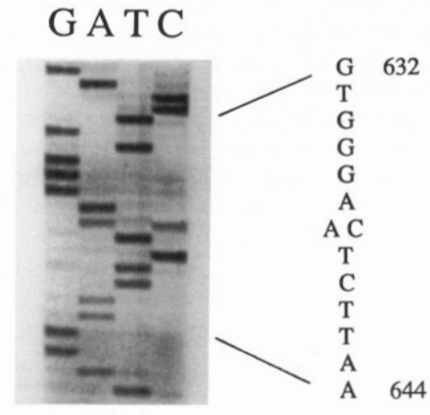


FIGURE 4: Nucleotide sequence across the point determined to contain a mutation in patient NF. A heterozygous A to C mutation is apparent at nucleotide 638 (the noncoding strand is shown, the wild type sequence has an A at this position), resulting in Leu205→Stop.

From this information the amino acid change was inferred. A summary of the mutations found and the predicted amino acid changes is given in Table I. The nucleotide numbers are taken from the cDNA sequence (Dahl et al., 1987), and the amino acid numbers include the initiating methionine residue, although this is not present in the mature protein (see below). All of the mutations found were single nucleotide substitutions resulting in the changing of one codon. There are four missense mutations and two nonsense mutations (AA and NF). Two of the mutations (NP, AA) are the result of a C to T transition at the C position of CpG doublets, proposed to be hotspots for mutation.

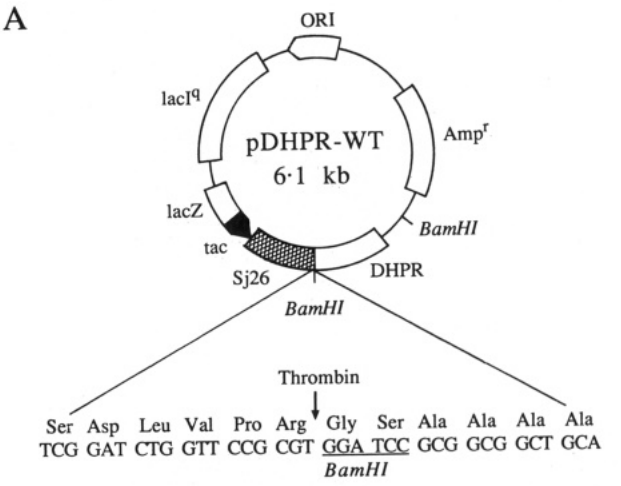
Apart from the mutation at amino acid 23, the changes reside in the 3′ half of the reading frame and, hence, in the C-terminal half of the protein. This is also true of most other DHPR mutations we have characterized (Howells et al., 1990; Blau et al., 1992; Dianzani et al., 1992) with the exception of Trp 108→Gly, which is in the N-terminal half of the protein.

In a previous paper we have reported the occurrence of a neutral mutation, G420A, which does not result in an amino acid substitution (Howells et al., 1990). Of the seven individuals examined here, two harbor this polymorphism. It does not appear to be associated with any specific disease causing mutations as demonstrated by the observation that while the parents of patient MZ (MMZ and FMZ) have the same (heterozygous) mutation only one parent harbors the polymorphism (band P, Figure 2).

Expression of Fusion Products. Large-scale preparations of the fusion protein yielded several milligrams of fusion protein per liter of culture. Figure 5B shows the purified fusion protein, before and after thrombin cleavage. After digestion with thrombin the two components of the fusion protein, the N-terminal glutathione S-transferase and C-terminal DHPR, are liberated. In this system the 26.7-kDa transferase has a higher mobility than the 25-kDa DHPR. The purified recombinant DHPR and the enzyme purified from human liver were electrophoresed and ran at indistinguishable mobilities (not shown).

Kinetic Characterization of the Enzymes. The enzymatic activity of the wild type and mutant DHPR enzymes was examined. The activity was measured on two separate occasions (using different protein preparations) in two ways. In the first all components except $DMPH_4$ were incubated for 5-10 min to allow equilibration to 37 °C and the reaction started by the addition of substrate. In the second, enzyme was added last. Comparison of the results will reveal any instability of the enzymes at 37 °C. It was found that the Gly23→Asp mutation rendered the enzyme completely inactive. The Trp108→Gly mutant, however, exhibited a high level of activity. The results obtained for the wild type protein and this mutant are shown in Table II. It can be seen that there is no instability of the wild type or mutant protein, as the values for V_{max} are similar for both methods. The mutant protein has reduced, but significant, activity compared to the wild type and a slightly higher affinity for the synthetic substrate. The third mutant, His158→Tyr, did not give reliable results using either system, and no kinetic constants could be calculated. It may be that this enzyme is unstable under the conditions of the enzyme assay.

The affinity of the wild type and Trp108→Gly enzymes for the cofactor NADH was also determined, using a $DMPH_4$ concentration of 50 μM. For the Trp108→Gly mutant, the k_m (NADH) was about double that of the wild type DHPR. This is in contrast to the slightly lower k_m (higher affinity) the mutant enzyme has for the pterin and may indicate that the binding of NADH is a significant factor in the observed lower activity.



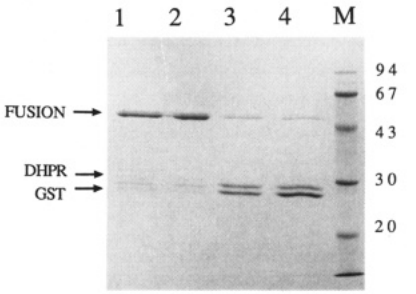


FIGURE 5: (A) Construct used for expression of the glutathione transferase–DHPR fusion protein. The diagram is based on that of Smith & Johnson (1988) and shows the DHPR cDNA inserted at the *BamHI* site. The sequence around the thrombin cleavage site is shown. The Gly-Ser dipeptide added to the N-terminus is apparent, followed by the DHPR amino acid sequence. (B) Cleavage of the fusion protein. Lanes 1 and 3 are WT fusion protein before and after thrombin digestion, and lanes 2 and 4 are a mutant. Size standards are shown in lane M.

Table II: Kinetic Parameters of DHPR Enzymes[a]

protein	V_{max}	K_m ($DMPH_4$)	K_m (NADH)
wild type (1)	333 ± 20	41 ± 7.0	11 ± 1.8
wild type (2)	301 ± 28	38 ± 9.7	ND
Trp108→Gly (1)	122 ± 9.3	25 ± 6.8	24 ± 7.1
Trp108→Gly (2)	143 ± 19	24 ± 9.8	ND
Gly23→Asp	0	NA	NA
His158→Tyr	ND	ND	ND

[a] V_{max} is in μM NADH oxidized/min/mg protein, k_m values are in μM. (1) and (2) refer to separate determinations as described in the text. NA = not applicable, ND = not determined.

Proteolytic Analysis of the Enzymes. During the course of protein purification it was discovered that the thrombin protease used to cleave the fusion protein into the transferase and DHPR moieties also caused degradation of two of the mutant DHPR enzymes. Extended incubation with thrombin results in a dramatic decrease in the levels of mutant DHPR relative to the levels of glutathione S-transferase. This is shown in Figure 6A. Incubation of the wild-type fusion protein with thrombin for 16 h at 37 °C does not diminish the levels of wild-type DHPR protein; however, the DHPR enzymes carrying either of the substitutions Trp108→Gly or His

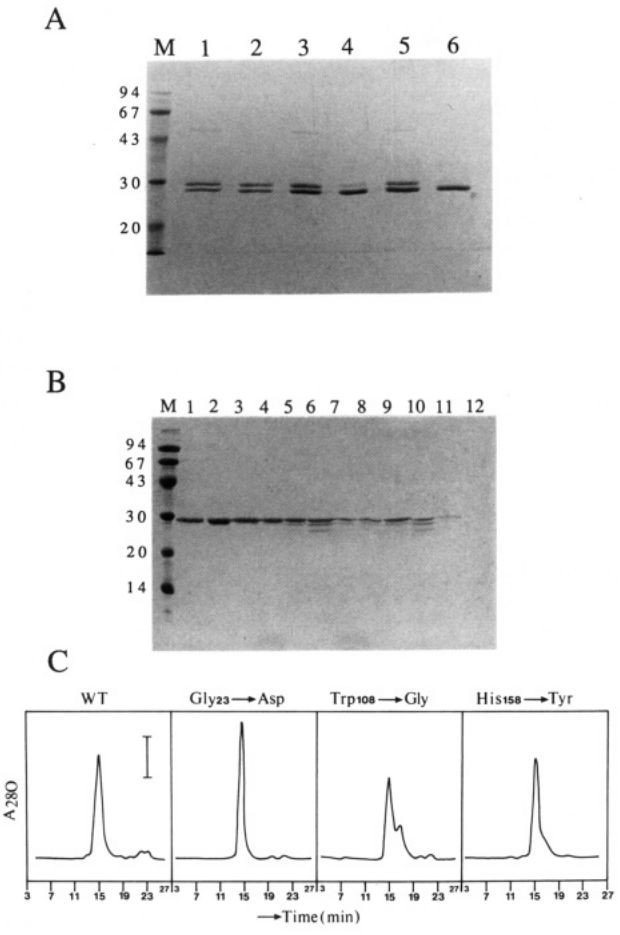


FIGURE 6: Characterization of the proteins. (A) Susceptibility of the fusion protein to thrombin digestion. Lanes 1 and 2, 3 and 4, and 5 and 6 are WT, His 158→Tyr, and Trp108→Gly, respectively, with odd-numbered lanes the result of 1 h thrombin digestion at 25 °C and even numbered lanes the result of 16 h digestion at 37 °C. (B) Susceptibility of the DHPR enzymes to chymotrypsin digestion. Lanes 1, 5, and 9 are WT, 2, 6, and 10 are the Gly23→Asp mutant, 3, 7, and 11 are Trp108→Gly, and 4, 8, and 12 are His158→Tyr. In lanes 1–4, the proteins were incubated at 37 °C for 1 h without enzyme. In lanes 5–8, proteins were incubated with chymotrypsin (1:50 w/w) for 15 min, and in lanes 9–12 for 1 h. (C) Gel filtration studies. Twenty μg of each protein was applied to the column, and the elution profiles are shown. The bar in the first panel represents 0.001 absorbance units. Size standards were run (not shown), and an estimation of molecular weights indicates that the main peak is a dimer of DHPR, with the smaller peak (visible only in panel 3, Trp108→Gly) being monomer.

158→Tyr are almost completely degraded by this treatment (the Gly23→Asp was not tested, see below).

The susceptibility of mutant enzymes to protease digestion was tested further using chymotrypsin. In this study all three mutants were examined (Figure 6B). In this experiment the DHPR enzyme has been purified from the glutathione S-transferase by thrombin digestion of the fusion protein while still bound to the gluthathione–agarose beads; hence, only the DHPR protein is present (see materials and methods). The two DHPR mutants which showed susceptibility to degradation by thrombin also exhibit marked sensitivity to chymotryptic digestion; after 1 h there is very little of the Trp108→Gly mutant enzyme and no detectable His158→Tyr mutant enzyme remaining.

In contrast, the Gly23→Asp mutant enzyme is relatively resistant to protease digestion. There appears to be slightly more digestion of this mutant than wild-type DHPR, but the extent of digestion does not approach that of the other two mutant enzymes examined.

Finally, the structure of the enzymes was studied using gel filtration. DHPR is known to form a dimer (Firgaira et al., 1981a), and it is possible that some mutations may retard this dimerization. The results are shown in Figure 6C. It can be seen that dimerized enzyme is present in all cases, however, the Tryp108→Gly mutation leads to the formation of a significant amount of monomer, which will presumably be inactive. The Gly23→Asp mutant is eluted as a slightly sharper peak than the other proteins (evidenced by the height of the peak—an equal amount of each protein was applied to the column).

DISCUSSION

This paper describes the characterization of the mutations of five patients diagnosed as DHPR deficient. In addition, the parents of a sixth patient, from whom material was not available, have been studied. Some of the DHPR mutations have been expressed in *E. coli* and the effects of the mutations on the structure and function of the enzymes studied by various methods. In this discussion we will first examine the nature of the amino acid substitutions found, with some speculation as to the consequences of the mutations, followed by an analysis of the results obtained by the *in vitro* protein studies. A summary of the mutations found and the probable reasons for enzyme deficiency resulting in the disease state is given in Table III.

In the analysis of mutant DHPR alleles, one known and five previously unknown DHPR mutations were found (Table I). There are two nonsense mutations, which would result in premature termination of the peptide chain. These mutations result in a loss of 40 amino acids (NF) or 24 amino acids (AA). The effect of a loss of C-terminal amino acids is often the degradation of the protein *in vivo*. This is certainly the case for NF, as this patient is known to have no cross-reacting material (CRM) in fibroblasts (Firgaira et al., 1983). Hence, both mutant proteins expressed from the two alleles are unstable. The second allele in this patient carries a Pro145→Leu mutation, which must also result in degradation. The mutation present in patient PM, by contrast, does not result in *in vivo* proteolysis as this patient has 100% CRM in fibroblasts (Cotton et al., 1986). However, evidence presented in this paper suggests that the protein structure is indeed perturbed by the His158→Tyr mutation (see below).

Patient NP has a homozygous Gly→Thr substitution. Glycine often has a defined role in the structure of the carbon backbone, allowing the chain to adopt a wide range of angles. Replacement with threonine will presumably inhibit this property. The sixth mutation determined in this study, and the only one found more than once, also involves mutation of a glycine residue. The patient LR and parents MMZ and FMZ harbor a Gly23→Asp substitution. This is the third conserved glycine residue in the dinucleotide binding consensus sequence Gly-X-Gly-X-X-Gly connecting the first β-strand to the α-helix in the βαβ fold (Wirenga et al., 1986). Replacing the small glycine residue with the larger, charged aspartic acid will presumably destabilize the formation of the βαβ fold, and the NADH cofactor will not be able to bind.

With the exception of the Gly23→Asp mutation, none have been observed more than once. This mutation has been observed in two unrelated Italian patients (MB, Dianzani et al., 1992, and LR) and in the (unrelated) Maltese parents of a third patient (MZ). Thus, it appears to come from a Mediterranean background. It is possible that as more DHPR mutations are identified various alleles will predominate in distinct ethnic and/or geographic communities. It is inter-

Table III: Characterization of the Mutations

patient	predicted AA change	activity in vitro	protease treatment	probable reason for deficiency
MMZ, FMZ, LR	Gly23→Asp	none	resistant	disruption of NADH binding site
DP	Trp108→Gly	50%	very susceptable	reduced dimerization leading to decreased protein levels and specific activity
PM	His158→Tyr	negligible	very susceptable	decreased specific activity
NP	Gly170→Thr	ND	ND	nonconservative AA substitution
AA	Arg221→Stop	ND	ND	premature termination, inactive or degraded protein
NF allele 1	Pro145→Leu	ND	ND	nonconservative AA substitution in dimerization helix
allele 2	Leu205→Stop	ND	ND	premature termination and degradation (patient is CRM negative)

esting to note that the common DHPR polymorphism (G420A) was observed in one of these parents carrying the Gly23→Asp mutation but not the other, and thus is not associated with the causative mutation (this mutation was also found in control samples; see Howells et al. (1990)).

The analysis of recombinant DHPR enzymes enables the effects of some of the mutations to be more clearly defined. This analysis involved three approaches: enzyme activity, susceptibility to protease digestion *in vitro*, and gel filtration to examine the quaternary structure of DHPR. Taking each mutant in turn, the properties revealed by these studies will be discussed. The Gly23→Asp mutation, proposed to alter a residue highly conserved in NADH binding domains, renders the enzyme completely inactive. This would be expected if the enzyme cannot bind the cofactor NADH. It was also observed that in gel filtration this mutant is eluted as a sharper peak than the wild-type enzyme and other mutants, indicating a somewhat different conformation. As it is known that the electrophoretic mobility of DHPR changes on the binding of NADH (Firgaira et al., 1981a) this suggests that NADH binding may be affected. This mutant is relatively resistant to digestion with chymotrypsin (Figure 6B), indicating that the overall structure of the folded protein is similar to the wild-type enzyme; hence, any change in conformation must be slight.

By contrast, from kinetic measurements it is apparent that the recombinant Trp108→Gly enzyme has an appreciable level of enzymatic activity and that the reduction in activity which is observed is due to a 2-fold decrease in the affinity for the NADH cofactor (Table II). Matthews et al., (1991) mutagenized the analogous residue in rat DHPR (Trp104→Phe) and found a slight increase in the k_m for NADH (27 μM vs 21 μM) but no decrease in the catalytic activity. It should be noted, however, that changing tryptophan to phenylalanine is a more conservative substitution than to glycine, as the ring structure is retained. The Trp108→Gly enzyme has between one-third and one-half of wild type activity *in vitro*. The cross-reacting material present in cultured fibroblasts is estimated at 38% (Firgaira et al., 1983). Hence, the activity of DHPR in cells would be expected to be approximately 15–20%, although no activity was detected in cultured fibroblasts (Firgaira et al., 1983). However, from the gel filtration data (Figure 6C) this mutant does not completely dimerize; hence, the CRM level of 38% may include a significant amount of monomer, which is probably not active. The inhibition of dimerization is due to the positioning of the mutation. In the recently published three-dimensional structure of rat DHPR (Varughese et al., 1992) it is proposed that two α helices (αE and αF) are involved in dimerization. As the rat and human enzymes share 94% identical residues, it is probable that the helices involved in the formation of homodimers will be the same. Trp108 (the equivalent of Trp104 in rat DHPR) is in helix αE.

Patient PM, harboring the His158→Tyr mutation, has 100% CRM, in contrast to the 38% CRM discussed above. However, there is no detectable activity in fibroblasts. In the enzyme assay described in this paper very little activity was observed, and reliable data enabling kinetic constants to be calculated could not be obtained. Gel filtration data shows that this enzyme is in the form of a homodimer, with little, if any monomer present. This mutant is very susceptible to protease digestion *in vitro*, indicating that the structure of the protein has been altered. From this data it is apparent that susceptibility to protease digestion *in vitro* does not correlate to CRM levels *in vivo*, as both the Trp108→Gly and His158→Tyr mutants are degraded *in vitro* but only the former has a reduced CRM level *in vivo*. It may be that the inability to completely dimerize results in *in vivo* degradation. It this context, it is interesting that a mutation previously found in this laboratory, the insertion of a threonine residue after amino acid 122 (Howells et al., 1990) is also in the region proposed by Varughese et al. (1992) to be responsible for the formation of a dimer. This patient has 30% CRM in fibroblasts. Furthermore, one of the mutations present in the CRM negative patient NF (Pro145→Leu) is also in this region. We intend to express this mutant protein to test dimer formation.

In summary, in this work we have identified five further mutations causing DHPR deficiency. The effects of three mutations have been probed by the expression of recombinant DHPR enzymes in *E. coli*. We have now identified a total of 10 mutations, with another found by Matsubara et al. (1992). Only one mutation has been found more than once, the Gly23→Asp substitution. It appears that it will not be possible to predict the course of the disease by a knowledge of the mutation carried by the patient. Thus, while diagnosis of DHPR deficiency is now possible by direct mutation detection, treatment of the disease will remain a case by case proposition. The expression and *in vitro* studies of mutant proteins will, however, reveal the mechanisms by which the amino acid substitutions affect the structure and function of the DHPR enzyme.

ACKNOWLEDGMENT

We wish to thank Drs. Henrik Dahl and Irma Dianzani for discussions, Ian Jennings for advice, and Gary Pfeifer for excellent technical assistance. We thank the clinicians who supplied fibroblasts (Table I) and Marjory Crawford for tissue culture. Thanks are also due to Denise Kirby, Haley Vogel and Karina Forshaw for synthesizing oligonucleotides and Kati Bromley for preparing the figures.

REFERENCES

Bachmann, B., Luke, W., & Hunsmann, G. (1990) *Nucl. Acids Res. 18*, 1309.

Blau, N., Heizmann, C. W., Sperl, W., Korenke, G. C., Hoffmann, G. F., Smooker, P. M., and Cotton, R. G. H. (1992) *Pediat. Res. 32*, 726–730.

Cotton, R. G. H. (1986) in *Folates and Pterins* (Blakely, R. L. & Whitehead, V. M., Eds.) Vol. 3, pp 359–412, Wiley and Sons, New York.

Cotton, R. G. H., Jennings, I. G., Bracco, G., Ponzone, A., and Guardamagna, O. (1986) *J. Inher. Metab. Dis. 9*, 239–243.
Cotton, R. G. H., Rodrigues, N. R., and Campbell, R. D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4397–4401.
Craine, J. E., Hall, E. S., and Kaufman, S. (1972) *J. Biol. Chem. 47*, 6082–6091.
Dahl, H. H-M., Hutchison, W., McAdam, W., Wake, S., Morgan, F. T., and Cotton, R. G. H. (1987) *Nucl. Acids Res. 15*, 1921–1932.
Danks, D. M., Schlesinger, P., Firgaira, F., Cotton, R. G. H., Watson, B. M., Rembold, H., & Hennings, G. (1979) *Pediat. Res. 13*, 1150–1155.
Dianzani, I., Howells, D. W., Ponzone, A., Saleeba, J. A., Smooker, P. M., and Cotton, R. G. H. (1992) *J. Med. Genet.*, in press.
Firgaira, F. A., Cotton, R. G. H., and Danks, D. M. (1979) *Clin. Chim. Acta 95*, 47–59.
Firgaira, F. A., Cotton, R. G. H., and Danks, D. M. (1981a) *Biochem. J. 197*, 45–53.
Firgaira, F. A., Choo, K. H., Cotton, R. G. H., and Danks, D. M. (1981b) *Biochem. J. 198*, 677–682.
Firgaira, F. A., Cotton, R. G. H., and Danks, D. M. (1983) in *Chemistry and Biology of Pteridines* (Blair, J. A., Ed.) pp 771–775, de Gruyter, Berlin.
Howells, D. W., Forrest, S. M., Dahl, H. H-M., and Cotton, R. G. H. (1990) *Am. J. Hum. Genet. 47*, 277–285.
Lazarus, R. A., Benkovic, S. J., and Kaufman, S. (1983) *J. Biol. Chem. 258*, 10960–10962.
Matsubara, Y., Hiroyuki, I., Endo, H., and Narisawa, K. (1992) *Nucl. Acids Res. 20*, 1998.
Matthews, D. A., Varughese, K. I., Skinner, M., Xuong, N. H., Hoch, J., Trach, K., Schneider, M., Bray, T., and Whitely, J. M. (1991) *Arch. Biochem. Biophys. 287*, 234–299.
Ramus, S. J., Forrest, S. M., and Cotton, R. G. H. (1992) *Hum. Mut.*, in press.
Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Scriver, C. R., Kaufman, S., and Woo, S. L. C. (1989) in *The Metabolic Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., & Valle, D., eds.) pp 495–546, McGraw Hill, New York.
Smith, D. B., and Johnson, K. S. (1988) *Gene 67*, 31–40.
Smith, I., Hyland, K., Kendall, B., and Leeming, R. (1985) *J. Inher. Metab. Dis. 8* (suppl. 1), 39–45.
Smith, I., Howells, D. W., and Hyland, K. (1986) *Postgrad. Med. J. 62*, 113–123.
Varughese, K. I., Skinner, M. M., Whitely, J. M., Matthews, D. A., and Xuong, N. H. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6080–6084.
Wirenga, R. K., Terpstra, P., and Hol, W. G. J. (1986) *J. Mol. Biol. 187*, 101–107.
Young, J. H., Walker, V., Tippet, P. A., Clayton, B. E., and Veall, R. M. (1983) *J. Inher. Metab. Dis. 6* (suppl. 2), 111–112.