

Cite this: *RSC Adv.*, 2017, 7, 36326

New findings about human dipeptidyl peptidase III based on mutations found in cancer†

M. Matovina,^a D. Agić,^b M. Abramić,^a S. Matić,^a Z. Karačić^a and S. Tomić^{*a}

Dipeptidyl peptidase III (DPP III) is a cytosolic enzyme belonging to the metallopeptidase family M49, involved in the final steps of protein catabolism. More than a hundred missense mutations can be found in the coding region of the human DPP3 gene when searching cBioPortal for Cancer Genomics. The role of two highly conserved residues in the family M49, whose mutations G313W and R510W were detected in human cancer, was investigated using combined experimental and computational approaches (substrate docking and MD simulations). Several mutants of human DPP III were expressed and purified as recombinant proteins, and their biochemical properties were determined. The conservative substitution of Arg510 with lysine mildly decreased enzyme activity for Arg-Arg-2-naphtylamide substrate, while the substitutions of Arg510 with glutamine and Gly313 with alanine substantially decreased enzyme activity, and tryptophan substitutions found in cancer, G313W and R510W, almost abolished enzyme activity. MD simulations showed that substitution of Gly313, and especially Arg510 with tryptophan, significantly increases the enzyme flexibility, particularly that of the binding site including the H⁴⁵⁰ELLGH⁴⁵⁵ motif, and influences the substrate interactions with the catalytic His568. The results clearly indicate that, besides the enzyme structure, its dynamics properties also significantly influence the human DPP III activity.

Received 3rd March 2017

Accepted 13th July 2017

DOI: 10.1039/c7ra02642k

rsc.li/rsc-advances

Introduction

The dipeptidyl-peptidase III (DPP III; EC 3.4.14.4) is a monozinc exopeptidase from the metallopeptidase family M49 (according to the MEROPS database, <http://www.merops.ac.uk>), found to be broadly distributed in mammalian tissues, and thought to contribute to the final steps of normal intracellular protein catabolism. There are strong indications of its role in the endogenous pain-modulation system,¹ as well as in the endogenous defence against oxidative stress.² Pathophysiological roles of human DPP III (h.DPP III) are indicated in malignant growth,^{3,4} and influenza virus infection.⁵ Recently, it was proven that DPP III contributes to the activation of transcription factor Nrf2, a constituent of the Nrf2-Keap1 signalling pathway, the main defence mechanism against many environmentally toxic agents and carcinogens in cells.^{6,7} However, (patho)physiological roles of DPP III are still not elucidated, and better understanding of its broad substrate specificity would be a step toward understanding its biological function.

From a structural point of view, the DPP III is a two-domain protein with the motifs HEXXGH and EE*XR(K)AE(D) considered as its trademark.^{8,9} Histidines from the first motif and

glutamate* from the second one take part in the zinc ion coordination, while the glutamate from the first motif is crucial for the catalytic activity of DPP III, namely the hydrolysis of dipeptides from the N-terminus of its substrates consisting of three or more amino acids.¹⁰

Until now several 3D structures of h.DPP III have been determined, ligand free and in complex with natural peptides. The structure of the ligand-free enzyme (PDB codes: 3FVY and 5EGY) was determined in the so-called open and closed form, while that of the liganded enzyme only in a more compact, closed, form (PDB codes: 3T6B, 3T6J, 5E33, 5E3A, 5E2Q, 5EHH, 5E3C).¹¹ The open, ligand-free enzyme structure is characterized with the wide inter-domain cleft (~40 Å wide and ~40 Å high) with the catalytic zinc ion bound at its "roof". Using computational approaches we have traced the protein closure,^{12,13} gained better insight into enzyme broad substrate specificity and determined the reaction mechanism of h.DPP III.^{14–16}

More than hundred missense mutations can be found in the coding region of h.DPP III gene by searching cBioPortal for Cancer Genomics (<http://www.cbioportal.org/>; accessed September 2014).^{17,18} Most of those mutations were predicted to have low or medium impact on protein function, however, two of them, G313W and R510W, which reside in the conserved regions 1 and 4 of DPP III,⁹ were predicted to have high influence on protein functionality. Those mutations were found in the tumour samples from lung adenocarcinoma, and colorectal adenocarcinoma samples, respectively. Mutation G313W was found in the provisional TCGA (The Cancer Genome Atlas)

^aDivision of Organic Chemistry and Biochemistry, Ruđer Bošković Institute, Bijenička cesta 54, Zagreb, Croatia. E-mail: sanja.tomic@irb.hr

^bJosip Juraj Strossmayer University of Osijek, Faculty of Agriculture in Osijek, Vladimira Preloga 1, Osijek, Croatia

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7ra02642k



study, and was removed from newer releases of cBioPortal (<http://www.cbioportal.org/>; accessed October 2015), however, this study was aimed at finding the cause of the DPP III inactivation by this mutation, and not at studying the potential role of these mutations in the development of cancer so we decided to proceed with the research of this mutant regardless of that. We investigated the influence of those mutations on the enzyme biochemical and physicochemical properties. Those mutants exhibited almost complete loss of activity towards synthetic substrate, Arg-Arg-2-naphthylamide (Arg₂-2NA; RRNA) compared to the wild type (wt) h.DPP III, and we set out to determine the cause of this activity loss by combining experimental approaches with computational simulations. In order to determine the root of the activity loss in these two mutants, we investigated three other amino acid substitutions, in which the native amino acid residues were substituted by an amino acid that differs less from the original than mutants found in tumour cells. In this way we were able to gain better understanding of how amino acid substitutions at particular position influence enzyme structure and activity. Altogether, we analysed 5 different mutants, G313W and R510W that were found in cancer, and the more similar (conserved) amino acid substitutions at the same positions, G313A, R510K and R510Q.

Results and discussion

Biochemical properties of h.DPP III mutants

We purified and biochemically characterized two recombinant protein mutants of h.DPP III, G313W and R510W, that were found in lung adenocarcinoma, and colorectal adenocarcinoma samples respectively (<http://www.cbioportal.org/>; accessed September 2014), in order to test their enzymatic activities in comparison to wt h.DPP III. Both residues are located in conserved regions of the family M49 (DPP III family), with R510 being the part of EECRAE motif of the active site, and G313 being the part of the first conserved region (amino acids 313 to 332).⁹ Both Gly313 and Arg510 are completely conserved among M49 family members (Fig. S1†). Since these amino acid substitutions, G313W and R510W, almost abolished h.DPP III activity towards its preferred Arg₂-2NA substrate (Table 1), we purified and biochemically characterized three more mutants

with more conservative amino acid substitutions at the same positions, G313A, R510K, and R510Q.

Measurements of specific activity of the wt h.DPP III and the mutants towards Arg₂-2NA showed that tryptophan mutants, G313W and R510W, have approximately 24 000, and 1600 fold lower specific activity than the wt h.DPP III, respectively. Substitution of alanine at amino acid residue 313 increased the activity of the protein compared to the G313W mutant, however, it was still considerably lower than the wt h.DPP III, showing that glycine at position 313 is essential for the h.DPP III activity. On the other hand, substitution by lysine instead of tryptophan at position 510 returned the specific activity of the mutant to approximately half of the activity of the wt h.DPP III, while substitution by glutamine raised enzyme activity comparing to the R510W mutant, but the mutant R510Q still had approximately 14 fold lower specific activity than the wt h.DPP III (Table 1). That result showed that for the efficient enzymatic activity h.DPP III requires a positively charged amino acid at position 510. The pH optimum for Arg₂-2NA hydrolysis was around 8.6 for R510 mutants, while G313W, and G313A had the optimum at pH 8.0, and 7.4, respectively. The studied substitutions slightly change the relative preferences for different dipeptidyl 2-naphthylamides (Arg₂-2NA, Ala-Arg-2NA and Phe-Arg-2NA) compared to the wt, however, Arg₂-2NA was still the preferred substrate for all mutants (Table 1).

The catalytic properties of the wt h.DPP III and all mutated forms were determined for the hydrolysis of characteristic synthetic substrate Arg₂-2NA from two or three independent measurements at pH 8.6 (Table 2), except for the G313A whose catalytic properties were determined from one measurement at pH 7.4. We did not determine the G313W kinetic parameters due to extremely low enzymatic activity of that mutant. All tested proteins showed similar K_M as the wt h.DPP III, except G313A that had more than 20 times higher K_M , while k_{cat} differed by 2–3 orders of magnitude for mutants G313A and R510W, respectively. The k_{cat} value of R510Q was 26 times lower than the wt, and R510K had similar k_{cat} as the wt.

We have revealed or confirmed the importance of several conserved amino acid residues situated outside of the two active-site motifs H⁴⁵⁰ELLGH⁴⁵⁵ and E⁵⁰⁷ECRAE⁵¹², in human DPP III (Tyr318, Trp300, His568, Asp496), so far.^{19–22} Among previously characterized h.DPP III mutants, H568N showed the

Table 1 Specific activity towards Arg₂-2NA, and substrate specificity of the h.DPP III, wt and the mutants^a

Protein	Specific activity/ nmol min ⁻¹ mg ⁻¹	RA Ala-Arg-2NA/%	RA Phe-Arg-2NA/%
h.DPP III			
WT	47 900	26	27
G313W	2	40	40
G313A	76	6	20
R510W	31	18	23
R510K	21 515	25	19
R510Q	3284	5	5

^a RA Relative activity towards substrates (normalized to that for Arg₂-2NA).

Table 2 Kinetic characterization of h.DPP III, wt and mutants^a

Protein h.DPP III	$K_M/\mu\text{M}$	k_{cat}/s^{-1}	$k_{cat}/K_M \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$
WT	5.45 ± 0.53	35.400 ± 6.364	6.475 ± 0.538
G313W	—	—	—
G313A	114.57	0.224	0.002
R510W	4.48 ± 1.49	0.012 ± 0.003	0.003 ± 0.000
R510K	4.06 ± 0.87	27.533 ± 9.905	7.066 ± 3.269
R510Q	2.10 ± 1.32	1.350 ± 0.365	0.864 ± 0.533

^a The values are the average ± st. dev. of 2 (WT and R510W) or 3 (R510K and R510Q) independent measurements at pH 8.6, except G313A for which we conducted 1 measurement at pH 7.4.



Table 3 The Zn^{2+} coordination and the range of R_{gyr} traced during simulations of different human DPP III variants. Data obtained during two 100 ns long MD simulations (see Table S1) are given. Details of all simulations performed within this work are given in Table S1. Letters 'm' and 'b' denote monodentate and bidentate coordination of Zn^{2+} by Glu451 and Glu508 carboxyl groups, respectively

h.DPP III variant	Zn^{2+} coordination				R_{gyr} Range (Å)
	H450	H455	E451	E508	
WT	+	+	m	m	26.9–28.4
G313W	+	+	m	m/b	26.2–28.6
G313A	+	+	m	m/b	26.4–28.5
R510K	+	\pm^a	m	m/b	26.3–28.2
R510W	+	+	m	m	26.2–28.3
R510Q	+	\pm^a	m	m	26.4–28.4
WT-closed	+	+	m	m/b	25.0–25.5
WT-RRNA	+	+	m	b	24.9–25.5
G313W-RRNA	+	+	m/b	b	24.8–25.6
G313A-RRNA	+	+	m/b	b	24.9–25.5
R510W-RRNA	+	+	m	b	24.9–25.7
R510Q-RRNA	+	+	m/b	m/b	24.8–25.5
R510K-RRNA	+	+	m	m/b	24.8–25.7

^a In the case of two simulation of variants, R510K and R510Q, His455 reoriented and during about 4.5% of the total simulation time (200 ns) have not coordinated the zinc ion.

lowest k_{cat} value for the hydrolysis of Arg₂-2NA, three orders of magnitude lower than the wt h.DPP III,²¹ without significant change in K_{M} value. According to steady-state kinetic parameters, this protein variant is similar to R510W characterized in our present study.

Molecular simulations

We simulated 6 different variants of h.DPP III, wild type and its mutants, G313A, G313W, R510K, R510Q and R510W. All

variants were simulated without substrate and in complex with the substrate Arg₂-2NA (RRNA), see Table 3. For each variant we performed four MD simulations (100 ns long), two for the ligand free enzyme variant and two for its complex with Arg₂-2NA, see Table S1.† Structure of the wt h.DPP III-RRNA complex is shown in Fig. 1 and positions of the amino acid residues mutated in this study are indicated. Influence of the mutations to the protein structure and flexibility was studied using the structure of the ligand free enzyme in its open form, while the influence of the point mutations on the ligand binding was studied using the closed form of the enzyme complexed with the preferred synthetic substrate Arg₂-2NA. Our earlier studies^{13,14,16} showed that such a closed enzyme form is the active one, *i.e.* it ensures environment suitable for the peptide hydrolysis. Orientation of Arg₂-2NA in the active site of the closed form closely resembles the position of Leu-enkephalin in the human DPP III binding site, for which the reaction mechanism was determined using QM/MM approach.¹⁶

For the G313W variant, two different orientation of the indole ring were considered. In one, the Trp side chain at position 313 points towards the active site and in the other it points toward the lower domain interior. However, since the orientation toward the lower domain interior turned out to be unfavourable, in this work we consider only results obtained with Trp313 pointing towards the interdomain cleft active site. According to the results of MD simulations, the point mutations at positions 313 and 510, G313A, G313W, R510K, R510Q, and R510W do not influence the metal ion coordination (Table 3). The simulations showed that, like in the wt protein,²⁵ the Zn^{2+} ion is mostly hexacoordinated in the simulated mutants, as well. In the ligand free variants the metal dication was coordinated by two histidines, H450 and H455, two glutamates, E451 and E508, and one or two water molecules, depending on the position of E451 and E508 carboxyl groups, during the

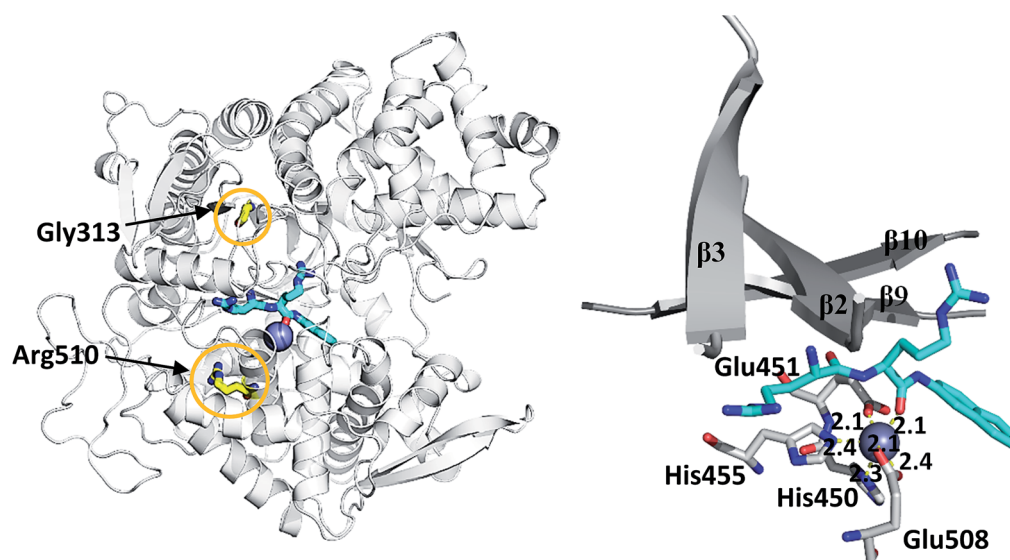


Fig. 1 (left) Structure of wild type DPP III (light grey) in complex with preferred synthetic substrate Arg₂-2NA (cyan), with marked positions of mutated residues (yellow) and the active site Zn^{2+} (grey sphere), (right) part of the beta-sheet core of the enzyme and the coordination of Zn^{2+} obtained after 100 ns of MD simulations. The distances (in Å) between coordinating atoms and Zn^{2+} are presented.



simulations. The water molecules coordinating Zn^{2+} often exchanged with bulk water during the simulations and they are not shown in Fig. 1. In the case of two simulation (variants R510K and R510Q) His455 reoriented, and during about 4.5% of the total simulation time have not coordinated the zinc ion. However, during the simulations of the other two replicas of the same variants the zinc coordination has been preserved during entire simulation time. Apparently, the zinc ion coordination is mostly preserved in all simulations and rarely occurring re-orientation of H455 can be attributed to the stochastic nature of the simulations.

MD-simulations of the ligand free h.DPP III variants

Although the mutations at positions 313 and 510 do not significantly influence the overall protein fold and the Zn^{2+} ion coordination, they induce local structural changes which distort the active site conformation, and in this way hinder substrate positioning in the orientation that is the most suitable for the enzymatic reaction. Gly313 is located in the β_6 sheet of the h.DPP III lower domain. At the end of the sheet there is a Glu316 residue, which upon protein closure approaches the substrate and interacts electrostatically with the N-terminal amino group of its first amino acid residue (Fig. S2†). The residue conservation within M49 family and large decrease of the G313W mutant enzyme activity, suggest that Gly residue at position 313 is essential for proper positioning of the substrate upon closing of the domains.

MD simulations revealed that, although Gly313 does not interact with the substrate, it dictates the position of Glu316.

Indeed, Trp at position 313 distorts the β_6 sheet conformation, changes the Glu316 position and prevents proper substrate positioning in the active site (Fig. 2 and S2†). The kinetic parameters of G313W mutant could not be determined due to its low activity and K_M of the more conservative mutant G313A was significantly increased compared to the wt (Table 2).

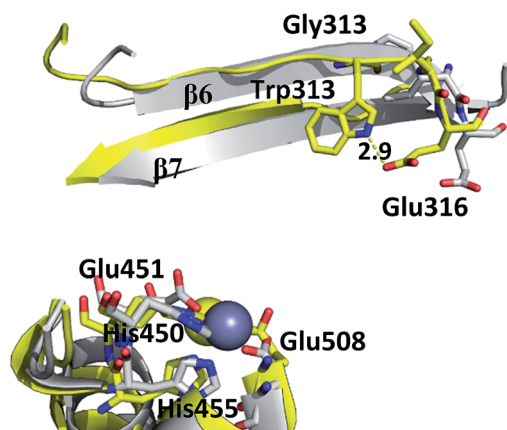


Fig. 2 Position of residues 313 (Gly and Trp, respectively) and Glu316 in β -sheets β_6 – β_7 in wt protein (grey) and mutant variant (yellow), in representative structures of 100 ns simulations. The distance (in Å) between Trp313 and Glu316 of mutant structure is labeled. Zn^{2+} is represented as a grey sphere and the residues of active site are shown as sticks.

This supports the finding that the mutation in G313 in general does not prevent the substrate binding, but disturbs its positioning in the active site in the catalytically productive orientation.

In order to get an insight into the overall protein flexibility, and to find out how the point mutations influence this flexibility, we computed the average Root Mean Square Fluctuations (RMSF) for the wild type protein and its mutants (Fig. 3). The results show that the lower (satellite) domain, especially its N and C terminal segments (residues 4 to 300 and 680 to 726, respectively) are the most flexible parts of the enzyme, followed by the substrate binding β -sheet region, consisting of two β -strands, β_9 and β_{10} . Analysis of the RMSF clearly indicated that mutations, either at position 313 or in position 510, increase protein flexibility, especially of the lower domain β -core (β -sheets β_2 – β_3 and β_9 – β_{10}). The R510K flexibility closely resembles that of the wild type protein.

Apparently, increased flexibility of the lower domain β -core, especially of β -sheets β_2 – β_3 and β_9 – β_{10} , is negatively correlated with the enzyme activity. The mutants with the highest fluctuations determined during MD simulations were also showing the lowest activity. On the other hand, both the activity and flexibility of R510K are very similar to those of the wild type protein. Further on, the RMSF profile revealed that substitution of Arg510 by Trp significantly increases fluctuations of the $\text{H}^{450}\text{ELLGH}^{455}$ motif and its surrounding (Fig. 3 and S3†).

Consequently, the interactions between the amino acid residues from this region with the rest of the protein are weaker

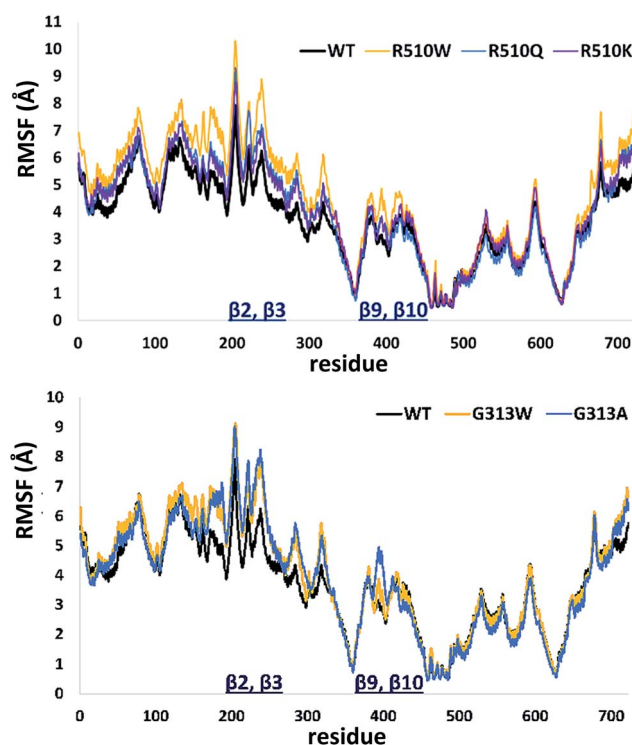


Fig. 3 Root Mean Square Fluctuations (RMSF) profiles of the human DPP III variants obtained by averaging over two 100 ns long simulations (see Table S1†).



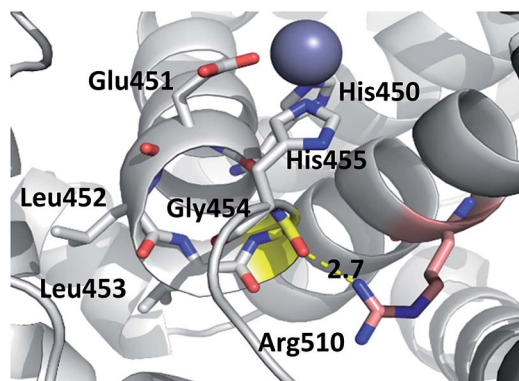


Fig. 4 Position of highly conserved residue R510 (pink) in the structure of h.DPP3 (PDB 3FVY) interacting with Gly454 (yellow, distance in Å) from the conserved H⁴⁵⁰ELLGH⁴⁵⁵ motif (shown as sticks) responsible for coordinating Zn²⁺ (grey sphere).

in mutants than in the wt h.DPP III. In the wt protein, Arg510 interacts electrostatically with carbonyl groups of Gly454, Leu453 and His455 (Fig. 4). The Arg510 to Trp and Gln mutations resulted with decrease of these interactions and as a consequence flexibility of this motif increased in accord with the RMSF results. The highest fluctuation of the H⁴⁵⁰ELLGH⁴⁵⁵ motif has been found in the R510W mutant, followed by R510Q, while the fluctuations in the R510K mutant and the wild type protein are similar (Fig. S3†). It seems that destabilization of the H⁴⁵⁰ELLGH⁴⁵⁵ motif does not disturb the Zn²⁺ coordination in the R510W and R510Q mutants but it significantly influences the enzymatic activity. This is not surprising if we know that Glu451 directly participates in the peptide bond hydrolysis, acting as a general base during the deprotonation of water molecule that attacks the scissile amide bond.¹⁶ The increased fluctuations of this motif in the Arg510 mutants has been observed in both open and closed protein forms. Apparently, the H⁴⁵⁰ELLGH⁴⁵⁵ instability can be accounted for the decreased peptidase activity of h.DPP III mutants.

Table 4 Population of the most relevant hydrogen bonds for the enzymatic reaction during two, 100 ns long MD simulations of the h.DPP III variants. The hydrogen bonds were calculated with CPPTRAJ using default parameters. However, only H-bonds with distances ≤2.5 Å are listed in the table

Residues	% H-bond					
	Variant					
	wt	G313W	G313A	R510W	R510Q	R510K
Y318–E327	66	87	52	31	78	34
H450–H455	22	22	19	17	19	14
E451–N406	59	41	11	49	52	55
H455–S457	2	3	3	2	3	16
H455–E507	84	89	86	63	77	73
E508–S504	8	11	6	5	4	8
E512–H568	68	74	32	73	70	61
X510–G454	78	80	74	35	12	13
X510–S457	62	50	72	6	1	4
X510–Q626	73	71	79	2	78	7

Additionally, the hydrogen bond between residue 510 and Ser457, present in the wild type enzyme, is significantly less stable in Arg510 mutants, while the Arg510–Gln626 hydrogen bond is preserved in the R510Q mutant, but not in R510W and R510K mutants (Table 4). Further on, the hydrogen bond between fully conserved Tyr318 and Glu327 is preserved in the R510Q but not in R510K and R510W mutants.

MD-simulations of the h.DPP III–Arg₂-2NA complexes

In the studied complexes two arginine residues of the substrate (Arg₂-2NA) form a β-strand that in the antiparallel fashion binds to the five-stranded β-core of DPP III (as shown in Fig. 1). Such binding closely resembles the tynorphin binding mode in the experimentally determined structure of the inactive DPP III mutant–tynorphin complex.²⁶ Binding of the substrate Arg₂-2NA into the active site of human DPP III stabilized the protein structure, especially the lower domain β-core (Fig. 3 and 5) and slightly changed the flexibility profile pattern. Apparently, binding of the synthetic substrate Arg₂-2NA to the β9 and β10 sheets decreases their mobility in the wt protein. However, despite the striking similarity of the substrate orientation in the simulated variants, the protein structure stabilization induced by the substrate binding is not the same in all variants (Fig. S4 and S5†), and the complex with the R510W mutant is

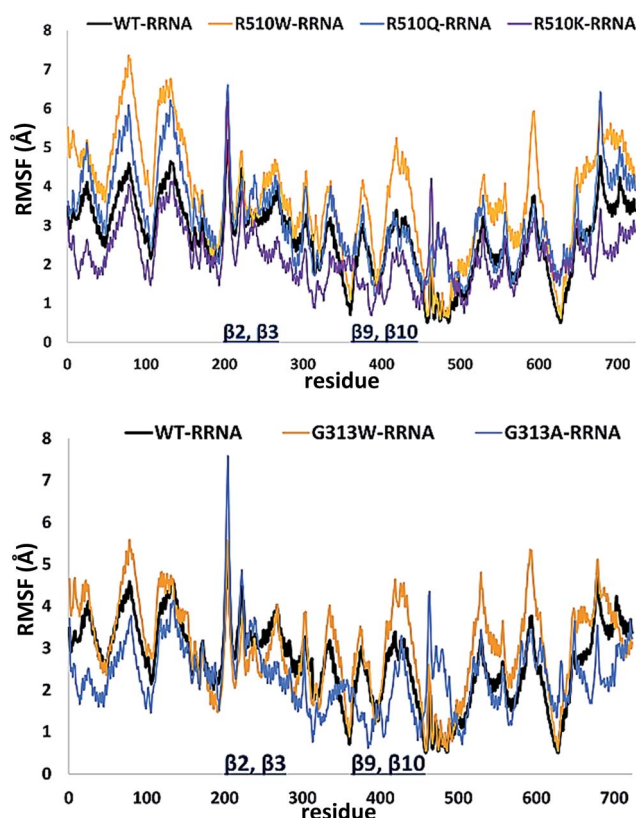


Fig. 5 Root Mean Square Fluctuations (RMSF) calculated for the 100 ns of MD simulations of the complexes between different human DPP III variants and Arg₂-2NA obtained by averaging over two 100 ns long simulations.



Table 5 Populations of the selected intra- and intermolecular hydrogen bonds averaged over two 100 ns long MD simulations of h.DPP III variants–Arg₂-2NA complexes. The hydrogen bonds were calculated with CPPTRAJ using default parameters. H-bonds with distances below 2.51 are listed in the table

H-bonds	WT-RRNA	R510W-RRNA	R510Q-RRNA	G313W-RRNA	G313A-RRNA
Y318–D496	<1	<1	40	3	97
Y318–E508	76	44	1	85	<1
Y318–H568	<1	<1	21	<1	1
X510–G454	57	34	<1	74	41
X510–Q626	68	<1	71	76	63
E512–H450	88	73	71	80	90
E316–RRNA	100	99	100	100	56
E329–RRNA	21/63	87	100	100	95
G385–RRNA	49	<1	5	<1	<1
G389–RRNA	54	77	74	55	<1
N391–RRNA	51	100	88	100	77
N394–RRNA	89	64	90	88	83
D396–RRNA	50	23	45	62	84
D496–RRNA	41	85	95	100	96
H568–RRNA	47	7	8	<1	<1

significantly more flexible than the other simulated complexes (Fig. S3†).

The hydrogen bond analysis revealed that, similarly to the ligand free protein, interaction between residues at position 510 and Gln626 is completely abolished in the R510W–Arg₂-2NA mutant complex, while the hydrogen bond between Arg510 and Gly454 has not been detected during the simulation of the R510Q complex (Table 5). Oppositely, the Tyr318 stabilization by Asp496 and His568 is the most stable in the complex with the G313A and the R510Q mutant, respectively (Fig. S7†) as confirmed by the hydrogen bond analysis (Table 5). We have showed that the 100% conserved Tyr318 is important for substrate stabilization in the active site.¹⁶ Its reorientation into direction of either Asp496 or His568 might be one of the reasons for the enzymatic inactivation of the R510Q mutant, also. Analysis of the substrate–protein hydrogen bonds revealed that

in general there are no significant differences among the complexes (Fig. 6, S4 and S5†). However, the stability of the hydrogen bond between the substrate and highly conserved residue G385 as well as the catalytic histidine H568 is the most stable in the complex with wt enzyme (Table 5 and Fig. 6 and S6†). It has been shown that His568 plays a significant role in the transition state stabilisation and the enzymatic reaction. By forming a stable hydrogen bond with the substrate carbonyl oxygen, His568 is dragging the negative charge away from the peptide C atom and in this way facilitates nucleophile attack of the activated water.¹⁶ So the lack of this hydrogen bond in the complexes with mutated protein may explain the enzymatic inactivity of these variants.

Conclusions

We expressed and biochemically characterized two mutant h.DPP III proteins, G313W and R510W, with mutations in the highly conserved regions of DPP III, to analyse their biochemical and physicochemical properties in comparison to the wild type h.DPP III. Aforementioned mutants exhibited dramatically lower activity towards the synthetic DPP III substrate, Arg₂-2NA, which prompted us to investigate the origins of that activity loss by combining experimental and computational approach. We also analysed 3 additional mutants with more conservative amino acid substitution at the same positions, G313A, R510K and R510Q. The results of these investigations gave new insight into biochemical and structural importance of conserved residues Gly313 and Arg510 for the enzyme activity. Experimental approach indicated that Gly313 to Ala mutation significantly influences the binding of the Arg₂-2NA substrate in contrast to the mutations of Arg510 that did not result in the significant change of *K_M*. In agreement with biochemical results, MD simulations revealed that Gly313 is essential for the proper positioning of the substrate, although there is no direct contact between them. However Gly313 dictates position of Glu316, conserved residue that interacts

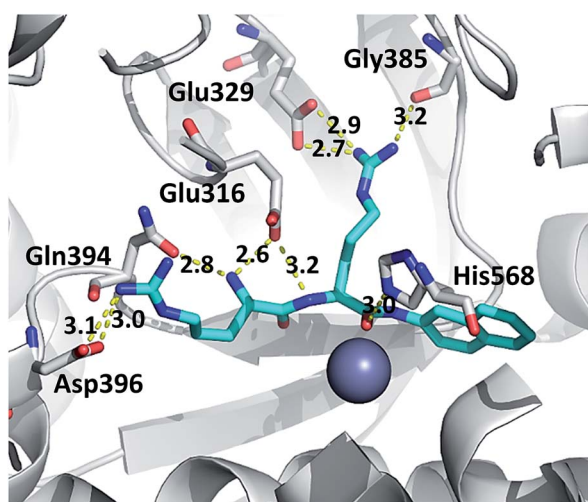


Fig. 6 The representative structure of the h.DPP III–Arg₂-2NA complex. The residues interacting with the substrate are presented as grey and the substrate as cyan sticks. The distances are given in Å.



electrostatically with the N-terminal amino group of the substrate. The efficiency of Arg₂-2NA hydrolysis (k_{cat}/K_M) was significantly decreased in all mutants except R510K. One possible reason for the enzymatic inactivity of the mutants is increased flexibility of their active site in comparison with the wild type enzyme, and the other is lack of the hydrogen bond between the substrate and catalytic His568, which is known to be important for the transition state stabilization.¹⁶

Apparently, the disrupted hydrogen bond between Arg₂-2NA and catalytic His568 in combination with the increased active site fluctuations, especially of the H⁴⁵⁰ELLGH⁴⁵⁵ motif provide possible explanation for inactivity of the G313W and R510W mutants.

This is the first report on negative correlation of DPP III activity with increased flexibility of the lower domain β -core.

Experimental

Protein expression and purification

Mutants of h.DPP III, G313W, G313A, R510W, R510K and R510Q were prepared by site-specific mutagenesis of wt h.DPP III His-tagged expression construct cloned in pET21b vector with QuikChange II XL Site-Directed Mutagenesis kit (Agilent technologies, USA). Proteins were expressed in *E. coli* strain BL21-CodonPlus(DE3)-RIL, and purified by Ni-NTA affinity chromatography, as described by Špoljarić *et al.* (2011).²³

Protein concentrations were determined using the protein-dye binding assay, with bovine serum albumin as a standard.²⁴

Enzyme activity assay and determination of kinetic parameters

The enzymatic activities of wt and mutant h.DPP IIIs were determined by a standard assay at 37 °C with Arg₂-2NA as a substrate, using the colorimetric method as previously described.²⁵ Kinetic parameters for hydrolysis of Arg₂-2NA were determined at 25 °C and at pH 8.6 for mutants R510W, R510K and R510Q, and at pH 7.4 for G313A, in the presence of 50 μM CoCl₂ by initial rate measurements, as previously described.²⁵ The kinetic parameters were calculated using Hanes–Woolf plot, with Arg₂-2NA concentrations from 2.5 to 40 μM for all mutants except G313A for which concentrations range from 20 to 240 μM was used. Kinetic parameters for mutant G313W were not determined due to low enzyme activity.

Molecular simulations

Model setup. In this work we used two h.DPP III conformations of different compactness, the compact, catalytic one (PDB code: 3T6B²⁶) and the less compact, so called ‘open’ conformation (PDB code: 3FVY). The chloride and magnesium ions present in these structures, as well as the crystalized water molecules, were removed, and the proper protonation of active site residues was set up using the tleap module (part of the AMBER14 suite of programs).²⁷ All Arg and Lys residues were positively charged and all Glu and Asp residues were negatively charged, as expected under the physiological conditions. Protonation states of histidine residues were determined

according to their ability to form hydrogen bonds with neighbouring amino acid residues. Mutated enzymes were prepared using model leap. For the purpose of selecting an amino acid rotamer at the mutated position we used the program Pymol (backbone dependent approach). However we have not selected the most populated rotamer, but the one that fits into the enzyme structure the best. For the G313W mutant two different Trp313 rotamers were considered, one pointing towards the inter-domain cleft and the other towards the lower domain. The first one (χ angles 182.1°, 180.0°) appeared to be more favourable during the preliminary simulations so it was selected for the further study. For the R510W mutant the most favourable rotamer, with the χ angles 164.9° and 257.4°, was considered. DPP III–RRNA complexes were constructed using utilities of the program Pymol (The PyMOL Molecular Graphics System, Version 1.7 Schrödinger, LLC.). We used the crystal structure of the DPP III mutant–tynorphin complex (PDB code: 3T6B) as a template, aligned RRNA to tynorphin and mutated Ala451 back to Glu in order to obtain the wild type enzyme. The complexes with the other variants were obtained by mutation of corresponding amino acid residues. The obtained complexes were energy-minimized and equilibrated during short MD simulations.

The enzyme variants, either ligand free or its complexes with the synthetic substrate Arg₂-2NA, were neutralized by adding Na⁺ ions and solvated in an octahedron box filled with TIP3P²⁸ water molecules ensuring an at least 11 Å thick water molecules buffer around the protein. The systems were parametrized by the AMBERTools14 modules antechamber and tleap, using GAFF²⁹ and ff14SB³⁰ force fields to parameterize the substrate and the protein, respectively. For the zinc cation, Zn²⁺, parameters were derived from our previous work¹² and modified according to the PDB survey.^{31,32}

Molecular dynamics simulations. The resulting system, consisting of ~95 000 (ligand free) and ~64 000 (complexes) atoms (~27 500 and ~17 500 molecules of water, respectively), were simulated using periodic boundary conditions. The electrostatic interactions were calculated using the particle-mesh Ewald method.³³ The PMEMD module running at GPU was used to conduct the molecular dynamics (MD) simulation. Before MD simulations, three rounds of geometry optimization, with different degree of the protein restraints, were performed by the module sander. In the first cycle 1500 optimization steps were performed, where the first 450 steps were of the steepest descent method, and the rest was the conjugate gradient. Both, the protein atoms and the metal ion, were constrained using a harmonic potential of 32 kcal (mol Å²)^{−1}, in order to equilibrate water molecules. In the second cycle 2500 steps of optimization was done. The metal ion and the protein backbone were constrained with 12 kcal (mol Å²)^{−1}. Finally, in the third cycle the same number of optimization steps was used as in the first cycle, and the protein backbone and the metal ion were constrained with 1 kcal (mol Å²)^{−1} and 12 kcal (mol Å²)^{−1}, respectively. The minimized system was heated from 0 to 300 K during 30 ps of MD simulation using a canonical ensemble (NVT), followed by equilibration stage of 30 and 100 ps (for the ligand free enzyme and complexes), during which the water



density was adjusted. The equilibrated system was then subjected to 100 ns of the productive MD simulations, 50 ns of conventional MD (cMD) followed by 50 ns of the dual boost accelerated MD (aMD) simulations. One boost was applied to torsional terms and the other to total potential. The latter facilitates diffusion of the explicit solvent molecules around the biomolecule and thus reduces hindrance that water induce on the large amplitude displacements of a solute. In this way more efficient conformational sampling is enabled.

Type and duration of the performed simulations is given in Table S1.[†]³⁴ The constant temperature (300 K) and pressure (1 atm) were ensured using Langevin dynamics with a collision frequency of 1 ps⁻¹, and Berendsen barostat, respectively.^{35,36} The time step during the periods of heating and cooling was 1 fs, and for the rest of the simulation 2 fs (to restrain the motion of hydrogens the SHAKE algorithm was used).

Data analysis. In order to analyse and to characterize influence of the mutations on the protein structure and flexibility, several geometric parameters were analysed such as the Zn²⁺ ion coordination, RMSD, RMSF and radius of gyration, R_{gyr} (RMSD and R_{gyr} were calculated for the backbone atoms and RMSF for C α atoms only). These analysis, in which the backbone atoms were used for calculations, as well as the analysis of the hydrogen bonds population, were performed using the program cpptraj, a part of the AMBER14 suite. For the purpose of selecting the representative structures for visualization we performed several types of clustering: based on the entire protein structure, based on the orientation of the mutated residue and based on the substrate binding mode. Figures were prepared using a structure from the most populated cluster obtained by one of the above listed methods. In the case of the wt, protein clustering based on the entire protein structure was used and in the case of mutants clustering was based on the orientation of the mutated amino acid residues. For the purpose of visualization of the ligand bound into the enzyme active site, clustering based on substrate RMSD (calculated for the all substrate atoms) was used to select the most representative binding mode.

Conflict of interest

There are no conflicts of interest to declare.

Acknowledgements

The work has been supported by Croatian Science Foundation under the project “7235 Flexibility, activity and structure correlations in the dipeptidyl peptidase III family” and by UKF grant agreement 21/15.

Notes and references

- 1 T. Chiba, Y.-H. Li, T. Yamane, O. Ogikubo, M. Fukuoka, R. Arai, S. Takahashi, T. Ohtsuka, I. Ohkubo and N. Matsui, *Peptides*, 2003, **24**, 773.
- 2 Y. Liu, J. T. Kern, J. R. Walker, J. A. Johnson, P. G. Schultz and H. Luesch, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 5205.
- 3 Š. Šimaga, D. Babić, M. Osmak, M. Šprem and M. Abramić, *Gynecol. Oncol.*, 2003, **91**, 194.
- 4 M. He, D. P. Mangiameli, S. Kachala, K. Hunter, J. Gillespie, X. Bian, H.-C. J. Shen and S. K. Libutti, *Clin. Cancer Res.*, 2010, **16**, 249.
- 5 V. A. Meliopoulou, L. E. Andersen, P. Brooks, X. Yan, A. Bakre, J. K. Coleman, S. M. Tompkins and R. A. Tripp, *PLoS One*, 2012, **7**, e37169.
- 6 B. E. Hast, D. Goldfarb, K. M. Mulvaney, M. A. Hast, P. F. Siesser, F. Yan, D. N. Hayes and M. B. Major, *Cancer Res.*, 2013, **73**, 2199.
- 7 B. E. Hast, E. W. Cloer, D. Goldfarb, H. Li, P. F. Siesser, F. Yan, V. Walter, N. Zheng, D. N. Hayes and M. B. Major, *Cancer Res.*, 2014, **74**, 808.
- 8 K. Fukasawa, K. M. Fukasawa, M. Kanai, S. Fujii, J. Hirose and M. Harada, *Biochem. J.*, 1998, **329**, 275.
- 9 M. Abramić, J. Špoljarić and Š. Šimaga, *Period. Biol.*, 2004, **2**, 161.
- 10 J. M. Chen and A. J. Barrett, Dipeptidyl-Peptidase III, in *Handbook of Proteolytic Enzymes*, ed. A. J. Barrett, N. D. Rawlings and J. F. Woessner, Elsevier, Academic Press, London, 2004, pp. 809–812.
- 11 P. Kumar, V. Reithofer, M. Reisinger, S. Wallner, T. Pavkov-Keller, P. Macheroux and K. Gruber, *Sci. Rep.*, 2016, **6**, 23787.
- 12 A. Tomić, M. González and S. Tomić, *J. Chem. Inf. Model.*, 2012, **52**, 1583.
- 13 A. Tomić, M. Berynsky, R. C. Wade and S. Tomić, *Mol. Biosyst.*, 2015, **11**, 3068.
- 14 A. Tomić and S. Tomić, *Dalton Trans.*, 2014, **43**, 15503.
- 15 J. Matić, F. Šupljika, N. Tir, P. Piotrowski, C. Schmuck, M. Abramić, I. Piantanida and S. Tomić, *RSC Adv.*, 2016, **6**, 83044.
- 16 A. Tomić, B. Kovačević and S. Tomić, *Phys. Chem. Chem. Phys.*, 2016, **18**, 83044.
- 17 E. Cerami, J. Gao, U. Dogrusoz, B. E. Gross, S. O. Sumer, B. A. Aksoy, A. Jacobsen, C. J. Byrne, M. L. Heuer, E. Larsson, Y. Antipin, B. Reva, A. P. Goldberg, C. Sander and N. Schultz, *Cancer Discovery*, 2012, **2**, 401.
- 18 J. Gao, B. A. Aksoy, U. Dogrusoz, G. Dresdner, B. Gross, S. O. Sumer, Y. Sun, A. Jacobsen, R. Sinha, E. Larsson, E. Cerami, C. Sander and N. Schultz, *Sci. Signaling*, 2013, **6**, pl1.
- 19 B. Salopek-Sondi, B. Vukelić, J. Špoljarić, Š. Šimaga, D. Vujaklija, J. Makarević, N. Jajčanin and M. Abramić, *Biol. Chem.*, 2008, **389**, 163.
- 20 J. Špoljarić, B. Salopek-Sondi, J. Makarević, B. Vukelić, D. Agić, Š. Šimaga, N. Jajčanin-Jozić and M. Abramić, *Bioorg. Chem.*, 2009, **37**, 70.
- 21 A. Tomić, M. Abramić, J. Špoljarić, D. Agić, D. M. Smith and S. Tomić, *J. Mol. Recognit.*, 2011, **24**, 804.
- 22 M. Abramić, Z. Karačić, M. Šemanjski, B. Vukelić and N. Jajčanin-Jozić, *Biol. Chem.*, 2015, **396**, 359.
- 23 J. Špoljarić, A. Tomić, B. Vukelić, B. Salopek-Sondi, D. Agić, S. Tomić and M. Abramić, *Croat. Chem. Acta*, 2011, **84**, 259.
- 24 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248.
- 25 M. Abramić, Š. Šimaga, M. Osmak, L. Čičin-Šain, B. Vukelić, K. Vlahoviček and L. J. Dolovčak, *Int. J. Biochem. Cell Biol.*, 2004, **36**, 434.



- 26 G. A. Bezerra, E. Dobrovetsky, R. Viertlmayr, A. Dong, A. Binter, M. Abramic, P. Macheroux, S. Dhe-Paganon and K. Gruber, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 6525.
- 27 D. A. Case, V. Babin, J. T. Berryman, R. M. Betz, Q. Cai, D. S. Cerutti, T. E. Cheatham III, T. A. Darden, R. E. Duke, H. Gohlke, A. W. Goetz, S. Gusarov, N. Homeyer, P. Janowski, J. Kaus, I. Kolossváry, A. Kovalenko, T. S. Lee, S. LeGrand, T. Luchko, R. Luo, B. Madej, K. M. Merz, F. Paesani, D. R. Roe, A. Roitberg, C. Sagui, R. Salomon-Ferrer, G. Seabra, C. L. Simmerling, W. Smith, J. Swails, R. C. Walker, J. Wang, R. M. Wolf, X. Wu and P. A. Kollman, *AMBER 14*, University of California, San Francisco, 2014.
- 28 W. L. Jorgensen, J. Chandrasekhar, J. D. Madura, R. W. Impey and M. L. Klein, *J. Chem. Phys.*, 1983, **79**, 926.
- 29 J. Wang, R. M. Wolf, J. W. Caldwell, P. A. Kollman and D. A. Case, *J. Comput. Chem.*, 2004, **25**, 1157.
- 30 J. A. Maier, C. Martinez, K. Kasavajhala, L. Wickstrom, K. E. Hauser and C. Simmerling, *J. Chem. Theory Comput.*, 2015, **11**, 3696.
- 31 I. Dokmanić, M. Šikić and S. Tomić, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2008, **64**, 257.
- 32 A. Tus, A. Rakipović, G. Peretin, S. Tomić and M. Šikić, *Nucleic Acids Res.*, 2012, **40**, W352.
- 33 T. Darden, D. York and L. Pedersen, *J. Chem. Phys.*, 1993, **98**, 10089.
- 34 D. Hamelberg, J. Mongan and J. A. McCammon, *J. Chem. Phys.*, 2004, **120**, 11919.
- 35 G. Grest and K. Kremer, *Phys. Rev. A: At., Mol., Opt. Phys.*, 1986, **33**, 3628.
- 36 H. J. C. Berendsen, J. P. M. Postma, W. F. van Gunsteren, A. DiNola and J. R. Haak, *J. Chem. Phys.*, 1984, **81**, 3684.

