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Contribution of amino acids in the active site of dipeptidyl peptidase 4 to the catalytic action of the enzyme

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Abstract

Dipeptidyl peptidase 4 (DP4)/CD26 regulates the biological function of various peptide hormones by releasing dipeptides from their N-terminus. The enzyme is a prominent target for the treatment of type-2 diabetes and various DP4 inhibitors have been developed in recent years, but their efficacy and side effects are still an issue. Many available crystal structures of the enzyme give a static picture about enzyme-ligand interactions, but the influence of amino acids in the active centre on binding and single catalysis steps can only be judged by mutagenesis studies. In order to elucidate their contribution to inhibitor binding and substrate catalysis, especially in discriminating the P_1 amino acid of substrates, the amino acids R125, N710, E205 and E206 were investigated by mutagenesis studies. Our studies demonstrated, that N710 is essential for the catalysis of dipeptide substrates. We found that R125 is not important for dipeptide binding but interacts in the P_1'position of the peptide backbone. In contrast to dipeptide substrates both amino acids play an essential role in the binding and arrangement of long natural substrates, particularly if lacking proline in the P_1 position. Thus, it can be assumed that the amino acids R125 and N710 are important in the DP4 catalysed substrate hydrolysis by interacting with the peptide backbone of substrates up- and downstream of the cleavage site. Furthermore, we confirmed the important role of the amino acids E205 and E206. However, NP Y, displaying proline in P_1 position, is still processed without the participation of E205 or E206.

Introduction

Dipeptidyl peptidase IV/CD26 (DP4, EC 3.4.12.5) is an extracellular serine protease with multiple physiological functions. The protein possesses a proteolytic activity which leads to the release of dipeptides from the N-terminus of polypeptides. The enzyme hydrolyses preferentially after proline in P_1 position but also accepts alanine or serine [1,2]. Consequently, DP4

has the potential to modulate cytokines, chemokines, neuropeptides, and growth factors containing these residues at the N-terminal penultimate position [3–7]. Furthermore, DP4 is implicated to be involved in several physiological processes by binding proteins such as adenosine deaminase, collagen, fibronectin, HIV coat protein gp120, plasminogen, HIV tat protein and the tyrosine phosphatase, CD45. However, these protein interactions do not affect the proteolytic activity of the enzyme [8–11].

DP4 is present on the surface of stimulated T cells, B cells and natural killer cells [12]. Additionally, it is found in a variety of tissues on epithelial, endothelial and acinar cells. In this context, the usage of the designation "CD26" refers to the discovery and its localisation as a cell surface antigen of lymphocytes. Within the hematopoietic system, the T cell activation marker, CD26, is an important factor in T cell-mediated immune response by modifying the activity of peptides involved in immune regulation [13].

The incretins, glucagon-like peptide 1 (GLP-1_{7-36}) and glucose-dependent insulinotropic peptide (GIP) are substrates of DP4 [14]. These gastrointestinal hormones stimulate the glucose-dependent insulin secretion [15,16]; inhibit glucagon release and gastric emptying and enhance growth and differentiation of ß-cells and insulin gene expression [17–19]. Inhibition of DP4 in wild type and diabetic mice leads to increased levels of unprocessed GLP-1 and GIP in the circulation, enhanced insulin secretion, and improved glucose tolerance. Selective inhibitors of DP4 improve plasma glucose levels in human type II diabetics [20]. Thus, the inhibition of DP4 is a promising concept to control blood glucose homeostasis in the treatment of type 2 diabetes patients [21,22]. Hence, many orally-administered DP4 inhibitors have been developed by various companies such as sitagliptin, vildagliptin, saxagliptin and linagliptin [23]. Since these inhibitors are suffering from side effects and efficacy issues, there is still an interest in discovering new anti-diabetic drugs [24]. To meet this objective, detailed information about key features of amino acids in the active centre of DP4 and their contribution to substrate and inhibitor binding will push this issue forward. Among the multitude of described DP4 inhibitors, there are also substrate-like dipeptide derivatives [25]. The natural occurring tripeptides diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) were also reported to have an inhibition effect on DP4 [26]. Rahfeld *et al.* [27] showed that these tripeptides are DP4 substrates and inhibit the enzyme competitively due to their slow turnover rate.

The catalytic mechanism of DP4 was extensively studied early by using small synthetic substrates. It was found, that the substrate hydrolysis is strict stereo specific. The scissile and P_2-P_1 bonds must be in *trans* configuration [28]. The P_2, P_1, and P_1' amino acid residues have to be in L-configuration. The rate limiting step for the DP4 catalysed hydrolysis of dipeptide derivates was found to be the deacylation reaction for proline substrates (proline in P_1 position), while it is the acylation reaction for alanine or serine substrates (alanine or serine in P_1 position) [29].

In 2003, the first crystal structure of DP4 in complex with the inhibitor valine-pyrrolidide was published by Rasmussen *et al.* [30]. Later on, the number of available DP4-crystal structures has steadily increased. Structures of DP4 co-crystallized with substrates, show important interaction sites within the active centre of the enzyme (Fig 1) [31,32].

The catalytic triad of DP4 consists of the residues S630, D708 and H740 and is found in a large cavity at the interface of the α/β- and the propeller domain. Typical for the catalysis mechanism of serine proteases is the formation of the so called oxanion hole which is formed by the backbone NH of Y631 and the side chain OH of Y547. Surrounding the catalytic triad are amino acids responsible for the formation of a recognition pocket which is favourable for proline residues due to its size and hydrophobic assembly. This S_1 specificity pocket is formed by the side chains of Y666, Y662, V711, V656, Y631 and W659. The environment is perfectly suited to accept proline and amino acids with small side chains. This is also indicated by the

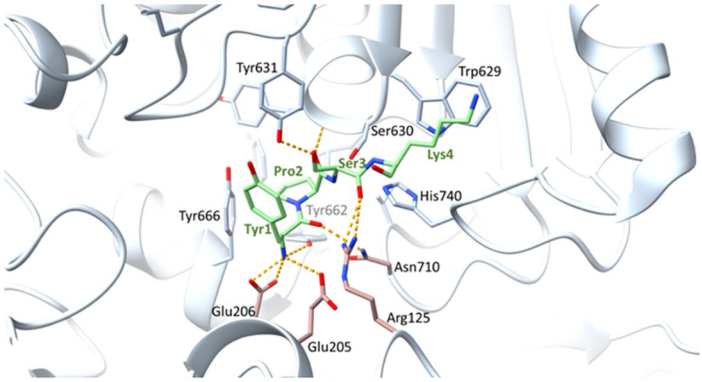

Fig 1. Interactions of the first 4 amino acids of NP Y bound to the active centre of DP4. DP4 amino acid side chains involved in substrate binding as well as secondary structure elements are presented in light grey. The amino acids investigated within the present study (Glu205, Glu206, Asn710 and Arg125) are highlighted in rose, the first residues of the substrate NP Y in green, H-bonds as yellow dotted lines. Picture was created by using the crystal structure PDB– 1r9n [32] and the molecular visualization system ChimeraX [33].

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preference of proline, alanine and to a lesser extend serine or glycine at the P_1 position [16]. Further recognition of substrates arises between their protonated N-terminus and two negatively charged site residues of E205 and E206. Replacing one of the two glutamic acids resulted almost in a loss of activity against dipeptide substrates [34]. The peptide backbone is stabilized by two amino acids namely N710 and R125 which form interactions to the peptide bond carbonyl of the substrates or inhibitors [35]. The S_1'site is described as flat and not well defined and does not mediate strong substrate-enzyme interactions. Aertgeerts *et al.* 2004 [32] showed by co-crystallizing the N-terminus of NP Y that the P_2'lysine is facing W629 and beyond that no further interactions are described. P_1'and P_2'face the solvent when bound in the tetrahedral complex. Additionally, with its freelance in these positions no favourable amino acids are described.

Despite the fact that DP4 belongs to the family of prolyl peptidases and prefers short peptide substrates showing proline in P_1, many of the supposed physiological DP4 substrates found in *in vitro* kinetic studies possess Ala or Ser at the penultimate position [11]. Furthermore, substrate and inhibitor binding not only depend on amino acid sequence, but also on peptide length [36]. In order to evaluate supporting mechanisms in the active centre of DP4, especially in regard to the hydrolysis of substrates lacking proline in P_1, we investigated the influence of the amino acids R125, E205, E206 and N710, which are in close orientation to the catalytic triad, on substrate hydrolysis and inhibitor binding. Therefore, the role of the depicted amino acids on the hydrolysis of substrates with varying length and different amino acids in the P_1 position was examined. This will provide a deeper insight into the mechanisms supporting the catalytic action of DP4, especially in regard to substrate lengths and the occupation of its P_1 position.

Results

In order to evaluate the role of distinct amino acids beyond the catalytic triad of DP4, seven enzyme variants were generated. The amino acids R125, N710, E206 and E205 in the catalytic site of DP4 were chosen in regard to specify their role in binding and hydrolysis of substrates and inhibitors. These variants were characterized by the determination of the kinetic parameters K_m, k_{cat}, k_{cat}/K_m and IC_{50} for dipeptidic and tripeptide substrates possessing different

amino acids in P_1. In order to analyse the role of these amino acids in the cleavage of longer peptides, we compared the turnover rates of the physiological DP4 substrates NP Y (proline in P_1), GIP (alanine in P_1) and PACAP38 (serine in P_1) by the enzyme and its variants. A focal point of this study was to investigate the role of the P_1 amino acid residue on the hydrolysis of natural high molecular weight substrates in comparison to synthetic low molecular weight substrates and inhibitors.

The side chains of R125 and N710 interact with carbonyl groups in the backbone of substrates and inhibitors [25,31,32,35]. Both amino acids are able to participate in a hydrogen bond network and provide flexibility in the orientation of their side chains. In order to investigate the influence of these side chains, both amino acids were mutated to alanine (R125A and N710A). Further, the variant R125K was used to examine the influence of the delocalisation of the positive charge of the side chain. N710 has been replaced by aspartic acid (N710D) to introduce a negative charge and furthermore to glutamine (N710Q) in order to extend the side chain by remaining its uncharged character.

The protonated substrate N-terminus is coordinated by two glutamic acids, E205 and E206. It has been shown in earlier studies that these glutamic acids are essential for DP4 to cleave Gly-Pro-p-nitroanilide [34]. In contrast to these studies we focussed on the characterization with inhibitors and substrates of varying length (derivatives of dipeptides, tripeptides and natural peptide substrates) and different amino acids in P_1 position. Both glutamic acids were replaced by alanine (E205A and E206A), consequently removing the negative charge of the side chain.

Characterization with derivatives of dipeptides

Dipeptides, coupled with a C-terminal fluorophore like 7-Amido-4-Methlycoumarin (AMC) are used as standard substrates to kinetically characterize DP4. The determination of the kinetic constants K_m, k_{cat} and k_{cat}/K_m for the hydrolysis of the dipeptide derivatives Ala-Pro-AMC and Ala-Ala-AMC by DP4 and its variants provides information about the amino acids interacting with the P_2- and P_1 position of substrates.

As shown in Table 1, the hydrolysis of Ala-Pro-AMC is slightly influenced by the mutagenesis of R125. However, the turnover of Ala-Ala-AMC is stronger impeded, especially the Km value, suggesting that R125 is rather involved in binding of substrates lacking proline in P_1 than in the hydrolysis step (Table 1, Fig 2). In contrast to R125, N710, E205 and E206 play an essential role in the cleavage of these compounds, very low or no more turnover rates could be observed after mutagenesis of this amino acids (Fig 2).

The specificity of DP4 for dipeptidic Xaa-Pro-substrates is about 10-fold higher than for substrates displaying alanine in P_1 position [37]. Therefore, two substrates, providing proline and alanine in P_1 position were chosen to evaluate whether the mutated amino acids are involved in the determination of this specificity. While Ala-Pro-AMC was cleaved by the three variants of N710 (N710A, N710D, N710Q), it was not possible to measure kinetic data for the release of AMC from Ala-Ala-AMC by N710Q. In comparison to the wild type enzyme, the K_m-values for the cleavage of Ala-Pro-AMC by the variants N710A and N710Q are 100-fold decreased, while the k_{cat}-values dropped about factor 30. Interestingly, N710A and N710D mutations have a minor impact on the Km-values for the cleavage of Ala-Ala-AMC, but the changes in k_{cat} are dramatically.

Since the glutamic acids E205 and E206 coordinate the protonated N-terminus in the P_2 position, effects in binding dipeptide derivatives, tripeptides and peptide hormones will be expected. The mutant E206A is nearly catalytic inactive against dipeptide derivative substrates. We were able to measure the turnover of Ala-Pro-AMC by E206A, albeit a cleavage of Ala-

Table 1. Hydrolysis of Ala-Pro-AMC and Ala-Ala-AMC by DP4 variants compared with wild type DP4. Experimental conditions: 100 mM HEPES-buffer (pH 7.6), T = 30 ˚C.

DP4- variant	Substrate	K_m [M]	k_{cat} [s^{-1}]	k_{cat}/K_m [$\mu M^{-1}s^{-1}$]
wild type	Ala-Pro-AMC	$(1.9 \pm 0.14) \cdot 10^{-5}$	32 ± 0.9	1.7 ± 0.13
	Ala-Ala-AMC	$(5.5 \pm 0.34) \cdot 10^{-4}$	5.7 ± 0.18	0.01 ± 0.0003
R125A	Ala-Pro-AMC	$(3.7 \pm 0.18) \cdot 10^{-5}$	48 ± 1.0	1.3 ± 0.07
	Ala-Ala-AMC	$(7.1 \pm 0.47) \cdot 10^{-4}$	2.3 ± 0.08	0.003 ± 0.0002
R125K	Ala-Pro-AMC	$(3.8 \pm 0.11) \cdot 10^{-5}$	37 ± 0.5	0.97 ± 0.03
	Ala-Ala-AMC	$(1.5 \pm 0.08) \cdot 10^{-3}$	2.6 ± 0.09	0.002 ± 0.0001
N710A	Ala-Pro-AMC	$(1.7 \pm 0.17) \cdot 10^{-3}$	0.74 ± 0.03	$(4.4 \pm 0.46) \cdot 10^{-4}$
	Ala-Ala-AMC	$(4.1 \pm 0.08) \cdot 10^{-4}$	$(9.4 \pm 0.06) \cdot 10^{-3}$	$(2.3 \pm 0.05) \cdot 10^{-5}$
N710Q	Ala-Pro-AMC	$(1.2 \pm 0.14) \cdot 10^{-3}$	0.23 ± 0.01	$(1.9 \pm 0.21) \cdot 10^{-4}$
	Ala-Ala-AMC	No hydrolysis at concentrations < 10 mM		
N710D	Ala-Pro-AMC	$(1.2 \pm 0.09) \cdot 10^{-3}$	19 ± 0.6	$(1.6 \pm 0.14) \cdot 10^{-2}$
	Ala-Ala-AMC	$(1.8 \pm 0.29) \cdot 10^{-3}$	0.05 ± 0.003	$(2.7 \pm 0.49) \cdot 10^{-5}$
E205A	Ala-Pro-AMC	$(2.4 \pm 0.09) \cdot 10^{-4}$	17 ± 0.2	$(7.1 \pm 0.27) \cdot 10^{-2}$
	Ala -Ala-AMC	$(4.7 \pm 0.84) \cdot 10^{-3}$	0.15 ± 0.015	$(3.2 \pm 0.64) \cdot 10^{-5}$
E206A	Ala-Pro-AMC	$(3.4 \pm 0.41) \cdot 10^{-3}$	0.8 ± 0.05	$(2.3 \pm 0.32) \cdot 10^{-4}$
	Ala -Ala-AMC	No hydrolysis at concentrations < 10 mM		

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Ala-AMC was not detectable. The k_{cat} value for E205A was affected as well as the K_m values, which increase 10-fold in comparison to the wild type enzyme.

Characterization with various inhibitors

In order to investigate the influence of the depicted amino acids of DP4 in the binding of several inhibitors, the IC_{50} values for these compounds were determined (Table 2). We used the dipeptide mimicking molecule Ile-thiazolidide [38] (S1 Fig) in comparison to several tripeptides. Tripeptides with a penultimate proline residue like diprotin A and B has been described to be substrates for DP4, but they show an apparent inhibition in a competitive substrate assay [27]. Thoma *et al.* published a crystal structure of DP4 in complex with diprotin A, showing interactions between R125 and the C-terminal carboxyl group of the tripeptide [31]. It was speculated that this interaction is only present during binding and hydrolysis of tripeptidic substrates and may stabilize the tetrahedral intermediate by the protection of the leaving group

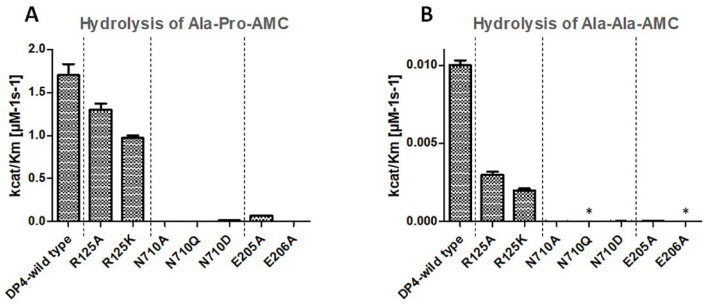

Fig 2. Relation between substrate P_1 amino acid and the influence of mutated residues in the active centre of DP4 on substrate cleavage. Experimental conditions: 100 mM HEPES buffer, pH 7.6, T = 30˚C, substrate concentrations varied from 4 x K_m to ¼ K_m, enzyme concentrations $5x10^{-9}$ M.

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Table 2. IC50 values of the dipeptide mimicking molecule Ile-thiazolidide and several tripeptides, measured with wild type DP4 and the generated DP4 variants. Experimental conditions: 100 mM HEPES-buffer (pH 7.6); T = 30 ˚C; substrate concentration (Gly-Pro-AMC) in the range of Km.

DP4-variant	inhibitor	IC_{50} [M]
wild type	Ile-thiazolidide	$(1.6 \pm 0.08) \cdot 10^{-7}$
	Diprotin B	$(3.7 \pm 0.13) \cdot 10^{-5}$
	Diprotin A	$(2.2 \pm 0.13) \cdot 10^{-6}$
	t-Bu-Gly-Pro-Ile	$(3.6 \pm 0.16) \cdot 10^{-6}$
	t-Bu-Gly-Pro-Ile-NH_2	$(6.7 \pm 0.14) \cdot 10^{-6}$
R125A	Ile-thiazolidide	$(3.1 \pm 0.41) \cdot 10^{-7}$
	Diprotin B	$(2.0 \pm 0.28) \cdot 10^{-4}$
	Diprotin A	$(4.0 \pm 0.53) \cdot 10^{-4}$
	t-Bu-Gly-Pro-Ile	$(4.0 \pm 2.00) \cdot 10^{-4}$
	t-Bu-Gly-Pro-Ile-NH_2	$(8.0 \pm 1.10) \cdot 10^{-5}$
R125K	Ile-thiazolidide	$(3.0 \pm 0.16) \cdot 10^{-7}$
	Diprotin B	$(1.0 \pm 0.09) \cdot 10^{-4}$
	Diprotin A	$(6.5 \pm 0.32) \cdot 10^{-5}$
	t-Bu-Gly-Pro-Ile	$(2.0 \pm 0.21) \cdot 10^{-4}$
	t-Bu-Gly-Pro-Ile-NH_2	$(5.3 \pm 0.29) \cdot 10^{-5}$
N710A	Ile-thiazolidide	$(4.0 \pm 1.00) \cdot 10^{-4}$
	Diprotin B	> 10 mM
	Diprotin A	> 10 mM
	t-Bu-Gly-Pro-Ile	> 10 mM
	t-Bu-Gly-Pro-Ile-NH_2	$(1.4 \pm 0.53) \cdot 10^{-4}$
N710Q	Ile-thiazolidide	$(2.0 \pm 0.50) \cdot 10^{-3}$
	Diprotin B	No inhibition
	Diprotin A	$(5.1 \pm 2.70) \cdot 10^{-3}$
	t-Bu-Gly-Pro-Ile	> 10 mM
	t-Bu-Gly-Pro-Ile-NH_2	> 10 mM
N710D	Ile-thiazolidide	$(3.0 \pm 0.15) \cdot 10^{-5}$
	Diprotin B	$(2.8 \pm 3.10) \cdot 10^{-3}$
	Diprotin A	$(1.1 \pm 0.40) \cdot 10^{-3}$
	t-Bu-Gly-Pro-Ile	$(6.0 \pm 0.46) \cdot 10^{-4}$
	t-Bu-Gly-Pro-Ile-NH_2	$(4.0 \pm 0.58) \cdot 10^{-4}$
E205A	Ile-thiazolidide	$(2.2 \pm 0.06) \cdot 10^{-5}$
	Diprotin B	$(4.1 \pm 2.10) \cdot 10^{-3}$
	Diprotin A	$(1.4 \pm 0.10) \cdot 10^{-3}$
	t-Bu-Gly-Pro-Ile	$(1.7 \pm 0.10) \cdot 10^{-3}$
	t-Bu-Gly-Pro-Ile-NH_2	$(1.3 \pm 0.05) \cdot 10^{-3}$
E206A	Ile-thiazolidide	> 10 mM
	Diprotin B	> 10 mM
	Diprotin A	> 10 mM
	t-Bu-Gly-Pro-Ile	> 10 mM
	t-Bu-Gly-Pro-Ile-NH_2	> 10 mM

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[31]. T-Butyl-Gly-Pro-Ile, with a free carboxyl terminus, was used in comparison to t-Butyl-Gly-Pro-Ile-NH_2, to investigate the role of R125 in the binding of tripeptides while interacting with the C-terminal carboxyl group.

The IC_{50} values of the dipeptide mimicking inhibitor Ile-thiazolidide were comparable between R125A, R125K and wild type DP4 (Table 2). These data confirmed our findings for

dipeptide derivative substrates, indicating that R125 has no essential influence on the binding of the amino acid residues in P_1- and P_2 position. However, we observed an effect for the variants R125A and R125K in the IC_{50} values of tripeptides, suggesting that the interaction between R125 and the C-terminal carboxyl group in P_1'position is important for the binding of these compounds. As already assumed, the influence of R125 mutation is more pronounced on binding t-Bu-Gly-Pro-Ile in contrast to t-Bu-Gly-Pro-Ile-NH_2.

In contrast to R125, the kinetic results of the variants from N710 revealed, that this amino acid is essential for binding both, tripeptides and the dipeptide analogue Ile-thiazolidide (Table 2). There was no inhibition of the DP4 variant E206A, while we were able to measure these data for E205A. This further demonstrates that E206 is apparently more important for the coordination of the N-terminus than E205.

Characterization with diprotin B as substrate

As described above, tripeptides with proline in P_1 position are substrates of DP4 with low turnover rate, maybe due to the stabilisation of the tetrahedral intermediate by interaction of R125 with the C-terminal carboxyl group.

We applied a LC-MS/MS assay to quantify the hydrolysis products of tripeptides in a time-dependent manner and directly assess the kinetic constants without using chromophore or fluorophore compounds. To evaluate the role of R125 in tripeptide binding and hydrolysis, we measured the kinetic constants K_m and k_{cat} for the cleavage of diprotin B by the variant R125A in comparison to the wild type enzyme (Table 3). As shown in Table 3, the k_{cat} value of the variant is comparable with the wild type enzyme whereas the K_m value increased, suggesting that an interaction between R125 and the C-terminal carboxyl group of tripeptides does not decelerate the rate-limiting step.

Characterization with peptide hormones

Besides characterizing the depicted amino acids with short substrates and inhibitors, their role in binding and processing of substrates with prolonged chain length was examined. The physiological substrates NP Y (proline in P_1 position), GIP (alanine in P_1 position) and PACAP38 (serine in P_1 position) were chosen to investigate the role of different amino acids in P_1 position. First, MALDI-TOF mass spectrometry analysis was used to examine the relative hydrolysis rates of these substrates by the DP4 variants (Table 4).

The kinetic data presented in Table 4 indicate that the depicted amino acids in the active centre of DP4 have a different relevance during the processing of long substrates. Depending on the amino acid in P_1 position, NP Y was still processed by the different DP4 variants. A cleavage of GIP was not observed by the variants E205A, E206A, N710A, N710Q and N710D. This demonstrates that N710, E205 and E206 are essential for the truncation of substrates with alanine in P_1 position. The hydrolysis of GIP catalysed by the DP4 variants R125A and R125K was detectable, but at a lower rate in comparison to the cleavage of NP Y (Fig 3). Interestingly, none of the DP4 variants was able to process PACAP38 with serine in P_1 position.

Table 3. Kinetic data of the cleavage of diprotin B by DP4 and the DP4-variant R125A. Experimental conditions: T = 30 ˚C; 100 mM Tris-HCl, pH 7.6; substrate concentration 25 μM.

DP4-variant	K_m [M]	k_{cat} [s^{-1}]	k_{cat}/K_m [$\mu M^{-1}s^{-1}$]
wild type	$(7.2 \pm 0.58) \cdot 10^{-6}$	7.0 ± 0.13	0,97 ± 0.08
R125A	$(3.7 \pm 0.26) \cdot 10^{-4}$	7.7 ± 0.13	$(2.1 \pm 0.15) \cdot 10^{-2}$

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Table 4. Hydrolysis of the peptide hormones NP Y, GIP and PACAP38 by DP4-variants in comparison to the DP4 wild type enzyme. +++ hydrolysis is comparable with the data obtained from the wild type enzyme. - hydrolysis below 0,01% of the wild type enzyme. Experimental conditions: T = 37 ˚C; 100 mM Tris-HCl, pH 7.6; substrate concentration 25 μM.

DP4-variant	Hydrolysis of NP Y (H_2N-Y-P-S. . .)	Hydrolysis of GIP (H_2N-Y-A-E. . .)	Hydrolysis of PACAP38 (H_2N-H-S-Q. . .)
Wild type	+++	+++	+++
R125A	++	+	-
R125K	+++	++	-
N710A	+	-	-
N710Q	+	-	-
N710D	++	-	-
E206A	+	-	-
E205A	++	-	-

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To investigate whether the observed effects on the substrate hydrolysis are related to differences in substrate binding or catalysis, we used the LC-MS/MS method to determine the K_m and k_{cat} values (Table 5).

As shown in Table 5, the k_{cat} values measured for NP Y were not decreased in comparison to the wild type enzyme. In contrast to NP Y, binding as well as the rate limiting step for processing GIP was affected by the exchange of R125.

Further, a variation of the amino acids N710, E205 and E206 affected the K_m- as well as the k_{cat} values for the hydrolysis of the physiological peptide substrates NP Y and GIP (Table 5). This agrees with our findings for the dipeptide derivative substrates, indicating that these amino acids are essential for substrate binding and hydrolysis.

Discussion

Several interactions between the active site of DP4 and its substrates/inhibitors were predicted on the basis of crystal structures of the enzyme. A crystal structure gives a static picture where the dynamics of enzyme-ligand interactions and the influence of amino acids in the active centre on catalysis and binding can hardly be judged. We performed mutagenesis studies and kinetic analysis to examine the role of amino acids around the catalytic triad in substrate catalysis and inhibitor binding.

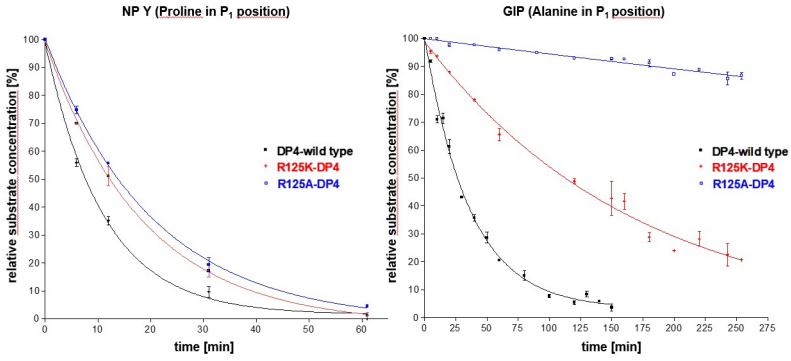

Fig 3. Hydrolysis of NP Y and GIP by DP4 and the DP4-variants R125A and R125K determined by MALDI-TOF mass spectrometry. Experimental conditions: 100 mM Tris-HCl-buffer (pH 7.6), T = 37 ˚C, substrate concentration 25 μM, enzyme concentration $5 \cdot 10^{-9}$ M.

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Table 5. Kinetic data of the hydrolysis of NP Y and GIP by DP4 variants, compared with wild type DP4. Experimental conditions: 10 mM Tris-buffer (pH 7.6), T = 30 ˚C.

Substrate	DP4-variant	K_m [M]	k_{cat} [1/s]	k_{cat}/K_m [μM^{-1} s^{-1}]
NP Y	wild type	$(1.3 \pm 0.5) \cdot 10^{-5}$	37 ± 2.7	2.85 ± 1.1
	R125A	$(1.5 \pm 0.2) \cdot 10^{-4}$	23 ± 0.9	0.15 ± 0.02
	R125K	$(5.2 \pm 0.2) \cdot 10^{-5}$	32 ± 0.5	0.61 ± 0.03
	N710A	$(8.3 \pm 1.7) \cdot 10^{-5}$	1.8 ± 0.08	0.02 ± 0.005
	N710Q	$(5.1 \pm 1.6) \cdot 10^{-4}$	2.0 ± 0.32	$(3.9 \pm 1.6) \cdot 10^{-3}$
	N710D	$(1.9 \pm 0.4) \cdot 10^{-4}$	12 ± 0.9	0.06 ± 0.01
	E205A	$(1.8 \pm 0.4) \cdot 10^{-4}$	11 ± 0.7	0.06 ± 0.01
	E206A	$(9.8 \pm 3.1) \cdot 10^{-5}$	1.9 ± 0.08	0.02 ± 0.005
GIP	wild type	$(8.4 \pm 1.2) \cdot 10^{-5}$	3.9 ± 0.2	$(4.6 \pm 0.7) \cdot 10^{-2}$
	R125A	$(1.2 \pm 0.8) \cdot 10^{-3}$	0.16 ± 0.08	$(1.3 \pm 1.0) \cdot 10^{-4}$
	R125K	$(1.7 \pm 0.2) \cdot 10^{-4}$	0.88 ± 0.08	$(5.2 \pm 0.5) \cdot 10^{-3}$

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DP4 is a member of a highly specialised protease-family, which recognizes preferentially proline in P_1 position. It is known that amino acids such as alanine or serine can also bind in the proline specific hydrophobic S_1 binding site. Interestingly, the change in the P_1 position of dipeptide derivative substrates is accompanied with a change in the rate-limiting step. The deacylation reaction is the rate-limiting process for the turnover of proline substrates while it is the acylation reaction for alanine or serine substrates. Many of the supposed physiological DP4 substrates, especially the incretins, whose prolonged half live is the main target in the treatment of human type II diabetics, possess Ala or Ser in P_1 position. This study was performed to elucidate the contribution of amino acids in the active site beside the catalytic triad to the enzymatic specificity and the process of substrate binding and catalysis. The focus was especially on the mechanism being involved in discriminating amino acids in the P_1 position of substrates. Further, the comparison of short synthetic substrates and longer physiological substrates should give detailed information about the relationship between substrate length and DP4 specificity.

Commonly, DP4 activity is characterized by measuring the release of a C-terminal fluorophor (e.g. AMC) or chromophor (e.g. pNA) from dipeptide derivatives. We evaluated the interactions between the catalytic side residues of DP4 and the amino acids in the P_2- and P_1 position by using the substrates Ala-Pro-AMC, Ala-Ala-AMC and the dipeptide mimicking inhibitor Ile-Thiazolidide respectively. Further, the use of Ala-Pro-AMC in comparison to Ala-Ala-AMC, allowed specifying the influence of the mutated side chains in the discrimination of the P_1 amino acid in dipeptide derivatives.

To verify the influence of the investigated DP4 amino acids on the interaction with the P_1'position, we extended the peptide length from 2 to 3 amino acids and kinetically characterized the DP4 variants with several tripeptides. As previously described, tripeptides with a proline residue in P_1 position are substrates of DP4 with a poor turnover rate, leading to a competitive inhibition of processing fluorogenic substrates. In order to evaluate the role of the varied amino acids, the apparent IC_{50} values for the inhibition of diprotin A, diprotin B, t-Butyl-Gly-Pro-Ile and t-Butyl-Gly-Pro-Ile-NH_2 by the DP4 variants were determined. Further we measured the K_m and k_{cat} values for the cleavage of diprotin B by the variants of R125, to examine the role of R125 in tripeptide binding and hydrolysis.

The influence of the varied amino acids in binding longer physiological DP4 substrates was also examined and compared with the kinetic data obtained for dipeptide derivatives and tripeptides. The role of the amino acid in P_1 position was evaluated by using the peptide

hormones NP Y (proline in P_1 position), GIP (alanine in P_1 position) and PACAP38 (serine in P_1 position). First, we examined by MALDI-TOF mass spectrometry analysis the ability of the DP4 variants to hydrolyse the peptide hormones. The k_{cat} and K_m values were determined by HPLC-MS/MS analytic to investigate whether the effects in substrate processing are related to substrate binding or the rate-limiting step.

Role of N710 in substrate binding and catalysis

Crystal structures of DP4 in complex with different dipeptide mimicking inhibitors revealed, that N710 together with R125 form a polar electrophilic environment for coordinating the P_2 carbonyl oxygen (Fig 1) [25,30,39]. The three generated variants of N710 (N710A, N710Q and N710D) displayed strong effects in binding and hydrolysis of dipeptide derivatives and tripeptides as well as peptide hormones. The exchange of asparagine to alanine (N710A) results in a loss of the capability to form hydrogen bonds. Thus, coordination of the P_2 carbonyl oxygen was not possible during substrate binding and hydrolysis, resulting in approximately 1000-fold decreased k_{cat}/K_m values for dipeptide derivatives. The variant N710Q showed similar effects as N710A in substrate/inhibitor binding and processing. Exchanging asparagine to glutamine, and extending the side chain by one methyl group, keeps the ability to serve as proton donor for hydrogen bond formation. However, the interaction between glutamine in position 710 and the P_2 carbonyl oxygen seems to be impossible probably due to steric changes. Further, we were not able to measure kinetic data for the hydrolysis of tripeptides and peptide hormones by the variants N710A and N710Q. In contrast, these substrates were cleaved by the variant N710D albeit with decreased k_{cat}/K_m values. It can be assumed that the carboxyl group of aspartic acid in position 710 serves as proton donor for hydrogen bond formation to the substrate carbonyl oxygen instead of N710. The lengths of the side chains are comparable between asparagine and aspartic acid albeit having different proton donor properties, resulting in varied K_m and k_{cat} values.

The variants of N710 showed effects on K_m, k_{cat} and IC_{50} values for substrates of all different lengths. This indicates that coordination of the P_2 carbonyl oxygen by N710 is in general important for substrate/inhibitor binding and hydrolysis. Our investigations with substrates offering different amino acids in P_1 position indicate the increasing importance of N710 during cleavage of substrates which possess amino acids with shorter side chains than proline (alanine or serine) in P_1 position. In this case, further interactions, e.g. with N710, are required for substrate binding and coordination of the scissile bond resulting in its cleavage. The proline in P_1 position of NP Y fits perfectly into the hydrophobic S_1 pocket in the active site of DP4, whereby the participation of N710 is not essential.

Role of R125 in substrate binding and catalysis

Co-crystallisation studies of DP4 with several dipeptidic inhibitors show, that R125 interacts with the carbonyl oxygen in P_2 position [25,30,39] and was therefore described as a secondary substrate binding hotspot in molecular docking analysis [24]. Thoma and co-workers published a crystal structure of DP4 in complex with diprotin A, whereby describing an interaction of R125 with the C-terminal carboxyl group of the tripeptide [31]. This interaction is only present in tripeptidic substrates due to the free carboxyl-terminus and may stabilize the tetrahedral intermediate by the protection of the leaving group. Further, the crystal structure of DP4 in complex with truncated NP Y (tNP Y, residues 1–10 of NP Y) shows an interaction between R125 and the carbonyl oxygen in P_1'position [32].

We measured similar kinetic data for the wild type enzyme and the variants of R125 in binding and hydrolysis of dipeptide derivatives and the dipeptide analogue inhibitor Ile-

Thiazolidide. Thus, we concluded that the interaction between R125 and the P_2 carbonyl oxygen described on the basis of several crystal structures has no important role in substrate/inhibitor binding or processing.

In contrast to the findings for dipeptide derivative substrates, our data show that the variants of R125 have an impaired binding ability for tripeptides. The IC_{50} values for tripeptide binding by the variants R125A and R125K were found to be increased in comparison to the wild type enzyme (Table 2). This indicates that R125 interacts with the amino acid in P_1'position. Tripeptides possess a free C-terminal carboxyl group, which belongs to an amide bond in longer substrates. To evaluate the influence of this P_1'carboxyl group on tripeptide binding, we measured the IC_{50} values for t-Butyl-Gly-Pro-Ile in comparison to t-Butyl-Gly-Pro-Ile-NH_2. The IC_{50} values dropped equally for the enzyme variants of N710, E205 and E206 when measured with both tripeptides. In contrast, the values for the binding of a tripeptide with a C-terminal carboxyl group (t-Butyl-Gly-Pro-Ile) by the variants of R125 were 100-fold decreased in comparison to the wild type enzyme (Table 2), whereas the IC_{50} values for t-Butyl-Gly-Pro-Ile-NH_2 dropped 10-fold. In case of t-Butyl-Gly-Pro-Ile-NH_2 a hydrogen bond between R125 and the P_1'carbonyl oxygen is most likely, whereas a salt bridge to the C-terminal carboxyl group could be assumed when binding t-Butyl-Gly-Pro-Ile. A salt bridge is an interaction with a higher binding energy in comparison to a hydrogen bond, explaining the differences in the impairment of the binding of t-Butyl-Gly-Pro-Ile and t-Butyl-Gly-Pro-Ile-NH_2 by the variants of R125.

It has been hypothesised that the interaction between R125 and the C-terminal carboxyl group of diprotin A stabilizes the tetrahedral intermediate during catalysis [31]. To evaluate the effect of R125 in tripeptide binding and hydrolysis, the K_m and k_{cat} values were determined for the truncation of diprotin B by the variant R125A. The kinetic data show that R125 plays an important role in tripeptide binding. This was demonstrated with the increase in the IC_{50} values of all used tripeptides (Table 2) as well as the K_m values of diprotin B (Table 3). Interestingly, the k_{cat} value for the hydrolysis of diprotin B by R125 was comparable to the wild type enzyme. These results confirm the findings based on the crystal structure of Thoma and colleagues, that R125 stabilizes the tetrahedral intermediate formation by interaction with the C-terminal carboxyl group of the tripeptide. Once cleavage occurred, binding of R125 to the substrate carboxyl group is not involved in the remaining acylation reaction. Hence, R125 does not decelerate the rate-limiting step in the hydrolysis of tripeptides showing a proline in P_1 position.

The cleavage of the physiological substrates NP Y, GIP and PACAP38 is also affected by the variation of R125. Interestingly, the kinetic investigations showed a strong influence of the amino acid in P_1 position. While PACAP38 (serine in P_1 position) was not hydrolysed, GIP (alanine in P_1 position) was cleaved by R125A and R125K, but with a significant lower hydrolysis rate as by the wild type enzyme. Fig 3 shows, that the hydrolysis rate of GIP also depends on the interaction with the amino acid placed on position 125. K125, as well as R125, is able to form a hydrogen bond to the carbonyl oxygen in P_1'position. The guanidino-group of arginine provides two centres to form hydrogen bonds whereas the ε-amino-group of lysine possesses only one centre. Consequently, the reduced hydrolysis velocity of GIP by R125K is probably caused by the weaker interaction between K125 and the carbonyl oxygen in P_1'position. In contrast, alanine in position 125 has not the ability to form a hydrogen bond to the P_1'carbonyl oxygen, which is reflected by a much slower hydrolysis rate.

As shown in Table 5, the K_m as well as the k_{cat} values changed for the cleavage of GIP by R125K and R125A. However, in case of NP Y both enzyme variants displayed an impaired binding while the k_{cat} values were not affected. The rate-limiting step for the hydrolysis of dipeptide derivative substrates with proline in P_1 position is the deacylation reaction, for

substrates with alanine in P_1 position the acylation reaction [29]. Our results confirm that R125 interacts with the amino acid in P_1'position. If the deacylation reaction is rate-limiting, R125 has no influence on this step, due to the fact that the C-terminal hydrolysis product (incl. amino acid in P_1'position) is already released. In contrast, if the acylation reaction would be rate-limiting, R125 would contribute by the interaction with the carbonyl oxygen in P_1'to the coordination and the cleavage of the scissile bond. Since variation of R125 has no influence on the k_{cat} value for substrates with proline in P_1 position (in our case NP Y and diprotin B) but for GIP (alanine in P_1 position), it seems that the rate-limiting step for the hydrolysis of longer DP4 substrates is comparable with the kinetics of dipeptide derivatives, and depends whether proline or alanine is in P_1 position [29].

Our investigations with the DP4 variants R125A and R125K further confirm the important role of the P_1 amino acid in substrates. Processing of proline-substrates is still carried out after removing the side chain of R125, because the interaction between proline and the hydrophobic S_1 pocket is still sufficient to enable substrate binding and catalysis. In contrast, processing of peptide hormones offering alanine (GIP) or serine (PACAP38) in P_1 position is strongly affected if the side chain of R125 does not participate in their binding by replacement to alanine.

Role of the glutamic acids E205 and E206 in substrate binding and catalysis

The enzymatic specificity of DP4 is usually dependent on the recognition of the P_1 amino acid by the hydrophobic S_1 binding pocket as well as the coordination of the positive charged N-terminus in P_2 position. Crystal structures show, that binding of the substrate N-terminus occur by the negative charged glutamic acids 205 and 206 [31,32].

Our results confirm that the loss of the negative charge by variation of E205 and E206 significantly affects the substrate binding of dipeptide derivatives. As previously described by Abbott and co-workers, the variant E206A has thereby stronger effects than E205A. Above characterization with dipeptide derivatives, we further examined the turnover of long peptide hormones. Interestingly, both variants were able to cleave NP Y, and not GIP and PACAP38. This again suggests a significant role of the amino acid in P_1 position. In case of NP Y, the recognition of proline by the hydrophobic S_1 pocket is sufficient for proper orientation of the scissile bond to allow the nucleophile attack of the active serine. Having a closer look on E206, our results also highlight the increasing independence from the coordination of the substrate N-terminus by this amino acid with growing substrate length. In comparison to the hydrolysis of NP Y, this interaction is not as essential as for the hydrolysis of dipeptidic substrates (Compare Tables 1 and 5).

In summary, we evaluated the importance of the amino acids R125, E205, E206 and N710 which are in close orientation to the catalytic triad in regard to their role in inhibition and binding of substrates with different length. It could be demonstrated that N710 is essential for processing substrates of all length while R125 supports the hydrolysis of peptide substrates consisting of more than two amino acids. Substrates displaying a proline in P_1 position are well suited to fit perfectly into the hydrophobic S_1 pocket by that strongly supporting their own catalysis. Alanine or serine in P_1 position are no perfect matches for the S_1 site. Nevertheless, many DP4 substrates offering smaller amino acids in P_1 position, show comparable turnover rates to substrates with proline in P_1. In that case, further stabilisation has to be provided by amino acids such as R125 and N710 or E205 and E206. This study provides evidence that especially R125 participates at the rate limiting step for the hydrolysis of substrates which are the main targets for type-2 diabetes treatment: GLP and GIP, both possessing alanine in P_1 position. A DP4 inhibitor, interacting with R125 would effectively diminish the cleavage of

these natural substrates, which would not be reflected by a conventional screening method using dipeptide substrates.

Materials and methods

Strains and plasmids

P.pastoris strain X-33 and the vector pPICZα C were purchased from Invitrogen (Thermo Fisher Scientific). *E.coli* XL-10 cells were provided by STRATAGENE EUROPE.

Site directed mutagenesis

Based on the protein sequence of soluble hDP4, Δ1–36 hDP4, within the vector pPICZα C [37], site directed mutagenesis according to manual from Stratagene (QuickChange™ Site-Directed Mutagenesis Kit) was carried out. In order to generate DP4 variants primers as listed in S1 Table were used.

Transformation and expression of DP4

The *P. pastoris* expression constructs were transformed following the protocols from Invitrogen (Thermo Fisher Scientific).

A 2 l fermentation was inoculated with a pre-culture following the method described by Bär *et al*. [37]. The fermentation procedure was carried out in a 5 l Biostat-B reactor (B. Braun, Melsungen, Germany).

Purification of DP4

Fermentation medium was centrifuged at 30,000 *g* for 20 minutes at 4˚C to pellet the yeast cells. The supernatant was filtered to remove any residual solids and concentrated, using a tangential flow filtration system from Sartorius, Göttingen, Germany (cut-off: 30 kDA). Affinity chromatography was carried out at 4˚C with a Ni-NTA sepharose column (Qiagen, Hilden, Germany). The column was pre-equilibrated with 50 mM NaH_2PO_4-buffer pH 7.6, 300 mM NaCl, 5 mM imidazole. The enzyme was eluted with increasing imidazole concentrations. The fractions with the highest DP4-content were pooled and concentrated to 0.5 ml using an Amicon ultrafiltration cell (cut-off: 10 kDA). This volume was applied to a superdex 200 gel filtration column (Cytiva life sciences). The column was equilibrated with 50 mM NaH_2PO_4-buffer pH 7.6 containing 300 mM NaCl with a flow rate of 0.25 ml/min. His(6)-37-766 hDP4 containing fractions were concentrated to 1 ml as described above.

Determination of kinetic parameters

K_m determination of dipeptide derivatives and determination of IC_{50} values. The reactions were performed in 100 mM HEPES buffer, pH 7.6 at 30˚C in a total assay volume of 270 μl by using 96-well plates. In order to measure K_m and k_{cat} values, the substrate concentrations varied from 4 x K_m to ¼ K_m, enzyme concentrations were set to $5x10^{-9}$ M. The kinetic parameters were calculated with non-linear regression analysis. For IC_{50} determination the inhibitor concentrations varied from 10 x IC_{50} to 1/10 IC_{50}, substrate Gly-Pro-AMC was used in the range of K_m. Fluorescence was detected with a SPECTRAFluor Plus (TECAN) at an excitation wavelength of 380 nm and an emission wavelength of 465 nm.

K_m determination by LC/MS-MS assay. Since there is no detectable fluorescence group released after cleavage of natural occurring substrates, kinetic constants (k_{cat} and K_m) for the cleavage of the diprotin A, diprotin B and the peptide hormones GIP and NP Y were determined by LC/MS-MS measurements. Therefore, DP4 was incubated with different substrate

concentrations in 10 mM Tris-buffer pH 7.6 at 30˚C. Enzyme concentrations and incubation times were chosen to obtain a linear product formation. After selected time points, samples were taken and the reaction stopped by addition of 19 volumes 0,1% HCOOH. Cleavage products were quantified in the respective samples by LC/MS-MS assay. Quantification of cleavage products occurred by using Ile-Pro-OH (diprotin A), Val-Pro-OH (diprotin B), Tyr-Pro-OH (NP Y) or Tyr-Ala-OH (GIP), respectively, as calibration standard (obtained from Bachem).

Determination of cleavage products by MALDI-TOF mass spectrometry. Cleavage assays of long natural substrates GIP, NP Y and PACAP38 were performed in 10 mM Tris-buffer, pH 7.6. Enzyme concentration 5 x 10^{-9} M, substrate concentration 25 μM, reaction time 1–3 hours, depending on the respective hydrolysis rate. Reactions were stopped by addition of 1 volume DHAP/DAHC-matrix, dissolved in 50% (v/v) acetonitrile, after certain time points. The matrix-sample mixture was placed in a vacuum, which caused the proteins to crystallize together with the matrix. Using the mass spectrometer "LD-TOF System G2025" from Hewlett Packard, the relative amount of substrate and respective product peaks in the samples could be determined. Relative hydrolysis rates were depicted by plotting relative substrate concentration against time.

Supporting information

S1 Fig. Chemical structure of Ile-thiazolidide.
(PDF)

S1 Table. List of primers. All primers to perform side directed mutagenesis were purchased from metabion (Planegg/Steinkirchen, Germany).
(TIF)

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