Investigation of the Dimer Interface and Substrate Specificity of Prolyl Dipeptidase DPP8

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DPP8 belongs to the family of prolyl dipeptidases, which are capable of cleaving the peptide bond after a penultimate proline residue. Unlike DPP-IV, a drug target for type II diabetes, no information is available on the crystal structure of DPP8, the regulation of its enzymatic activity, or its substrate specificity. In this study, using analytical ultracentrifugation and native gel electrophoresis, we show that the DPP8 protein is predominantly dimeric when purified or in the cell extracts. Four conserved residues in the C-terminal loop of DPP8 (Phe822, Val833, Tyr844, and His859), corresponding to those located at the dimer interface of DPP-IV, were individually mutated to Ala. Surprisingly, unlike DPP-IV, these single-site mutations abolished the enzymatic activity of DPP8 without disrupting its quaternary structure, indicating that dimerization itself is not sufficient for the optimal enzymatic activity of DPP8. Moreover, these mutations not only decreased $k_{cat}$, as did the corresponding DPP-IV mutations, but also dramatically increased $K_{m}$. We further show that the $K_m$ effect is independent of the substrate assayed. Finally, we identified the distinctive and strict substrate specificity of DPP8 for hydrophobic or basic residues at the P2 site, which is in sharp contrast to the much less discriminative substrate specificity of DPP-IV. Our study has identified the residues absolutely required for the optimal activity of DPP8 and its unique substrate specificity. This study extends the functional importance of the C-terminal loop to the whole family of prolyl dipeptidases.

The prolyl dipeptidases have attracted extensive investigation in recent years because of their unique ability to cleave the peptide bond after a penultimate proline residue (1). Proline is an amino acid that is not cleavable by many cellular proteases in vivo. The biological importance of the prolyl dipeptidases is underscored by the fact that many peptide hormones, chemokines, and neuropeptides contain one or more proline residues. Consequently, the processing and degradation of such biologically important regulatory peptides requires proline-cleaving proteases. The prolyl dipeptidase family includes dipeptidyl peptidase IV (DPP-IV), fibroblast activation protein (FAP), DPP8, DPP9, and DPP2 (1). DPP-IV is a validated drug target for type II diabetes, based on its ability to process proteolytically in vivo two insulin-sensing hormones, glucagon-like peptide-1 and glucose-dependent insulinoتروopic polypeptide (2–4). Chemical inhibitors of DPP-IV have been found effective in the treatment of type II diabetes in human clinical trials (5, 6). Determining the structures and catalytic properties of the prolyl dipeptidases is important both to discover DPP-IV selective inhibitors for disease treatments and to elucidate the biological functions of these prolyl-cleaving proteases in vivo.

The biochemical properties of DPP-IV and FAP are significantly different from those of the classical serine proteases, such as trypsin, chymotrypsin, and subtilisin, or the monomeric prolyl-cleaving prolyl oligopeptidase (7). Both DPP-IV and FAP are homodimeric enzymes with highly mutually superimposable structures (8–12) (Fig. 1A). Each monomer consists of an $\alpha/\beta$ hydrolase domain and a $\beta$-propeller domain, with the active site located between them (8–12). Both the C-terminal loop in the $\alpha/\beta$ hydrolase domain and the propeller loop that extends from strand 2 of the fourth blade in the $\beta$-propeller domain interact with the same region on the other monomer across a 2-fold axis at the dimer interface (8–12) (Fig. 1A). The C-terminal loop, consisting of the last 50 amino acids, is highly conserved among the prolyl dipeptidases (13) (Fig. 1B). It has two $\alpha$-helices (amino acids 713–725 and 745–763) connected by one $\beta$-sheet (amino acids 726–744) (8–12) (Fig. 1A). We have previously shown that this loop is critical for the dimerization of DPP-IV (13, 14). Single site mutation in the C-terminal loop disrupts DPP-IV dimer formation (13, 14). Moreover, the DPP-IV dimeric structure is essential for its optimal enzymatic activity. Only a fraction of enzymatic activity is retained by any of the DPP-IV monomers studied so far, whereas the dimeric mutant proteins retain the same activity as that of the wild-type dimer (13, 14). Thus, the enzymatic activity of DPP-IV correlates with its quaternary structure (13, 14). Unlike the classical serine proteases, the prolyl dipeptidases have a conserved and unique Glu-Glu motif.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Fig. 15.

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2 The abbreviations used are: DPP, dipeptidyl peptidase; pNA, p-nitroanilide; AUC, analytical ultracentrifugation; FAP, fibroblast activation protein; HA, hemagglutinin.
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**The in vitro substrate specificity of several prolyl dipeptidases has been investigated with position-scanning combinatorial dipeptide libraries, which become fluorogenic upon cleavage by the protease (17–19).** DPP-IV cleaves regardless of the Pro at the P1 position was found for DPP-IV, FAP, and DPP2. The enzymatic activity (15, 16) of DPP-IV, FAP, and DPP2 is dependent upon the dipeptide library, which becomes fluorogenic upon cleavage by the protease (17–19). A nearly absolute requirement for Ala at the P1 site (3, 4, 20, 21). Using an X-Pro-7-amino-4-methyl-coumarin library, in which Pro is fixed at P1 and X is any amino acid, it was found that X = Ala at the P1 site (3, 4, 20, 21).

**FIGURE 1. DPP-IV structure and the alignment of the C-terminal loop.** A, ribbon representation of homodimeric DPP-IV. The two subunits were colored purple and blue, respectively. B, alignment of the C-terminal loop in the DPP family. The residues mutated in this study (F822A, V833A, Y844A, and H859A) are marked with blue triangles, and the catalytic triad residues of DPP8 (Asp817 and His849) are marked with red stars. The identical or similar residues were highlighted with different colors (Vector NT software). The sequences have the following GenBank accession numbers: NP_001926 (DPP-IV), Q12884 (FAP), NP_001927 (DPP6), AAG29766 (DPP8), AAL47179 (DPP9), and P42658 (DPP10). C, enlarged view of one monomer of DPP-IV with the C-terminal loop in green and the residues involved in substrate binding (S1 pocket) (Val711, Tyr666, Tyr662, Trp659, Val656, and Tyr668) in red, as identified in crystallization studies (9, 11). A and C were drawn with the DeepView Program version 3.7 (available on the World Wide Web at www.expasy.org/spdbv) with the structure of DPP-IV (Protein Data Bank code 1N1M).

**TABLE 1.**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence (residues)</th>
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<tr>
<td>DPP8</td>
<td>(774) ALAFRKPKQTSIQEYQWLKLYRNLGSRFAALKL</td>
</tr>
<tr>
<td>DPP4</td>
<td>(865) ALAFRKPKQTSIQEYQWLKLYRNLGSRFAALKL</td>
</tr>
<tr>
<td>FAP</td>
<td>(595) ALAFRKPKQTSIQEYQWLKLYRNLGSRFAALKL</td>
</tr>
<tr>
<td>DPP6</td>
<td>(890) ALAFRKPKQTSIQEYQWLKLYRNLGSRFAALKL</td>
</tr>
<tr>
<td>DPP9</td>
<td>(765) ALAFRKPKQTSIQEYQWLKLYRNLGSRFAALKL</td>
</tr>
<tr>
<td>DPP10</td>
<td>(751) ALAFRKPKQTSIQEYQWLKLYRNLGSRFAALKL</td>
</tr>
</tbody>
</table>

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**Ala are preferred by DPP2 (18).** Apart from its dipeptidase activity, FAP also has prolyl-cleaving endopeptidase activity, which is absent from DPP-IV and DPP2 (8, 22–24). Compared with DPP-IV and FAP, relatively little is known of DPP8. DPP8 contains 882 amino acids with 27% identity or 51% homology to DPP-IV (25). The expression of DPP8 is up-regulated in stimulated T cells (25). Several potent inhibitors targeting both DPP8 and DPP9 have been discovered by us (26) and by Lankas et al. (27), with an isoidoline core structure at the P1 site, suggesting that the S1 pocket of DPP8 is much bigger than that of DPP-IV (26). Chemical inhibition by an isoidoline-derivative compound results in the attenuation of T-cell activation (27). Moreover, administration of the inhibitor to animals results in severe toxicity and various pathological symptoms (27). In vitro, DPP8 hydrolyzes substrates with proline at the penultimate position (25, 28). The Glu-Glu motif (Glu<sup>256</sup>-Glu<sup>257</sup>) is also present in DPP8 and is necessary for its dipeptidase activity; mutations at this motif result in the inactivation of DPP8 enzymatic activity (16). Unlike the membrane-bound and glycosylated exoproteases DPP-IV and FAP, DPP8 is located in the cytoplasm, with no glycosylation present at the active site (15). This motif is critical for their dipeptidase activity; mutations at the motif abolish this enzymatic activity (15, 16).

**The in vitro substrate specificity of several prolyl dipeptidases has been investigated with position-scanning combinatorial dipeptide libraries, which become fluorogenic upon cleavage by the protease (17–19).** A nearly absolute requirement for Pro at the P1 position was found for DPP-IV, FAP, and DPP2 (18, 19). A weak activity directed against Ala at P1 site has been observed for DPP-IV (18), which is consistent with the observation that several in vivo substrates of DPP-IV, such as glucagon-like peptide-1, glucose-dependent insulinotropic polypeptide, growth hormone-releasing hormone, and peptide histidine methionine, contain Ala at the P1 site (3, 4, 20, 21). Using an X-Pro-7-amino-4-methyl-coumarin library, in which Pro is fixed at P1 and X is any amino acid, it was found that DPP-IV, FAP, and DPP2 have slightly different substrate preferences at the P2 site (18, 19). DPP-IV cleaves regardless of the residues at the P2 site, with a preference for hydrophobic > basic > neutral > acidic residues (2, 18). On the contrary, Ile, Pro, and Arg are preferred by FAP (19), whereas Lys, Met, and detected on DPP8 protein purified from baculovirus-infected insect cells (25, 28). DPP8 was found to be monomeric when prolyl dipeptidase activity; mutations at this motif result in the inactivation of DPP8 enzymatic activity (16). Unlike the membrane-bound and glycosylated exoproteases DPP-IV and FAP, DPP8 is located in the cytoplasm, with no glycosylation...
**EXPERIMENTAL PROCEDURES**

**Materials**—The chromogenic dipeptide substrates were purchased from Bachem. X-Pro-pNA and Ala-X-pNA libraries, in which X is any amino acid, were custom synthesized by GL Biochem Ltd. (Shanghai, China). Fetal bovine serum was purchased from Hyclone. The nickel affinity column was from Novagen. Lipofectin and insect culture media (Grace’s medium and Express Five medium) were from Invitrogen. The human liver cDNA library and linear viral vector were from Clontech. The Western detection kit Western Lightning™ was from PerkinElmer Life Sciences. Q-Sepharose™ High Performance was from Amersham Biosciences.

**Plasmid Construction**—Oligonucleotides containing the sequence encoding the His tag and the factor Xa cleavage site (5’-GGATCCATGGGCACACCATCATCATCAGCGCGGGATTTGAAGTGCTCTAGA-3’ and 5’-TCTAGAGCCACCTTACATCCGCTTGAAGTCATGATGATGATGGTGTCCCATGGATCC-3’) were annealed and cloned into pTOPO-II vector (Invitrogen), producing the plasmid pTOPO-MHF. The full-length cDNA of human DPP8 was amplified by PCR from a human liver cDNA library (Clontech) with 5’TCTAGAGCT- and 5’-CATGGATCC-3’ primers, producing the plasmid pTOPO-MHF. The estimated molecular mass of this tagged DPP8 is 103.4 kDa. Site-directed mutagenesis of DPP8 was performed as described previously (13). The primers used were 5’-GAGAATGTCCATGCTGCATCATCATCATCACGGCGGGATTTGAAGTGCTCTAGA-3’ and 5’-GGCTTTCAAGCTTTTATCATCATTAGAGCGCAGC-3’ and cloned into the XbaI and NotI sites of pTOPO-MHF, producing pTOPO-MHF-DPP8. The BamHI-NotI fragment of pTOPO-MHF-DPP8 was further subcloned into the BamHI and NotI sites of pBAC-PAK8 (28), generating plasmid pBAC-MHF-DPP8 for the expression of DPP8 protein in baculovirus-infected insect cells. The estimated molecular mass of this tagged DPP8 is 103.4 kDa. Site-directed mutagenesis of DPP8 was performed as described previously (13).

**Native Gel Electrophoresis and Western Blot Analysis**—Plasmids pEF-DPP8, pEF-HA-DPP8, or the vector-only control were transfected into the human 293T embryonic kidney cell line with the calcium phosphate method in 6-well plates. After transfection, 10° cells were lysed with 100 μl of buffer containing 50 mm Hepes-KOH (pH 7.4), 150 mm NaCl, 1% Triton X-100, and protein inhibitor mixture (Roche Applied Science). The cell lysate was cleared by centrifugation and mixed with loading buffer (final composition of the loading buffer: 50 mm Tris/HCl, pH 6.8, 0.1% bromphenol blue, and 10% glycerol). The lysate was fractionated on 8% polyacrylamide gel. The cell lysate was cleared by centrifugation and mixed with loading buffer (final composition of the loading buffer: 50 mm Tris/HCl, pH 6.8, 0.1% bromphenol blue, and 10% glycerol). The loaded samples were centrifuged at 15,000 g for 10 min at 4 °C versus R plot

**Prolyl Dipeptidase DPP8**

To examine the subunit composition of DPP8 inside the cells, we constructed an expression plasmid for the expression of DPP8 in mammalian 293T cells. The DPP8 cDNA fragment was released from pTOPO-MHF-DPP8 by PstI digestion, blunt-ended with T4 DNA polymerase, and further digested with XbaI. The PstI (blunted)-XbaI fragment was then ligated into pcDNA3-hemagglutinin (pcDNA3-HA) at the Apal (blunted) and XbaI sites. The HA-DPP8 fragment was released from pcDNA-HA-DPP8 by digestion with HindIII and NotI, before ligation into the pEF-SCM vector (30), to generate pEF-HA-DPP8. Digestion with HindIII and self-ligation resulted in pEF-DPP8 without the HA tag.

**Insect Cell Culture, DNA Transfection, Virus Selection, and Amplification**—Sf9 cells were grown in Grace’s medium supplemented with 10% fetal bovine serum at 27 °C. The transfection of DNA into Sf9 cells and the selection and amplification of the recombinant viruses were carried out as described previously (28). Sf9 cells were infected at a multiplicity of infection of 1, determined to be the optimal condition for protein expression, as described previously (28), and the cells were harvested 72 h after infection.

**Purification of DPP8 Protein**—Recombinant DPP8s were purified as described previously, with modifications (28). The insect cell pellets were resuspended and sonicated in buffer A (50 mm sodium phosphate, pH 7.5, and 0.5 mm NaCl). The cell lysate was cleared by centrifugation and filtration through a 0.45-μm filter before it was applied to a nickel-nitrioltriacetic acid affinity column. The column was equilibrated and washed with 10 bed volumes of buffer A, followed sequentially by 10 bed volumes of buffer B containing 20 mm and then 40 mm imidazole. Finally, the proteins were eluted with buffer C containing 250 mm imidazole. The eluate of the eluate was changed to buffer B, containing 50 mm Tris (pH 8.0), before it was applied to a Q-Sepharose column. The DPP8 proteins were eluted with a gradient of 0 to 0.5 m NaCl in buffer B. Gel filtration chromatography was performed with a Superdex 200 HR column, as described previously (13).

**Sedimentation Velocity and Equilibrium Analysis**—The sedimentation velocity experiment was performed using a Beckman-Coulter XL-A analytical ultracentrifuge with an An60Ti rotor at 40,000 rpm at 20 °C with standard double-sector aluminum centerpieces as described previously (13, 14, 31). Concentrations of DPP8s around 0.1–0.2 mg/ml (1.2–2.3 mg/ml) were used. The UV absorption of the scans was scanned every 10 min for 4 h. Sedimentation equilibrium experiments were performed with six-channel epon charcoal-filled centerpieces, as described previously (31, 32). The loaded samples were centrifuged at speeds of 6,000, 9,000, 12,000, and 15,000 rpm, each for 14–18 h at 20 °C. In our study, DPP8 proteins achieved an equilibrium state after 14 h. At each channel, a single base-line parameter was included as a floating parameter common to all rotor speeds. The time invariant and radial invariant noise were also fitted for the better fitting quality. The scans at different rotor speeds were then globally fitted to a noninteracting discrete species model, as described previously (31, 32). The Ultracent 7.2 program was used to calculate the In C versus R² plot (33). Multiple scans at different time points were also fitted to a continuous size distribution model using the SEDFIT program.
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(Version 9.3) to calculate the molecular weights and sedimentation coefficients. The Sednterp program (version 1.07) was used to calculate solvent density, viscosity, Stokes’ radius ($R_s$), and the anhydrous frictional ratio ($f/f_o$).

**Determination of Substrate Specificities and Kinetic Constants of DPP8s**: DPP-IV protein was purified as described previously (13). For substrate scanning experiments, purified DPP8 (80 nM) or DPP-IV (20 nM) proteins were incubated in phosphate-buffered saline with 10 µM individual chromogenic substrate X-Pro-pNA or Ala-X-pNA, where X is any of the 20 amino acids. The initial rate was measured by monitoring the absorbance at 405 nm for the first 3–5 min.

The kinetic constants were determined as described previously using freshly purified enzymes (13). For Ala-X-pNA series of the substrates, except Ala-Pro-pNA, the concentration of wild type DPP8 used was 0.5 µM. For X-Pro-pNA series of the substrates, except Asp-Pro-pNA, the enzyme concentrations used were 0.01 µM for wild-type DPP-IV, 0.005–0.01 µM for wild-type DPP8, 0.02–0.05 µM for H859A, 0.4 µM for V833A, 0.2–1 µM for F822A, and 1 µM for Y844A, respectively. For measurements against Asp-Pro-pNA, 0.2, 0.5, and 1 µM DPP8, H859A, and F822A, respectively, were used. The initial rate was measured with less than 10% substrate depletion for the first 10–300 s. The steady-state parameters, $k_{cat}$ and $K_m$, were determined from initial velocity measurements at 0.5–5 $K_m$ of the substrate concentration. The kinetic parameters were analyzed using nonlinear regression of the Michaelis-Menten equation. Correlation coefficients better than 0.99 were obtained throughout.

**RESULTS**

**Human DPP8 Protein Is Predominantly Dimeric**: We had engineered a factor Xa cleavage site between the amino-terminal His tag and the DPP8 coding sequence and expressed and purified DPP8 proteins from baculovirus-infected insect cells (see “Experimental Procedures”). No differences were observed in the biochemical properties, including the quaternary structures and enzymatic activity, of this version of DPP8 and that previously described with only a His tag (data not shown) (28).

DPP8 protein was purified to homogeneity with a molecular mass of 105 kDa on SDS-PAGE (Fig. 2A, lane 1), which is consistent with its calculated molecular mass (estimated molecular mass of tagged DPP8 is 103.4 kDa). The sedimentation velocity and sedimentation equilibrium, measured by AUC, were used to determine the quaternary structures of purified DPP8 (Fig. 3 and Table 1). DPP8 was dimeric, with a molecular mass of 210 kDa, a Stokes’ radius of 5.8 nm, and a sedimentation coefficient of 8.6 S (Table 1). A small fraction of tetramer (11%) was present in the purified proteins (Fig. 3A), with a molecular mass of 427 kDa, a Stokes’ radius of 7.9 nm, and a sedimentation coefficient of 12.9 S (Table 1). The $f/f_o$ ratios of both the dimeric and tetrameric proteins were 1.5, indicating that the protein is globular but not spherical (Table 1). A tiny amount of monomeric DPP8 was also detected (Fig. 3A). It was unfolded and non-globular because the Stokes’ radius was unusually long (7.5 nm), with an $f/f_o$ ratio of 2.4 (data not shown). The high fitting quality of the AUC experiment was demonstrated in Fig. 3B and supplementary Fig. 1S. Therefore, purified DPP8 exists predominantly in the dimeric form with a small quantity of tetramers present.

DPP8 is known to be a cytoplasmic protein (25). Next, we set out to determine the oligomeric state of DPP8 inside the cells using native gel electrophoresis coupled to Western blot analysis. Native gel electrophoresis was used previously to determine the subunit composition of DPP-IV (13). A DPP8-specific antibody that specifically recognizes purified DPP8 proteins in Western blot analysis was generated in house for this purpose (see “Experimental Procedures”) (Fig. 2B, lanes 1 and 4). Purified DPP8 without boiling ran at mainly two positions (Fig. 2B, lane 1); the lower band corresponds to the dimeric form, and the upper band corresponds to the tetrameric form based on its relative quantity shown in AUC. After being boiled, DPP8 protein ran faster at the position different from the unboiled...
FIGURE 3. Purified DPP8 is dimeric determined by AUC. A, sedimentation velocity analysis of DPP8 with AUC. The three panels represent the trace of absorbance at 280 nm during sedimentation, the residues of model fitting, and the sedimentation coefficient distribution for all species. B, sedimentation equilibrium analysis of DPP8 with AUC. Centrifugation rates for the equilibrium experiments were 6,000 rpm (circle), 9,000 rpm (triangle), 12,000 rpm (square), and 15,000 rpm (diamond). The solid lines are the best fit distributions from global analysis to a noninteracting discrete species model, assuming a single species, drawn with SEDPHAT. The residual of the fit is shown below the panels and has a local root mean square error of 0.0082.
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Single Site Mutations in the C-terminal Loop Inactivate DPP8—Previously, we have shown that several hydrophobic and hydrophilic residues in the highly conserved C-terminal loop are important for DPP-IV dimerization and enzymatic activity (13, 14). Because of the DPP sequence homology in the region, we investigated whether the corresponding residues of DPP8 are also critical for its enzymatic activity (Fig. 1B). We chose three hydrophobic and one hydrophilic residues, shown previously to impart differential effects on DPP-IV dimerization and enzymatic activity (13, 14), and mutated them to Ala in the wild-type DPP8 (Fig. 2A, lanes 2–5). Compared with the vector-only control, DPP8 proteins inside the cells ran at a position corresponding to the dimeric form of the protein (Fig. 2B, lanes 2 and 3). HA-tagged DPP8 also ran at the same position (Fig. 2B, lane 5), indicating that tagging at the N terminus did not affect the quaternary structure of DPP8. Combined this and the AUC study, we concluded that DPP8 exists predominantly as a dimer inside the cell and tagging at the amino terminus did not affect its subunit composition.

TABLE 2

Hydrodynamic properties of wild-type and mutant DPP8s

<table>
<thead>
<tr>
<th></th>
<th>Wild-type dimer</th>
<th>Wild-type tetramer</th>
<th>F822A dimer</th>
<th>V833A dimer</th>
<th>Y844A dimer</th>
<th>H859A dimer</th>
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<tr>
<td>Stokes radius ($R_g$) (nm)</td>
<td>5.8</td>
<td>7.9</td>
<td>5.9</td>
<td>5.9</td>
<td>6.3</td>
<td>5.9</td>
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<td>Sedimentation coefficient ($s_{20, w}$) (S)</td>
<td>8.6</td>
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<td>8.2</td>
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<tr>
<td>Molecular mass (kDa)</td>
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<td>427</td>
<td>220</td>
<td>220</td>
<td>218</td>
<td>213</td>
</tr>
<tr>
<td>Anhydrous frictional ratio ($f/f_o$)</td>
<td>1.5</td>
<td>1.6</td>
<td>1.5</td>
<td>1.5</td>
<td>1.6</td>
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</table>

* The calculated molecular mass of tagged monomeric DPP8 is 103.4 kDa.

Substrate Specificity of DPP8 at P1 and P2 Sites—Unlike the corresponding mutations of DPP-IV (13, 14), single-site mutations of DPP8 resulted in unique and dramatic increments in $K_m$ values (Table 2). Whether this $K_m$ effect is dependent on the substrate assayed remains unclear. The substrate preference of DPP8 at the P1 and P2 sites, as defined by Berger and Schechter (34), has not been investigated across the full spectrum of amino acids. Only a handful of substrates from different laboratories have been assayed previously (28, 29), making it difficult to draw any conclusion. Therefore, we first scanned the substrate specificity of DPP8 using a custom-synthesized positional scanning library, which is a powerful tool for determining the substrate specificities of proteases (17). The library consists of Ala-$X$-$p$NA and X-Pro-$p$NA, where $X$ is any one of the 20 amino acids. Upon cleavage by the protease, chromogenic $p$NA is detectable by its absorbance at 405 nm ($A_{405}$).

Using the Ala-$X$-$p$NA library, we determined that DPP8 prefers Pro at the P1 position (Fig. 5A), like DPP-IV (Fig. 5B). Notably, the activity of DPP8 against Ala-$X$-$p$NA was lower than that of DPP-IV, which is consistent with the measurement of their kinetic constants (Fig. 5, $y$ axis, and Table 2) (28). Based on this initial scanning, we measured the kinetic constants against substrates with Ala, Thr, Met, Trp, or Gly at the P1 position. The DPP8 activities against these substrates were less than 0.4% to the optimal enzymatic activity of DPP8. Depending upon the mutation site, $K_m$ and $k_{cat}$ values are affected to different extents.

Inactive DPP8 Proteins Remain Dimeric—To determine whether these decreases in enzymatic activity are due to changes in the quaternary structure like DPP-IV, we analyzed the quaternary structures of these mutant proteases by both sedimentation equilibrium and sedimentation velocity analyses with AUC. To our surprise, all mutant proteases remained dimeric (Fig. 4, B–E). There was no change in molecular mass, Stokes’ radius, or sedimentation coefficient compared with those of the wild-type DPP8 protease, in any of the mutant proteins studied. All mutant proteins had a molecular mass of 213–220 kDa, a Stokes’ radius of 5.9–6.3 nm, and a sedimentation coefficient of 8.2–8.8 S, which are typical of dimeric DPP8 (Table 1). The frictional ratios for these dimeric mutants were about 1.5, indicative of their nonspherical globular structure. Interestingly, these mutant proteases also contained small amounts of tetramers with sedimentation coefficients of about 13 S (Fig. 4, B–E, and data not shown). The percentages of the tetramers present for wild type DPP8, F822A, V833A, Y844A, and H859A were 11, 17, 15, 8.5, and 7.4%, respectively. The monomers observed were unfolded nonglobular proteins, similar to the structure of monomeric wild-type DPP8 (data not shown).

Substrate Specificity of DPP8 against the P1 and P2 Sites—Unlike the corresponding mutations of DPP-IV (13, 14), single-site mutations of DPP8 resulted in unique and dramatic increments in $K_m$ values (Table 2). Whether this $K_m$ effect is dependent on the substrate assayed remains unclear. The substrate preference of DPP8 at the P1 and P2 sites, as defined by Berger and Schechter (34), has not been investigated across the full spectrum of amino acids. Only a handful of substrates from different laboratories have been assayed previously (28, 29), making it difficult to draw any conclusion. Therefore, we first scanned the substrate specificity of DPP8 using a custom-synthesized positional scanning library, which is a powerful tool for determining the substrate specificities of proteases (17). The library consists of Ala-$X$-$p$NA and X-Pro-$p$NA, where $X$ is any one of the 20 amino acids. Upon cleavage by the protease, chromogenic $p$NA is detectable by its absorbance at 405 nm ($A_{405}$).

Using the Ala-$X$-$p$NA library, we determined that DPP8 prefers Pro at the P1 position (Fig. 5A), like DPP-IV (Fig. 5B). Notably, the activity of DPP8 against Ala-$X$-$p$NA was lower than that of DPP-IV, which is consistent with the measurement of their kinetic constants (Fig. 5, $y$ axis, and Table 2) (28). Based on this initial scanning, we measured the kinetic constants against substrates with Ala, Thr, Met, Trp, or Gly at the P1 position. The DPP8 activities against these substrates were less than 0.4%
of that against Ala-Pro-pNA (supplemental Table 1S). For Ala-Ala-pNA, Ala-Thr-pNA, and Ala-Met-pNA, the \( k_{\text{cat}} \) values decreased from 27- to 717-fold, whereas the \( K_m \) values increased from 2- to 11-fold. No activity was detected with Trp or Gly at the P1 position under these assay conditions. Overall, the substrate preference of DPP8 at P1, as quantified by \( k_{\text{cat}}/K_m \) values, is Pro >> Ala/Met/Thr >> Trp/Gly. Therefore, DPP8 prefers Pro at the P1 position substantially more than any other amino acid. The DPP8 activity against Ala-Ala-pNA was roughly similar to that against Asp-Pro-pNA, the least preferred of all the X-Pro-pNA substrates assayed (see below; Table 3).

Next, we investigated the substrate preference of DPP8 at the P2 position, with Pro fixed at P1. As shown in Fig. 5C, the residues most favored by DPP8 at the P2 site were hydrophobic residues, and the least favored were acidic residues, which is similar but not identical to the substrate preference of DPP-IV (Fig. 5D). The kinetic constants for a selected group of the substrates, including small (Ala, Gly, and Ser), hydrophobic (Leu and Met), basic (Arg and Lys), and acidic (Asp) residues at the P2 position, were measured (Table 3). To our knowledge, this represents one of the most comprehensive kinetic studies of DPP8 (28, 29) using chromogenic substrates. According to the \( k_{\text{cat}}/K_m \) values, Asp-Pro is the least preferred substrate for both DPP8 and DPP-IV, with \( k_{\text{cat}}/K_m \) values of 0.3 and 227 mM s\(^{-1}\), respectively. Interestingly, there was a difference of over 1,700-fold selectivity between the most preferred (the hydrophobic residues Leu and Met or basic residues Arg and Lys at the P2 position) and the least preferred (Asp-Pro) substrates of DPP8, whereas there was only an 11-fold difference between the most preferred (Ala-Pro) and the least preferred (Asp-Pro) substrates of DPP-IV (Table 3). Strikingly and interestingly, these preferred substrates of DPP8 also had the lowest \( K_m \) values (around 16–30 \( \mu \)M), making this finding physiologically relevant. In contrast, DPP-IV did not have such a distinct selectivity at the P2 site, readily and indiscriminately cleaving all the substrates tested (Table 3). In fact, there was only a 4-fold difference in both the \( K_m \) (30 \( \mu \)M against Ala, Leu, and Met versus 130 \( \mu \)M against Gly, Lys, and Asp), and \( k_{\text{cat}} \) values (29 s\(^{-1}\) against Asp versus 108 s\(^{-1}\) against Arg and Lys) (Table 3). This suggests that DPP-IV cleaves even the acidic residues at P2 indiscriminately, whereas DPP8 does not. Therefore, the overall preference of DPP8 at the P2 site, as supported by the highest \( k_{\text{cat}}/K_m \) and the lowest \( K_m \) values, is Arg/Met/Leu/Lys >> Ala/Ser/Gly >> Asp, with strict selectivity for hydrophobic and basic residues at the P2 site. We have demonstrated for the first time that DPP8 is a much more discriminating enzyme with regard to its substrates than is DPP-IV, since it has strict selectivity for hydrophobic and basic residues at the P2 site.

**Effects of Mutations on \( K_m \) Are Independent of the Substrate Assayed**—Next, we determined whether the effects of the mutations on \( K_m \) are contributed by the substrate assayed. Two mutant DPP8 proteins with differential effects on \( K_m \) and \( k_{\text{cat}} \), F822A and H859A, were studied by measuring their kinetic constants (Table 3). Consistent with the conclusions drawn with Ala-Pro-pNA (Table 2) and irrespective of the substrates assayed, H859A mainly affected the \( K_m \) values, with less effect on the \( k_{\text{cat}} \) values, whereas F822A affected both \( K_m \) and \( k_{\text{cat}} \) dramatically. For example, the \( K_m \) values against Leu-Pro-pNA increased to 348 and 390 \( \mu \)M for H859A and F822A, respectively, compared with 23 \( \mu \)M for wild-type DPP8 (Table 3). Therefore, for each different substrate assayed, the \( K_m \) values increased unanimously for both mutant proteins relative to that of wild-type DPP8. This indicates that the amino acid residues and not the substrates are the deciding factors in the decrease in mutant enzyme activity. Moreover, both mutant proteins retained substrate preferences at the P2 site similar to that of wild-type DPP8, with Met-Pro and Leu-Pro being the most potent substrates and Asp-Pro the least potent substrate. Therefore, irrespective of the substrate assayed, both the H859A and F822A mutations resulted in a loss of enzymatic activity but affected \( K_m \) and \( k_{\text{cat}} \) to different extents. These data demonstrate that the decrease in enzymatic activity results solely from the interface mutation, independent of the substrate assayed.
Prolyl Dipeptidase DPP8

DISCUSSION

Because of their unique proline-cleaving properties, the prolyl dipeptidases, including DPP-IV, FAP, DPP8, DPP9, and DPP2, are exciting new targets that have attracted intensive study in recent years. Whereas the function of DPP8 in vivo requires further investigation, the intriguing observation that DPP8/9 inhibitors induce severe toxicity and pathological symptoms in animals (27) makes it urgent and important to understand the structure and functional relationships of DPP8. In this study, we determined the quaternary structure of DPP8, identified the amino acid residues essential for its optimal enzymatic activity, explored its substrate specificity, and investigated the relationship between its quaternary structure and enzymatic activity. Our work revealed significant differences in the biochemical properties of DPP8 and those of the homologous enzyme DPP-IV.

We have demonstrated, for the first time, that an intact dimer interface is absolutely essential for the optimal enzymatic activity of DPP8. Unlike DPP-IV (13, 14), DPP8 dimerization is not sufficient for its optimal enzymatic activity, indicating that the interplay between the enzymatic activity and quaternary structure is distinctly different from that of DPP-IV. The dimeric mutant DPP8 enzymes have little enzymatic activity, and not only is $k_{cat}$ affected but also $K_m$ (Tables 2 and 3). The $K_m$ effect is unique to DPP8, because all the mutations at the dimer interface of DPP-IV studied so far do not affect the $K_m$ values at all (13, 14). We have also shown that this $K_m$ effect is independent of the substrates assayed (Table 3). This conclusively demonstrates that the amino acid residues at the interface,

![Figure 5](image_url)

**Figure 5.** Substrate specificity of human DPP8 proteins determined with positional scanning dipeptide libraries. A and B, screening against the Ala-X-pNA library, with DPP8 (A) and DPP-IV (B), respectively. X is indicated in single-letter code on the x axis. C and D, screening against the X-Pro-pNA library with DPP8 (A) and DPP-IV (B), respectively. Data in each column were calculated from at least three independent measurements. Some error bars are too small to show.

| TABLE 3 |

| Kinetic constants of DPP8s and DPP-IV against X-Pro-pNA |

The experiments were carried out as described under "Experimental Procedures." Amino acids are listed as single-letter code. Data were calculated from four independent measurements with two different batches of enzymes.

<table>
<thead>
<tr>
<th>P2-P1</th>
<th>DPP8 wild type</th>
<th>K_m $\mu M$</th>
<th>k_cat $s^{-1}$</th>
<th>k_cat/K_m</th>
<th>P2-P1</th>
<th>DPP-IV wild type</th>
<th>K_m $\mu M$</th>
<th>k_cat $s^{-1}$</th>
<th>k_cat/K_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-P</td>
<td>127 ± 7</td>
<td>22 ± 0.3</td>
<td>173 ± 7</td>
<td></td>
<td></td>
<td>30 ± 6</td>
<td>72 ± 4</td>
<td>2,430 ± 320</td>
<td></td>
</tr>
<tr>
<td>G-P</td>
<td>528 ± 38</td>
<td>25 ± 0.7</td>
<td>47 ± 2.0</td>
<td></td>
<td></td>
<td>129 ± 14</td>
<td>102 ± 9</td>
<td>787 ± 19</td>
<td></td>
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<tr>
<td>S-P</td>
<td>238 ± 42</td>
<td>19 ± 1.1</td>
<td>80 ± 9</td>
<td></td>
<td></td>
<td>75 ± 0.4</td>
<td>73 ± 0.1</td>
<td>977 ± 4</td>
<td></td>
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<tr>
<td>L-P</td>
<td>23 ± 4</td>
<td>7.0 ± 0.4</td>
<td>308 ± 30</td>
<td></td>
<td></td>
<td>32 ± 2</td>
<td>61 ± 0.6</td>
<td>1,895 ± 119</td>
<td></td>
</tr>
<tr>
<td>M-P</td>
<td>29 ± 1</td>
<td>15 ± 0.01</td>
<td>520 ± 25</td>
<td></td>
<td></td>
<td>41 ± 4</td>
<td>54 ± 0.3</td>
<td>1,320 ± 122</td>
<td></td>
</tr>
<tr>
<td>R-P</td>
<td>27 ± 1</td>
<td>14 ± 0.1</td>
<td>524 ± 03</td>
<td></td>
<td></td>
<td>62 ± 3</td>
<td>108 ± 0.3</td>
<td>1,313 ± 43</td>
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<tr>
<td>K-P</td>
<td>16 ± 4</td>
<td>3.2 ± 0.01</td>
<td>201 ± 54</td>
<td></td>
<td></td>
<td>125 ± 11</td>
<td>107 ± 5</td>
<td>856 ± 14</td>
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<tr>
<td>D-P</td>
<td>2,668 ± 331</td>
<td>0.9 ± 0.1</td>
<td>0.3 ± 0.01</td>
<td></td>
<td></td>
<td>128 ± 7</td>
<td>29 ± 0.5</td>
<td>227 ± 9</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>P2-P1</th>
<th>DPP8 H859A</th>
<th>K_m $\mu M$</th>
<th>k_cat $s^{-1}$</th>
<th>k_cat/K_m</th>
<th>P2-P1</th>
<th>DPP8 F822A</th>
<th>K_m $\mu M$</th>
<th>k_cat $s^{-1}$</th>
<th>k_cat/K_m</th>
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<td>2,720 ± 85</td>
<td>8.0 ± 0.2</td>
<td>3 ± 0.01</td>
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<td>1,957 ± 83</td>
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<tr>
<td>G-P</td>
<td>2,731 ± 483</td>
<td>1.4 ± 0.07</td>
<td>0.6 ± 0.06</td>
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<td></td>
<td>4,599 ± 199</td>
<td>0.2 ± 0.07</td>
<td>0.05 ± 0.006</td>
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<tr>
<td>S-P</td>
<td>7,462 ± 519</td>
<td>13.8 ± 0.27</td>
<td>1.8 ± 0.02</td>
<td></td>
<td></td>
<td>2,249 ± 118</td>
<td>0.1 ± 0.003</td>
<td>0.04 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>L-P</td>
<td>348 ± 36</td>
<td>3.8 ± 0.2</td>
<td>11 ± 0.5</td>
<td></td>
<td></td>
<td>390 ± 5</td>
<td>0.1 ± 0.007</td>
<td>0.2 ± 0.02</td>
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</tr>
<tr>
<td>M-P</td>
<td>325 ± 31</td>
<td>3.0 ± 0.2</td>
<td>9 ± 0.2</td>
<td></td>
<td></td>
<td>988 ± 96</td>
<td>0.3 ± 0.02</td>
<td>0.3 ± 0.01</td>
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<tr>
<td>R-P</td>
<td>270 ± 21</td>
<td>1.2 ± 0.1</td>
<td>4 ± 0.2</td>
<td></td>
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<td>1,673 ± 6</td>
<td>0.4 ± 0.01</td>
<td>0.01 ± 0.003</td>
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<tr>
<td>K-P</td>
<td>507 ± 115</td>
<td>2.4 ± 0.3</td>
<td>5 ± 0.5</td>
<td></td>
<td></td>
<td>1,358 ± 214</td>
<td>0.2 ± 0.01</td>
<td>0.1 ± 0.008</td>
<td></td>
</tr>
<tr>
<td>D-P</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

*ND, not detectable for the enzymatic activity under the assay condition described under “Experimental Procedures.”
including Phe^{822}, Val^{833}, Tyr^{844}, and His^{859}, are essential for the optimal enzymatic activity of DPP8. The effect of the mutations on $k_{\text{cat}}$ is consistent with the fact that two of the catalytic triads of DPP8 (Asp^{817} and His^{849}) are nearby or in the C-terminal loop (Fig. 1B). Thus, the mutations might affect the position and orientation of the catalytic triad, resulting in the decrease in $k_{\text{cat}}$. The effects of the mutations on $K_m$ are likely to be because the C-terminal loop of DPP8 is not only involved in its dimerization, like that of DPP-IV, but it also interacts directly with the substrate or is part of the S1 pocket. Although the C-terminal loop of DPP-IV is very close to the S1 pocket, it is not involved in substrate binding (Fig. 1C). The S1 site of DPP8 might be bigger than that of DPP-IV, as demonstrated in our previous study by its preference for isoidoline rather than a five-ring structure at the S1 site (26). Whether the C-terminal loop of DPP8 is directly involved in its interaction with substrates must be confirmed by crystallographic analysis of the protein complexed with its substrates/inhibitors. Our findings regarding DPP8 in this study are probably also applicable to DPP9 because of the high sequence homology between these two proteins.

Another striking discovery is the strict and distinctive substrate selectivity of DPP8 for hydrophobic and basic residues at the P2 site (Table 3). This preference is significantly different from that of DPP-IV, which cleaves indiscriminately at P2, as demonstrated with chromogenic substrates and substrates in vivo, including chemokines, hormones, and neuropeptides (3, 4, 20, 21, 35). DPP8 is localized in the cytoplasm. The cellular substrates of DPP8 are unknown but are expected to differ from those of DPP-IV. Under biological conditions, where all substrates are at low concentrations, DPP8 would preferentially cleave the substrates with the lowest $K_m$ values (i.e., hydrophobic residues or basic residues at the P2 site) (Table 3). More importantly, the $K_m$ values (16–30 $\mu$M) against these preferred DPP8 substrates are within the range reported for glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide (4.5–39 $\mu$M) (20, 21), the two validated substrates of DPP8. The catalytic efficiency of DPP8 against these preferred substrates is on the order of $10^{-5}$–$10^{-6}$ M$^{-1}$ s$^{-1}$ (Table 3), which is meaningful and significant under physiological conditions, as supported by previous studies of the physiological substrates of DPP-IV (3, 4, 20, 21, 35). Thus, the substrate specificity of DPP8 explored in this study is important for the discovery of the biological substrates of DPP8, which will allow us to understand its biological function. It will also facilitate the discovery of therapeutically potent and selective DPP8 inhibitors, possibly through a designed interaction between the bulky hydrophobic or basic moieties at the P2 site with the S2 pocket of DPP8. The development of potent and selective DPP8 inhibitors will be useful for probing and understanding its biological roles in vivo.

Our study has extended the functional importance of the C-terminal loop to the whole family of prolyl dipeptidases. It is highly likely that DPP8 assumes a topology and configuration similar to those of DPP-IV, FAP, and DPP6 (8–11, 36), with the C-terminal loop and the propeller loop forming the dimer interface (Fig. 1A). Notably, we have discovered that DPP8 contains detectable amounts of the tetrameric form. Interestingly, the crystal structure of porcine DPP-IV is also tetrameric, forming a dimer of dimers (9). Its propeller domain (Asn^{279}–Gln^{286}) interacts with the same area on the other monomer, with a 222 symmetric assembly (9), and with adenosine deaminase (through Ile^{287}–Asp^{297} and Asp^{331}–Gln^{344} (37). Because of the similarity in their sequences and quaternary structures, we speculate that the tetrameric form of DPP8 might be a dimer of dimers, assembled through an interaction between the propeller domains of dimeric DPP8, similar to that of porcine DPP-IV (9). Thus, the propeller domain of the prolyl dipeptidases might be a general protein-protein interaction module. Apart from its role in forming tetramers, other cellular proteins might interact with the propeller domain of DPP8 to regulate its biological function in vivo. The C-terminal loop is critical for the enzymatic activity and/or the quaternary structure of both DPP-IV (13, 14) and DPP8 (this study). This may explain why the addition of either V5, His, or green fluorescent protein tag to the C-terminal region of DPP8, DPP9, or FAP inactivates the protease, produces only a monomeric form, or inhibits the expression of the protein inside the cell (16, 25, 29, 38).

Our systematic approach to determining the structure, substrate specificity, and regulatory mechanisms of the enzymatic activity of DPP8 will facilitate the identification of its in vivo substrates, which will help us to understand its function in vivo and accelerate the discovery of potent and selective chemical inhibitors of both DPP-IV and DPP8.

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Prolyl Dipeptidase DPP8


Investigation of the Dimer Interface and Substrate Specificity of Prolyl Dipeptidase DPP8
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Gu-Gang Chang and Xin Chen

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