Novel disease-causing mutations in the dihydropyrimidine dehydrogenase gene interpreted by analysis of the three-dimensional protein structure

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Dihydropyrimidine dehydrogenase (DPD) deficiency is an autosomal recessive disease characterized by thymine-uraciluria in homozygous deficient patients. Cancer patients with a partial deficiency of DPD are at risk of developing severe life-threatening toxicities after the administration of 5-fluorouracil. Thus, identification of novel disease-causing mutations is of the utmost importance to allow screening of patients at risk. In eight patients presenting with a complete DPD deficiency, a considerable variation in the clinical presentation was noted. Whereas motor retardation was observed in all patients, no patients presented with convulsive disorders. In this group of patients, nine novel mutations were identified including one deletion of two nucleotides [1039-1042delTG] and eight missense mutations. Analysis of the crystal structure of pig DPD suggested that five out of eight amino acid exchanges present in these patients with a complete DPD deficiency, Pro86Leu, Ser201Arg, Ser492Leu, Asp949Val and His978Arg, interfered directly or indirectly with cofactor binding or electron transport. Furthermore, the mutations Ile560Ser and Tyr211Cys most likely affected the structural integrity of the DPD protein. Only the effect of the Ile370Val and a previously identified Cys29Arg mutation could not be readily explained by analysis of the three-dimensional structure of the DPD enzyme, suggesting that at least the latter might be a common polymorphism. Our data demonstrate for the first time the possible consequences of missense mutations in the DPD gene on the function and stability of the DPD enzyme.

Key words: uracil, thymine, 5-fluorouracil, pyrimidines, *DPYD*.

INTRODUCTION

Dihydropyrimidine dehydrogenase (DPD, EC 1.3.1.2) is the initial and rate-limiting enzyme in the catabolism of the pyrimidine bases and it catalyses the NADPH-dependent reduction of uracil and thymine to 5,6-dihydrouracil and 5,6-dihydrothymine, respectively. The activity of DPD is exclusively present in the cytosol [1] and the enzyme seems to be ubiquitously expressed with the highest activity being found in the liver [2] and peripheral blood monocytes [3]. The enzyme has been purified and extensively characterized from liver tissues of rat, pig, bovine and man [4–7]. These studies demonstrated that the native mammalian enzyme has a molecular mass of approximately 210 kDa and is composed of two identical subunits. DPD contains various tightly associated prosthetic groups including two FMN, two FAD and eight [4Fe–4S] clusters [8]. Recently, the crystal structure of recombinant pig DPD has been resolved and it revealed a highly modular organisation of the subunits, consisting of five domains with different folds [9].

DPD is also responsible for the breakdown of the widely used antineoplastic agent 5-fluorouracil (5FU). The catabolic route plays a significant role as more than 80 % of the administered 5-fluorouracil is catabolized by DPD [10]. In this light, a pharmacogenetic disorder has been described concerning cancer patients with a complete or partial deficiency of DPD suffering from severe toxicity, including death, following the administration of 5FU [11,12]. Recently, it has been shown that a number of these patients were genotypically heterozygous for a mutant allele of *DPYD*, the gene encoding DPD [11–13]. Considering the pivotal role of DPD in chemotherapy using 5FU, the identification of novel mutations underlying a DPD deficiency is of the utmost importance since it will allow the identification of patients at risk.

In this respect, the analysis of the *DPYD* gene of paediatric patients with a complete deficiency of DPD has contributed significantly to the identification of disease-causing mutations [14]. In children, a deficiency of DPD is often accompanied by a neurological disorder, but a considerable variation in the clinical presentation among these patients has been reported [14,15]. The underlying mechanism for the observed clinical abnormalities is still not known. In these patients, a large accumulation of uracil and thymine has been detected in urine, blood and in cerebrospinal fluid, whereas no activity of DPD could be detected in fibroblasts and mononuclear blood cells. To date, seven different mutations have been reported in 17 families presenting 22 patients with a complete deficiency, including: two deletions (295-298delTCAT, and 1897delC), one splice-site mutation (IVS14+1G > A) and four missense mutations (85T > C, 703C > T, 2657G > A, and 2983G > T) [14]. It has been suggested that the

Abbreviations used: DPD, dihydropyrimidine dehydrogenase; PBM cells, peripheral blood mononuclear cells; 5FU, 5-fluorouracil.

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85T > C mutation might be a common polymorphism, although functional analysis of this mutation in *Escherichia coli* demonstrated that the 85T > C mutation resulted in a mutant DPD protein without significant residual enzyme activity [13,16].

Recently, the crystal structure of pig DPD has been determined at a resolution of 1.9 Å [9]. This allows for the first time the possibility of investigating novel missense mutations in a three-dimensional framework. In this paper, we report the clinical and genetic findings of eight patients presenting with a complete DPD deficiency, as well as the analysis of the mutations at the structural protein level.

MATERIALS AND METHODS

Materials

[4-^{14}C]Thymine (1.85–2.22 GBq/mmol) was obtained from Moravek Biochemicals (Brea, CA, U.S.A.). $\alpha,\alpha,\alpha,6$-$^{2}H_4$-Thymine and 1,3-$^{15}N_2$-uracil were obtained from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). Lymphoprep™ (specific gravity 1.077 g/ml, 280 mOsm) was obtained from Nycomed Pharma AS (Oslo, Norway). LeucoSep tubes were supplied by Greiner (Frickenhausen, Germany). Fetal-calf serum was obtained from BioWhittaker (Walkersville, MD, U.S.A). Ham's-F10 medium with 20 mMHepes was obtained from Gibco BRL (Breda, The Netherlands). AmpliTaq Taq polymerase, BigDye™-Terminator-Cycle-Sequencing-Ready Reaction kits and BigDye™-Primer-Cycle-Sequencing-Ready Reaction kits were supplied by PerkinElmer (Foster City, CA, U.S.A.). A Qiaquick Gel Extraction kit was obtained from Qiagen (Hilden, Germany). All other chemicals used were of analytical grade.

Analysis of pyrimidine bases

Urine samples were centrifuged at 10000 ***g*** for 5 min to remove debris. An internal standard solution (20 μl), containing 1 mM of $\alpha,\alpha,\alpha,6$-$^{2}H_4$-thymine and 1,3-$^{15}N_2$-uracil was added to 200 μl of clear urine. Subsequently, 20 μl of this urine was injected into the HPLC–MS/MS system. The metabolites of interest were separated on a Phenomenex Aqua analytical column (250 × 4.6 mm, 5 μm particle size, Phenomenex, Torrance, CA, U.S.A.) with a linear gradient from 50 mM acetic acid, adjusted to pH 4.0 with NH_4OH (eluent A) to a mixture of 50 mM acetic acid (pH 4.0)/methanol (1:1 [v/v], eluent B) [17]. A splitter between the HPLC column and the MS was used to introduce the eluent at a flow rate of 10–20 μl/min into the MS. The eluent from 4.1 to 9 min was introduced into the MS, using an electrically operating valve. A Quattro II tandem MS was used in the positive electrospray ionization mode and nitrogen was used as the nebulizing gas. The collision gas was argon and the cell pressure was 0.25 Pa. The source temperature was set at 80 °C and the capillary voltage was maintained at 3.5 kV. Multiple-reaction monitoring was used to detect the specific m/z transition of 127 to 110 for thymine and 113 to 70 for uracil [17].

Culture conditions of human fibroblasts

Fibroblasts were cultured from skin biopsies obtained from controls and the index patient. Biopsies were incubated at 37 °C in Ham's nutrient mixture F-10, supplemented with 20 mM Hepes and 15 % (v/v) fetal-calf serum in 25 cm² cell-culture flasks until an adequate number of proliferating cells was obtained. Subsequently, cells were cultured in Ham's nutrient mixture F-10 supplemented with 20 mM Hepes and 10 % (v/v) fetal-calf serum. Fibroblasts were harvested with 0.25 % (w/v) trypsin and, after washing the cells once with PBS (9.2 mM Na_2HPO_4, 1.3 mM NaH_2PO_4, 140 mM NaCl, pH 7.4) and twice with 0.9 % (w/v) NaCl, the cells were collected by centrifugation (175 ***g***, at 7 °C for 5 min) and the supernatant was discarded. The pellets were stored at −80 °C.

Isolation of human peripheral blood mononuclear cells (PBM cells) and granulocytes

PBM cells were isolated from 15 ml EDTA-anticoagulated blood by centrifugation over Lymphoprep™ and the cells from the interface were collected and treated with ice-cold NH_4Cl to lyse the contaminating erythrocytes, as described previously [18]. The pellet from the centrifugation step over Lymphoprep™, containing the granulocytes and erythrocytes, was diluted with 7 ml of supplemented PBS (9.2 mM Na_2HPO_4, 1.3 mM NaH_2PO_4, 140 mM NaCl, 0.2 % (w/v) BSA, 13 mM sodium citrate, 5 mM glucose, pH 7.4) and centrifuged at 800 ***g*** at 24 °C for 10 min. To lyse the erythrocytes, the pellet was resuspended in 7 ml ice-cold ammonium chloride solution (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) and kept on ice for 5 min. After the addition of 10 ml of ice-cold supplemented PBS, the solution was centrifuged at 250 ***g*** at 4 °C for 10 min. The pellet was collected and subjected to another lysis step as described above. The pellet containing the granulocytes was washed once more with supplemented PBS and the final cell pellet was frozen in liquid nitrogen and stored at −80 °C until further analysis.

Determination of the DPD activity

The activity of DPD was determined in a reaction mixture containing 35 mM potassium phosphate, pH 7.4, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 250 μM NADPH and 25 μM [4-^{14}C]thymine [18]. Separation of radiolabelled thymine from radiolabelled dihydrothymine was performed isocratically (50 mM NaH_2PO_4, pH 4.5, at a flow rate of 2 ml/min) by HPLC on a reversed-phase column (Alltima C18, 250 × 4.6 mm, 5 μm particle size, Alltech Associates, Nicholasville, KY, U.S.A.) and protected by a guard column (Supelguard LC-18-S, 5 μm particle size, 20 × 4.6 mm, Supelco, Bellafonte, PA, U.S.A.) with online detection of the radioactivity, as described previously [18]. Protein concentrations were determined with a copper-reduction method using bicinchoninic acid, essentially as described by Smith et al. [19].

PCR amplification of coding exons

DNA was isolated from fibroblasts or granulocytes using standard procedures. PCR amplification of all 23 coding exons and flanking intronic regions was carried out using the primer sets, as described before [11]. Amplification of all exons, except exon 1 and 14, was carried out in 50 μl reaction mixtures containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 10 pmol of each primer, 200 μM of each dNTP and 2 units of Taq polymerase (PerkinElmer). Amplification of exon 1 was performed in the presence of 0.75 mM $MgCl_2$ and 5 % DMSO with a hot start. Amplification of exon 14 was performed in the presence of 2 mM $MgCl_2$. After initial denaturation at 95 °C for 5 min, amplification was carried out for 30–35 cycles (95 °C for 1 min, 50–65 °C for 1 min, 72 °C for 1 min). PCR products were separated on 1 % (w/v) agarose gels, visualized with ethidium bromide and purified using a Qiaquick Gel Extraction kit (Qiagen, Hilden, Germany) or used for direct sequencing.

Sequence analysis

Sequence analysis of genomic fragments amplified by PCR was carried out on an Applied Biosystems model 377 automated

DNA sequencer using the dye-primer or dye-terminator method (PerkinElmer).

Stereoviews of the point mutation sites

The Figures showing the stereoviews of the point mutation sites and their environment in the DPD protein were generated using the programs BOBSCRIPT [20,21] and RASTER3D [22].

RESULTS

Clinical phenotype of patients with DPD deficiency

Investigation of the clinical symptoms in eight patients showed a considerable variation in the clinical presentation among these patients (Table 1). Motor retardation was observed in all patients and mental retardation was noted in six out of eight patients. Growth retardation, microcephaly, dysmorphy, autism and ocular abnormalities were less frequently observed. Surprisingly, no patients presented with convulsive disorders such as seizures and epileptic insults. Patient 5, however, suffered from convulsions 20 months after the diagnosis (Table 2). In all patients, the onset of the clinical phenotype occurred during childhood (Table 2).

Biochemical phenotype of patients with a DPD deficiency

Analysis of urine samples for abnormalities in purine and pyrimidine metabolism showed the presence of strongly elevated levels of uracil and thymine. The uracil and thymine levels were 23- to 90-fold and 58- to 1132-fold, respectively, higher compared with those observed in urine of controls, which is indicative of a complete deficiency of DPD (Table 3). The DPD activity was determined in PBM cells or in fibroblasts established from a skin biopsy. In all patients presenting with thymine-uraciluria, the activity of DPD proved to be undetectably low (Table 3).

DPD mutations in patients with DPD deficiency

Analysis of the genomic sequences of all 23 exons of the DPD gene, in the eight patients with a complete DPD deficiency, revealed the presence of nine novel mutations (Table 3). Patients 1, 3, 4, 6 and 8 were homozygous for the 257C > T (Pro86Leu), 632A > G (Tyr211Cys), 1108A > G (Ile370Val), 1475C > T (Ser492Leu) and 2933A > G (His978Arg) mutation, respectively. Patients 2 and 7 were compound heterozygous for the previously identified splice donor site mutation IVS14+1G > A, leading to the skipping of exon 14, and the novel mutations 601A > C (Ser201Arg) and 2846A > T (Asp949Val), respectively. Furthermore, patient 2 proved to be homozygous for the previously identified 85T > C (Cys29Arg) mutation. Patient 5 was compound heterozygous for a two base-pair deletion and a novel missense mutation 1679T > G (Ile560Ser). Thus, five patients were homozygous for one of the identified novel mutations, whereas the other three patients were compound heterozygotes.

Table 1 Phenotype of eight patients with DPD deficiency at diagnosis (− none, + mild, + + severe)

Clinical phenotype reported after diagnosis in individual patients 1–8, respectively: (1) muscular hypotonia, large platelets, macrocephaly; (2) history of regression; (3) unspecific neurological symptoms of mild muscular weakness; (4) obese, puberty onset at age 9 years, sleep apnoea, adenoidectomy; (5) failure to thrive, central cataracts, hypertonicity, two episodes with diarrhoea, abnormalities at the EEG, MRI brain at the age of 22 months showed mild abnormalities, convulsions since the age of 2 years, at the age of 4 the patient is not able to walk, speak or eat alone but only smiles; (6) abnormal MRI; (7) pseudostrabismus, ugly formed, coarse, notched and fawn-coloured teeth [25]; (8) mild hypertonia, speech and language delay, hoarse voice, solitary play, asthma. Ethnicity is given in parenthesis.

	Phenotype at diagnosis							
Patient	Convulsions	Motor retardation	Mental retardation	Growth retardation	Microcephaly	Dysmorphy	Autism	Ocular abnormalities
1 (Eritrean–Ethiopian)	−	+	−	−	−	−	−	−
2 (Scottish)	−	+	+	−	−	−	−	−
3 (Turkish)	−	+	−	−	−	−	−	−
4 (Asian)	−	+ +	+ +	−	−	−	−	−
5 (Greek–Ukrainian)	−	+ +	+ +	+	+ +	+ +	−	+
6 (Pakistani)	−	+	+	−	−	−	+	−
7 (Dutch)	−	+	+	+	−	+	−	+
8 (Pakistani)	−	+	+	−	−	+	+	−

Table 2 Onset phenotype of patients with DPD deficiency

n.d., not determined.

Patient	Age of onset of symptoms	Age at diagnosis	Epilepsy in family	Consanguinity	Treatment
1	10 months	11 months	No	n.d.	No
2	2 years	3 years, 11 months	No*	No	No
3	3 months	3 months	No	Yes	No
4	3 years, 2 months	3 years, 9 months	n.d.	n.d.	No
5	At birth	6 months	No	No	Yes†
6	8 months	2 years, 2 months	No	Yes	No
7	At birth	1 year, 10 months	n.d.	No	No
8	3 years, 3 months	4 years	No	Yes	n.d.

* Malignant hyperthermia.

† Vigabatrin for 6 months from age 26 months onwards and condition was stable for three months, recurrent convulsions with average of one per week, treated with frizium (clobazam).

Table 3 Mutations and DPD activity in patients with DPD deficiency

Genotype (nomenclature according to Antonarakis [26]), location (according to Wei et al. [27]), and effect of the mutation on DPD protein or mRNA in DPD deficient patients. Data for patients 1, 2, 4, 5, 7 and 8 have also been published elsewhere [28]. Values are means ± S.E.M., n = number of individuals.

Patient	Genotype	Effect	Location	DPD activity*† (nmol/mg/h)	Tissue	Uracil‡ (μmol/mmol creatinine)	Thymine§ (μmol/mmol creatinine)
1	257C > T/257C > T	Pro86Leu	EX 4	< 0.011	Fibroblasts	578	342
2	IVS14+1G > A/601A > C 85T > C/85T > C	Del (EX 14)/Ser201Arg Cys29Arg	EX 6 EX 2	< 0.05	PBM cells	327	258
3	632A > G/632A > G	Tyr211Cys	EX 6	< 0.002	Fibroblasts	901	679
4	1108A > G/1108A > G	Ile370Val	EX 10	< 0.05	PBM cells	177	110
5	1039-1042delTG/1679T > G	Frameshift/Ile560Ser	EX 10/EX 13	< 0.02	PBM cells	441	151
6	1475C > T/1475C > T	Ser492Leu	EX 12	< 0.005	PBM cells	480	356
7	IVS14+1G > A/2846A > T	Del (EX 14)/Asp949Val	EX 22	< 0.003	Fibroblasts	234	35
8	2933A > G/2933A > G	His978Arg	EX 23	< 0.05	PBM cells	223	178

* DPD activity in fibroblasts of controls, 0.89 ± 0.56 nmol/mg/h (n = 21).
† DPD activity in PBM cells of controls, 10.0 ± 3.4 nmol/mg/h (n = 22).
‡ Uracil levels in urine of controls, 10.0 ± 9.5 μmol/mmol creatinine (n = 20) (range 1–37 μmol/mmol creatinine).
§ Thymine levels in urine from controls, 0.6 ± 0.4 μmol/mmol creatinine (n = 20) (range 0.06–1.7 μmol/mmol creatinine).

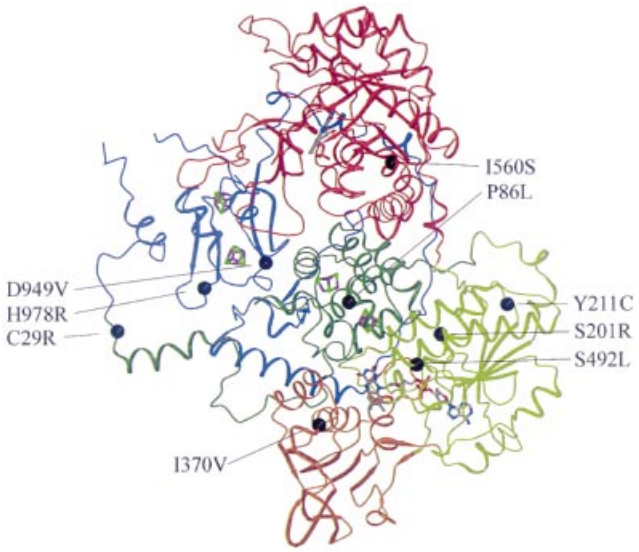

Figure 1 Localization of the point mutation sites in the DPD crystal structure

For clarity, the Figure shows only one subunit of the DPD homodimer, with the N-terminal FeS-cluster domain coloured in green, the FAD-binding domain in olive-green, the NADPH-binding domain in orange, the FMN-binding domain in red, and the C-terminal FeS-cluster domain coloured in blue. Cofactors are shown as ball-and-stick models. Black spheres indicate the positions of the newly identified point mutations. All mutation sites are labelled.

DPD mutations interpreted by crystallography

The amino acid sequences of pig and human DPD are 93% identical, and all eight positions affected by the newly identified mutations, as well as their close environment, are conserved between both enzymes [9]. Therefore, the inactivation of the human enzyme caused by these point mutations can be interpreted in a three-dimensional framework provided by the crystal structure of the pig enzyme. The localization of the point mutations sites in the DPD crystal structure is shown in Figure 1.

Five out of the eight amino acid exchanges, Pro86Leu, Ser201Arg, Ser492Leu, Asp949Val and His978Arg, most likely interfere, directly or indirectly, with cofactor binding or electron transport. Pro86 is located in close proximity to one of the iron–sulfur clusters in the N-terminal domain (nFeS1), and adjacent to Asn141 (Figure 2a). The introduction of a leucine at this position, with a side-chain conformation closest to that resembling the structure of proline, would cause a steric clash with the side-chain of Asn141 and the FeS-cluster itself and would, therefore, prevent cluster binding. An accommodation of the structure to the larger side-chain by adoption of an alternative side-chain conformation or movements of Pro86Leu and Asn141 is impossible. The space for another side-chain conformation for Pro86Leu is restricted by the bulky residue Trp41, protruding towards Pro86 from the other subunit in the dimer, and even small movements of both residues would influence the positions of their neighbours, Cys87 and Cys140, two of the cluster ligands.

The inactivity of the DPD proteins containing arginine instead of a serine at position 201, or instead of histidine at position 978, is attributable to space limitations for the larger side-chain of arginine. Both residues participate in the formation of domain-interfaces in close proximity to FAD and cluster nFeS2, or nFeS1 and cFeS1, respectively. Consequently, these mutations most likely lead to a serious disturbance of the electron flow between the individual prosthetic groups and might even prevent proper folding of these proteins.

The effect of the Ser492Leu mutation can also readily be explained from the structure. This serine is located in the binding site for the cofactor FAD and the distances between its hydroxyl group and the atoms O3* and O5* of the ribityl moiety, and the OP2 of one of the phosphates of FAD are 3.5 Å, 3.5 Å, and 3.8 Å, respectively (Figure 2b). The distances are greater than those for strong hydrogen bonds, but clearly show that the exchange to the larger and hydrophobic side-chain of leucine would prevent binding of the FAD.

By exchange of aspartate 949 for valine, the carboxyl group involved in a hydrogen bond to the main-chain nitrogen of Glu951 is lost (Figure 2c). This hydrogen bond is of structural importance as it anchors a helical turn following a β-strand in the C-terminal FeS-cluster domain of DPD, which subsequently positions the loop carrying the cluster ligands Cys953 and Cys956 close to the cluster cFeS1. The loss of the hydrogen bond is likely to have negative effects on the cluster formation.

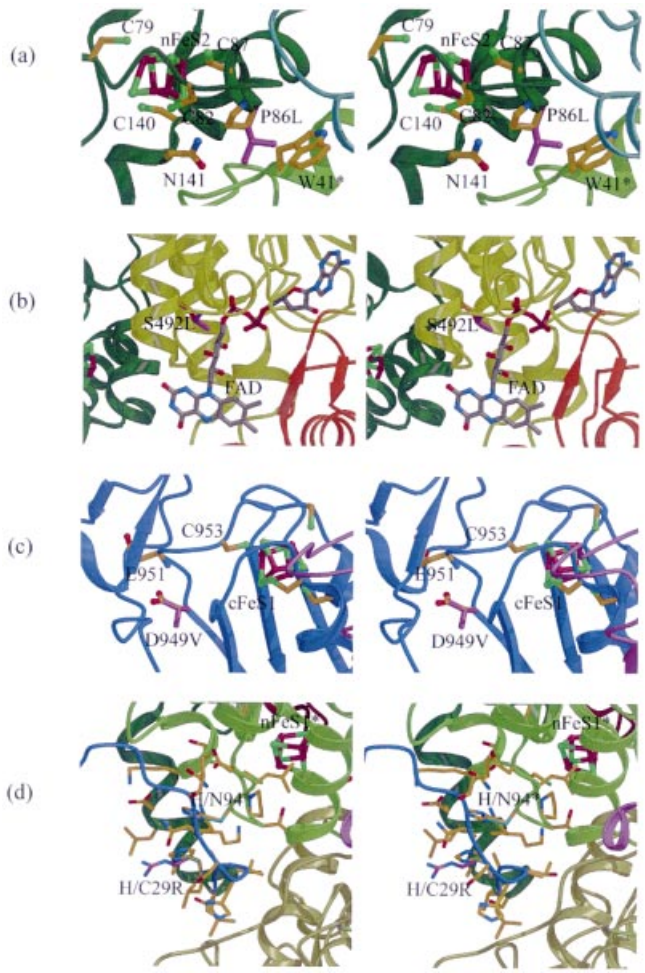

Figure 2 Stereoviews of the point mutation sites and their environment

Point mutations at (**a**) position 86 (P86L), (**b**) position 492 (S492L), (**c**) position 949 (D949V), and (**d**) position 29 (C29R). Secondary structure elements are coloured dark green for the N-terminal FeS-cluster domain of DPD, olive-green (**b**) for the FAD-binding domain, orange for the NADPH-binding domain, and dark blue for the C-terminal FeS-cluster domain. Those belonging to the other subunits in the dimer are shown in bright green (N-terminal FeS-cluster domain), bronze (**d**) (FAD-binding domain), brown (NADPH-binding domain), pink (FMN-binding domain), or bright blue (C-terminal FeS-cluster domain), respectively. Cofactors and amino acid side-chains are shown as ball-and-stick models, sulphur atoms of the FeS-clusters in green, iron atoms in dark pink. Amino-acid side-chains as seen in the crystal structure of pig DPD are shown with ochre carbon atoms. Carbon atoms in cyan indicate models of amino acid side-chains in human DPD, when they are different from those in the pig enzyme due to sequence deviations. Side-chain models with carbon atoms in magenta represent residues introduced into the human DPD by point mutations. All residues mentioned in the text are labelled, those originating from the second subunit in the dimer are additionally marked with an asterisk.

For the remaining three point mutations, the effect of Ile560Ser might be due to the introduction of a hydrophilic residue into a hydrophobic environment, leading to the destabilization of an especially sensitive region of the protein. The isoleucine belongs to one of the α/β-barrel helices in the FMN-binding domain of DPD, its side-chain pointing towards the barrel strands. Similar destabilizing effects due to the loss of two hydrogen bonds and several hydrophobic contacts in the FAD-binding domain could explain the protein inactivation by point mutation at position 211 (Tyr211Cys).

Only the effect of the Ile370Val mutation cannot be explained by the analysis of the three-dimensional structure of pig DPD alone. The exchange is conservative; isoleucine differs from valine only by an additional methyl group. The side-chain contributes to a hydrophobic cluster extending across the dimer-interface and connecting the NADPH-binding domains of each subunit in the dimer. The residues in closest proximity to Ile370 are Phe363 and Ala340. The latter is via the main-chain nitrogen directly involved in the binding of the 3′-hydroxyl group of the adenine ribose of NADPH, whereas Phe363 is the preceding residue to Arg364 which neutralizes the negative charge of the 2′-phosphate of NADPH. It is possible though, that the mutation destabilizes the tightly packed hydrophobic cluster or causes small structural changes preventing proper binding of the NADPH substrate.

Recently, a few cancer patients have been identified suffering from severe toxicity following the administration of 5FU, who were homozygous for a Cys29Arg point mutation with normal activity of DPD [13]. In contrast, recombinantly expressed protein containing the same mutation did not possess any residual activity [16]. Examination of the crystal structure of pig DPD does not reveal reasons for the non-functionality of the recombinant mutant protein. Interestingly, Cys29 is not conserved between both enzymes. The histidine found in pig DPD is located in a solvent-accessible region of the dimer interface (Figure 2d). The only interaction of its side-chain is a water-mediated hydrogen bond with the imidazol-ring of His94 from the other subunit in the dimer. This residue is also not conserved, but rather is replaced by an asparagine in the human enzyme, which is able to maintain all hydrogen-bond interactions seen for His94 in pig DPD, including a water-mediated hydrogen bond to the cystein at position 29 in the human enzyme. It could therefore be assumed that this interface region is not substantially altered between both proteins. Owing to the location of residue 29 on the protein surface, the modelling of a significantly larger arginine at this position does not result in any unfavourable close contacts to surrounding residues, since its side-chain can easily be pointed towards the solvent.

DISCUSSION

DPD deficiency is an autosomal recessive disease characterized by thymine-uraciluria in homozygous deficient patients and has been associated with a variable clinical phenotype. Despite the fact that the first patient was diagnosed over 15 years ago, only a limited number of patients have been reported since. A thorough investigation of the clinical abnormalities encountered in the eight patients showed that all patients presented with motor retardation whereas mental retardation was observed in six out of eight patients. A conspicuous finding was the observation that none of the patients presented with convulsive disorders such as seizures or epileptic attacks. In a recent survey of 17 families presenting 22 patients with a complete deficiency, convulsive disorders proved to be one of the most common clinical abnormalities encountered [14]. It should be noted that almost all of these patients were from Northern Europe, which is in sharp contrast to the origin of the patients presented in this study. To date, the pathological mechanism underlying the various clinical abnormalities is still not known, although the wide variation in clinical presentation, and perhaps also the racial and regional differences, suggest that additional factors play a role in the clinical manifestation of the disease.

DPD is generally considered to be the rate-limiting step in the catabolism of the pyrimidine bases uracil and thymine. Under normal conditions, a low DPD activity is still sufficient to

maintain uracil and thymine homoeostasis because obligate heterozygotes do not excrete elevated levels of pyrimidine bases [23]. Thus, the persistently increased levels of uracil and thymine in urine are in line with the presence of a complete deficiency of DPD in these patients.

Until now, seven different mutations have been reported in 17 families presenting 22 patients with a complete deficiency [14]. Analysis of the prevalence of the various mutations among DPD patients has shown that the G > A point mutation in the invariant splice donor site is by far the most common (52%). Recently, we showed that 1.8% of the Dutch population is heterozygous for the IVS14+1G > A mutation [12]. On this basis, one can estimate, therefore, that 1.2 individuals per 10000 would be homozygous for this mutation. Thus, compared with other frequently occurring inborn errors of metabolism, such as phenylketonuria (1:20000), the expected prevalence of a DPD deficiency is high.

To understand the genetic basis underlying the DPD deficiency in eight patients presenting with thymine-uraciluria, we sequenced the entire *DPYD* gene comprising 23 exons. In this group of patients we could identify nine novel mutations including eight amino acid substitutions and one deletion of two nucleotides. The frameshift mutation leads to premature termination of translation before the FAD and uracil/thymine binding sites and thus to a non-functional protein. Mammalian DPD appears to be relatively well conserved throughout evolution, since a comparison of the deduced amino acid sequences of human DPD with that of pig and bovine DPD disclosed a homology of 93% [9]. Recently, the crystal structure of pig DPD has been determined, and for the first time, we were able to predict the effects of disease causing mutations on the protein conformation and/or the binding of the various cofactors and substrates [9]. In this way, the analysis of the three-dimensional protein structure suggested that five out of the eight amino acid exchanges, Pro86Leu, Ser201Arg, Ser492Leu, Asp949Val and His978Arg, present in patients with a complete DPD deficiency, interfered directly, or indirectly with cofactor binding or electron transport. Furthermore, the two point mutations Ile560Ser and Tyr211Cys most likely destabilize the DPD protein. Only the effect of the Ile370Val mutation could not readily be explained by analysis of the three-dimensional structure of the DPD enzyme. Thus, it remains to be established whether the Ile370Val mutation is responsible for the lack of DPD activity or if additional mutations might be present in, for example, the promoter region of the *DPYD* gene [24].

Patient 2 also proved to be homozygous for the previously identified Cys29Arg mutation. Expression of the missense mutations Arg235Trp and Cys29Arg in *E. coli* showed that these mutations resulted in mutant DPD proteins without significant residual activity [16]. Recently, however, it has been suggested that the Cys29Arg mutation might be a common polymorphism, as suggested by the high prevalence of this mutation in cancer patients experiencing severe 5FU toxicity. In addition, homozygosity for this mutation was noted in two individuals with almost normal DPD activity [13]. This observation appears to be supported by analysis of the DPD protein structure, indicating that the Cys29Arg mutation would not introduce major changes in protein configuration or the binding of the various cofactors and substrates. In contrast, the Arg235Trp mutation directly affected the binding of the cofactor FAD [9]. Thus, the study of the crystal structure of DPD provides a powerful tool to analyse the effects of amino acid substitutions on the function and stability of the enzyme.

Defects in the degradation of pyrimidines have been associated with a variable clinical phenotype, whereas the same defects can lead to severe life-threatening toxicities when (partially) deficient individuals are treated with the pyrimidine analogue 5FU [11–14]. The identification of novel disease-causing mutations in patients with a DPD deficiency will not only augment our knowledge about structure–function relationships, but also provide an important and reliable tool for carrier detection of patients at risk.

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