Short Communication

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Aspartate 496 from the subsite S2 drives specificity of human dipeptidyl peptidase III

Abstract: Human dipeptidyl peptidase III (hDPP III) is a member of the M49 metallopeptidase family, which is involved in intracellular protein catabolism and oxidative stress response. To investigate the structural basis of hDPP III preference for diarginyl arylamide, using site-directed mutagenesis, we altered its S2 subsite to mimic the counterpart in yeast enzyme. Kinetic studies revealed that the single mutant D496G lost selectivity due to the increase of the K_m value. The D496G, but not S504G, showed significantly decreased binding of peptides with N-terminal arginine, and of tynorphin. The results obtained identify Asp496 as an important determinant of human DPP III substrate specificity.

Keywords: metallopeptidase; site-directed mutagenesis; substrate specificity; zinc enzyme.

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Human dipeptidyl peptidase III (DPP III; EC 3.4.14.4) is a constituent of the human central proteome (Burkard et al., 2011), and a member of the M49 family of metallopeptidases (DPP III family), which catalyze the hydrolytic cleavage of dipeptides from the amino termini of their oligopeptide substrates (Abramić et al., 2004a; Chen and Barrett, 2004; Rawlings et al., 2012). These zinc-dependent enzymes are considered to be implicated in intracellular protein catabolism, oxidative stress response, and, in mammals, opioid peptides degradation (Abramić et al., 1988; Fukasawa et al., 1998; Baršun et al., 2007; Liu et al., 2007). Human DPP III is also related to pathological processes of cataractogenesis and tumor growth (Zhang et al., 2001; Šimaga et al., 2003). It is a monomeric protein with 737 amino acids in the polypeptide chain. Crystal structure of ligand-free human and yeast DPP III has been solved, revealing elongated protein molecule with two domains separated by a wide cleft, and very similar overall fold (Baral et al., 2008; Bezerra et al., 2012). We have recognized five evolutionary conserved regions in the primary structure of M49 peptidases, embedded in a stretch of about 300 amino acids (Abramić et al., 2004a), which in human DPP III starts with Gly313 (Figure 1).

The first 3-D structure of human DPP III inactive mutant E451A in complex with pentapeptide tynorphin revealed the formation of closed enzyme's active site during the substrate binding, due to the domain movement (Bezerra et al., 2012). The same crystal structure has exposed the amino acid residues forming five deep subsites (S2 to S3′), consisting of seven (S2′) to 13 (S2) residues.

Most recently, we have found that yeast and human enzyme differ significantly in specificity towards dipeptidyl arylamide substrates of X-Arg-arylamide type, and in catalytic efficiency (Jajčanin-Jozić and Abramić, 2013). The yeast counterpart was found to be less active catalyst with broader specificity.

To investigate the underlying structural basis of human DPP III preference for Arg-Arg-arylamide, a hallmark substrate of this type of mammalian tissue dipeptidyl peptidase (Ellis and Nuenke, 1967), we compared the amino acid sequence of human and yeast enzyme (non-selective towards dipeptidyl naphthylamides) by multiple sequence alignment (MSA). Interestingly, we found identity in structurally equivalent residues forming the S1, S1′ and S2′ subsites, and a significant difference in two, out of 13, constituents of the S2 subsite: in human DPP III Asp496 and Ser504 correspond to Gly505 and Gly 513 in the yeast enzyme (Jajčanin-Jozić and Abramić, 2013). There was a difference in S3′ subsite constituents as well, but rather conservative (Ile422, Arg675, and Phe677 in yeast are replaced by structurally equivalent Val412, Lys670, and Ile672 in human DPP III). As the dipeptidyl arylamide

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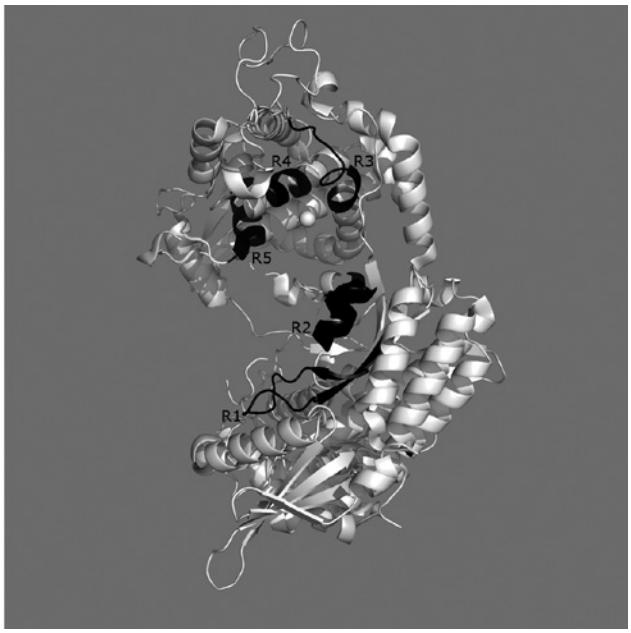

Figure 1 Conserved regions in human dipeptidyl peptidase III (DPP III).
Five evolutionary conserved regions of the M49 family are presented as black areas (R1 to R5) in the 3-D structure of ligand-free human DPP III (PDB: 3FVY). Structure was generated using PyMOL (www.pymol.org). The zinc ion is represented as a white sphere.

substrates occupy the subsites S2, S1 and S1′, in this study, we focused to the structural variation observed in the S2 subsite. We assumed that the observed difference in substrate specificity of human and yeast DPP III originates in a single mutation of Asp496 to Gly, or Ser504 to Gly, or in double mutation of both Asp496 and Ser504 to glycine. To prove our hypothesis, we prepared single mutant proteins of human DPP III, D496G, and S504G, and a double mutant D496G/S504G by site-directed mutagenesis, and examined their properties.

The structural characteristics of purified recombinant mutant proteins were examined through the circular dichroism (CD) analysis and compared to the wild-type DPP III. Almost identical CD spectra were obtained (Figure 2), suggesting that the substitution of Asp496 or Ser504, or both Asp496 and Ser504, with a glycine residue did not cause a change in protein secondary structure.

Hydrolytic activity of the purified mutated enzymes was examined towards three dipeptidyl 2-naphthylamide substrates differing in the amino acid residue in P2 position (Arg-Arg-2NA, Ala-Arg-2NA, and Phe-Arg-2NA), and compared to the wild-type. All mutant proteins were enzymatically active. The single mutant S504G exhibited very similar kinetic properties to the wild-type: pronounced preference for Arg-Arg-2NA, due to the significantly lower K_m value for that substrate (Table 1). Catalytic efficiency (k_{cat}/K_m) of S504G was somewhat (20%–40%) higher for Arg-Arg-2NA and Ala-Arg-2NA, as compared to the wild-type human DPP III (Table 1).

In contrast to the replacement of Ser504, the substitution of Asp496 with glycine residue dramatically influenced the specificity, but not the hydrolytic activity, of mutated enzyme. The K_m values for all three X-Arg-2NA were significantly changed in D496G: 11-fold increased K_m for Arg-Arg-2NA, and 6-fold and 4.8-fold lowered K_m values for Ala-Arg-2NA and Phe-Arg-2NA, respectively. The k_{cat} values for the hydrolysis of these substrates by D496G did not change (Table 1).

A double mutant's D496G/S504G kinetic properties for the hydrolysis of Ala-Arg- and Phe-Arg-2NA, and the K_m value for Arg-Arg-2NA, were almost identical to that of the single mutant D496G. The only difference between these two enzyme forms was that the double mutant D496G/S504G, but not D496G, displayed a significantly (2-fold) lower k_{cat} value, compared to the wild-type (Table 1). The double mutant was omitted from further investigation.

The results obtained with dipeptidyl 2-naphthylamide substrates pointed to the Asp at the position 496 as the residue critical for human DPP III substrate specificity. We further investigated whether the Asp496 is the basis for the greater preference of human DPP III for peptide substrates with a basic residue at P2. Interaction with peptides was assayed at physiological pH (7.4). Polypeptide aprotinin (58 amino acids) was chosen as its sequence starts with an Arg. The K_i values were determined using Arg-Arg-2NA (with the wild-type and S504G) or Phe-Arg-2NA (with the wild-type and D496G) as an alternative substrate. Therefore, at first, the K_m values were determined at pH 7.4 for the corresponding synthetic substrates and the wild-type and both single mutants.

As shown in Table 2, the affinities (K_i values) of the wild-type human DPP III and S504G for selected peptides were very similar: the highest affinity was determined for pentapeptide tynorphin (K_i about 4 nM), followed by the heptapeptide angiotensin III (wild-type DPP III K_i=21 nM). Notably, both these enzyme forms showed affinity in micromolar concentration range for polypeptide aprotinin, which was comparable to that shown for pentapeptide Leu-enkephalin (Table 2). However, the mutant with replaced Asp496, D496G, differed significantly. Its binding potency was 10-fold lower for angiotensin III (K_i value of 208 nM) and 12.6-fold decreased for aprotinin (K_i=148 μM), and almost 40-fold reduced for tynorphin. Interestingly, the K_i value for another pentapeptide, Leu-enkephalin, was almost unchanged, compared to the

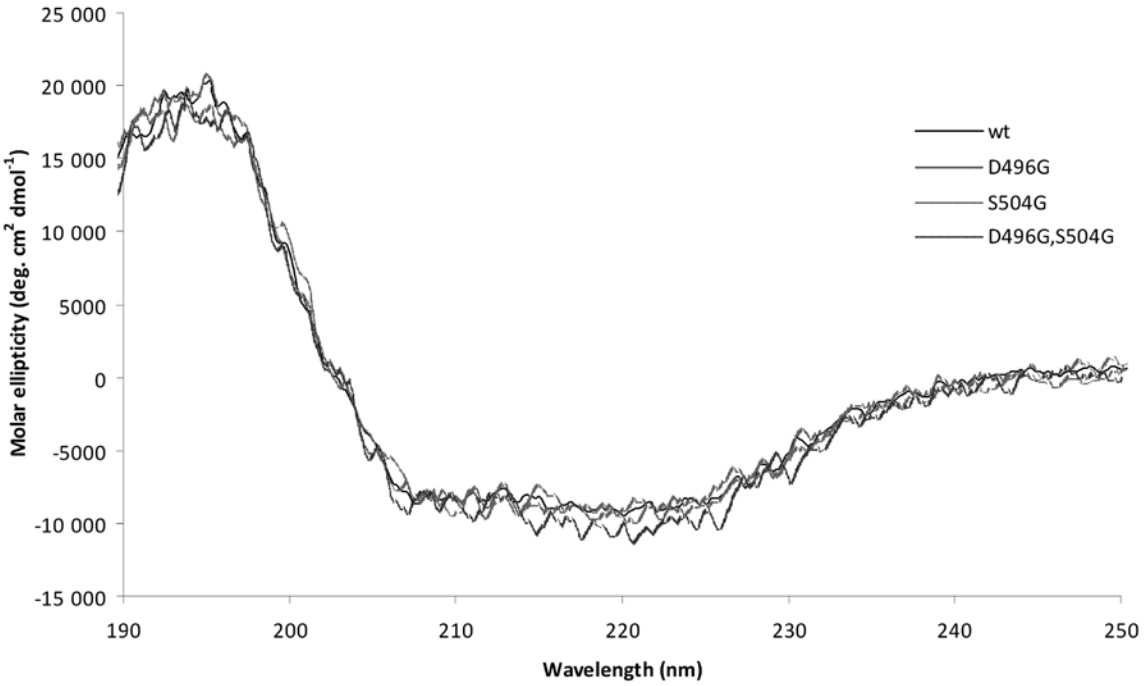

Figure 2 Circular dichroism (CD) spectra of wild-type human dipeptidyl peptidase III and mutants (D496G, S504G, and D496G/S504G). CD spectra were recorded on a Jasco J-815 spectropolarimeter (JASCO, Easton, MD, USA). Purified enzyme solutions (0.3–0.5 mg protein/ml) were measured in 20 mM sodium phosphate buffer, pH 7.4.

wild-type enzyme. A small decrease (1.9-fold) in D496G affinity was observed for tripeptide Arg-Phe-Ala (Table 2).

In search of submolecular determinants of distinct substrate specificity of human DPP III towards diarginyl arylamide, we started from our finding that yeast enzyme is non-selective in this respect (Jajčanin-Jozić and Abramić, 2013) and the fact that human and yeast DPP III proteins have a very similar fold. The three-dimensional structure of a human DPP III inactive variant (E451A) – tynorphin (Val-Val-Tyr-Pro-Phe) complex (Bezerra et al., 2012) enabled definition of enzyme subsites S2–S3′. The S2 subsite of human DPP III is formed by 13 residues, majority belonging to the evolutionary conserved regions (R) of M49 family (Figure 1): Glu316 (R1), Ile390 (R2), Asn391 (R2), Ile392 (R2), Asn394 (R2), Asp396 (R2), Arg399 (R2), His455 (R3), Trp495, Asp496, Ser504, Glu507 (R4), and Glu508 (R4).

Considering the constituents of the subsites that accommodate dipeptidyl arylamide substrate, that is, those building the S2, S1 and S1′ subsite, human and yeast DPP III differ only in two amino acid residues from the subsite S2 (Asp496 and Ser504 in human are structurally equivalent to Gly505 and Gly513 in yeast enzyme, respectively). By the application of site-directed mutagenesis, we have altered the S2 subsite of human DPP III to mimic its counterpart in yeast enzyme. Kinetic studies revealed that already single mutant D496G has lost selectivity for Arg-Arg-2NA, a well-known preferred substrate of human (and mammalian) DPP III (Table 1). This was due to the change of K_m value (observed for all three X-Arg-2NA examined), indicating that Asp496 is crucial for human DPP III specificity towards diarginyl arylamide substrate. According to the obtained results, Ser504 would not be important for selectivity of human DPP III with this type of substrate.

Interestingly, the double mutant with substituted both Asp496 and Ser504 by Gly showed 3-fold lower catalytic efficiency towards Arg-Arg-2NA than the single mutant D496G which indicates that Ser504 has a role in obtaining the full catalytic activity of human DPP III.

It is known that the yeast ortholog is much less effective catalyst for the hydrolysis of dipeptidyl arylamides than the human enzyme (Jajčanin-Jozić and Abramić, 2013). The double mutant's catalytic efficiency towards Arg-Arg-2NA is 24-fold lower compared to the human DPP III wild-type, but 16-fold higher than the yeast enzyme (Jajčanin-Jozić and Abramić, 2013). This indicates that, except the difference in the constituents of the S2 subsite, there are some other, yet unknown, determinants that govern yeast DPP III catalytic power.

Significant influence of one amino acid residue from the substrate binding site on the enzyme specificity is not rare phenomenon in the field of peptidases. The S2-P2

Table 1 Kinetic analysis of human dipeptidyl peptidase III (DPP III) enzyme variants.

	Wild-type	D496G	S504G	D496G/S504G
Arg-Arg-2NA				
K_m (μM)	3.7±1.1	41.3±15.3	3.9±1.0	42.2±9.1
k_{cat} (s^{-1})	21.5±2.1	30.5±8.3	33.5±4.5	10.4±1.8
k_{cat}/K_m (mM^{-1} s^{-1})	5810.8	738.5	8589.7	246.4
Ala-Arg-2NA				
K_m (μM)	100.9±3.0	16.5±5.0	106.8±11.7	22.7±12.2
k_{cat} (s^{-1})	14.3±1.1	10.1±1.3	25.0±0.5	10.7±1.8
k_{cat}/K_m (mM^{-1} s^{-1})	141.7	612.1	234.1	471.4
Phe-Arg-2NA				
K_m (μM)	20.9±5.2	4.4±0.2	21.7±1.4	6.2±0.4
k_{cat} (s^{-1})	8.0±3.7	4.2±1.1	8.7±3.0	4.2±0.2
k_{cat}/K_m (mM^{-1} s^{-1})	382.8	954.5	400.9	677.4

K_m and k_{cat} values are the averages of three determinations±SD. Experimental procedures: the construct containing full cDNA for human DPP III with affinity C-terminal six-histidine tag (His_6) was cloned into pET-21a vector (Novagen), between *NdeI* and *XhoI* restriction sites, according to procedure described by Špoljarić et al. (2011). Point mutations of the human DPP III gene: D496G and S504G were carried out with the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), using the pET-21a-hDPP III-His_6 construct as a template and the primers, custom synthesized by Sigma (St. Louis, MO, USA): D496G forward primer: 5′-GGA GCG GGG AGA CCT GG**G GT**A GCA AGT TCA GCA CC-3′; D496G reverse primer: 5′-GGT GCT GAA CTT GCT **ACC** CCA GGT CTC CCC GCT CC-3′; S504G forward primer: 5′-GTT CAG CAC CAT CGC C**GG T**AG CTA CGA AGA GTG CCG G-3′; S504G reverse primer: 5′-CCG GCA CTC TTC GTA GCT **ACC** GGC GAT GGT GCT GAA C-3′. The double mutant D496G/S504G was obtained by performing site-directed mutagenesis using D496G primers on the pET-21a-DPPIIIHisS504G vector as a template. Each mutant sequence was confirmed by DNA sequencing of the entire DPPIII gene (Macrogen sequencing service, Amsterdam, The Netherlands). The wild-type human DPP III and all mutant enzymes (D496G, S504G, D496G/S504G) were expressed in *Escherichia coli* as recombinant proteins containing an C-terminal His_6-tag and purified by metal affinity chromatography. Heterologous expression was performed according to Špoljarić et al. (2011) with minor modifications. Constructs containing His-tagged human DPP III, wild-type or mutants, were ïntroduced by electroporation into the *E. coli* expression strain BL21-CodonPlus(DE3)-RIL (Stratagene) grown in Luria-Bertani broth with ampicillin (Fermentas, St. Leon-Rot, Germany) (100 μg/ml), and induction of protein expression was carried overnight using 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside, Fermentas) at 18°C. Cell harvesting and lysis, and protein purification from the soluble fraction of the cell lysate, by metal affinity chromatography, was performed according to established procedure (Jajčanin-Jozić et al., 2010). Protein purity was confirmed by gel electrophoresis under denaturing conditions, SDS-PAGE, performed on a Mini-PROTEAN Tetra System (Bio-Rad, Hercules, CA, USA). Protein bands were stained with Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany). SDS-PAGE demonstrated that the wild-type and mutant proteins appeared as bands with similar molecular mass of about 82 kDa (not shown). Protein concentration was determined, using the bovine serum albumin (Serva) as a standard, following the Bradford method (Bradford, 1976). Fractions of high purity (according to the SDS-PAGE and the enzymatic activity) were pooled, desalted and stored as described (Špoljarić et al., 2011). The standard assay of enzyme activity of the human DPP III wild-type and protein variants was performed, to follow protein purification, with Arg-Arg-2-naphthylamide as substrate spectrophotometrically as described (Abramić et al., 2004b). Kinetic analysis was performed at 25°C and at pH 8.0, in the presence of 50 μM $CoCl_2$, with the dipeptidyl 2-naphthylamides (Arg-Arg-2NA, Ala–Arg-2NA and Phe-Arg-2NA, products of Bachem, Bubendorf, Switzerland) as substrates, by continuous measurement of the fluorescence of the free 2-naphthylamine, a product of enzymatic hydrolysis, as described (Abramić et al., 2004b). The kinetic parameters K_m and k_{cat} were determined from the initial reaction rates using Hanes plots (Jajčanin-Jozić et al., 2010) and a nonlinear regression program (GraphPad Prism version 5; GraphPad, La Jolla, CA, USA).

site interactions largely determine the primary specificity of papain-like proteinases papain family of cysteine peptidases). Interestingly, Brömme et al. have shown that a single mutation in the S2 subsite of these cysteine peptidases, which is defined by six amino acid residues and commonly displays preference for occupation by a bulky hydrophobic side chain, can change their specificity (Brömme et al., 1994). Thus, the replacement of Phe205 of cathepsin S with its cathepsin B equivalent Glu, results in the enzyme form that efficiently hydrolyzes substrates containing a basic P2 residue. Cathepsin B differs from cathepsin S exactly by the ability to hydrolyze such basic substrates. Additional evidence of the importance of one amino acid residue for the variation in substrate specificity of papain family peptidases has provided the study of Chan et al. (1999). These authors altered Gly234 at the S2 subsite of the *Leishmania major* cathepsin B-like protease, to Glu, which is found at the corresponding site in mammalian cathepsin B. This substitution shifted the *Leishmania* enzyme specificity towards that of cathepsin B.

The importance of one amino acid residue for substrate specificity has also been shown for the representatives of several metallopeptidase families: aminopeptidase B (M1 family) (Pham et al., 2011), thimet oligopeptidase, and neurolysin (M3 family) (Oliveira et al., 2003; Kadonosono et al., 2008). In the M4 family (thermolysin-like proteases,

Table 2 Interaction of human dipeptidyl peptidase III (DPP III) enzyme forms with peptides.

Peptide sequence (name)	K_i (μM)		
	Wild-type	D496G	S504G
Arg-Val-Tyr-Ile-His-Pro-Phe (angiotensin III)	0.021±0.003	0.208±0.031	0.011±0.002
Arg-Phe-Ala	17.02±0.12	31.9±2.12	n.d.
Arg-Pro-Asp-Phe-Cys-Leu... (aprotinin, 58 amino acids)	11.72±2.36	147.70±3.51	7.40±0.55
Val-Val-Tyr-Pro-Trp (tynorphin)	0.0043±0.0002	0.1600±0.0064	0.0041±0.0004
Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin)	4.32±0.40	2.23±0.14	2.89±0.26

All peptides were pure substances obtained from Bachem (Bubendorf, Switzerland), composed of L-amino acids; aprotinin was purchased from ThermoFisher Scientific (Waltham, MA, USA). The K_i values, which are presented as mean±standard deviation, were determined according to Chu and Orlowski (1985). The hydrolysis of 5 μM substrate (Arg-Arg-2NA with S504G and the wild-type DPP III, K_m values: 3 μM and 6 μM, respectively; Phe-Arg-2NA with D496G, K_m value: 9 μM) in 20 mM Tris-HCl buffer pH 7.4 in the presence and absence of peptide was followed at 25°C, and the K_i value was calculated as described by Baršun et al. (2007). K_m for each dipeptidyl-2NA was determined under the same pH and temperature conditions, and was calculated from the initial reaction rates using a nonlinear regression program (GraphPad Prism version 5; GraphPad, La Jolla, CA, USA). n.d., not determined.

TLPs), the S1′ pocket has been demonstrated as a major determinant of the substrate specificity. TLPs exhibit a preference for large hydrophobic P1′ residues (Leu or Phe). However, the differences in specificity and activity exist between the individual members (e.g., TLP from *Bacillus stearothermophilus* prefers Phe over Leu at the P1′ position of its dipeptide and tripeptide substrates in contrast to thermolysin, TLN), and the site-directed mutagenesis of one of the S1′ subsite residues (Phe133 substituted by Leu) converted the catalytic characteristics of this variant into that of TLN (S1′ subsite of TLN is composed of the side chains of Phe130, Leu133, Val139 and Leu202) (de Kreij et al., 2000).

Our present results on the kinetics of dipeptidyl arylamides hydrolysis implied the important role of Asp496 in the preferential binding of the basic amino acid residue from the N-terminus of the substrate. To test this, we examined the interaction of the wild-type human DPP III and both single mutants with four biologically active peptides and a tripeptide Arg-Phe-Ala. Replacement of Ser504 by Gly did not influence the binding of selected peptides. In contrast and in accord to our prediction, the mutant D496G showed significantly decreased binding of peptides with N-terminal arginine (angiotensin III, aprotinin, Arg-Phe-Ala). However, this enzyme form exhibited dramatically lower binding (about 40-fold increased K_i value) of pentapeptide tynorphin (Val-Val-Tyr-Pro-Trp). This finding, which points to the crucial role of Asp496 in human DPP III interaction with tynorphin, is unexpected, considering that known crystal structure (Bezerra et al., 2012) revealed that in the direct polar interactions with ligand are engaged Glu316, Tyr318, Ala388, Gly389, Ile390, Asn391, Asn394, His568, and Arg669, but not Asp496. The N-terminus of the bound pentapeptide is anchored by polar interactions to the side chains of Glu316 and Asn394, and to the main-chain carbonyl group of Asn391 (Bezerra et al., 2012, Figure 2C).

To explain the importance of Asp496 in binding of tynorphin, we inspected the crystal structure of human DPP III-tynorphin complex (PDB entry: 3T6B) and found that the oxygen of Asp496 carboxylate is 9 Å apart from the N-terminal nitrogen of Val 1 of tynorphin. However, the same carboxylate oxygen is within the hydrogen bond distance (3.15 Å) with the hydroxyl oxygen of Ser317 (Figure 3). This serine is a neighboring residue to Glu316, which forms crucial polar interaction with the N-terminus of tynorphin (Figure 3).

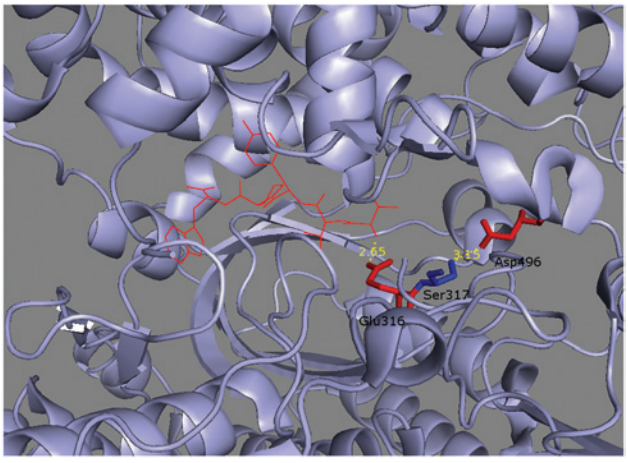

Figure 3 Asp496-Ser317 distance in human dipeptidyl peptidase III complex with tynorphin (PDB: 3T6B).
Structure was generated using PyMOL (www.pymol.org). Amino acid residues (Asp496, Ser317, and Glu316) are given by the ball-and-stick representation. The bound peptide (tynorphin, Val-Val-Tyr-Pro-Trp) is shown as (red) lines.

Therefore, the contribution of Asp496 to the binding of small amino acid residues (Val in tynorphin, Ala in Ala-Arg-2NA) could be exerted indirectly, through H-bonding interaction with Ser317. The absence of this interaction (like in D496G) could influence the positioning of Glu316, an evolutionary conserved residue of the S2 subsite, which is essential for the binding of the terminal amino-group of the substrate. Interestingly, in the ligand-free (open state) human DPP III (PDB entry: 3FVY), Asp496 (residue from the «upper» domain) and Ser317 (residue from the «lower» domain) are 25.31 Å apart (Supplementary Figure 1). Bezerra et al. (2012) have revealed that an exceptionally large domain motion (rotation of about 60°) occurs upon ligand binding to human DPP III whereby the N-terminus of the bound peptide tynorphin is completely buried by the enzyme. The domain movement brings Asp496 close to Ser317, and this is proven by crystal structure determination (Bezerra et al., 2012).

It is logical to anticipate that Asp496, as one of 13 residues forming the S2 subsite (being situated deeper in the S2 subsite) could interact directly through polar interaction with the N-terminal basic residue (e.g., arginine in angiotensin III). Having in mind the complexity of S2 subsite, bulkier hydrophobic P2 residues of the substrate (like Tyr in Leu-enkephalin), compared to Val from tynorphin, could form greater number of direct interactions with other amno acid residues – constituents of the S2 subsite. This could explain the lack of Asp496 influence on binding of Leu-enkephalin to human DPP III.

To examine our hypothesis that Asp496-Ser317 interaction significantly contributes to the high affinity of human DPP III for tynorphin, we have prepared the human DPP III mutant S317A by the site-directed mutagenesis, as is described in the legend to Table 1, by using the primers 5′-CAT CGG GTT CAT CGA GGC GTA CCG CGA CCC CTT TGG (forward) and 5′-CCA AAG GGG TCG CGG TAC GCC TCG ATG AAC CCG ATG (reverse), and affinity purified the recombinant protein. The kinetic parameters for hydrolysis of Arg-Arg-2NA, and the K_i values for peptide binding were determined at pH 7.4 and at 25°C. As expected, the substitution of Ser317 with Ala did not change the kinetic parameters (K_m=7.5±0.6 μM; k_{cat}=5.0±0.07 s^{-1}), nor the affinity for peptides with basic residue at P2 position (the K_i values of 17.4±0.6 nM and 20.3±4.4 μM were determined for angiotensin III and aprotinin, respectively). However, this mutant exerted identical high affinity (K_i=3.8±0.8 nM) interaction with tynorphin as the wild-type (Table 2). Therefore, we conclude that the significant contribution of Asp496 in tynorphin binding to human DPP III does not depend on the H-bonding interaction Asp496-Ser317. In the crystal structure of enzyme-peptide complex, this aspartic acid residue faces towards tynorphin, and its substitution by Gly can influence change to the size and polarity of this binding pocket with a concomitant change to the substrate binding. Therefore, further studies by computational approach and/or by the determination of the mutant D496G-tynorphin complex crystal structure are needed to obtain a valid answer to this intriguing question.

There are many examples of the importance of one particular aspartic acid residue for the catalytic activity and/or substrate specificity of an enzyme. Apart from Asp being a part of the catalytic triad of serine peptidases, among the proteolytic enzymes, the classical paradigm is Asp189, situated at the base of the S1 substrate binding pocket of trypsin, a residue critical for this enzyme's specificity for Arg and Lys in P1 position of its substrates (Evnin et al., 1990).

In the M49 family, there is no evolutionary conserved aspartic acid residue, and only one conserved Glu, at the position 316 in the sequence of human DPP III. Our results reveal Asp496, a constituent of S2 subsite, as important structural determinant of human DPP III substrate specificity and give the guidance for further investigation of the family M49.

The S2 subsite is of crucial importance for substrate binding and hydrolytic activity of M49 peptidases. Elucidation of the X-ray structure of human DPP III in complex with pentapeptide tynorphin identified that the N-terminus of the bound peptide is completely buried by the enzyme and anchored by polar interactions to the three conserved constituents of the S2 subsite – the side chains of Glu316 and Asn394 and to the main-chain carbonyl group of Asn391 (Bezerra et al., 2012). It is known that DPP III does not accept peptides with modified amino-end as substrates (Abramić et al., 1988).

The majority of S2 constituents are situated in the evolutionary conserved regions of the M49 family, and Asp496, Trp495, and Ser504 are not. Based on the MSA of 16 DPP III amino acid sequences (including four bacterial) selected to cover a broad phylogenetic range (Supplementary Figure 2), Asp496 is found conserved in the majority (7/12) of eukaryotic orthologs, and replaced by Ser (in roundworm *Caenorhabditis elegans*, protozoan parasite *Giardia intestinalis* and starlet sea anemone *Nematostella vectensis*) or Gly (in *Aspergillus niger* and *Saccharomyces cerevisiae*). MSA revealed that Trp495 is conserved in eight eukaryotic proteins, and substituted by Tyr in the remaining four eukaryotic sequences. Instead of Ser504 (conserved in 12 sequences, three bacterial among them), a Cys, Asn, or Gly are found in identical sequence position.

Due to the lack of data on prokaryotic DPP III 3-D structure, and the gaps introduced by the MSA (Supplementary

Figure 2), it is difficult to predict if structurally equivalent Asp (or Glu) exists in the S2 subsite of prokaryotic orthologs. Based on the MSA alone, *Bacteroides thetaiotaomicron* DPP III contains an aspartic acid residue (Asp465) close to the position of Asp496 in human DPP III (and the ortholog from *Stigmatella aurantiaca* does not), which might imply that *B. thetaiotaomicron* DPP III, similar to its human counterpart prefers Arg_2-2NA as a synthetic substrate. For that, we have reported experimental evidence (Vukelić et al., 2012).

The results obtained in our present study indicate that the variation of constituent of the S2 subsite, corresponding to the Asp496 in human DPP III, which is observed in some lower eukaryotes, could influence their substrate specificity. As mentioned before, similar variations are reported for several other (metallo)peptidase families.

Interestingly, three non-conserved constituents of the S2 subsite of human DPP III (Trp495, Asp496, and Ser504) are situated in the spacer region between the evolutionary conserved regions R3 and R4 of the family M49. As we reported earlier, the length of spacer region is much shorter in bacterial proteins, compared to the eukaryotic DPPs III (Abramić et al., 2004a). The spacer region of the human DPP III sequence comprises linear motif E^{480}TGE (Supplementary Figure 2). Hast et al. (2013) have shown that DPP III binds Keap 1 protein at cellular level via the ETGE motif, thus activating the Keap1-Nrf2 signaling pathway, which is very important in cellular defense to oxidative and electrophilic stress. No such motif is found in lower eukaryotic and prokaryotic DPP III sequences (Supplementary Figure 2), which means that DPPs III from different origins might vary in their physiological functions. The role of human DPP III in the Nrf2-Keap1 pathway is exerted through protein-protein interactions and it does not require this enzyme's activity. This supports the notion that the complete range of physiological functions of DPP III remains to be discovered.

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