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Dihydropyrimidinase deficiency: Phenotype, genotype and structural consequences in 17 patients

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ABSTRACT

Dihydropyrimidinase (DHP) is the second enzyme of the pyrimidine degradation pathway and catalyses the ring opening of 5,6-dihydrouracil and 5,6-dihydrothymine. To date, only 11 individuals have been reported suffering from a complete DHP deficiency. Here, we report on the clinical, biochemical and molecular findings of 17 newly identified DHP deficient patients as well as the analysis of the mutations in a three-dimensional framework. Patients presented mainly with neurological and gastrointestinal abnormalities and markedly elevated levels of 5,6-dihydrouracil and 5,6-dihydrothymine in plasma, cerebrospinal fluid and urine. Analysis of *DPYS*, encoding DHP, showed nine missense mutations, two nonsense mutations, two deletions and one splice-site mutation. Seventy-one percent of the mutations were located at exons 5–8, representing 41% of the coding sequence. Heterologous expression of 11 mutant enzymes in *Escherichia coli* showed that all but two missense mutations yielded mutant DHP proteins without significant activity. Only DHP enzymes containing the mutations p.R302Q and p.T343A possessed a residual activity of 3.9% and 49%, respectively. The crystal structure of human DHP indicated that the point mutations p.R490C, p.R302Q and p.V364M affect the oligomerization of the enzyme. In contrast, p.M70T, p.D81G, p.L337P and p.T343A affect regions near the di-zinc centre and the substrate binding site. The p.S379R and p.L7V mutations were likely to cause structural destabilization and protein misfolding. Four mutations were identified in multiple unrelated DHP patients, indicating that DHP deficiency may be more common than anticipated.

1. Introduction

Pyrimidine nucleotides are essential for a vast number of biological processes such as synthesis of RNA, DNA, phospholipids, glycogen and sialylation and glycosylation of proteins [1]. In addition, altered homeostasis of pyrimidines and their degradation products might impair neurological function [2–4]. In humans, the pyrimidine bases

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uracil and thymine are degraded via a three-step pathway to β-alanine and β-aminoisobutyric acid, respectively (Fig. 1). Dihydropyrimidine dehydrogenase (DPD, EC 1.3.1.2) is the initial and rate-limiting enzyme, catalysing reduction of uracil and thymine to 5,6-dihydrouracil and 5,6-dihydrothymine, respectively. Subsequently, dihydropyrimidinase (DHP, EC 3.5.2.2) catalysis the hydrolytic ring opening of the dihydropyrimidines. Finally, the resulting *N*-carbamyl-β-alanine and *N*-carbamyl-β-aminoisobutyric acid are converted in the third step to β-alanine and β-aminoisobutyric acid, ammonia and CO_2 by β-ureidopropionase (EC 3.5.1.6).

The enzymes of the pyrimidine degradation pathway are also involved in degradation of the antineoplastic agent 5-fluorouracil (5FU) [5]. DPD deficient patients have an increased risk of developing severe toxicity after 5FU administration [5,6]. Similarly, patients with a DHP deficiency have developed severe 5FU-associated toxicity [7–10].

DHP has been purified and characterised in eukaryotic species and the DHP crystal structures from the yeast *Saccharomyces kluyveri* (^{Sk}DHP), the slime mold *Dictyostelium discoideum* (^{Dd}DHP) and human DHP have recently been determined [11–15]. These studies revealed a high structural resemblance between eukaryotic DHP proteins and those of hydantoinases, the bacterial counterparts of DHP as well the DHP-like non-catalytic proteins involved in neuronal development [16–22].

The gene encoding DHP, *DPYS*, maps to chromosome 8q22 and consists of 10 exons spanning >80 kb of genomic DNA [23]. The cDNA coding for human DHP contains an open reading frame of 1560 nucleotides, corresponding to a protein of 519 amino acids with a calculated molecular weight of 56,629 Da [23].

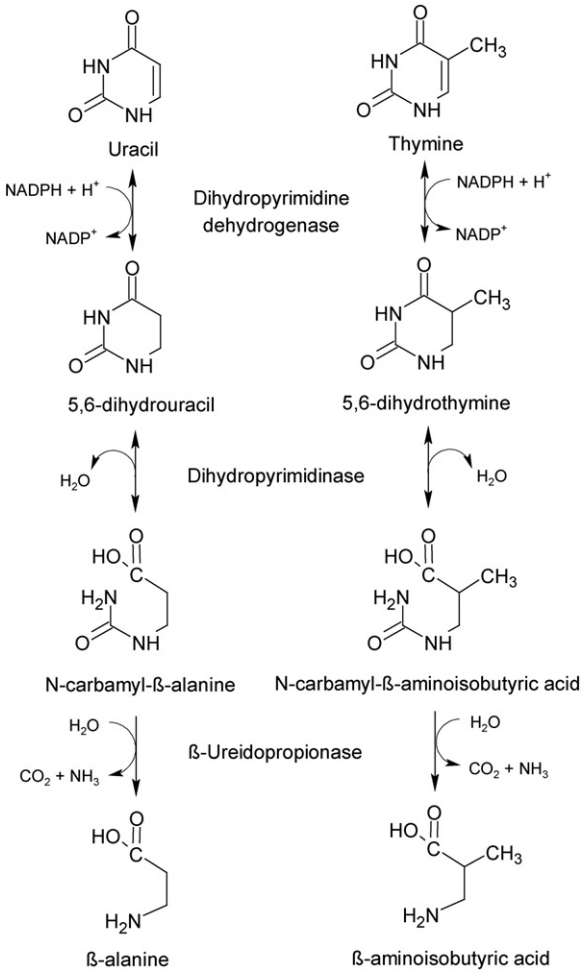

Fig. 1. Catabolic pathway of the pyrimidine bases uracil and thymine.

DHP deficiency (MIM 222748) is an autosomal recessive disease characterized by a large accumulation of dihydrouracil and dihydrothymine in urine, blood and cerebrospinal fluid. To date, only 11 patients have been described with a complete DHP deficiency, including five symptomless individuals who were identified by a screening program for inborn errors of pyrimidine degradation [7,23–31]. Therefore, the clinical, biochemical and genetic spectrum of patients with a DHP deficiency is still largely unknown. The clinical phenotype of five affected patients included seizures, mental retardation, growth retardation and dysmorphic features.

Here, we report on the clinical, biochemical and molecular findings of 17 newly identified patients and 3 previously characterised patients [23,31] with a complete DHP deficiency and the functional and structural consequences of the mutations at the protein level.

2. Materials and methods

2.1. Patients

The patients were collected in seven European countries and Australia. Criteria to test for a metabolic disorder in general or specifically for a disturbed purine/pyrimidine metabolism vary in the various countries. General testing for a metabolic disorder is considered indicated in patients suffering from developmental delay, neurological manifestations (such as ataxia or dystonia), enlarged liver and spleen or thickened gums, skeletal dysostosis and in patients with prolonged signs and symptoms of the hematological, immunological, ophthalmological and gastrointestinal systems. In this way, 17 patients were identified in whom a dihydropyrimidinase deficiency was likely. Subsequent analysis of family members of the affected patients revealed another three probably affected persons. Samples of all patients with a possible abnormality were forwarded to the Laboratory for Genetic Metabolic Diseases in Amsterdam for further analysis.

2.2. Analysis of pyrimidines in body fluids

Concentrations of uracil, thymine, dihydrouracil, dihydrothymine, *N*-carbamyl-β-alanine and *N*-carbamyl-β-aminoisobutyric acid in urine, plasma and CSF were determined using reversed-phase HPLC combined with electrospray tandem mass spectrometry [32].

2.3. PCR amplification of coding exons

DNA was isolated from EDTA blood by standard procedures. PCR amplification of all nine coding exons and flanking intronic regions was carried out using the primer sets as described previously [31]. PCR products were separated on 1.5% agarose gels, visualized with ethidium bromide and used for direct sequencing.

2.3.1. Expression of DHP in E. coli

To obtain an expression plasmid containing wild-type human DHP cDNA (pSE420-DHP), the EcoRI-BamHI insert of a plasmid comprising the complete coding region of the human DHP gene was subcloned into the pSE420 vector [33]. Mutations were introduced into the human DHP sequence using the megaprimer technique and the resulting mutant genes cloned into the pSE420 vector. Resulting clones were sequenced completely to verify the presence of the mutation and to exclude the presence of random mutations introduced by PCR artifacts. Expression plasmids were introduced into the *E. coli* strain BL21 and wild-type DHP and mutant DHPs were expressed as described before [8]. Cells were washed with an isolation buffer (35 mM potassium phosphate pH 7.4 and 2.5 mM $MgCl_2$ and resuspended in 300 μl of isolation buffer. The cell suspension was

frozen for 16 h at −20 °C, thawed on ice and lysed by sonication. The crude lysate was centrifuged at 18,000 *g* for 15 min and the resulting supernatant was stored at −80 °C.

The activity of DHP was determined at 37 °C in a standard assay mixture containing an aliquot of cell supernatant, 100 mM Tris–HCl (pH 8.0), 1 mM dithiothreitol and 500 μM [6-^{14}C]dihydrouracil with subsequent separation of radiolabeled dihydrouracil from *N*-carbamyl-β-alanine with reversed-phase HPLC [34].

2.4. Western blot analysis

Cell supernatants (10 μg) were fractionated on a 7.5% (w/v) SDS-polyacrylamide gel and transferred to a nitrocellulose filter. Detection of DHP proteins was performed using a polyclonal antibody against rat DHP [8]. Antigen–antibody complexes were visualized using anti-rabbit antibody conjugated to alkaline phosphatase.

2.5. Sequence analysis

Sequence analysis of genomic fragments amplified by PCR and expression plasmids was carried out on an Applied Biosystems model 3730 automated DNA sequencer using the Big dye-terminator method (Perkin Elmer Corp., Foster City, CA). *DPYS* sequence of patients was compared to that observed in controls and the reference sequence of *DPYS* (Ref Seq NM_001385.2; Ensembl ENST 00000351513).

2.6. Crystal structure analysis

The analysis of the crystal structure of human DHP (PDB accession code: 2vr2) was performed using WinCoot [35,36]. The crystal structures of slime mold, yeast and *Sinorhizobium meliloti* DHP, as well as of homologous collapsin-response mediator proteins and hydantoinases were used for comparative analyses (PDB accession codes: 2ftw and 2fty [11], 3dc8 [37], 1kcx [21], 2gse [22], 1yny [16], 1k1d [18], 1nfg [20], 1gkp [17], 1gkr [19]). The figures showing the point mutation sites and their environment of the human DHP protein were generated using the program PyMOL (DeLano, W.L. The PyMOL Molecular Graphics System (2002), url: http://www.pymol.org).

3. Results

3.1. Clinical phenotype

Clinical features in the 20 DHP deficient patients showed a considerable variation in presentation (Table 1). Mental retardation, hypotonia and seizures were observed in 63%, 45% and 30% of the patients, respectively. Growth retardation, failure to thrive, microcephaly and autism were observed less frequently. Forty-five percent of the patients presented with gastrointestinal problems (feeding problems, cyclic vomiting, gastroesophageal reflux, malabsorption with villous atrophy). One patient suffered from a combined deficiency of DHP and a tyrosinemia type II and another patient had both DHP deficiency and congenital microvillous atrophy [28]. Phenotypic variability of DHP deficiency was demonstrated in two families in which the index patient was clinically affected whereas the same genotype did not lead to overt symptoms in siblings.

3.2. Biochemical phenotype

Urine and plasma samples of the patients showed strongly elevated levels of dihydrouracil and dihydrothymine and moderately elevated levels of uracil and thymine (Fig. 2). The dihydrouracil and dihydrothymine concentrations were 16- to 135-fold and 15- to 197-fold, respectively, higher compared to the mean concentration observed in urine of controls. In plasma, the concentrations of dihydrouracil and dihydrothymine were 5- to 25-fold and 7- to 29-fold, respectively, higher compared to the mean concentration observed in controls. Analysis of CSF also showed highly elevated levels of dihydrouracil (20 ± 6 μM, $n = 4$; controls 2.2 ± 0.9, $n = 16$) and dihydrothymine (27 ± 4 μM, $n = 4$; controls 0.8 ± 0.2 μM, $n = 16$). In DHP deficient patients, a strong correlation existed between urinary concentration of dihydrouracil and dihydrothymine

Table 1
Phenotype of 20 patients with DHP deficiency (−, none; +, mild; ++, severe).

Patient[a]	Age (years)	Gender	Growth retardation	Failure to thrive	Feeding problems	Microcephaly	Mental retardation	Seizures	Hypotonia	Autism	Other	Consanguinity
1 (MA)	3.0	F	−	−	+	−	++	+	+	−	1	yes
2.1 (TR)	4	M	−	−	−	−	−	−	−	−	2	no
2.2 (TR)	3	M	−	−	−	−	−	−	−	−		
3 (TR)	0.7	M	−	+	+	−	+	−	+	−	3	yes
4 (TR)	1.6	M	−	−	+	−	−	+	−	−	4	yes
5 (TR)	0.6	F	−	+	+	−	+	−	+	−	5	yes
6.1 (HT)	23	F	−	−	−	−	+	+	−	−	6.1	no
6.2 (HT)	17	F	−	−	−	−	−	−	−	−		no
6.3 (HT)	5	F	−	−	−	−	−	−	−	−		no
7 (TR)	14	F	++	−	−	−	+	−	−	−	7	yes
8 (TR)	9	F	−	−	−	++	++	++	+	+	8	yes
9 (TR)	2	M	−	−	+	−	+	−	++	−	9	yes
10 (IT)	5	F	−	−	+	−	+	−	−	++	10	no
11 (SE)	11	F	+	−	−	−	−	−	−	−	11	no
12 (GR)	5.9	F	++	++	−	++	++	++	+	−	12	no
13 (DE)	11	F	−	−	−	−	+	−	−	−	13	no
14 (VE)	2.0	F	−	−	−	−	−	−	−	−	14	no
15 (LY)	7	F	−	−	−	−	+	−	+	−	15	yes
16 (UK)	0.1	M	++	++	++	++	?	+	+	−	16	yes
17 (TR)	3.5	M	−	−	−	−	+	−	+	+	17	no
Total			20% (4/20)	20% (4/20)	35% (7/20)	15% (3/20)	63% (12/19)	30% (6/20)	45% (9/20)	15% (3/20)		

[a] The nationality of the patient is given in parenthesis. [1]Anal atresia, underdeveloped distal phalanges of 3rd fingers, coarse face, broad nasal bridge, prominent and simple ears, chorea, progressive neuronal atrophy, MRI cortical atrophy [27,61]; [2]MRI mild parieto-occipital white matter loss, severe delay in speech development [31]; [3]Congenital microvillous atrophy [28]; [4]data from [24,25]; [5]Tyrosinemia type II, asymptomatic at the age of 10; [6.1]Protein S deficiency, cerebral thrombophlebitis leading to hemiparesia, antigliadin negative malabsorption with villous atrophy; [7]Gall stones, hypermetropia; [8]MRI porencephaly; [9]Gastroesophageal reflux, [10]Mild facial coarsening; [11]Cyclic vomiting; [12]Ventricular septum defect, hypertelorism, flat nasal bridge, short philtrum, thin upper lip vermillion, fifth finger clinodactyly; [13]Macrocephaly, obesity, Paroxysmal theta waves on EEG; [14]Cardiomyopathy; [15]Ataxia, cataract; [16]In vitro fertilisation pregnancy, severe respiratory problems of infancy, demise at 4 weeks of age; [17]hyperactive behavior.

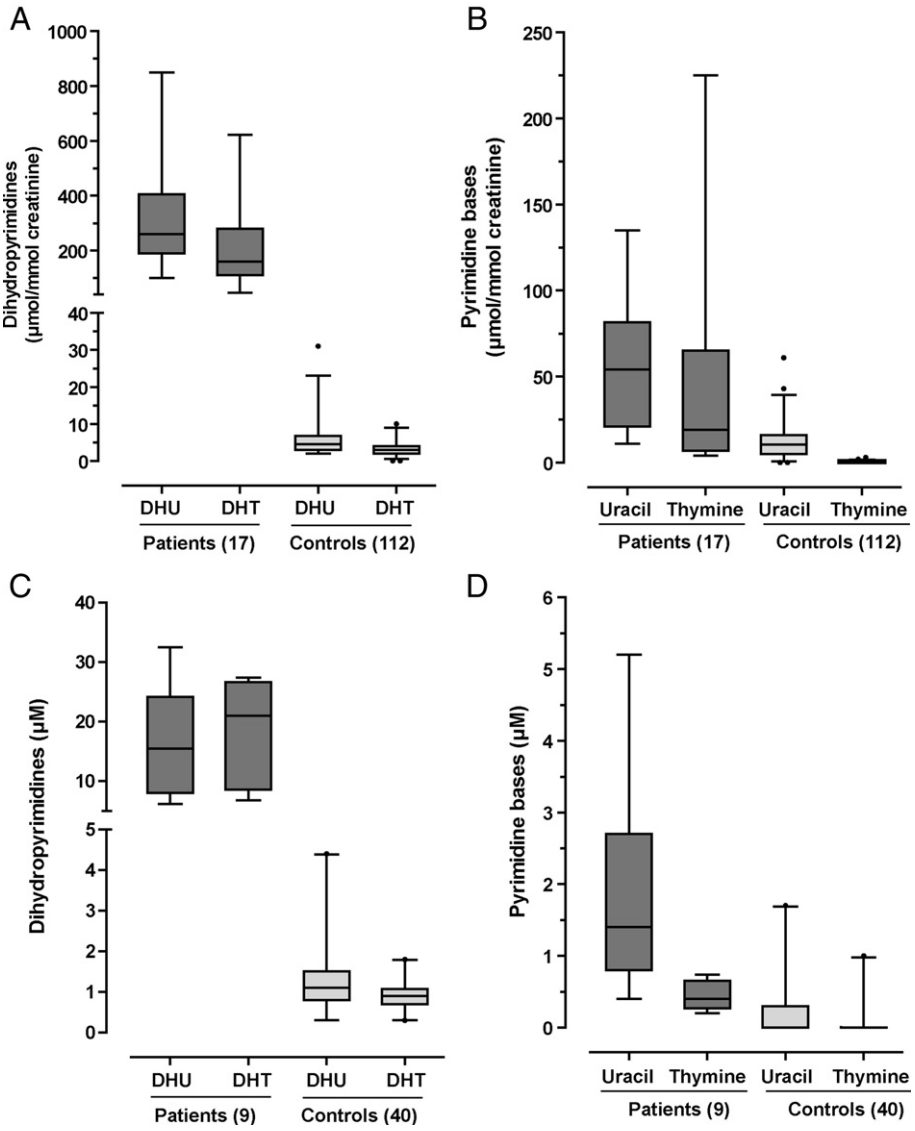

Fig. 2. Concentrations of dihydropyrimidines and pyrimidine bases in DHP patients and controls. Panel A shows the urinary levels of dihydrouracil (DHU) and dihydrothymine (DHT) and panel B shows the concentrations of the pyrimidine bases in urine. The plasma levels of the dihydropyrimidines and the pyrimidine bases are shown in panels C and D, respectively. The top, bottom and line through the middle of a box correspond to the 75th percentile, 25th percentile and 50th percentile, respectively. The whiskers on the bottom extend from the 2.5th percentile and top 97.5th percentile. The circles represent outliers.

($R^2 = 0.87$, $P = 0.000007$). A similar correlation existed between dihydrouracil and dihydrothymine concentration in plasma ($R^2 = 0.72$, $P = 0.028$). Thus, dihydropyrimidinuria in these patients strongly suggested DHP deficiency.

3.3. Genotype

DPYS analysis in the 17 hitherto uncharacterized patients showed one previously described missense mutation (L7V) and 13 novel mutations, including eight missense mutations, two nonsense mutations, two deletions and one splice-site mutation (Table 2). Homozygosity for one of the identified mutations was observed in 11 patients. The mutations c.1078T>C, c.1027A>G and c.1092 + 3delG were each present in two unrelated patients and c.1137C>A was identified in four unrelated patients. Fig. 3 shows the distribution of *DPYS* mutations over the exons. In particular exons 5–8, representing 41% of the coding sequence, harbour 71% of the mutations. An analysis for the presence of the 14 mutations in 90 controls did not identify individuals either heterozygous or homozygous for one of these mutations.

3.4. Functional analysis of mutant DHP enzymes

To investigate the effect of the nine missense and two nonsense mutations on the activity of DHP, the mutations were introduced into a pSE420-DHP vector by site-directed mutagenesis and expressed in *E. coli*. No endogenous DHP activity (<0.3 nmol/mg/h) could be detected in the *E.coli* strain used for the expression of the constructs. Introduction of wild-type DHP construct increased DHP activity more than 300-fold above the background level. Expression of DHP constructs containing the mutations p.W360R, p.V364M, p.D81G, p.L7V, p.M70T, p.L337P and p.R465X yielded no detectable DHP activity (<0.2%) (Fig. 4). Expression of the mutant DHP constructs containing the p.S379R and p.R490C and p.R475X mutation yielded a residual activity of 0.2–0.9%. Significant residual DHP activities were detected for mutant DHP enzymes containing the p.R302Q (3.9% activity) and p.T343A mutation (49% activity).

To exclude the possibility that the lack of DHP activity was the result of an inability to produce the mutant protein in *E. coli*, the expression levels were analysed by immunoblotting. Fig. 4 shows that

Table 2
Results of mutation analysis in 20 patients with DHP deficiency.

Patient	Genotype	Effect	Location
1[a]	c.[1078T>C]+[1078T>C]	p.[W360R]+[W360R]	Ex 6
2.1[b]	c.[1078T>C]+[1235G>T]	p.[W360R]+[R412M]	Ex 6, Ex 7
2.2[b]	c.[1078T>C]+[1235G>T]	p.[W360R]+[R412M]	
3	c.[905G>A]+[905G>A]	p.[R302Q]+[R302Q]	Ex 5
4	c.[1137C>A]+[1137C>A]	p.[S379R]+[S379R]	Ex 7
5	c.[476delA]+[476delA]	p.[K159RfsX12]+[K159RfsX12]	Ex 3
6.1	c.[19C>G]+[209T>C]	p.[L7V]+[M70T]	Ex 1
6.2	c.[19C>G]+[209T>C]	p.[L7V]+[M70T]	Ex 1
6.3	c.[19C>G]+[209T>C]	p.[L7V]+[M70T]	Ex 1
7	c.[1468C>T]+[1468C>T]	p.[R490C]+[R490C]	Ex 9
8	c.[1423C>T]+[1423C>T]	p.[R475X]+[R475X]	Ex 8
9	c.[1092+3delG]+[1092+3delG]	unknown	IVS6
10	c.[1092+3delG]+[1092+3delG]	unknown	IVS6
11	c.[1010T>C]+[1010T>C]	p.[L337P]+[L337P]	Ex 6
12	c.[1090G>A]+[1137C>A]	p.[V364M]+[S379R]	Ex 6, Ex 7
13	c.[242A>G]+[1027A>G]	p.[D81G]+[T343A]	Ex 1, Ex 6
14	c.[1027A>G]+[1393C>T]	p.[T343A]+[R465X]	Ex 6, Ex 8
15	c.[1137C>A]+[1137C>A]	p.[S379R]+[S379R]	Ex 7
16	c.[1254_1255delTC]+[1254_1255delTC]	p.[His419SerfsX5]+[His419SerfsX5]	Ex 8
17	c.[1137C>A]+[1137C>A]	p.[S379R]+[S379R]	Ex 7

[a] Data obtained from [23].
[b] Data obtained from [31].

the mutant proteins were expressed in comparable amounts as the wild-type protein. Only expression levels of DHP enzymes containing the mutation p.S379R, p.W360R and p.L7V were lower than that observed for the wild-type DHP enzyme. Expression constructs containing the two nonsense mutations p.R465X and p.R475X yielded shortened DHP proteins. Thus, the lack of DHP activity of mutant DHP enzymes in *E. coli* is not due to rapid degradation of the various mutant DHP proteins in the *E. coli* lysates.

3.5. Structural effects of DHP mutations

Fig. 5A depicts the location of all presently reported point mutation sites within the subunit of human DHP. None of the affected residues is located within the active site, excluding direct interference with substrate binding. However, some of the residues are engaged in interactions that may be important for maintaining structural integrity of the active site or of subunit interfaces in the DHP tetramer (Fig. 5B).

R302 is almost strictly conserved in DHP homologues. Its side chain forms hydrogen bonds and/or charged interactions with E218, E226 and R220, thus connecting the loop leading to the seventh barrel-helix with several secondary structure elements that are strongly engaged in subunit contacts (Fig. 6A). The R302Q mutation abolishes most hydrogen bonding possibilities and does not provide any charge compensation for the E218 and E226 side chains. The likely consequence is increased local flexibility with potentially disturbed subunit interactions.

A similar consequence is expected for the mutation of V364, belonging to a helix-loop-helix motif that interacts with residues from another subunit. In DHPs homologs, its environment shows higher sequence variance than residue 364 itself but the hydrophobic character of both is always retained. The V364M mutation exchanges a smaller by a larger hydrophobic side chain, resulting in steric clashes with residues in its proximity such as W360 and F374 (Fig. 6B). Some of these clashes may be prevented by other conformations of the methionine, but readjustments of nearby side chains to optimize their packing are required in either case. Previously, another mutation with activity-abolishing effects has been identified and localized in the same area (W360R) [31], suggesting that it is very sensitive to structural disturbances.

The side chain of R490 anchors the C-terminal tail of one subunit to the surface of another via van der Waals interactions and hydrogen bonds to E215, Y284 and N286 (Fig. 6C). A mutation to cysteine (R490C) is expected to increase the flexibility of the C-terminal tails, leading to disturbances in tetramer association. This is in line with the observation that in microbial hydantoinases, C-terminal regions are not essential for catalysis but affect oligomeric structure [38] and can explain the very low residual activity of truncated human DHP protein (p.475X), lacking the last 45 amino acids.

L7 belongs to the β-sandwich domain and is distant from the active site and subunit interfaces. Its side chain involves in hydrophobic interactions with nearby residues (Fig. 6D). The apolar character of the interactions is conserved in most structural homologues. Exchange of L7 to valine shortens the side chain but retains its

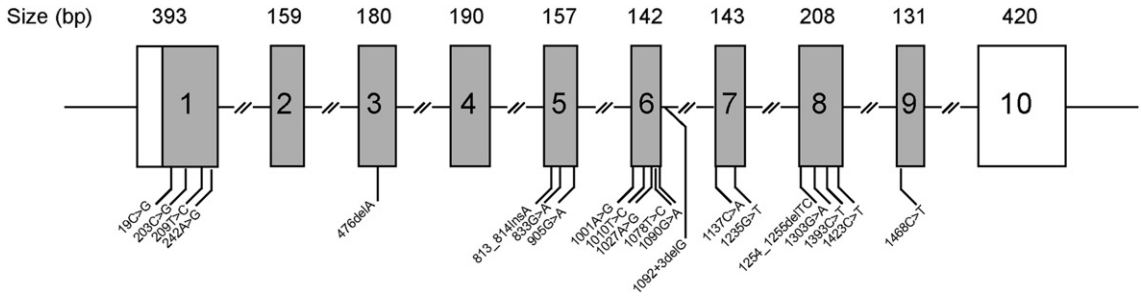

Fig. 3. Genomic organisation and mutation analysis of *DPYS*. *DPYS* consists of 10 exons encoding an open reading frame of 1560 bp (depicted in gray). The various mutations identified in DHP deficient patients are indicated, numbers correspond to the cDNA position.

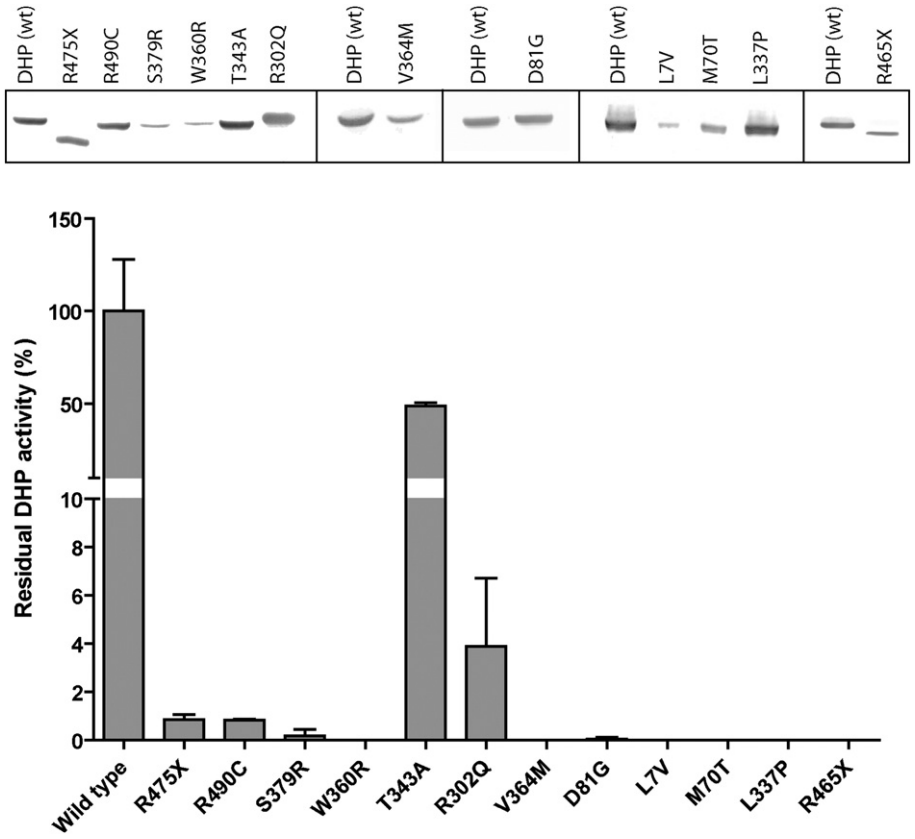

Fig. 4. DHP activity and immunoblot analysis of wild-type and mutant DHPs expressed in *E. coli*. The results represent the mean of three to six independent measurements, the error bar represents 1 SD.

hydrophobic properties. Structural effects of the mutation are not obvious. Possible, loss of the methyl group weakens hydrophobic interactions by leaving a hole in the otherwise tightly packed cluster of apolar side chains. It is uncertain whether the resulting increased local flexibility is sufficient to cause global protein instability or to influence folding dynamics.

S379 is located in the tightly packed interface between the catalytic and the β-sandwich domain (Figs. 5A, 6E) opposite a strictly conserved glycine (G62) that connects the third strand of a β-sandwich sheet with the first strand of the $(\beta/\alpha)_8$-barrel. The latter precedes the HxH motif important for binding of the di-zinc centre. In homologs of human DHP the serine is either conserved or replaced by cysteine or alanine (Table 3), indicating there is no space for a larger side chain. Thus, the S379R mutation might cause enzyme misfolding.

M70T, D81G, L337P, and T343A have indirect effects on zinc ion or substrate binding by perturbing the fine-tuned geometry within and near the active site. M70 directly follows the zinc-binding HxH motif and engages in hydrophobic interactions with e.g. I98, V352, F83, T68 and T87 (Fig. 6F). The nearby D100 forms hydrogen bonds with M70 and T68, thus fixating the position of both neighbours of zinc-ligand H69. The M70T mutation is predicted to have one or several of the following consequences, each affecting enzyme structure and stability: 1. inability of the shorter, polar side chain of threonine to form favourable hydrophobic contacts with its environment; 2. steric clashes with F83 and other nearby amino acids; 3. disturbance of the native interactions of D100 due to formation of a hydrogen bond with T70.

Mutation of D81 to glycine would abolish both hydrogen bonds involving the aspartate side chain, i.e., those to Q421 and T329 (Fig. 6G). The aspartate is conserved in most homologues of human DHP (Table 3), while in *A. aurescens* hydantoinase the carboxyl group of a nearby aspartate is placed at a spatially equivalent position, forming the same interactions. This underlines the crucial role of D81 in interconnecting domains as well as secondary structure elements carrying zinc-ligating residues.

The mutation sites L337 and T343 are spatially close to each other in one of the long loops protruding from the top of the barrel that shapes the entrance to the active site (Fig. 6G). This loop contains helical elements and leads into a conserved sequence motif, ^{345}IPNG^{348} in human DHP. N347 of this motif is a substrate binding residue, interacting with atom N1 of the dihydropyrimidine and the following glycine is involved in positioning the substrate binding residue G298. L337 and T343 are located at the outmost periphery of the protein in two consecutive 3_{10}-helical turns. Replacement of L337 by other residues than proline most likely has modest effects; the side chain is oriented towards the bulk solvent and only involved in weak hydrophobic interactions. Proline, however, tends to break or kink helices due to its conformational rigidity and inability to donate an amide hydrogen bond. Partial unwinding of its helical components may cause considerable increase in flexibility or disorder of the long loop segment participating in constructing the substrate binding site. T343 seems to play a role in the structural fixation of the tip of this loop. Mutation to alanine eliminates the hydrogen bond to D341 and weakens hydrophobic interactions with F342 and residues extending from the closest barrel-helix. However, the hydrogen bond formed between D341 and K344 may provide sufficient structural stability to this region allowing tolerance the T343A exchange. This would explain the significant residual activity of the mutant.

4. Discussion

DHP deficiency is an inborn error of the pyrimidine degradation pathway, affecting the ring opening of both dihydrouracil and

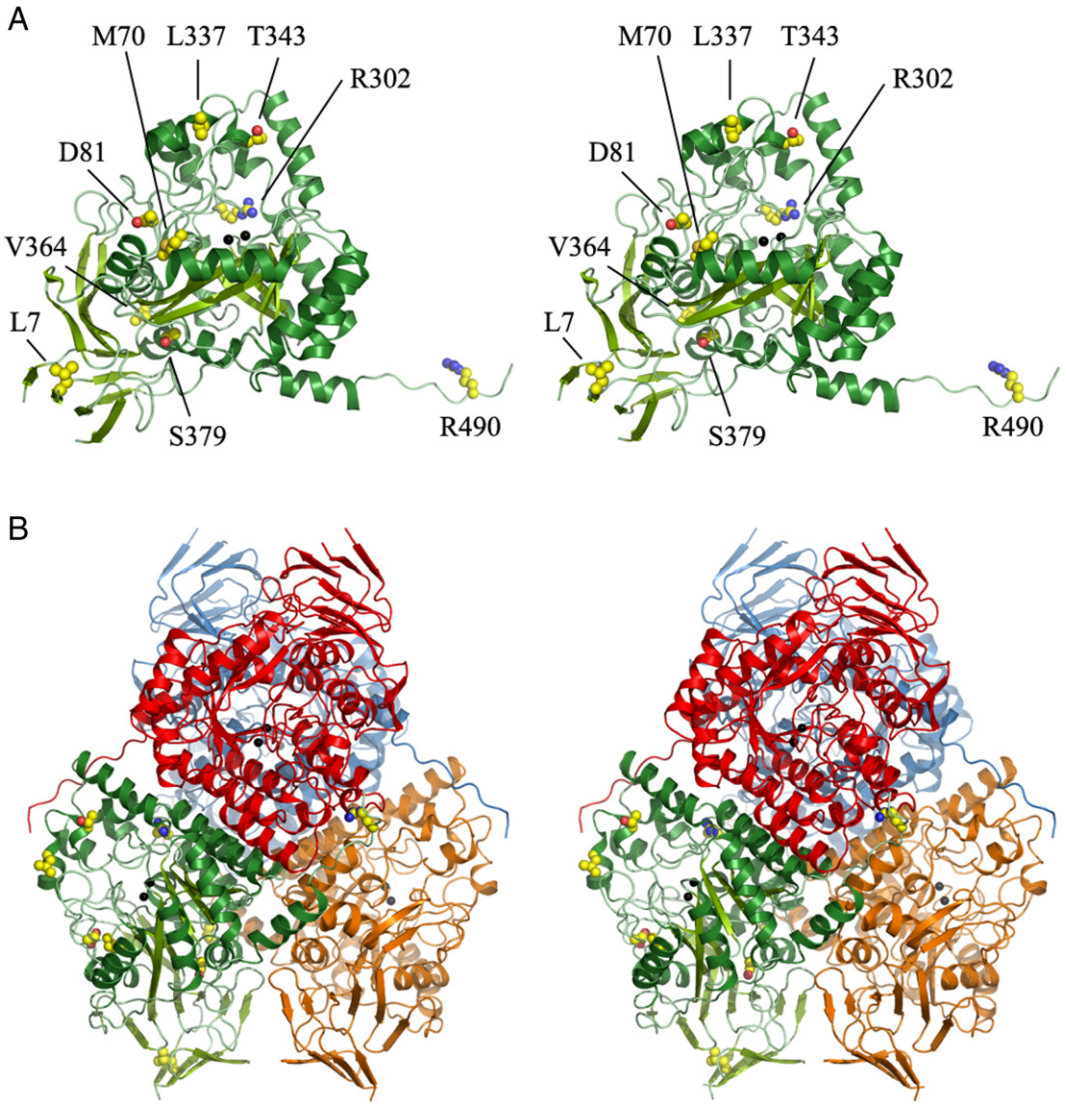

Fig. 5. Structure of human DHP and location of point mutation sites. (A) Stereoview of the human DHP subunit in cartoon representation, with secondary structure elements depicted in different shades of green and zinc ions of the di-metal centre as black spheres. Location of the point mutation sites reported herein is indicated by space-fill representation and labelling of the corresponding residues. (B) Stereoview of the human DHP homotetramer in cartoon representation. One subunit is shown as depicted in panel A, including mutation sites, while the other subunits are depicted in orange, red and blue. Zinc ions are shown as black spheres.

dihydrothymine. Patients with complete DHP deficiency present with strongly elevated levels of dihydropyrimidines in urine, plasma and CSF. The most frequently observed clinical features in the present study were mental retardation, hypotonia and seizures which is comparable to that observed for patients with a DPD deficiency [39–41]. An unexpected finding was the high frequency of gastrointestinal problems in DHP deficient patients. The identification of individuals with complete DHP deficiency without any clinical manifestations and the marked intrafamilial variability in phenotype, indicate that additional (epi)genetic and environmental factors are likely to be involved.

The pathogenesis underlying the various clinical manifestations remains as yet unknown. Biochemically, patients with a deficiency in one of the three enzymes of the pyrimidine degradation pathway show moderately decreased levels of β-alanine and strongly decreased levels of β-aminoisobutyric acid [42–44]. β-Alanine is a structural homologue of γ-aminobutyric acid and glycine, which are the major inhibitory neurotransmitters in the central nervous system. β-alanine itself has been suggested to be involved in synaptic transmission and has been shown to regulate dopamine levels [39,45]. In addition, β-alanine is the rate-limiting substrate for the synthesis of carnosine. Oral administration of β-alanine increases the carnosine content in muscles and delays the onset of neuromuscular fatigue while improving endurance performance and lean body mass [46–49]. β-Aminoisobutyric acid is a partial agonist of the glycine receptor [50]. Recently, it was demonstrated that β-aminoisobutyric acid is able to increase the excretion of leptin and stimulate fatty acid oxidation [51]. Leptin itself has been shown to have neuroprotective, cognitive and anticonvulsant effects [52–54]. Thus, it is conceivable that the altered homeostasis of β-aminoisobutyric acid in patients with DHP deficiency might affect leptin levels and fatty acid oxidation and contribute to the development of mental retardation and hypotonia in the patients.

Mammalian DHP uses not only 5,6-dihydrouracil and 5,6-dihydrothymine as substrates but also catalyses the hydrolysis of a variety of other compounds [13,55–58]. The broad substrate specificity of DHP could implicate that DHP is involved in detoxification of endogenous compounds and xenobiotics. In addition, DHP is highly homologous to the non-catalytically active dihydropyrimidinase-like proteins which play an important role in neuronal development

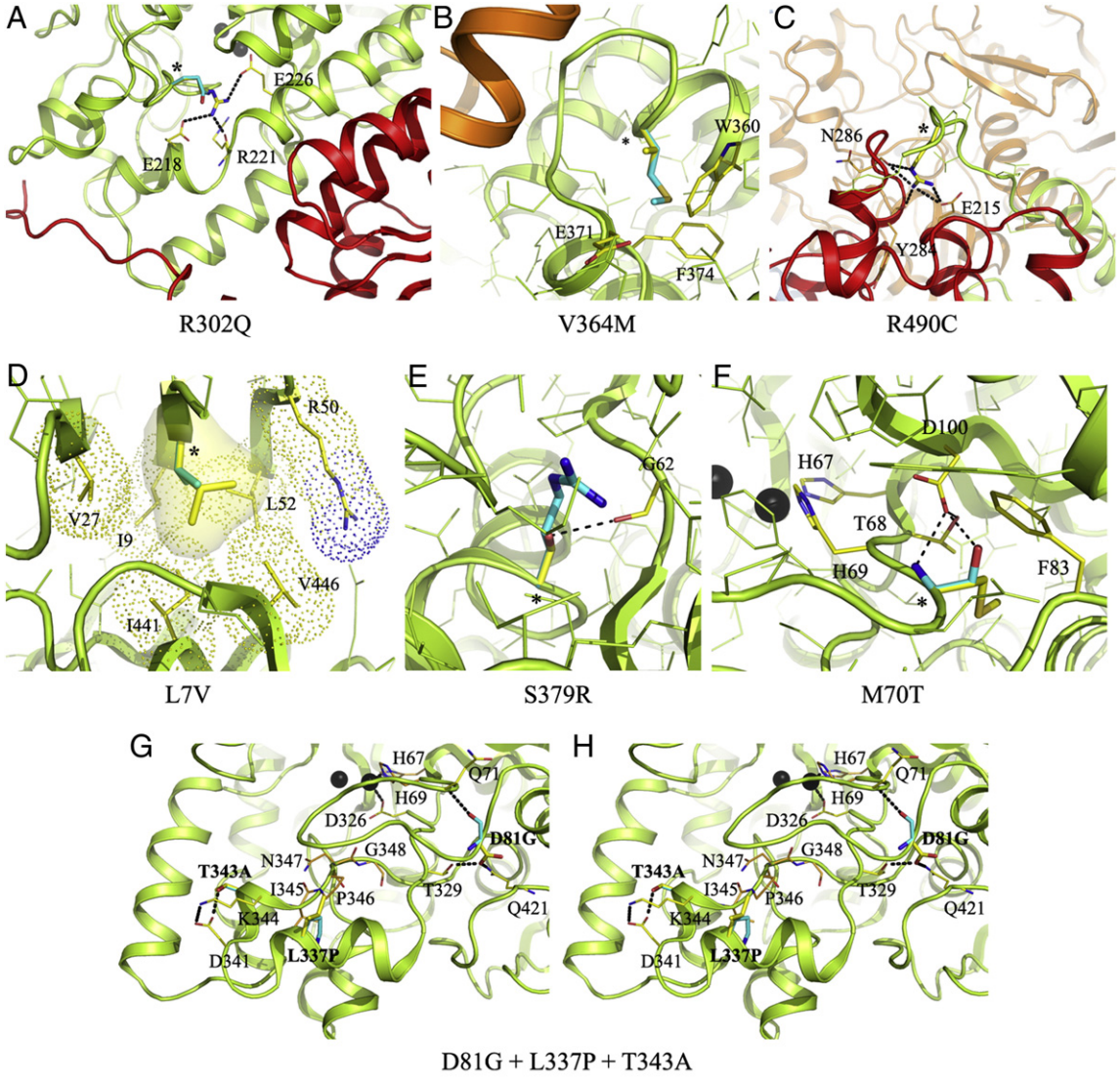

Fig. 6. Mutation sites and their environment in human DHP crystal structure. The cartoon representation of the human DHP backbone is depicted in green. Otherwise not further discussed side chains are shown in green as thin sticks. Zinc ions of the di-metal centre are depicted as black spheres. Residues are labelled and shown with thicker sticks and oxygen atoms in red, nitrogen atoms in blue and sulphur atoms in dark yellow. For residues in wild-type human DHP, carbon atoms are coloured yellow. Carbon atoms in cyan mark side chains introduced by the point mutations. Identity of various mutation sites is indicated below each figure. In figures (A)–(F) the sites are labelled by asterisks. Hydrogen bonds are indicated by dotted black lines. The (energetically preferred) side chain conformation causing the least clashes and most optimal interactions with surrounding residues was chosen. (A) Distant view of the R302Q mutation site showing its location close to an oligomerization interface within the DHP tetramer. For clarity, only the side chain at the mutation site and of its interaction partners are depicted. One subunit is shown in green, another in red. (B) Close-up view of the V364M mutation site. The C-terminal tail of another subunit that interacts with the loop-region following the mutation site is shown in orange. (C) Close up view of the R490C mutation site. Stick representation is only used for the residues of the C-terminal tail of one subunit (in green), the residue at the mutation site (yellow carbon atoms for the arginine of the wild-type DHP and cyan carbon atoms for the cysteine of the mutant), and for interacting residues of a neighbouring subunit (orange carbon atoms). The subunit harbouring the depicted R490 is shown in green, the others in red, orange and blue. (E) Close-up view of the L7V mutation site. The yellow surface represents the van der Waals sphere of the mutated residue, while corresponding spheres of neighbouring residues are depicted by yellow and blue dots. (E)–(F) Close-up views of the S379R and M70T mutation sites. (G) Stereo view of the D81G, L337P and T343A mutation sites. For clarity, stick representation is only used for residues mentioned in the text. Residues belonging to the conserved sequence motif involved in substrate binding and the zinc-ligating H67 and H69 are shown with orange carbon atoms.

[21,22,33]. To date, it is not known whether the DHP protein itself might have similar additional functions as well.

Mutation analysis of *DPYS* showed 14 mutations, including nine missense mutations, two nonsense mutations, two deletions and one splice-site mutation. Systematic analysis of the sequences present in the vicinity of the splice junctions has led to consensus sequences for both the splice acceptor and splice donor sites [59]. A scoring system developed by Shapiro and Senapathy showed that most naturally occurring splice acceptor and splice donor sites yielded scores above 0.70. Analysis of the consensus sequence of the authentic splice donor site of exon 6 of *DPYS* showed that the c.1092 + 3delG mutation reduces the consensus value from 0.73 (wild-type) to 0.61 (mutant). Heterologous expression of 11 mutant enzymes in *E. coli* showed that all but 2 missense mutations yielded mutant DHP proteins without significant activity (<1%). Only DHP containing the mutations p.R302Q and p.T343A possessed 3.9% and 49% residual enzymatic activity, respectively. This observation is in line with the fact that the two patients who were compound heterozygous for the p.T343A mutation presented with relatively low urinary concentrations of the dihydropyrimidines.

The crystal structure of human DHP showed that the native enzyme is composed of four identical subunits containing two zinc ions [12]. Each subunit consists of two domains, a larger $(\beta/\alpha)_8$-barrel domain that binds the catalytic di-zinc centre, and a smaller domain with a β-sandwich fold. The core elements of the latter are a seven-stranded and a four-stranded mixed β-sheet (Fig. 5A). Both domains

Table 3
Sequence conservation amongst DHPs and homologs at selected positions.

Protein[a]	Mutation sites in human DHPase and corresponding residues								
^{Hs}DHPase	**L**7	**M**70	**D**81	**R**302	**L**337	**T**343	**V**364	**S**379	**R**490
^{Dd}DHPase	I	F	**D**	**R**	M	**T**	**V**	**S**	V
^{Sk}DHPase	**L**	V	**D**	**R**	R	R	Y	C	-
^{Dm}DHPase*	V	**M**	**D**	**R**	**L**	**T**	**V**	**S**	E
^{At}DHPase*	I	L	**D**	**R**	**L**	R	**V**	**S**	P
^{Mm}DHPase*	**L**	**M**	**D**	**R**	**L**	**T**	**V**	**S**	**R**
^{Rn}DHPase*	**L**	**M**	**D**	**R**	**L**	**T**	**V**	**S**	**R**
^{Sm}DHPase	T	L	**D**	**R**	F	**T**	**V**	**S**	**R**
^{Mm}CRMP-1	**L**	L	**D**	S	V	**T**	**V**	**S**	**R**
^{Hs}CRMP-2	**L**	F	**D**	S	V	**T**	**V**	**S**	**R**
^{B9}DHyd	K	L	**D**	**R**	**L**	**T**	**V**	**S**	-
^{Bs}DHyd	K	L	**D**	**R**	**L**	**T**	**V**	**S**	-
^{Bp}DHyd	I	V	**D**	**R**	R	R	**V**	A	-
^{Ts}DHyd	**L**	I	**D**	**R**	**L**	**T**	**V**	**S**	-
^{Aa}LHyd	V	I	G	**R**	-	-	**V**	C	-

Residues corresponding to the mutation sites in human DHP were identified using structure-based sequence alignments for all homologs of known structure. A hyphen indicates the absence of a corresponding residue, either because of a shorter protein sequence or because of significant structural deviations from the ^{Hs}DHP. Bold letters indicate sequence conservation to human DHP. An asterisk marks enzymes for which no 3D structural data are available.

[a] Abbreviations correspond to the following: Hs, *Homo sapiens*; Dd, *Dictyostelium discoideum*; Sk, *Saccharomyces kluyveri*; Dm, *Drosophila melanogaster*; At, *Arabidopsis thaliana*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*; Sm, *Sinorhizobium meliloti* CECT4114; B9, *Bacillus* sp. AR9; Bs, *Bacillus stearothermophilus*; Bp, *Burkholderia pickettii*; Ts, *Thermus* sp.; Aa, *Arthobacter aurescens*; DHyd, D-stereospecific hydantoinase; LHyd, L-stereospecific hydantoinases; CRMP, (non-enzymatic) collapsin-response mediator protein.

contribute to intermolecular contacts within the physiological relevant homotetramers (Fig. 5B). Analysis of the environment of the point mutation sites within the crystal structure of human DHP allowed us to predict the effects of amino acid exchanges on structure and function of the enzyme. The point mutations R490C, R302Q and V364M prevent or disturb oligomerization to the physiologically relevant homotetramer. In contrast, M70T, D81G, L337P and T343A affect regions near the di-zinc centre and the substrate binding site influencing active site geometry and metal-binding. S379R impairs packing of the catalytic and β-sandwich domain against each other which might cause protein misfolding. Only the potential effect of the L7V exchange was less obvious, but may be based on structural destabilization of the hydrophobic core of the β-sandwich domain.

Recently, it has been shown that patients with a partial DHP deficiency are prone to the development of severe toxicity after the administration of 5-fluorouracil [7–10]. The present finding that individuals with very low DHP levels can be symptomless urges for careful analysis of cancer patients before 5FU administration. In addition, the finding of four mutations in multiple DHP patients of different nationalities may indicate that a DHP deficiency might be not as rare as generally considered. The prevalence of a DHP deficiency in Japan has been estimated to be 1 in 10,000 which is comparable to the estimated frequency of patients with a DPD deficiency in the Netherlands [7,60]. The present results should facilitate identification of further individuals with decreased DHP activity.

Acknowledgments

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