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Effects of naturally occurring missense mutations and G525V in the hydratase domain of human D-bifunctional protein on hydratase activity

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ABSTRACT

D-bifunctional protein (D-BP) deficiency is thought to lead to severe lipid metabolism disorders. To investigate the effect of naturally occurring missense mutations in the hydratase domain in D-BP, we constructed several D-BP hydratase variants and measured their activities. Missense mutations at sites whose conservation rates among 30 eukaryotes were < 70% did not affect hydratase activity. We predicted that missense mutations of highly conserved amino acids would markedly reduce activity. However, R562H and R562L, naturally occurring missense mutations of highly conserved amino acids, did not reduce activity. This result suggests that a missense mutation in a highly conserved amino acid does not always lead to severe lipid metabolism disorders. We also investigated the effect of G525V, which had been found in a mildly symptomatic patient with D-BP deficiency who was heterozygous for G525 and G658X. G525V markedly reduced hydratase activity. We had predicted that heterozygous G525V and G658X would lead to severely disordered lipid metabolism. However, the symptoms were inconsistent with this prediction. Characterizing mutations in the D-BP gene and the symptoms of D-BP deficiency may require pleiotropy, not only in vitro, studies.

1. Introduction

D-bifunctional protein (D-BP, often called multifunctional enzyme type 2: MFE2, or 17-β-hydroxysteroid dehydrogenase 4: HSD17B4) is a 79.7-kDa protein harboring a 2-enoyl-CoA hydratase domain, a 3-hydroxyacyl-CoA dehydrogenase domain, and a sterol-carrier protein (SCP)-2 like domain (Fig. 1A). The enzyme catalyzes the second and third steps of peroxisomal β-oxidation. The enzyme yields 3-oxoacyl-CoA from *trans*-2-enoyl-CoA via 3*R*-hydroxyacyl-CoA (Fig. 1B) [1]. The peroxisomal β-oxidation system participates in the degradation of very long-chain fatty acids and branched-chain fatty acids and in the side-chain cleavage of bile acid intermediates. In general, children with congenital defects in this system, such as those with Zellweger syndrome, present a high level of abnormal lipids in plasma, hypotonia, developmental delay, seizure, hepatic dysfunction, renal failure, and facial and skull dysmorphism, among other symptoms, and die at a few years of age [2]. In cases with D-BP deficiency alone, the same symptoms are observed [3]. Accordingly, D-BP is believed to be necessary for life.

Multiple alignment using the Peroxisome Database [4], which provides comprehensive information on peroxisomes, showed that the amino acid sequences constituting the dehydrogenase and hydratase motifs in D-BP are highly conserved among diverse eukaryotes. We accordingly inferred that the enzyme group containing these sequences plays an essential role in various eukaryotes, including humans. However, an analysis of single-nucleotide polymorphisms (SNPs) using the National Center for Biotechnology Information SNP database (NCBI dbSNP), showed that SNPs causing naturally occurring missense mutations are found in the conserved amino acid sequences of D-BP [5]. Ordinarily, missense mutations in conserved amino acid sequences have major detrimental effects on the structure or function of the protein. However, the association between these naturally occurring missense mutations and D-BP deficiency has remained unclear, partly because a simple evaluation method for discerning the effects of these mutations on D-BP deficiency has not yet been developed. In this study we attempted to apply our previously developed method to such an evaluation.

To aid in the investigation of the physiological significance of the stereospecificity of D- and L-bifunctional protein (L-BP), we have developed a system for separate quantification of 3*R*- and 3*S*-hydroxyacyl-CoA, which is a product of L-BP hydratase, using high-performance liquid chromatography (HPLC) with a chiral separation column [6]. This method enables us to analyze the activity of hydratase even if D-BP and L-BP are both present in the experimental system. In addition, we have cloned human D-BP hydratase domain and expressed it as a fusion protein with green fluorescent protein (GFP), which we named GFP-MFE2H, to demonstrate the utility of the HPLC method [7]. This fusion

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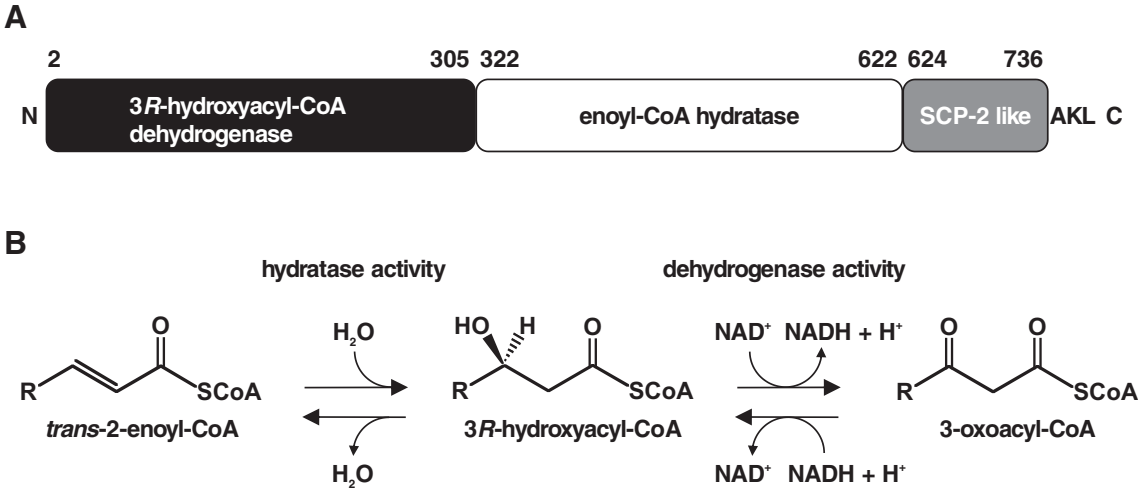

Fig. 1. Primary structure (A) and function (B) of D-BP. The first methionine is numbered 1. "AKL" at the C terminus indicates an amino acid sequence (Ala–Lys–Leu) that is a peroxisome-targeting signal.

protein is easy to purify and assay because of its traceability under ultraviolet (UV) light.

We focused on nine amino acids in the hydratase domain of D-BP that were suggested (by ≥70% identity in multiple alignment) to show homology among 30 eukaryotes. These amino acids contain the following naturally occurring missense mutations in humans: A353V, I380V, G421E, G468S, R555I, K557M, R562H, R562L, K557I, Q587E, and L622F (Fig. 2). This surprising finding of missense mutations in conserved amino acids led us to investigate the effects of these mutations on hydratase activity by site-directed mutagenesis using GFP-MFE2H and HPLC.

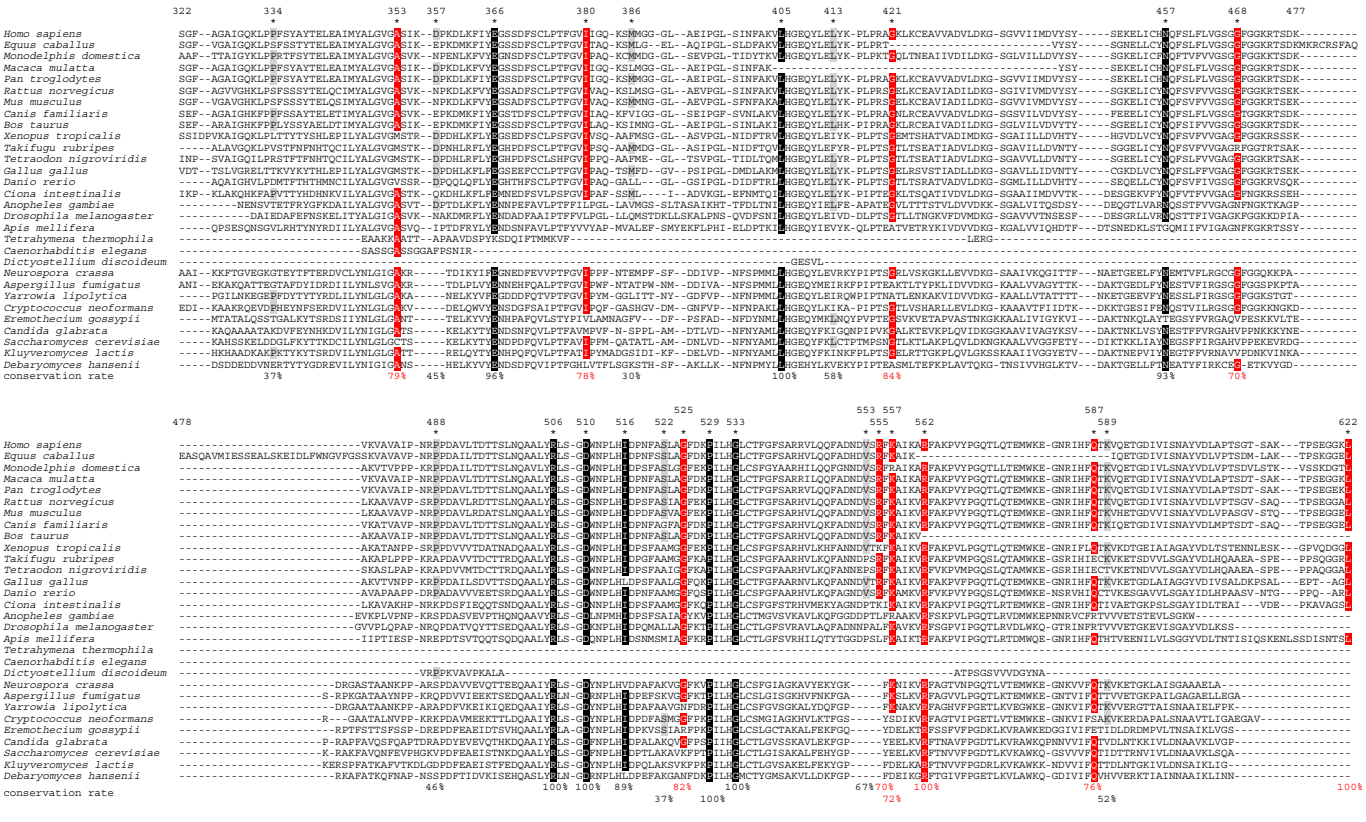

Fig. 2. Multiple alignment of the hydratase domain of D-bifunctional protein. Amino acid sequences of human D-BP domain (amino acids 322–622) and its multiple alignment among 30 eukaryotes were taken from the Peroxisome Database. Asterisks (*) show the locations of SNPs that cause missense mutations in humans. Gray indicates amino acids with conservation rates among eukaryotes of <70%. Black and red indicate amino acids with conservation rates > 70%. Black indicates amino acids for which hydratase activities of variants have already been investigated [7]. Red indicates amino acids for which the effects of missense mutations are not clear. Accession numbers are as follows, *Homo sapiens*, NP_000405; *Equus caballus*, NP_001075370; *Monodelphis domestica*, XP_001378211; *Macaca mulatta*, XP_001087837; *Pan troglodytes*, XP_003310890, *Rattus norvegicus*, NP_077368; *Mus musculus*, NP_032318; *Canis familiaris*, XP_531860; *Bos taurus*, NP_001007810; *Xenopus tropicalis*, NP_001027490; *Takifugu rubripes*, NP_058850; *Tetraodon nigroviridis*, NP_570602; *Gallus gallus*, NP_990274; *Danio rerio*, NP_956430; *Ciona intestinalis*, XP_002124954; *Anopheles gambiae*, XP_314766; *Drosophila melanogaster*, NP_573109; *Apis mellifera*, XP_393475; *Tetrahymena thermophile*, XP_001021425; *Caenorhabditis elegans*, NP_509146; *Dictyostelium discoideum*, XP_635235; *Neurospora crassa*, XP_962039; *Aspergillus fumigatus*, XP_ 746577; *Yarrowia lipolytica*, XP_503980; *Cryptococcus neoformans*, XP_571589; *Eremothecium gossypii*, NP_986606; *Candida glabrata*, XP_448864; *Saccharomyces cerevisiae*, NP_012934; *Kluyveromyces lactis*, XP_454574; *Debaryomyces hansenii*, XP_456704.

We also investigated the hydratase activity of G525V, which was found recently [8] in a patient with D-BP deficiency but mild symptoms.

2. Material and methods

2.1. Material and instruments

A plasmid encoding GFP-MFE2H (pGFP-MFE2H) [7] was used as a template for site-directed mutagenesis [9]. The substrate (*trans*-2-hexadecenoyl-CoA) and the product (3*R*-hydroxyhexadecanoyl-CoA) were synthesized chemically as described previously [6]. All other materials and chemicals were of analytical grade. Site-directed mutagenesis was performed using a thermal cycler (MyCycler, Bio-Rad Lab. Inc., Hercules, California, US). DNA sequencing was performed with an automated DNA sequencer (ABI PRISM 310 genetic analyzer, Applied Biosystems, Foster City, California, US). The fluorescence intensity of GFP was measured with a microplate fluorescence reader (MTP-2000, Corona Electric Co., Ltd., Hitachinaka, Japan) in a 96-well white microplate (Nunc, Roskilde, Denmark). HPLC was performed with a Shimadzu LC-10ADvp system equipped with a SPD-10vp (Shimadzu Co., Kyoto, Japan) detector, and equipped with a chiral separation column (CHIRALPAK AD-RH, 4.6 × 150 mm, 5 μm, Daicel Chemical Ind. Ltd., Tokyo, Japan).

2.2. Site-directed mutagenesis

GFP-MFE2H variants were constructed by site-directed mutagenesis using pGFP-MFE2H as a template. Temperature cycling was performed with specific primers (Table 1). The reaction mixture contained approximately 50 ng of pGFP-MFE2H, 1 μM of each primer, 250 μM of each dNTP, 1× reaction buffer, and 2.5 units of PfuTurbo DNA polymerase (Agilent Technologies Inc., CA, US) to a final volume of 50 μL. Site-directed mutagenesis using a thermal cycler was performed by initial denaturation at 95 °C for 2 min, followed by 14 cycles of 95 °C for 30 s; 55 °C for 1 min; 68 °C for 13 min; and a final extension at 68 °C for 13 min. After digestion of template DNA (pGFP-MFE2H) with 2 units of *Dpn*I (37 °C for 1 h), constructed plasmids were introduced into competent cells of *Escherichia coli* JM109. The transformation of *E. coli* JM109 and expression and purification of GFP-MFE2H variants were performed as described in our previous report [7].

2.3. Incubation and HPLC

Incubation and HPLC were performed as described in our previous report [7] as follows: 40 μL of 100 μM *trans*-2-hexadecenoyl-CoA was added to 10 μL of enzyme solution (15–25 units) and incubated at 30 °C for 10 min. To stop the reaction, the mixture was placed on ice and 10 μL of 0.1 M HCl was added. A 20-μL aliquot was injected onto an HPLC column. HPLC conditions were as follows: mobile phase, 10 mM phosphate buffer (pH 5.0)/methanol, 35/65; flow rate, 0.5 mL/min; column temperature, 25 °C; and detection, 260 nm. 3*R*-hydroxyhexadecanoyl-CoA, the product of GFP-MFE2H, was quantified using a standard calibration curve of peak area vs. concentration, constructed using the authentic compound (3*R*-hydroxyhexadecanoyl-CoA). One unit of GFP-MFE2H and its variants was defined as one unit of fluorescence intensity. Hydratase activity (pmol/unit/min) was also calculated. The relative activities (%) of the variants were calculated using the hydratase activity of the wild type as 100%.

Table 1
GFP-MFE2H variants, primers, and hydratase activities.

GFP-MFE2H variants	Primers[a]	Conservation ratio (%)[b]	Hydratase activity (pmol/unit/min)[c]	Relative activity (%)[d]
Wild	–	–	1.59 ± 0.27	100
A353V	5′-CTTGGAGTGGGAGTGTCAATCAAGG-3′ 5′-CCTTGATTGACACTCCCACTCCAAG-3′	79	2.68 ± 0.14	168.1
I380V	5′-CCCACCTT CGGAGTTGTCATAGGTCAGAAATC-3′ 5′-GATTTCTGACCTATGACAACTCCGAAGGTGGG-3′	77.8	1.32 ± 0.07	83.0
G421E	5′-CACTTCCCAGAGCAGAAAAATTAAAATGTG-3′ 5′-CACATTTTAATTTTTCTGCTCTGGGAAGTG-3′	84	1.01 ± 0.07	63.6
G468S	5′-CTTGTTGGCTCTGGAAGCTTTGGTGGAAAAC-3′ 5′-GTTTTCCACCAAAGCTTCCAGAGCCAACAAG-3′	70.4	2.24 ± 0.28	141.0
G525V	5′-GTCTAGCAGTTTTTGACAAG-3′ 5′-CTTGTCAAAAACTGCTAGAC-3′	81.5	0.07 ± 0.02	4.2
R555I	5′-GATAATGATGTGTCAATATTCAAGGC-3′ 5′-GCCTTGAATATTGACACATCATTATC-3′	72.2	2.07 ± 0.17	130.1
K557M	5′-GATGTGTCAAGATTCATGGCAATTAAGGC-3′ 5′-GCCTTAATTGCCATGAATCTTGACACATC-3′	70.4	1.72 ± 0.20	108.2
R562H	5′-GGCAATTAAGGCTCATTTTGCAAAACCAG-3′ 5′-CTGGTTTTGCAAAATGAGCCTTAATTGCC-3′	100	6.69 ± 0.82	420.4
R562L	5′-GGCAATTAAGGCTCTTTTTGCAAAACCAG-3′ 5′-CTGGTTTTGCAAAAAGAGCCTTAATTGCC-3′	100	3.45 ± 0.46	216.9
Q587E	5′-CAGAATTCATTTTGAAACCAAGGTCCAAG-3′ 5′-CTTGGACCTTGGTTTCAAAATGAATTCTG-3′	76	2.75 ± 0.35	172.5
L622F	5′-GGGCGGGAAGTTTTAATGAAAGCTT-3′ 5′-AAGCTTTCATTAAAACTTCCCGCCC-3′	100	1.46 ± 0.10	91.7

[a] Upper and lower sequences are forward and reverse primers, respectively. Missense mutations were underlined.
[b] Among eukaryotes (see Fig. 2).
[c] Means ± S.D. (n = 3).
[d] Hydratase activity of wild type was taken as 100%.

3. Results and discussion

3.1. Naturally occurring missense mutations

We have previously reported the effects of missense mutations in the hydratase domain of D-BP, which is associated with congenital lipid metabolism disorder, on its activity in vitro [7]. It was clear that missense mutations in highly conserved amino Table 1.

Acids markedly decreased activity (Table 1). This result suggested that the loss of the activity evokes D-BP deficiency type II. Gloerich et al. classified D-BP deficiency into the following three subtypes according to the defect: type I, a complete deficiency of D-BP; type II, an isolated D-BP enoyl-CoA hydratase deficiency; and type III, an isolated D-BP 3-hydroxyacyl-CoA dehydrogenase deficiency [10]. GFP-MFE2H variants corresponding to the missense mutations that cause D-BP deficiency type II showed little or no hydratase activity. For example, the relative hydratase activities of N457Y and N457D found in patients were 9.8% and 3.4%, respectively. R506C and R506H, which were also found in patients [11], did not exhibit hydratase activity. Other missense mutations such as A348T, A427V, and W511R, which have been reported as naturally occurring missense mutations, did not affect hydratase activity [7]. The conservation rates of N457 and R506 among eukaryotes were found to be 92.6% and 100%, respectively, but those of A348, A427, and W511 were 62.1%, 60%, and 48.1%, respectively. These results suggested that the consensus amino acids conserved among various species are indispensable, and that their replacement by other amino acids abolishes protein activity. In particular, we hypothesized that activity would be affected by changes of amino acids with conservation rates of approximately 90% and above.

In the present study, we investigated the effects of other missense mutations reported as naturally occurring, with conservation rates of approximately 70–100%, on hydratase activity. We predicted, for mutations with low conservation rates, that their hydratase activities would not change in comparison with Table 1 the wild type. The results are shown in Table 1. In contrast, we predicted that the activities of R562H and R562L would be very low or absent, given that the conservation rate of R562 is 100%. However, contrary to our prediction, their activities were increased by 4 and 2 times, respectively, compared to that of the wild type (Table 2). Koski et al. proposed based on a simulated structural study that R562 is a functionally important residue located in a substrate-binding pocket. They proposed that R562 forms interactions with the CoA moiety of 2-enoyl-CoA via van der Waals stacking interaction with the π electrons of the adenine ring of 3′-phosphate ADP [12]. Histidine has an aromatic ring. Even if R562 is changed to a histidine, it may interact with the adenine ring as well as arginine. For this reason, R562H did not affect activity. In contrast, leucine (L) does not contribute to π–π interactions. We suggest that K560 and F563, which adjoin L562, compensate for the deficiency of stacking interaction. The conservation rate of K560 and F563 is 100%, as is that of R562. To form the substrate-binding pocket, R562 may cooperate with K560 and F563.

Considering the crystal structure of the hydratase 2 unit of human D-BP (PDB entry 1S9C) and D-BP of fruit fly (PDB entry 3OML) from the Protein Data Bank (PDB) [13], we found that the amino acids A353, I380, G421, G468, R555, K557, R562, and Q587, which were the focus of this study, are located distantly from the hydratase motif or dimerization site (Fig. 3). Crystal structure information supports the inference that amino acid changes do not affect the hydratase activity.

In this study we focused on naturally occurring missense mutations of the D-BP hydratase domain. We investigated the effects of the mutations on hydratase activity in vitro. The results suggested that not all of the naturally occurring missense mutations found at highly conserved amino acid positions evoke severe lipid metabolism disorders. Characterizing the relevance of these mutations to lipid metabolism disorders awaits further in vivo investigation. In the next subsection, we describe a case in which in vivo investigation is important.

Table 2
Relative activities of GFP-MFE2H variants corresponding to missense mutations found in human D-BP.

Conservation ratio (%)[a]	Missense mutation	Relative activity (%)[b]	D-BP deficiency type II[c, d]
100	L405P	n.d.	+
100	R506C	n.d.	+
100	R506H	n.d.	+
100	D510Y	n.d.	+
100	P529L	n.d.	+
100	H532R	n.d.	+
100	G533R	n.d.	+
100	R562H	420.4	
100	R562L	216.9	
100	L622F	91.69	
96.4	E366G	n.d.	+
92.6	N457D	3.4	+
92.6	N457Y	9.8	+
88.9	I516T	1.4	+
84.0	G421E	63.6	
81.5	G525V	4.2	+
79.0	A353V	168.1	
77.8	I380V	83.0	
76.0	Q587E	172.5	
72.1	R555I	130.1	
70.4	G468S	141.0	
70.4	K557M	108.2	
63.0	I559V	81.8	
62.1	A348T	95.8	+
60.0	A427V	125.9	
57.1	A491T	103.0	
48.1	W511R	76.6	
16.0	A606S	69.5	

[a] Among eukaryotes (see Fig. 2).
[b] Hydratase activity of wild type was taken as 100%. "n.d." means "not detected".
[c] Plus sign (+) indicates missense mutations found in patients with D-bifunctional protein deficiency type II.
[d] Gray area indicates the results of our previous article[7].

3.2. G525V variant

Recently, Mizumoto et al. reported a novel D-BP deficiency caused by compound heterozygosis of G525V and G658X (a nonsense mutation) [8]. The level of transcription of the D-BP gene was markedly lower than that of a healthy subject, and expression of D-BP in fibroblasts was not observed by immunostaining. However, the symptoms induced by the mutations were mild. G525V and G658X lie in the coding regions of the 2-enoyl-CoA hydratase and SCP-2 like domains, respectively. It thus seems unlikely that these mutations affect the transcription of the D-BP gene and its subsequent expression. In addition, if the expression level of D-BP were low, its transcription should be enhanced to compensate for the loss. But our results contradict this hypothesis.

In addition to the report of Mizumoto, McMillan et al. have reported other mild cases of D-BP deficiency [15]. They identified compound heterozygous mutations, A34V and I516T, in the D-BP gene. In our investigation, I516T markedly reduced hydratase activity (<10%). Thus, in a patient with heterozygous A34V and I516T, it is possible that the mild symptoms were caused by alleviation of the affects of I516T by D-BP harboring A34V, which preserves hydratase activity. We suggest that the homozygosity of mutations that markedly reduce D-BP activity evokes the severe symptoms. Accordingly, we predicted that G525V would markedly reduce D-BP activity and that G658X would not affect this activity. We investigated the effects of G525V on activity and found that the hydratase activity of G525V was decreased to approximately 4.2% in comparison with the wild type. The patient could be classified as type II. With respect to the factors accounting for the mild symptoms of G525V/G658X, we find it likely that G658X did not affect D-BP activity to compensate for the loss of function associated with G525V. The

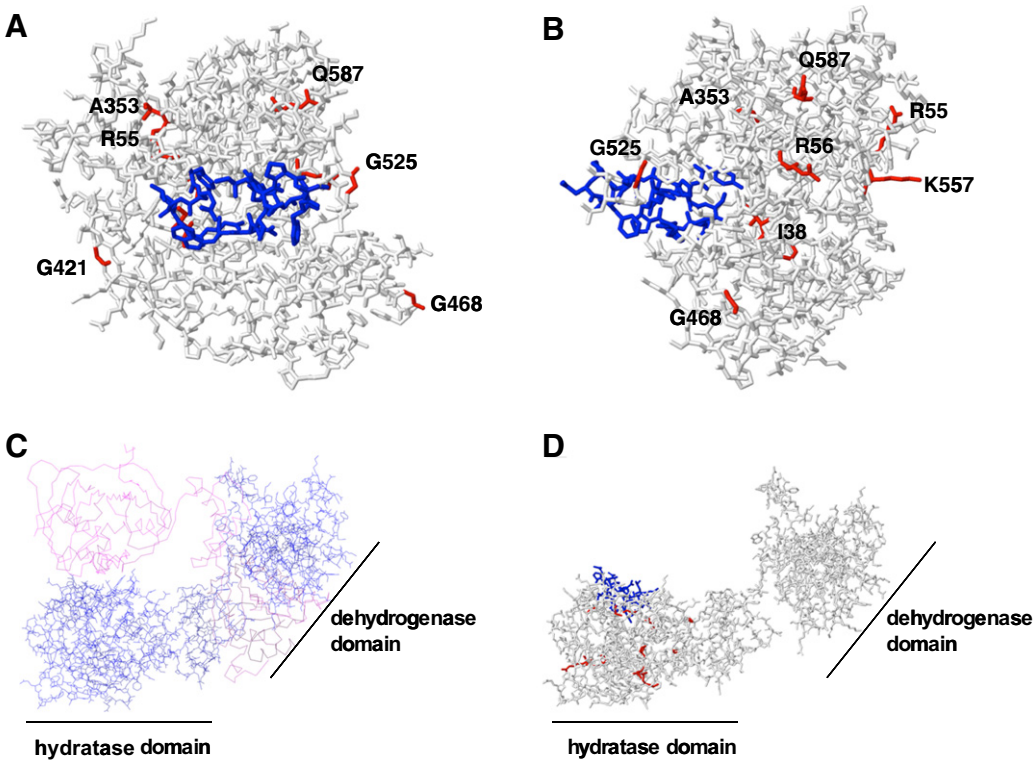

Fig. 3. Three-dimensional structure of human D-BP hydratase unit (A and B) and fruit fly D-BP (C and D). The enoyl-CoA hydratase 2 unit of human D-BP (PDB entry 1S9C) is shown. Blue indicates residues of the enoyl-CoA hydratase motif. Red indicates residues at which the effects of missense mutations were investigated in this study (A). Three-dimensional structure of the panel, A is rotated around the y-axis (B). The D-BP dimer of fruit fly (PDB entry 3OML). Magenta and blue indicate each monomer of the D-BP molecule (C). D-BP monomer indicated in blue in panel C is shown in stick style. As in panel A, blue and red indicate the enoyl-CoA hydratase motif and amino acid residues, respectively (D). All three-dimensional structures were drawn on the basis of data from PDB using the software Strap (Interactive Structure based Sequences Alignment Program) [14].

mutant protein derived from G658X does not carry amino acid 658 and the downstream amino acid sequence, accordingly lacks the peroxisome targeting signal (PTS) sequence, Ala–Lys–Leu, at the C terminus, and is not transferred to the peroxisome [11]. Briefly, although we surmised that heterozygous G525V and G658X would evoke a drastic lipid metabolism disorder, the symptoms of the patient with the mutations were mild.

Ferdinandusse et al. reported a similar case [11]. A patient with heterozygous N457D and I516T lived 13.5 years, although a patient with N457Y and I516T lived only 11 months. However, our results suggested that both compound heterozygotes should experience severe disease, given that the hydratase activities of N457D and N457Y were markedly reduced (to < 10%), as well as that of I516T.

The discrepancy between our results and the symptoms of the patients might be explained by the existence of a pathway compensating for the defect of D-BP, such as via L-BP or another metabolic pathway present in enteric bacteria. The question remains unresolved and merits further study.

4. Conclusions

A naturally occurring missense mutation in a highly conserved amino acid of the hydratase domain in D-BP did not abolish its activity, suggesting that the loss of hydratase activity does not invariably evoke severe lipid metabolism disorders in vitro. The deactivation associated with G525V suggested the existence of some metabolic pathway compensating for D-BP deficiency.

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