Tyrosine 547 Constitutes an Essential Part of the Catalytic Mechanism of Dipeptidyl Peptidase IV

Jais R. Bjelke‡ ‡‡, Jesper Christensen§§, Sven Branner¶, Nicolai Wagtmann¶, Christina Olsen§§, Anders B. Kanstrup**, and Hanne B. Rasmussen §§ §§

From the ‡ Medical Institute of Biochemistry and Genetics, Panum Institute, University of Copenhagen, Blegdamsvej 3C, DK-2200 Copenhagen, ‡‡ Protein Science, Cancer and Immunobiology, §§ Medicinal Chemistry, and §§ Protein Structure, Novo Nordisk A/S, Novo Allé, DK-2890 Bagsvaerd, Denmark

Human dipeptidyl peptidase IV (DPP-IV) is a ubiquitously expressed type II transmembrane serine protease. It cleaves the penultimate positioned prolyl bonds at the N terminus of physically important peptides such as the incretin hormones glucagon-like peptide 1 and glucose-dependent insulino tropic peptide. In this study, we have characterized different active site mutants. The Y547F mutant as well as the catalytic triad mutants S630A, D708A, and H740L showed less than 1% wild type activity. X-ray crystal structure analysis of the Y547F mutant revealed no overall changes compared with wild type apoDPP-IV, except the abolition of the hydroxyl group of Tyr547 and a water molecule positioned in close proximity to Tyr547. To elucidate further the reaction mechanism, we determined the crystal structure of DPP-IV in complex with diisopropyl fluorophosphate, mimicking the tetrahedral intermediate. The kinetic and structural findings of the tyrosine residue are discussed in relation to the catalytic mechanism of DPP-IV and to the inhibitory mechanism of the 2-cyclopentylidine class of potent DPP-IV inhibitors, proposing an explanation for the specificity of this class of inhibitors for the S9b family among serine proteases.

Dipeptidyl peptidase IV (DPP-IV,1 CD26, EC 3.4.14.5) is a human serine protease belonging to the S9b protein family, which was first identified by Hopps-Havanaugh and Glener (1) as glycylproline β-naphthylamidase. It is characterized by an unusual ability to cleave prolyl peptide bonds at penultimate positions at the N terminus and exists both as a type II integral transmembrane lipid raft-associated glycoprotein and as a soluble 210–290-kDa homodimeric form (2–4).

Many peptides have been identified as DPP-IV substrates in vitro and in vivo, including chemokines, neuropeptides, and incretin hormones, and DPP-IV has therefore been proposed as an important regulator of different physiological and pathological conditions (5). There is a considerable pharmacological interest in DPP-IV, because the enzyme has been shown to inactivate the incretin hormones glucagon-like peptide 1 and glucose-dependent insulino tropic peptide in vivo (6, 7). This makes DPP-IV an important regulator of glucose homeostasis, as glucagon-like peptide 1 and glucose-dependent insulino tropic peptide have glucose-dependent insulino tropic as well as neogenetic effects on the pancreatic β-cells. The use of DPP-IV inhibitors in diabetes is being explored, and the first short term treatments of diabetes mellitus type 2 patients with DPP-IV inhibitors have demonstrated clinical proof of the concept (8, 9).

The published crystal structure of human recombinant DPP-IV in complex with the substrate analog valine pyrrolidide (ValPyr) (Fig. 1) has revealed many important details regarding the inhibitor binding in the active site cavity, which by analogy illustrate substrate binding (10). The active site is positioned in a large cavity, formed at the interface of an α/β hydrolase domain and an eight-bladed β-propeller domain. The catalytic triad has been identified by site-directed mutagenesis in mouse DPP-IV, which by homology corresponds to Ser630, Asp708, and His740 in human DPP-IV (11). The catalytic triad is arranged in a topological fold and sequential order, which defines the α/β hydrolase domain. Furthermore, the catalytically essential Ser630 is located in a so-called “nucleophile elbow” consisting of the sequence Gly628–Trp629–Ser630–Tyr641–Gly632, a consensus sequence characteristic for all serine peptidases in the SC clan, i.e. GXXSXXG (12, 13). Furthermore, the crystal structure determinations have suggested detailed information of the catalytic mechanism of DPP-IV. For example, we now understand the essential function of the residues Glu605 and Glu606 for coordination of the N-terminal amine of the substrate, which had been demonstrated by use of site-directed mutagenesis (14). In addition, the residues Arg125 and Asn710 appear essential for coordination of the carbonyl of the N-terminal amino acid residue of the substrate and, together with the two glutamates, align the substrate optimally for the nucleophilic attack by Ser630. A negatively charged tetrahedral oxyanion intermediate is generated in the transition state and is stabilized by a so-called oxyanion hole. This is a recognized mechanism among serine proteases. Based on analysis of the structure and sequence alignment to the homolog S9 protein family member prolyl oligopeptidase (POP), this oxyanion hole has been suggested to be formed via hydrogen bonding to Tyr457-OH and the backbone NH of Tyr457 (10, 15–18). In this study, we have investigated the oxyanion stabilization of DPP-IV catalysis by removing the hydroxyl group of Tyr547 by means of site-specific mutagenesis to the phenylalanine equivalent. The kinetic data of the Y547F mutant show that Tyr547 is essential.
for DPP-IV catalysis. Determination and comparison of the crystal structures of (i) the mutant Y547F, (ii) a complex between DPP-IV and the covalent inhibitor diisopropyl fluorophosphate (DFP), and (iii) the apo forms of DPP-IV (in-house as well as previously published) confirm the oxyanion stabilizing role of the hydroxyl group of Tyr547 in the catalytic mechanism of DPP-IV. In addition, these results suggest that the inhibitory mechanism of these pharmacologically important class of DPP-IV inhibitors, the 2-cyanoypyridinones, is conducted via proton acceptance from Tyr547 resulting in stable complexed.

MATERIALS AND METHODS

Bioinformatics—Analyses of the structure of recombinant human DPP-IV were performed using the Quanta software (Accelrys Inc.). The Vector NTI Suite 6.0 (InforMax Inc.) was used for sequence analysis, gene alignments, and primer design.

Chemicals and Reagents—QIAprep Miniprep System and Qiagen Gel Extraction II kits were purchased from Qiagen (San Diego). The baculovirus transfer vector pBluebac4.5 was from Invitrogen. Mouse anti-CD26 monoclonal antibody clones MA2600 and MA261 were from Endogen (Rockford, IL) and Bender MedSystems (Vienna, Austria), respectively. Horseradish peroxidase-conjugated rabbit anti-mouse IgG was from Dako (Glostrup, Denmark). Spodoptera frugiperda 9 (SF9) and High five insect cells were grown in Grace Insect medium supplemented with fetal calf serum ranging from 0 to 10%, yeastolate, 20 mM glutamine, and 0.25 µg/ml gentamycin in either tissue culture flasks or glass spinner bottles at 28 °C. Chromatographic columns and materials (CNBr-activated Sepharose 4B matrix, MonoQ ion-exchange column, and Q-Sepharose high performance resin) were from Amersham BioSciences. Adenosine deaminase (ADA) protein was from Roche Applied Science. DFP was from Sigma.

Site-directed Mutagenesis of DPP-IV—Introduction of point mutations in the recombinant human DPP-IV baculovirus transfer vector CDS/DPP-IV-pBluebac4.5 (see Ref. 10) was performed using the QuickChange™ Site-directed Mutagenesis kit from Stratagene (La Jolla, CA). The following primers were used for introduction of mutations (forward primers are shown): Y547F, 5′-GAA ATA TCC TCT ATT AGA GTT GTT TGC AGG CCC ATT TAG TGC-3′; S630A, 5′-TTT GGG GCT GGG CAT ATG GAG GGT A; D708A, 5′-GGT ATA CTG ATG AAG ACC TTG GAA-3′. Previously published DNA Sequencing Ready Reaction from Applied Biosystems.

Crystallographic Data Collection and Handling—Crystallographic data collection was performed at the synchrotron beamlines MaxLab 711 (Lund, Sweden), ESRF ID 14-4 (Grenoble, France) and on an in-house rotating anode Rigaku RU300. Data reductions were performed with the HKL2000 software package (20). The structures were solved by the molecular replacement method using wild type DPP-IV as a search model (Protein Data Bank code 1N1M). Model building was performed using Quanta software (Accelrys Inc.) and iterative refinement (initially performed as a rigid body refinement) using CNS (21). Structure validation and handling were performed with Procheck (22) and Moleram.

RESULTS

Structural and Sequence Analysis of the Active Site of DPP-IV—The hydroxyl group of the side chain of Tyr547 is coordinated via a water molecule to the hydroxyl group of Ser630 (Fig. 1) and has, together with the main chain NH of Tyr547, been suggested as a stabilizer of the oxyanion intermediate during catalysis (10). To test the catalytic effect of the hydroxyl group, the Y547F mutant variant lacking the para-positioned hydroxyl group was constructed.

The detailed crystallographic data of human DPP-IV have already been published in previous communications (33–35) and will not be described here in detail.

Expression and Characterization of DPP-IV Mutants—The DPP-IV mutants were generated using a PCR-based site-directed mutagenesis method directly in CD5/DPP-IV-pBlueBac. This construct encodes a soluble recombinant human form of DPP-IV lacking the cystolic and transmembrane domains. The open reading frame of this construct is fused to the leader secretion signal of CD5. Thus, expressed protein is secreted to the cell supernatants after post-translational modifications. All PCR-introduced sequence errors by complete DNA sequencing.

G. J. Kleywegt, unpublished information.
High titer baculovirus stocks produced in Sf9 insect cells were used for expression studies in High5 insect cells (multiplicity of infection >1). Expression levels were analyzed for intracellular levels by use of SDS-PAGE-Coomassie (Fig. 2A) and for secreted protein to cell supernatant by use of an ADA-sandwich ELISA. From SDS-PAGE analysis of total cell lysates, protein bands with an electrophoretic mobility equal to purified DPP-IV were observed and interpreted as DPP-IV expressed protein. No protein bands with similar electrophoretic mobility could be observed with insect cell expression controls. Intracellular DPP-IV protein accounted for ~10–30% of total cellular protein (~20–60 μg/10⁶ cells). The level of secreted protein in the cell supernatants was significantly lower compared with intracellular DPP-IV (i.e., 0.5–5 μg per ml ~1–10% of intracellular amounts assuming ~10⁶ cells/ml supernatant). Sandwich ELISA titration of the cell supernatants indicated that the secreted mutant DPP-IV proteins bound to ADA and anti-human CD26 monoclonal antibodies. Altogether, these data verified that structurally intact DPP-IV mutants had been expressed. Only cell-secreted DPP-IV was used for further studies.

All DPP-IV mutants were normalized directly in the cell supernatants to three different protein concentrations (i.e., 0.11, 0.16, and 0.54 μM) by using ELISA, and enzymatic activity levels were characterized at these concentrations by using substrate analogs (Table I). The three catalytic triad mutants S630A, D708A, and H740L exhibited less than 1% specific activity compared with wild type DPP-IV. Surprisingly, the Y547F mutant showed a root mean square of 0.86 Å of the Y547F mutant, which was not a result of an active site collapse and/or conformational changes within the active site or the whole protein as such. Inspection of the electron density of the mutated residue revealed a strongly defined phenylalanine, positioned exactly as the phenyl moiety of the tyrosine residue (Fig. 4, A and B). Most interesting, coordination of a water molecule (Wat123, see Fig. 4A) by the tyrosine was observed in different apo structures (previously published human DPP-IV (Protein Data Bank code 1PFQ (24) and Protein Data Bank code 1J2E (25)) and porcine DPP-IV (Protein Data Bank code 1ORV (26))) as well as our own in-house information but not in the phenylalanine mutant. In one of the previously published human apo structures (Protein Data Bank code 1NU6 (27)), this water

Kinetically, k_{cat} dropped for the Y547F mutant by ~50-fold by using the putative substrate Gly-Pro-pNA, whereas K_{m} values increased ~30-fold compared with wild type, resulting in an overall drop of more than 1,500-fold for the second-order rate constant k_{cat}/K_{m} (Fig. 3 and Table II). Similar results were obtained with other substrate analogs, showing no differences in substrate specificity as a result of the mutation.

X-ray Crystallography Structure Determination—The x-ray crystal structures of the apoDPP-IV, the complex DFP-DPP-IV, and the DPP-IV mutant Y547F were determined. Diffraction data sets were collected at 2.0 Å resolution for the apoDPP-IV, 2.7 Å for the DFP-DPP-IV complex, and 2.2 Å for the Y547F mutant. Crystallographic data collection and refinement statistics are listed in Table III. All structures were solved by molecular replacement using the previously published DPP-IV structure (Protein Data Bank code 1N1M) as a search model excluding inhibitor and water molecules.

From analysis of the active site cavity and the overall structure of the Y547F mutant, it was clear that the overall structure of the mutant was conserved, and comparison of active site residues showed no conformational changes, neither main chain nor side chain, from wild type DPP-IV. Superimposition of C α trace of the dimer structure of wild type DPP-IV and the Y547F mutant showed a root mean square of 0.86 Å² (1530 C α atoms). Thus, the decreased enzyme activity of the Y547F mutant was not a result of an active site collapse and/or conformational changes within the active site or the whole protein as such. Inspection of the electron density of the mutated residue revealed a strongly defined phenylalanine, positioned exactly as the phenyl moiety of the tyrosine residue (Fig. 4, A and B). Most interesting, coordination of a water molecule (Wat123, see Fig. 4A) by the tyrosine was observed in different apo structures (previously published human DPP-IV (Protein Data Bank code 1PFQ (24) and Protein Data Bank code 1J2E (25)) and porcine DPP-IV (Protein Data Bank code 1ORV (26))) as well as our own in-house information but not in the phenylalanine mutant. In one of the previously published human apo structures (Protein Data Bank code 1NU6 (27)), this water
molecule is absent or not included. Besides Tyr\textsuperscript{547}-OH, this water molecule coordinates Ser\textsuperscript{630}-OH, main chain NH of Tyr\textsuperscript{547}, and a neighboring water molecule, making it part of a network of water molecules within the active site cavity. The DPP-IV-ValPyr complex (Protein Data Bank code 1N1M (10)) also has a water molecule at the same position. The structural deviations seem to be species related, because the previously published human apo structures are similar to the previously published porcine and human apo structures showed few differences, e.g. side chain positions of Tyr\textsuperscript{105}, Phe\textsuperscript{208}, Tyr\textsuperscript{439}, Tyr\textsuperscript{534}, Cys\textsuperscript{551}, and Trp\textsuperscript{639}. The structural deviations seem to be species related, because the previously published human apo structures are similar to what we observe. Only the human apo structure INU6 showed a difference, because this structure lacked the water molecule positioned between Tyr\textsuperscript{547} and Ser\textsuperscript{630} (26).

In the structurally and functionally related endopeptidase POP (member of the S9a family), a similar mechanism using a tyrosine moiety for oxygen stabilization has been suggested based on mutagenesis and analysis of kinetic data. This protein consists of an \(a/\beta\) hydrolase fold, encompassing the catalytic triad composed of Ser\textsuperscript{554}, His\textsuperscript{680}, and Asp\textsuperscript{641}, and a seven-bladed tunnel-forming \(\beta\)-propeller, i.e. notably different from the homologue's eight-bladed domain in DPP-IV (18, 28). Furthermore, POP is an endopeptidase with a different substrate specificity profile compared with DPP-IV (29). Tyr\textsuperscript{473} in the active site cavity of POP, which is homologous to Tyr\textsuperscript{547} of DPP-IV, has been suggested as an oxyanion coordination site functioning as a nucleophilic water molecule. Furthermore, the complex structure between human DPP-IV and the covalent inhibitor DFP showed that the phosphonate P=O was within hydrogen bonding distance with the Tyr\textsuperscript{547}-OH and the main chain NH of Tyr\textsuperscript{547}. DFP is well accepted as a mimic of the tetrahedral enzyme-substrate intermediate, and taken together with the structures of the mutant and the apo form, this strongly implies that the oxyanion hole is indeed comprised of Tyr\textsuperscript{547}-OH and the main chain NH of Tyr\textsuperscript{547}. Note, structural alignments between the previously published porcine and human apo structures showed few differences, e.g. side chain positions of Tyr\textsuperscript{105}, Phe\textsuperscript{208}, Tyr\textsuperscript{439}, Tyr\textsuperscript{534}, Cys\textsuperscript{551}, and Trp\textsuperscript{639}. The structural deviations seem to be species related, because the previously published human apo structures are similar to what we observe. Only the human apo structure INU6 showed a difference, because this structure lacked the water molecule positioned between Tyr\textsuperscript{547} and Ser\textsuperscript{630} (26).

In this study, we have shown that the residue Tyr\textsuperscript{547} of DPP-IV is essential for catalysis. The analysis of the mutant structure as well as the apo and DFP complex of DPP-IV supports the suggestion that the role of Tyr\textsuperscript{547} is to stabilize the tetrahedral oxyanion intermediate. Exchanging Tyr\textsuperscript{473} with phenylalanine resulted in a vast drop in activity of the same magnitude as alanine/leucine mutants of the catalytic triad residues (11). Structure determination of the Y547F mutant revealed a completely intact protein structure, and the only difference observed was the absence of the para-positioned hydroxy group and a coordinating water molecule, Wat\textsuperscript{123}, Wat\textsuperscript{236}.

**DISCUSSION**

In this study, we have shown that the residue Tyr\textsuperscript{547} of DPP-IV is essential for catalysis. The analysis of the mutant structure as well as the apo and DFP complex of DPP-IV supports the suggestion that the role of Tyr\textsuperscript{547} is to stabilize the tetrahedral oxyanion intermediate. Exchanging Tyr\textsuperscript{473} with phenylalanine resulted in a vast drop in activity of the same magnitude as alanine/leucine mutants of the catalytic triad residues (11). Structure determination of the Y547F mutant revealed a completely intact protein structure, and the only difference observed was the absence of the para-positioned hydroxy group and a coordinating water molecule, Wat\textsuperscript{123}, Wat\textsuperscript{236}.

Structural alignment of the mutant, apo, and DFP complex structures showed no differences in the overall fold or side chain conformations. Most interesting, Wat\textsuperscript{123} is found at a similar position in the DPP-IV-ValPyr complex, inbetween the Ser\textsuperscript{630}-OH and the Tyr\textsuperscript{547}-OH motif in close proximity (~3.2 Å) to the proline-mimicking moiety of the inhibitor ValPyr (Fig. 1), thereby not excluding a direct catalytic role in the mechanism, e.g. functioning as a nucleophilic water molecule. Furthermore, the complex structure between human DPP-IV and the covalent inhibitor DFP showed that the phosphonate P=O was within hydrogen bonding distance with the Tyr\textsuperscript{547}-OH and the main chain NH of Tyr\textsuperscript{547}. DFP is well accepted as a mimic of the tetrahedral enzyme-substrate intermediate, and taken together with the structures of the mutant and the apo form, this strongly implies that the oxyanion hole is indeed comprised of Tyr\textsuperscript{547}-OH and the main chain NH of Tyr\textsuperscript{547}. Note, structural alignments between the previously published porcine and human apo structures showed few differences, e.g. side chain positions of Tyr\textsuperscript{105}, Phe\textsuperscript{208}, Tyr\textsuperscript{439}, Tyr\textsuperscript{534}, Cys\textsuperscript{551}, and Trp\textsuperscript{639}. The structural deviations seem to be species related, because the previously published human apo structures are similar to what we observe. Only the human apo structure INU6 showed a difference, because this structure lacked the water molecule positioned between Tyr\textsuperscript{547} and Ser\textsuperscript{630} (26).

In the structurally and functionally related endopeptidase POP (member of the S9a family), a similar mechanism using a tyrosine moiety for oxygen stabilization has been suggested based on mutagenesis and analysis of kinetic data. This protein consists of an \(a/\beta\) hydrolase fold, encompassing the catalytic triad composed of Ser\textsuperscript{554}, His\textsuperscript{680}, and Asp\textsuperscript{641}, and a seven-bladed tunnel-forming \(\beta\)-propeller, i.e. notably different from the homologue's eight-bladed domain in DPP-IV (18, 28). Furthermore, POP is an endopeptidase with a different substrate specificity profile compared with DPP-IV (29). Tyr\textsuperscript{473} in the active site cavity of POP, which is homologous to Tyr\textsuperscript{547} of DPP-IV, has been suggested as an oxyanion coordination site using a similar mutagenesis strategy as employed in this study of DPP-IV (30). Szeltner et al. (30) demonstrated that the POP mutant Y473F had at least an 8-fold reduction in the kinetic specificity rate constant \(k_{\text{cat}}/K_m\) depending on which putative substrate was used. They concluded on this basis that Tyr\textsuperscript{473} contributes to the transition state stabilization via an oxyanion binding capacity and speculated on whether the hydroxy group of Tyr\textsuperscript{473} also interacts with the substrate carbonyl oxygen in the Michaelis-Menten complex formation. We observed a drop of more than 1,500-fold in \(k_{\text{cat}}/K_m\) for the DPP-IV mutant of the homologues compared with wild type, i.e. a 50-fold drop and a 30-fold increase for \(k_{\text{cat}}\) and \(K_m\), respectively. Thus the POP mutant retained a significantly higher intrinsic activity compared with the homologue's mutant of DPP-IV, an intrinsic activity that was suggested to be the result of bulk water occupying and substituting the position of the ablated hydroxy group. This notion is not confirmed by the crystallographic
results presented here on DPP-IV, where the crystal structure determination of the Y547F mutant on the contrary revealed less bound water. A spatial structural search in the Protein Data Base using the program SPASM (31) revealed that the Tyr547 residue together with the catalytic triad constitute a unique structure-activity relationship, because the only serine proteases with three-dimensional structures known to date having the spatial arrangement Tyr547, Ser630, Asp708, and His740 (i.e. numbering according to DPP-IV) were identified as DPP-IV and POP. Thus, only peptidases of the S9 subfamilies seem to contain this catalytic motif, and from the kinetic data obtained in this study on DPP-IV the motif seems even more important for optimal activity than the homologous motif in POP. In addition, the main chain Tyr631-NH coordination of the

![Kinetic plot: Y547F and wild type](image)

**FIG. 3.** Kinetic plot of the purified wild type and the Y547F mutant using the putative substrate Gly-Pro-pNa. Replicate activity measurements for wild type (●) and Y547F (○) as well as directly fitted Michaelis-Menten curves are displayed.

**TABLE II**

|          | WT       | Y547F    |
|----------|----------|----------|
| $v_{\text{max}}$ ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) | 198 ± 21 | 3.9 ± 0.1* |
| $K_m$ ($\text{mM}$) | 1.43 ± 0.23 | 44.08 ± 0.01* |
| $k_{\text{cat}}$ ($\text{s}^{-1}$) | 279,000 ± 29,500 | 5,560 ± 110* |
| $k_{\text{cat}}/K_m$ ($\text{s}^{-1} \text{mM}^{-1}$) | ~195,000,000 | ~128,000* |

* Significantly different when compared with purified wild type DPP-IV values as determined by Student's $t$ test ($p < 0.005$).

**TABLE III**

X-ray crystallographic data collection and refinement statistics

|          | Y547F Apo | Y547F DFP |
|----------|-----------|-----------|
| Space group | $P2_1;2_1;2_1$ | $P2_1;2_1;2_1$ |
| Unit cell (Å) | 118.8 | 119.3 |
| $a$ | 121.4 | 122.4 |
| $b$ | 129.1 | 129.8 |
| $c$ | 129.1 | 129.8 |
| Wavelength (Å) | 0.995 | 0.980 |
| Resolution range (Å) | 40–2.20 (2.28–2.20) | 30–2.00 (2.07–2.00) |
| No. measured reflections | 327,518 | 437,212 |
| No. unique reflections | 84,656 | 124,645 |
| Redundancy | 3.9 | 3.5 |
| Completeness (%) | 86.0 (36.4) | 97.0 (89.3) |
| $R_{\text{merge}}$ (%) | 11.6 (1.8) | 9.9 (2.6) |
| $R_{\text{merge}}^{\text{all}}$ (%) | 9.2 (33.7) | 11.8 (42.3) |
| $R_{\text{merge}}^{\text{free}}$ (%) | 21.7/26.9 | 22.9/27.2 |
| No. of atoms | | |
| Non-hydrogen atoms | 13,215 | 13,536 |
| Water molecules | 862 | 1,212 |
| Average B-factor (all atoms, Å²) | 30.9 | 26.9 |
| Root mean square deviation | | |
| Bond lengths (Å) | 0.006 | 0.005 |
| Angles (°) | 1.3 | 1.4 |

* $R_{\text{merge}}$ indicates $\sum_{\text{hkl}} |I_{\text{obs}}| - |I_{\text{calc}}|/\sum_{\text{hkl}} |I_{\text{obs}}|$, where $I_{\text{obs}}$ and $I_{\text{calc}}$ are the diffraction intensity values of the individual measurement and the corresponding mean value for reflection $hkl$, respectively.

* $R$ indicates $\sum_{\text{hkl}} |F_{\text{obs}}| - |F_{\text{calc}}|/\sum_{\text{hkl}} |F_{\text{obs}}|$, where $F_{\text{obs}}$ and $F_{\text{calc}}$ are the observed and calculated structure factor amplitudes for reflection $hkl$. $R_{\text{free}}$ is a cross-validation set of 5% omitted reflections from refinement.
Tyr\textsuperscript{547} Is an Essential Part of Catalytic Mechanism of DPP-IV

FIG. 4. Close-up of active site residues. The initial $F_o - F_c$ electron density maps are overlaid the apoDPP-IV (A), Y547F mutant (B), and complex DFP-DPP-IV (C, slightly different view, relative to A and B) contoured at 2\(\sigma\) (cyan), 3\(\sigma\) (red), 5\(\sigma\) (purple), only contoured in the apo structure), and 8\(\sigma\) (blue, only contoured in the DFP structure). The initial $2F_o - F_c$ electron density map is overlaid the complex DFP-DPP-IV contoured at 1\(\sigma\) (gray). Structural inspections of the active site of the Y547F mutant reveals a missing water molecule, clearly seen in the wild type apo structure (i.e. hydrogen bonds between Tyr\textsuperscript{547}-OH, Ser\textsuperscript{630}-OH, and Tyr\textsuperscript{601}-NH are indicated). The mutated residue (Phe\textsuperscript{547}) is positioned exactly as the tyrosine residue. The water molecule designated Wat\textsuperscript{258} and Wat\textsuperscript{421} in the apo and the Y547F mutant structure, respectively, is moved 0.5 Å away from the 547 residue and 0.3 Å (2.9 versus 3.2 Å) closer to the neighboring Tyr\textsuperscript{601}-OH (not shown) in the mutant structure. The complex between DFP and DPP-IV showed that the organo-phosphorous inhibitor was covalently bound to Ser\textsuperscript{630}, mimicking the tetrahedral intermediate.

This would lead to the inhibitor being bound to Ser\textsuperscript{630} as an imidate, which is a stable entity. Intuitively, the location of a protonated species in an oxyanion hole would seem disfavored. However, stable covalent complexes with imidate geometry have been observed in crystal structures of DPP-IV with such inhibitors, both by us\textsuperscript{8} and others (24), under conditions where stable acyl-enzyme intermediates have not been observed previously. The observation of such stable acyl-enzyme mimetics, presumed to be neutral imidates, thus supports the possibility that Tyr\textsuperscript{547} may function both as a hydrogen bond donor forming the anion hole, and as a hydrogen bond acceptor stabilizing a covalently bound imidate, thereby providing a plausible explanation as to why the therapeutically very promising cyano-pyrrolidines are specific for the S9b family among serine proteases.

The conserved and structurally well defined Wat\textsuperscript{123} may be involved in catalysis but could also just be located in the very favorable oxyanion site in the absence of ligands that utilize this functionality. The observation that Wat\textsuperscript{123} is present in the DPP-IV-Val-Pyr structure and the apo structure but absent in the DPP-IV-DFP structure and in the acyl-enzyme mimetic structures points to the latter possibility.

In conclusion, the Y547F mutation decreased activity to less than 1% of wild type, a decrease of the same magnitude as knock-out mutants of the catalytic triad residues Ser\textsuperscript{630}, Asp\textsuperscript{708}, and His\textsuperscript{740}. Structure analysis of the Y547F mutant revealed an intact active site with only a single water molecule absent, which leads us to conclude that the residues Tyr\textsuperscript{547}, Ser\textsuperscript{630}, Asp\textsuperscript{708}, and His\textsuperscript{740} are equally important for the catalytic mechanism of DPP-IV. The crystal structure of DPP-IV in complex with DFP, mimicking the tetrahedral intermediate, shows Tyr\textsuperscript{547} to strongly participate in the coordination of the intermediate, and thereby indicate the participation of Tyr\textsuperscript{547} in the oxyanion hole formation. Altogether, it is concluded that...
Tyr547 is not only important but is essential for cleavage of the prolyl peptide bond.

Acknowledgments—We thank Brian Rosenberg, Connie Winther, and Ulla Toftegaard for excellent technical assistance. We also thank Henning Ralf Stennicke, Ole Hvilsted Olsen, and Richard D. Carr for fruitful discussions and comments on the manuscript.

REFERENCES

1. Hopsu-Havu, V. K., and Glenner, G. G. (1966) Histochemie 7, 197–201
2. Abbott, C. A., and Gorrell, M. D. (2002) in Ectopeptidases: CD13/Aminopeptidase N and CD26/Dipeptidylpeptidase IV in Medicine and Biology (Langner, J., and Anzorge, S., eds) pp. 171–184, Kluwer/Plenum Publishing Corp., New York
3. Lambeir, A. M., Diaz Pereira, J. F., Chacon, P., Vermeulen, G., Heremans, K., Devreese, B., Van Beeumen, J., De Meester, I., and Scharpe, S. (1997) Biochim. Biophys. Acta 1340, 215–226
4. Duke-Cohan, J. S., Morimoto, C., Rocker, J. A., and Schlossman, S. F. (1996) J. Immunol. 156, 1714–1721
5. Mentlein, R. (1999) Regul. Pept. 85, 9–24
6. Holst, J. J., and Deacon, C. F. (1998) Diabetes 47, 1663–1670
7. Holst, J. J., and Orskov, C. (2001) Scand. J. Clin. Lab. Invest. 234, (suppl.) 75–85
8. Ahren, B., Simonsen, E., Larsson, H., Landin-Olsson, M., Torgerisson, H., Jansson, P. A., Sandqvist, M., Bavenholm, P., Eferding, S., Eriksson, J. W., Dickson, S., and Holms, D. (2002) Diabetes Care 25, 869–875
9. Ahren, B., Landin-Olsson, M., Jansson, P. A., Svensson, M., Holms, D., and Schweizer, A. (2004) J. Clin. Endocrinol. Metab. 89, 2078–2084
10. Rasmussen, B. H., Branner, S., Wiberg, F. C., and Wagtmann, N. (2003) Nat. Struct. Biol. 10, 19–25
11. Marguet, D., Bernard, A. M., Vivier, I., Darmoul, D., Naquet, P., and Pierres, M. (1992) J. Biol. Chem. 267, 2200–2208
12. Bernard, A. M., Mattei, M. G., Pierres, M., and Marguet, D. (1994) Biochemistry 33, 15204–15214
13. Abbott, C. A., Baker, E., Sutherland, G. R., and McCaughan, G. W. (1994) Immunogenetics 40, 331–338
14. Abbott, C. A., McCaughan, G. W., and Gorrell, M. D. (1999) FEBS Lett. 458, 278–284
15. Brandt, W. (2000) Adv. Exp. Med. Biol. 477, 97–101
16. Reva, B., Finkelstein, A., and Topol, S. (2002) Proteins 47, 180–193
17. Abbott, C. A., Yu, D. M., Wollatt, E., Sutherland, G. R., McCaughan, G. W., and Gorrell, M. D. (2000) Eur. J. Biochem. 267, 6149–6150
18. Fulop, V., Bocskei, Z., and Polgar, L. (1998) Cell 94, 161–170
19. de Meester, I., Vanhoof, G., Lambeir, A. M., and Scharpe, S. (1996) J. Immunol. Methods 199, 99–105
20. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
21. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J. J., Nissly, M. P., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 905–921
22. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
23. Hughes, T. E., Mone, M. D., Russell, M. E., Weldon, S. C., and Villhauer, E. B. (1999) Biochemistry 38, 11597–11603
24. Oefner, C., D’Aracy, A., Mac, S. A., Pierau, S., Gardiner, R., and Dale, G. E. (2003) Acta Crystallogr. Sect. D Biol. Crystallogr. 59, 1206–1212
25. Hiramatsu, H., Kyono, K., Hagashiyama, Y., Fukushima, C., Shima, H., Sugiyama, S., Inaka, K., Yamamoto, A., and Shimizu, R. (2003) Biochem. Biophys. Res. Commun. 302, 849–854
26. Engel, M., Hoffmann, T., Wagner, L., Wermann, M., Heiser, U., Kiefersauer, R., Huber, R., Bode, W., Demuth, H. U., and Brandstetter, H. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5063–5068
27. Thomas, R., Loffler, B., Stihle, M., Huber, W., Ruf, A., and Hennig, M. (2003) Structure (Lond.) 11, 947–959
28. Fulop, V., Seidtner, Z., and Polgar, L. (2000) EMBO Rep. 1, 277–281
29. Polgar, L. (1992) Biochemistry 31, 7729–7735
30. Seidtner, Z., Renner, V., and Polgar, L. (2000) Protein Sci. 9, 353–360
31. Kleywegt, G. J. (1999) J. Mol. Biol. 285, 1887–1897
32. Carter, P., Abrahamsson, L., and Wells, J. A. (1991) Biochemistry 30, 6142–6148
33. Siezen, R. J., and Leunissen, J. A. (1997) Protein Sci. 6, 501–523
34. Waskell, A., Navashin, G., Susman, F., and Hwang, J. R. (1989) Biochemistry 28, 3629–3637
35. Asheth, B. (1983) Biochemistry 22, 117–122
Tyrosine 547 Constitutes an Essential Part of the Catalytic Mechanism of Dipeptidyl Peptidase IV

Jais R. Bjelke, Jesper Christensen, Sven Branner, Nicolai Wagtmann, Christina Olsen, Anders B. Kanstrup and Hanne B. Rasmussen

J. Biol. Chem. 2004, 279:34691-34697.
doi: 10.1074/jbc.M405400200 originally published online June 2, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405400200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 5 of which can be accessed free at http://www.jbc.org/content/279/33/34691.full.html#ref-list-1