The X-ray Crystal Structure of Human Aminopeptidase N Reveals a Novel Dimer and the Basis for Peptide Processing⁎‡§

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Abstract: Human aminopeptidase N (hAPN) is a dimeric cell surface protease involved in peptide processing, cell adhesion, endocytosis, and signal transduction. Crystal structures of peptide and inhibitor complexes were determined. Unlike other family members, hAPN shows substrate-dependent loop ordering and a novel dimer structure. A model for catalysis and conformational changes provides mechanistic insights into how hAPN mediates its functional roles.

Human aminopeptidase N (hAPN/hCD13) is a dimeric membrane protein and a member of the M1 family of zinc metallopeptidases. Within the rennin-angiotensin system, its enzymatic activity is responsible for processing peptide hormones angiotensin III and IV. In addition, hAPN is also involved in cell adhesion, endocytosis, and signal transduction and it is an important target for cancer therapy. Reported here are the high resolution x-ray crystal structures of the dimeric ectodomain of hAPN and its complexes with angiotensin IV and the peptidomimetic inhibitors, amastatin and bestatin. Each monomer of the dimer is found in what has been termed the closed form in other M1 enzymes and each monomer is characterized by an internal cavity surrounding the catalytic site as well as a unique substrate/inhibitor-dependent loop ordering, which in the case of the bestatin complex suggests a new route to inhibitor design. The hAPN structure provides the first example of a dimeric M1 family member and the observed structural features, in conjunction with a model for the open form, provide novel insights into the mechanism of peptide processing and signal transduction.

Aminopeptidase N (APN)† (also known as CD13) is a cell surface membrane protein that plays important roles in a wide range of normal physiological functions including the processing of peptide hormones, such as angiotensin III and IV (1), neuropeptides important in pain (2), and chemokines involved in inflammation and angiogenesis (3). APN is also known to mediate cell adhesion and endocytosis (4–7), it is involved in cancer progression (1, 8–10), and it serves as receptor for various mammalian coronaviruses (11, 12). Although typically described as a cell surface dimer, APN is also found as monomers on the cell surface (13) and a soluble form of the ectodomain constitutes a major component of its activity in blood (14).

APN functions in the rennin-angiotensin system to remove the N-terminal arginine residue from the peptide hormone angiotensin III (AngIII; RVYIHPF) to generate angiotensin IV (AngIV; VYIHPF). It can further degrade AngIV into smaller peptides although the physiological role, if any, of these degraded forms is unknown (7). AngIII is the main effector in the brain rennin-angiotensin system for vasopressin release (15), whereas AngIV has been shown to cause vasodilatation, hypertrophy, and activation of NF-κB, and it is involved in memory (16–18). The rennin-angiotensin system is a multi-component system of peptide hormones and signaling receptors important in blood pressure regulation and electrolyte balance and there is now much evidence to support the fact that it is dysregulated during malignancy (1). Direct support for the role of APN in cancer stems from work done with the APN knock-out mouse and cancer models showing that up-regulation of APN promotes angiogenesis, tumor growth, and metastasis (8–10). Because of its overexpression on tumor cells (19–21), human APN (hAPN) has been targeted for the development of anti-cancer therapeutics (22). The hAPN inhibitor bestatin, for example, has been shown to increase the survival rates of post-operative cancer patients (23) and hAPN-specific cyclic peptides containing the Asn-Gly-Arg (NGR) motif are being developed as a means of targeting tumor cells (24–28).

hAPN (EC 3.4.11.2) is a member of the M1 family of aminopeptidases, zinc metallopeptidases represented in all kingdoms of life. Members of the family fall into two structural categories with either a three- or four-domain organization and in all cases domain II possesses the thermolysin-fold (29). They are characterized by conserved HEXXH/HX18E zinc-binding and GXMXEN catalytic motifs and all share mechanistic features with thermolysin. Typically these M1 enzymes possess relatively broad specificity for the N-terminal amino acid (P1) of their peptide substrates and members of the M1 family possess an internal cavity surrounding the catalytic site that has been argued to confer on these enzymes specificity for small peptide substrates (30). Although the means by which substrates gain access to the catalytic site and products are released has been the subject of some debate (30–32), human endoplasmic reticulum aminopeptidase; GnT1, N-acetylgalactosamine transferase 1.

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This article contains supplemental Figs. S1–S6 and Table S1.

The atomic coordinates and structure factors (codes 4FYQ, 4FYR, 4FYS, and 4FYI) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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2 The abbreviations used are: APN, aminopeptidase N; AngIII, angiotensin III; AngIV, angiotensin IV; NGR, Asn-Gly-Arg; ERAP, endoplasmic reticulum aminopeptidase; GnT1, N-acetylgalactosaminyltransferase 1.

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† This article contains supplemental Figs. S1–S6 and Table S1.

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peptidase (ERAP) 1 (33, 34) and archael tricorn interacting factor F3 (35) can exist in an open form where the cavity and catalytic site are exposed to bulk solvent. In the case of the plasmodium M1, the tricorn interacting factor F3, and the bacterial PepN, the enzymes are thought to degrade small peptides to amino acids (30, 32, 35). This is to be contrasted with mammalian APN and ERAP1 and -2, enzymes that can generate defined peptide products (33, 34, 36) such as AngI and peptides trimmed for presentation by major histocompatibility complex class I proteins. Interestingly, APN is also thought to be involved in peptide degradation in the renal proximal tube and the small intestine (37, 38) and it too has been found to have a relatively broad substrate specificity at the P1 position with preference for the removal of small hydrophobic or basic amino acids (39). To shed light on the ability of hAPN to process various peptide substrates including AngIII and AngIV we determined its x-ray crystal structure in the presence of AngIV and two peptidomimetic inhibitors, amastatin and bestatin. The structure represents the first example of a dimeric M1 enzyme and has provided novel insights into the mechanism of peptide processing and signal transduction.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—A soluble form of hAPN (residue 66–967) was expressed and purified from a stably transfected HEK 293S GnT-1 cell line (40) essentially as previously described (41). Cells were grown in DMEM/F-12 supplemented with 3% FBS (Invitrogen), 1× penicillin-streptomycin (Invitrogen), 1 mg/liter of doxycycline (Sigma), and 1 mg/liter of aprotinin (Bioshop Canada). The harvested medium was concentrated 10-fold and the fusion protein was purified by IgG-Sepharose affinity chromatography. The protein A tag was removed by on-column tobacco etch virus protease digestion

5.5. The deglycosylated hAPN was then purified by Q-Sepharose ion exchange chromatography. The resultant hAPN was removed by on-column tobacco etch virus protease digestion and the liberated hAPN was further purified by Q-Sepharose ion exchange chromatography. The resultant hAPN was treated with endo-β-N-acetylglucosaminidase A (42) in 10 mM HEPES, 50 mM NaCl, pH 7.5, at 37 °C, followed by jack bean α-mannosidase (Prozyme) in 50 mM MES, 0.4 mM ZnSO₄, pH 5.5. The deglycosylated hAPN was then purified by Q-Sepharose ion exchange chromatography and Superdex 200 gel filtration chromatography in 10 mM HEPES, 50 mM NaCl, pH 7.4, and concentrated to 20 mg/ml. The selenomethionine-labeled protein was expressed by supplementing methionine-free media with 30 mg/liter of selenomethionine as previously described (41).

**Protein Crystallization**—The deglycosylated native and selenomethionine-labeled hAPN was crystallized by the hanging drop method. Protein stock solutions at 20 mg/ml in 10 mM HEPES, pH 7.5, and 50 mM NaCl were mixed 1:1 with well solution containing 2 M (NH₄)₂SO₄, 10% glycerol, and 100 mM sodium acetate, pH 5.0. Crystals were cryoprotected with well solution containing 25% glycerol. For crystallization of the AngIV complex, hAPN was preincubated for 3 days with 2.5 mM EDTA, and AngIII (Anaspec) was then added at 300 μM. Crystals were grown for approximately 1 week before cryoprotection and data collection. Complexes of bestatin (300 μM) (Bioshop Canada) and amastatin (300 μM) (Bioshop Canada) with the zinc-bound native enzyme were obtained by co-crystallization.

**Data Collection, Structure Determination, and Refinement**—Data were collected at the Canadian Light Source, Saskatoon (Beamline CMCF-08ID-1). A single-wavelength anomalous dispersion experiment was performed at the peak (0.9795 Å) of the selenium absorption edge. Diffraction images were processed and scaled using HKL2000 (43); 5% of each dataset was flagged for the calculation of Rmerge. A summary of statistics is provided in Table 1. The SHELX (44) program suite was used to determine the selenium atom positions and to determine phases. Automated model building using ARP/wARP resulted in a model that was 95% complete. Alternate rounds of manual rebuilding using COOT (45) and automated refinement using REFMAC (46) and Phenix (47) were performed. Geometric parameters for bestatin and amastatin were obtained from the Ligand expo database. Ramachandran analysis of all four structures (native, AngIV, bestatin, and amastatin complex) showed that 92% of the residues are in the most favored region, with 8% in the additionally allowed region. All of the residues in the substrate/inhibitor structured loop also fall in the most favored and additionally allowed regions of Ramachandran space. Figures were generated using the program PyMOL. Interface calculations were done using the PISA server.

**Surface Plasmon Resonance Analysis, Analytical Ultracentrifugation, and Enzyme Kinetics**—The ectodomain was used without deglycosylation for the C18 HPLC-based kinetics assay, the sedimentation equilibrium analysis, the surface plasmon resonance peptide binding assay, and the colorimetric enzyme assay. hAPN enzymatic activity was assayed using L-leucine-ρ-nitroanilide (Sigma) in 10 mM MES, pH 6.5. Initial velocities were obtained at 298 K over a range of substrate concentrations at an enzyme concentration of 10 nM. The generation of ρ-nitroanilide was monitored at 405 nm. Kinetic analysis of the removal of the first amino acid from AngIII, AngIV, and the peptides, VVYIHPF and RYIHPF, was performed by measuring the loss of the substrate using a C18 reverse phase HPLC assay. Various concentrations of peptides were mixed with hAPN (0.5 nM) in 10 mM MES, pH 6.5, at 298 K, and the digest was stopped with 5% phosphoric acid at various time points to obtain initial velocities. Each stopped reaction was loaded onto a C18 column (Vydac 218TP) and eluted isocratically with 85 mM phosphoric acid, adjusted to pH 3 with triethanolamine, containing 15 (VVYIHPF) or 17% (the others) acetonitrile. Peptides were quantitated at 195 nm based on a standard curve generated with known peptide concentrations. AngIII, AngIV, Ang/II(4–8), VVYIHPF, and RYIHPF are all standard curve generated with known peptide concentrations. AngIII, AngIV, and the peptides, VVYIHPF and RYIHPF, was performed by measuring the loss of the substrate using a C18 reverse phase HPLC assay. Various concentrations of peptides were mixed with hAPN (0.5 nM) in 10 mM MES, pH 6.5, at 298 K, and the digest was stopped with 5% phosphoric acid at various time points to obtain initial velocities. Each stopped reaction was loaded onto a C18 column (Vydac 218TP) and eluted isocratically with 85 mM phosphoric acid, adjusted to pH 3 with triethanolamine, containing 15 (VVYIHPF) or 17% (the others) acetonitrile. Peptides were quantitated at 195 nm based on a standard curve generated with known peptide concentrations. AngIII, AngIV, Ang/II(4–8), VVYIHPF, and RYIHPF are all baseline separated. Sedimentation equilibrium analysis of hAPN at concentrations of 0.1, 0.25, and 0.5 mg/ml were performed at speeds of 7000 and 9000 rpm at 277 K. Surface plasmon resonance was performed on CM-5 chips coupled with hAPN that had been preincubated with 2.5 mM EDTA (1 day) and analysis was performed in buffer containing 2.5 mM EDTA. Binding plateau values as a function of AngIII and AngIV concentration were used to compute the dissociation constants assuming a 1:1 binding model.
RESULTS

Overall Structure of the hAPN Dimer—hAPN is a 967-residue type-2 membrane glycoprotein as shown in Fig. 1a. The ectodomain was expressed and shown to possess a $K_d$ of 0.3 ± 0.05 mM for the hydrolysis of leucine-\(p\)-nitroanilide, a value similar to that obtained for the rabbit and porcine enzymes purified from tissue (48). The intact membrane protein exists as dimers and monomers on the cell surface in rabbit (13) and using analytical ultracentrifugation we have determined that the ectodomain expressed alone dimerizes with a $K_d$ of 0.8 \(\mu\)M. The ectodomain is also dimeric in the crystal (data collection and refinement statistics in Table 1). As shown in Fig. 1b, each monomer possesses the four-domain structure (domains I-IV) characteristic of the four-domain M1 metallopeptidases whose structures have been determined to date (30–36, 49). Domain II possesses the thermolysin-fold and contains both the zinc binding site and the catalytic site, as well as the characteristic consensus motifs, $^{388}$HEXXH$_{19}$E$^{371}$ and $^{352}$GXXMEN$^{356}$. The dimer interface is mediated by hydrophobic interactions and a hydrogen bond and salt-bridge network, and it buries ~840 \(\AA^2\) of surface area on each monomer (Fig. 2). In each monomer, the catalytic site is exposed to a large internal cavity (~2800 \(\AA^3\)), which is inaccessible to the bulk solvent. The native, peptide bound, and inhibitor bound structures are very similar (root mean square deviation over all protein atoms of 0.13–0.17 \(\AA\)) except for a flexible loop in domain IV that is structured by substrate and inhibitor binding as discussed below. Fig. 1b shows a model for the orientation of hAPN on the cell surface. The dimer possesses dimensions of 131 \(\AA\) \(\times\) 62 \(\AA\) in projection, values very close to those measured by negative stain electron microscopy (135 \(\AA\) \(\times\) 55 \(\AA\)) for intact porcine APN in reconstituted lipid vesicles (50).

Peptide Binding and the Catalytic Site—To shed light on the structural basis for substrate binding and catalysis we determined the x-ray crystal structure of hAPN in its native form and in complex with peptide substrate. The latter was obtained by the co-crystallization of zinc-depleted hAPN with 300 \(\mu\)M AngIII (RVYIHPF), although the clearly defined electron density of the first three residues shows that only AngIV (VYIHPF) is bound in the catalytic site (Fig. 3a). Zinc-depleted hAPN binds both AngIII (5 ± 0.4 \(\mu\)M) and AngIV (15 ± 4 \(\mu\)M) with similar affinity (Table 2 and supplemental Fig. S1). Fig. 3b shows an overlay of the zinc-bound native enzyme with that of the zinc-free AngIV complex in the vicinity of the zinc binding site.
site. In the native enzyme the zinc ion is coordinated by His^{388}, His^{392}, and Glu^{411} (of the HEXHxHE motif) and both oxygen atoms of an acetate molecule from the crystallization buffer (supplemental Table S1). In the AngIV complex the N-terminal Val residue is deeply buried, an observation consistent with the fact that these M1 enzymes are exopeptidases. The carbonyl oxygen atom of the valine occupies the same position as that of the OD1 acetate oxygen atom in the native structure (there is no bound acetate in the peptide complex) and at the same time it accepts a hydrogen bond from Tyr^{477}, a residue proposed to stabilize the oxyanion generated in the transition state (51). In addition, the α-amino group of the Val residue is hydrogen bonded to Glu^{355} (of the GXXG motif), Glu^{411} and Gln^{215} (a cis peptide conserved among members of the family), residues all implicated in substrate binding and/or transition state stabilization (51). Taken together, it is clear that the bound peptide is poised for catalysis and represents a substrate complex. Further support for this suggestion stems from the fact that the scissile bond (between the Val (P1) and Tyr (P1')) straddles the side chain of Glu^{389}, a residue proposed to shuttle a proton from the hydrolytic water molecule to the amide nitrogen of the scissile bond (51). Indeed, the side chain of Glu^{389} forms a hydrogen bond to the more weakly coordinated OD2 acetate oxygen atom (supplemental Table S1), an atom whose position approximates that of the hydrolytic water molecule prior to nucleophilic attack. Because the zinc ion is thought to both activate the hydrolytic water molecule and coordinate the oxyanion in the transition state (supplemental Fig. S2), we propose that our native acetate-bound zinc structure, in conjunction with the zinc-free peptide complex, serves as a good model for that of the Michaelis complex.

**Peptide Binding, Loop Ordering, and Specificity**—Both the electron density and temperature factors show that the first three amino acids (residue positions P1-P1') of the bound AngIV peptide are the most well defined, whereas the last three (residue positions P3'-P5') are increasingly disordered (Fig. 3a). The binding of AngIV buries ~470 Å² of hAPN, and the
binding site is mainly composed of residues from domain II (336 Å²) and domain IV (134 Å²) (Fig. 3c). In addition, comparison of the peptide complex with that of the native enzyme shows that peptide binding leads to the ordering of an eight-residue flexible loop (891 YGGGSFSF 898) that is not observed in the electron density maps of the native structure. The loop further buries the bound substrate and although the interactions between the loop and the bound peptide are not that extensive, the electron density describing it is strong and its temperature factors (25–35 Å²) are comparable with that of non-loop residues in the vicinity (supplemental Fig. S3a).

The Val (P1) and Tyr (P1′) residues make extensive interactions with hAPN and both are completely buried (Fig. 3, b and c). In addition to the key interactions between the Val amino group and its carbonyl oxygen atom (as discussed above), its side chain sits in an apolar pocket formed by Gln211, Gln213, Ala351, Met354, and Phe472 and the side chain of loop residue Ala351, Met354, and Phe472 and the side chain of loop residue Ala351, Met354, and Phe472 and the side chain of loop residue Ala351, Met354, and Phe472 and the side chain of loop residue Ala351, Met354, and Phe472 and the side chain of loop residue. The flexible loop in the bestatin complex is well ordered (average temperature factors are 30–40 Å²)( supplemental Fig. S3a). In contrast to that observed for the amastatin complex, the interactions between hAPN and both are very similar to those observed in the bestatin complexes of other M1 enzymes (30–34, 53). Amastatin binding also structures the flexible loop (average temperature factors are 25–30 Å²) around the side chain of the LeuβN[αOH] moiety in a fashion very similar to that seen for Val (P1) in the AngIV complex (supplemental Fig. S3b and S4c). In addition, the hydrogen bonding pattern between the loop and other hAPN residues is essentially the same as that observed in the AngIV complex.

In contrast to that observed for the amastatin complex, the interactions between bestatin and hAPN do not correspond to that of a substrate complex (Fig. 4, b and d). The PheβN[αOH] moiety of bestatin is pushed deeply into the S1 pocket that accommodates the Val (P1) side chain in the AngIV complex of its amino group, carbonyl oxygen atom, and α-hydroxy group make only water-mediated hydrogen bonds to hAPN. The carboxyl group of the C-terminal Leu residue coordinates the zinc ion in a fashion similar to that of acetate in the native complex (supplemental Table S1), and a tetrahedrally coordinated water molecule, also observed in the native structure, occupies the pocket filled by the Val (P1) α-amino group in the AngIV complex. The side chain of the C-terminal Leu residue occupies the S1′ pocket that accommodates the Tyr (P1′) side chain of AngIV and the Val (P1′) side chain of amastatin, in their respective complexes. Despite the differences in binding geometry, both bestatin and amastatin would block substrate binding, an observation consistent with the fact that they are both competitive inhibitors.

The flexible loop in the bestatin complex is well ordered (average temperature factors are 30–40 Å²) (supplemental Fig. S3c) but now found to assume a conformation very different from that observed in the AngIV and amastatin complexes (Fig. 5). Loop residue Phe896, which formerly capped the S1 pocket has been repositioned to accommodate the Phe side chain of the PheβN[αOH] moiety (supplemental Fig. S4d) and the interactions involving loop residues Gly894 and Ser895, observed in the AngIV and amastatin complexes, are replaced by both

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**Table 2**

**Summary of kinetics and binding data**

| P1′ | P1 | P2 | P3 | P4′ | P5 | P6 | V<sub>max</sub> | K<sub>m</sub> | K<sub>d,apo</sub> | k<sub>cat</sub> |
|-----|----|----|----|-----|----|----|------------|------|-------------|-----|
| Arg | Val | Tyr | Ile | His | Pro | Phe | AngIII 29.8 ± 4.8 6.2 ± 2.0 5 ± 0.4 | 54 | |
| Val | Tyr | Ile | His | Pro | Phe | AngIV 9.4 ± 0.4 5.7 ± 1.9 ND | 17 | |
| Val | Tyr | Ile | His | Pro | Phe | AngIV 9.7 ± 1.6 2.1 ± 0.3 15 ± 4.0 | 16 | |
| Arg | Tyr | Ile | His | Pro | Phe | AngIV 52.1 ± 4.1 2.5 ± 0.5 ND | 89 | |

*ND, not determined.*
direct and water-mediated interactions between loop residue Ser\textsuperscript{895} and non-loop residues Asp\textsuperscript{189}, Leu\textsuperscript{190}, Asp\textsuperscript{216}, and Gln\textsuperscript{213} in domain II. The Phe side chain sits in a pocket formed by both loop and non-loop residues. Although the bestatin binding geometry does not mimic a true substrate complex, the loop conformation observed does provide a model for how substrates with bulky side chains at the P1 position might be accommodated (supplemental Fig. S4\textsuperscript{e}).

**Kinetic Analysis of Angiotensin III and IV and Related Peptides**—To gain insight into the basis for the ability of hAPN to process both AngIII and AngIV we measured the kinetic parameters for the removal of the first amino acid from AngIII, AngIV, and two related peptides where the first amino acids of AngIII and AngIV were swapped for that of the other (Table 2 and supplemental Fig. S5). The data show that although $k_{\text{cat}}$ for the removal of Arg from AngIII is about 3-fold higher than that of Val from AngIV, a compensatory increase in $K_m$ leads to similar catalytic efficiencies. Comparison of the control peptide pairs which differ only in the N-terminal amino acid shows that $k_{\text{cat}}$ for the removal of Arg is 3–5-fold higher than that of Val and that the nature of the N-terminal amino acid does not significantly affect $K_m$.  

**FIGURE 4.** Substrate binding and catalytic site of the bestatin and amastatin complexes. a, chemical structure of amastatin and bestatin. The rectangular box encloses the $\alpha$-hydroxyl group. b, $F_o - F_c$ omit maps contoured at 2.0 $\sigma$ for amastatin and bestatin. Carbons, oxygens, and nitrogens are colored gray, red, and blue, respectively. c, stereoview of the amastatin binding site. hAPN carbon atoms are colored purple. The zinc ion is shown as a green sphere. The substrate-induced loop is colored red. d, stereoview of the bestatin binding site. hAPN carbon atoms are colored teal. The zinc ion is shown as a green sphere and water molecules as red spheres. Selected hydrogen bonds are shown as red dashed lines. The substrate-induced loop is colored red.
Access to the Catalytic Site and a Model for the Open Conformation—As discussed above, the catalytic site of hAPN is exposed to a large internal cavity that is not connected to bulk solvent by an appreciable channel or opening (Fig. 6a). Moreover, structural alignment shows that both monomers of the hAPN dimer correspond to that of ERAP1 in its closed conformation (33, 34). To explore the possibility that hAPN might be able to access the ERAP1 open conformation, domains (I + II) and (III + IV) of hAPN were treated as rigid bodies and superimposed on domains I and IV of ERAP1 in the open conformation. Each monomer in the open conformation was then superimposed on the hAPN dimer through domain IV. The resultant models are free of steric clashes and with both monomers in the open conformation, the dimer takes on an S-shaped configuration, each lobe of which corresponds to a monomer whose internal cavity and catalytic site are exposed to bulk solvent (Fig. 6b). Although a large protein interface between domain (I + II) and IV is broken on conversion from the closed to the open form, the interface possesses a large percentage of polar residues. Conversion to the open form pulls the catalytic residues in domain II away from both the loop and non-loop residues in domain IV (see Fig. 3c), in this way pulling apart the residues that serve to sandwich the peptide in the binding site. In the open form of ERAP1, the equivalent of Tyr^477 in hAPN is rotated away from its catalytically active conformation, a perturbation thought to render the open form catalytically inactive (33–35) and it is likely that the same would occur in hAPN.

DISCUSSION

The work reported here has provided much insight into the basis for peptide recognition and catalysis by hAPN. Significantly, we have found that an eight-residue loop, structured only on substrate or inhibitor binding, forms a cap over the side chain of the N-terminal Val of AngIV in the hAPN-AngIV complex. The loop is structured in a very similar way in the amastatin complex but assumes a fundamentally different conformation in the bestatin complex. Moreover, the loop conformation observed in the hAPN-AngIV complex would not be able to accommodate the bulky N-terminal Arg residue found in AngIII, whereas that observed in the bestatin inhibitor complex would (supplemental Fig. S4, b and e). It follows that substrate-dependent loop ordering and the observed plasticity may reflect a requirement for broad specificity at the P1 position of the bound substrate. Notably, differences in the conformation of a single side chain in the S1 site have served to accommodate different N-terminal amino acids in other M1 family members (31, 32, 54). In addition to its role in processing AngIII and AngIV, hAPN has a number of other physiological peptide substrates and it shows relatively broad specificity when assayed with amino acid analogues (39). Our kinetics data show that the identity of the amino acid in the P1 position does not profoundly affect $K_{m}$ or $k_{cat}$ when measured in the context of AngIII, AngIV, and related control peptides (Table 2). The ability of hAPN to process a range of peptide substrates is also reflected in the fact that interactions with the side chains at peptide substrate positions, P1’ and P2’, are such that a range of amino acid types could be accommodated and that beyond the third amino acid there is little chemical and steric complementarity between the enzyme and the substrate. These structural features are consistent with previous enzyme kinetic analysis that suggested that the active site of rat APN is composed of subsites recognizing the three N-terminal residues (55). This is to be contrasted with that of ERAP1 where recognition of the C-terminal end of the substrate is thought to ensure the production of a peptide product of defined length (33, 34, 56). Finally, the suggestion that hAPN is catalytically active only in the closed form supports a model where hAPN, like other members of the M1 family, is designed to limit its specificity to peptides, substrates that can be accommodated by the internal cavity formed in the closed form.

Comparison of the hAPN-AngIV complex with that of the native enzyme has provided new insight into the mechanism of peptide hydrolysis by M1 family members. Because the geometry of the hAPN-AngIV complex observed is sterically compatible with the presence of a bound zinc ion, it follows that peptide binding, as observed, might lead directly to the transition state with no requirement for a significant change in atomic positions. On peptide binding the zinc-bound water molecule (observed in the absence of acetate (57)), would in a concerted process be displaced by the substrate carboxyl oxygen atom and re-positioned for in-line nucleophilic attack and hydrolysis. The OD2 atom of the bound acetate in the native structure and the $\alpha$-hydroxyl group in the amastatin complex provide models for the position of the water molecule prior to the transition state. This concerted process is to be contrasted with the two-step model stemming from work on the only other peptide complex of an M1 enzyme available (58). Using a catalytically inactive leukotriene A4 hydrolase mutant, it was found that the deprotonated form of the $\alpha$-amino group of the bound substrate was found to coordinate the zinc ion in what was assumed to be an initial binding event.

Although amastatin is found to bind hAPN in a fashion very similar to that of AngIV, the binding geometry observed for bestatin does not correspond to that of a substrate complex or that observed in other known bestatin complexes (30–33). In those complexes, the bestatin carboxyl oxygen atom and the
α-hydroxyl group are found to coordinate the zinc ion as seen in our amastatin complex. In the hAPN-bestatin complex these groups make only water-mediated interactions with hAPN and the C-terminal carboxyl group of bestatin coordinates the zinc ion in a fashion similar to that observed for the acetate ion in our native structure. Similar zinc-carboxylate interactions have been observed in the structures of many other zinc-dependent proteases (59). As shown in supplemental Fig. S4f, the Phe side chain of the bestatin PheB8N[αOH] moiety clashes with the side chain of loop residue Phe896 when modeled with the binding geometry and loop conformation observed in the amastatin complex. The novel binding geometry observed presumably reflects the fact that in all of the M1-bestatin complexes determined, to date, the loop in those structures does not block bestatin from binding in the canonical fashion either because it is too short or it differs in sequence and conformation from that observed in the AngIV and amastatin complexes of hAPN (supplemental Fig. S6). Given the novel binding geometry and the importance of the loop, the hAPN-bestatin complex would be expected to facilitate the development of specific hAPN inhibitors for use as anti-cancer agents and analgesics for pain management (2, 22). Building on the key interactions with the bestatin phenyl and carboxyl groups, while at the same time eliminating the α-hydroxyl and carbonyl functional groups, might provide a means of generating specific inhibitors that do not inhibit other human M1 family members.

Analysis of the hAPN-AngIV complex shows that residues in domains II and IV serve to sandwich the peptide substrate in the closed form (Fig. 3c) and that conversion to the open form would be required for both AngIII binding and AngIV release. Because interactions between the substrate-structured loop and residues in domains II and IV would also be expected to stabilize the closed form, a change in loop conformation or loop ordering, on peptide bond cleavage, might promote conversion to the open form and product release. At the same time, we see no structural reason to exclude the possibility that a peptide product might rebind for another round of cleavage, while the enzyme is still in the closed conformation. Both ordered and disordered loop conformations, as well as peptide-bound and peptide-free structures were obtained in the closed form and the volume required to allow the cleaved peptide to rebind in a shifted register is certainly available. In this way, the internal cavity would also serve to provide a means of trapping peptides for their processive degradation to amino acids or very small peptides. Indeed, a recent molecular dynamics simulation of the plasmodium M1 enzyme has provided evidence of processive peptide degradation in the closed conformation (60).

**FIGURE 6. hAPN and the modeled open form.** a, open-book view of a surface representation of hAPN sectioned to illustrate the internal cavity and catalytic site. The structured loop is colored red and the AngIV peptide is shown in yellow stick representation. b, open-book view of hAPN with both monomers modeled in the open form. c, surface representation of hAPN viewed from the membrane. d, surface representation of hAPN with both monomers modeled in the open form viewed from the membrane. e, overlay of hAPN in c (green) with that of the model in d (blue) viewed parallel to the membrane surface. The N-terminal amino acids that connect to the transmembrane region of each monomer are colored black. Numerical values refer to the distance between the N-terminal amino acid of each monomer.
X-ray Structure of Human Aminopeptidase N

The hAPN dimer provides the first example of an M1 metallo-apeptidase in dimeric form and its structure provides further insights into how hAPN mediates its many functions. In addition to its roles in peptide processing, hAPN is also involved in cell adhesion, endocytosis, and signal transduction (4–7, 61), processes often associated with changes in conformation and/or oligomeric state. As shown in Fig. 1b, the dimer interface is made up exclusively of residues from the C-terminal domain (domain IV) of each monomer. Given that hAPN is a type-2 membrane glycoprotein and as such possesses an N-terminal membrane anchor, this arrangement leads to an arch-like structure on the cell surface. This architecture is of particular significance given the suggestion that each monomer can also assume both an open and a closed conformation. As shown in Fig. 6, c–e, conversion from the open/open dimer to the closed/closed dimer leads to a large conformational change that could form the basis of a signal transduction event. Notably, this conversion results in a large change (~50 Å) in the distance between the membrane anchoring N termini of the two monomers of the dimer. Bradykinin is a known competitive inhibitor of hAPN (62) and its binding would be expected to stabilize the closed form, a conformation that might in turn be responsible for its ability to mediate uptake of the bradykinin receptor complex (6). Given that the membrane-bound dimer possesses two points of membrane attachment it is also conceivable that the rate of interconversion between the open and closed forms, and hence the catalytic activity of hAPN, would be different in the membrane-bound dimer from what it is in either the membrane-bound monomer or soluble forms of the ectodomain. Our structures suggest that a rapid interconversion between the open and closed forms would promote the efficient binding and release of AngII and AngIV, whereas a slower rate of interconversion might lead to progressive degradation. Taken together the novel dimeric structure of hAPN and the resulting models for catalysis and signal transduction are expected to stimulate new research directions.

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