

Crystal structure of prostate-specific membrane antigen, a tumor marker and peptidase

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Prostate-specific membrane antigen (PSMA) is highly expressed in prostate cancer cells and nonprostatic solid tumor neovasculature and is a target for anticancer imaging and therapeutic agents. PSMA acts as a glutamate carboxypeptidase (GCPII) on small molecule substrates, including folate, the anticancer drug methotrexate, and the neuropeptide *N*-acetyl-L-aspartyl-L-glutamate. Here we present the 3.5-Å crystal structure of the PSMA ectodomain, which reveals a homodimer with structural similarity to transferrin receptor, a receptor for iron-loaded transferrin that lacks protease activity. Unlike transferrin receptor, the protease domain of PSMA contains a binuclear zinc site, catalytic residues, and a proposed substrate-binding arginine patch. Elucidation of the PSMA structure combined with docking studies and a proposed catalytic mechanism provides insight into the recognition of inhibitors and the natural substrate *N*-acetyl-L-aspartyl-L-glutamate. The PSMA structure will facilitate development of chemotherapeutics, cancer-imaging agents, and agents for treatment of neurological disorders.

crystallography | folate hydrolase | glutamate carboxypeptidase II | Naaladase I

Prostate-specific membrane antigen (PSMA), also known as glutamate carboxypeptidase II (EC 3.4.17.21), *N*-acetyl- α -linked acidic dipeptidase I [Naaladase (NLD) I], or folate hydrolase, is a 750-residue type II transmembrane glycoprotein that is highly expressed in prostate cancer cells and in nonprostatic solid tumor neovasculature (1) and expressed at lower levels in other tissues (2, 3), including healthy prostate, kidney, liver, small intestine, and brain (reviewed in refs. 4 and 5). PSMA expression increases with prostate disease progression and metastasis (6), thus PSMA is the target of a Food and Drug Administration-approved prostate cancer-imaging agent, the ¹¹¹In-labeled 7E11 monoclonal antibody (Prostascint, Cytogen, Princeton, NJ), which binds an intracellular epitope exposed upon cell death. The extracellular domain of PSMA is targeted by monoclonal antibodies such as J591, which is in clinical trials for specific delivery of imaging agents or therapeutics to cells overexpressing PSMA (7).

In addition to its role as a tumor marker, PSMA contains a binuclear zinc site and is active as a glutamate carboxypeptidase, catalyzing the hydrolytic cleavage of α - or γ -linked glutamates from peptides or small molecules (8). Its substrates include poly- γ -glutamated folates, which are essential nutrients, and the poly- γ -glutamated form of the anticancer drug methotrexate (Fig. 1A) (9), in which case cleavage renders it less efficacious. The enzymatic activity of PSMA can be exploited for the design of prodrugs, in which an inactive glutamated form of the drug is selectively cleaved and thereby activated only at cells that express PSMA (10).

PSMA also cleaves and inactivates the abundant neuropeptide *N*-acetyl-L-aspartyl-L-glutamate (α -NAAG) (11) (Fig. 1A), which is an inhibitor of the NMDA ionotropic receptor and an agonist of the type II metabotropic glutamate receptor subtype 3 (12). A breakdown of the regulation of glutamatergic neurotransmission by α -NAAG is implicated in schizophrenia, seizure disorders, Alzheimer's disease, Huntington's disease, and amy-

trophic lateral sclerosis (13). Thus, inhibition of PSMA potentially confers neuroprotection both by reducing glutamate and increasing α -NAAG. Indeed, the subnanomolar inhibitor 2-(phosphonomethyl) pentanedioic acid has been shown to provide neuroprotection in cell culture and/or animal models of ischemia, diabetic neuropathy, drug abuse, chronic pain, and amyotrophic lateral sclerosis (14).

PSMA is a member of a superfamily of zinc-dependent exopeptidases, which include carboxypeptidases with a mononuclear zinc active site (e.g., carboxypeptidase A) (15) and carboxy- and aminopeptidases with a binuclear zinc active site [e.g., carboxypeptidase G₂ (CPG₂) (16), peptidases T and V (PepT and PepV) (17, 18), *Streptomyces griseus* aminopeptidase (Sgap) (19), and *Aeromonas proteolytica* aminopeptidase (AAP) (20)]. In addition to a limited region of homology with these soluble single-domain (e.g., AAP, Fig. 1C) (19, 20) or double-domain (e.g., CPG₂, Fig. 1D) (16–18) zinc-dependent exopeptidases, the entire sequence of PSMA is homologous to at least four other human proteins: NLDL (expressed in ileum; 35% identity) (2, 21), NLD2 (expressed in ovary, testis, and brain; 67% identity) (2, 21, 22), transferrin receptor (TfR) 1 (TfR1; expressed in most cell types; 26% identity) (23) (Fig. 1B), and TfR2 (expressed predominantly in liver; 28% identity) (24). Of these homologs, only the 3D structure of TfR1 is available; it is a type II transmembrane glycoprotein with three extracellular domains: the protease-like, apical, and helical domains (29%, 25%, and 23% identical to PSMA, respectively) (23). The helical and apical domains have no counterparts in the single- and double-domain zinc-dependent exopeptidases (Fig. 1). TfR1 binds the hemochromatosis protein HFE and iron-loaded transferrin at overlapping binding sites (25). Both TfR1 and PSMA are internalized into endosomes via cytoplasmic YXRF and MXXXL motifs, respectively (26, 27). Whereas TfR1 delivers iron-loaded transferrin and HFE to cells, PSMA may internalize folate or other ligands, a property that can be exploited for targeted delivery of chemotherapeutics. Indeed, the binding of PSMA to the monoclonal antibody J591 increases its internalization rate and potential uptake of tethered ligands (28).

Here we present the 3.5-Å crystal structure of the PSMA ectodomain (residues 44–750), which corresponds approximately to the alternatively spliced PSM' protein expressed in the cytosol (residues 60–750) (29). The structure reveals a two-fold symmetric homodimer with overall similarity to TfR1, but PSMA has a more compact dimer interface and an enzymatically active protease domain. The active site of PSMA contains two zinc atoms coordinated by histidine and glutamate/aspartate residues and a water molecule, similar to active sites in Sgap,

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Abbreviations: PSMA, prostate-specific membrane antigen; AAP, *A. proteolytica* aminopeptidase; AMP1, *A. thaliana* PSMA; CPG₂, carboxypeptidase G₂; α -NAAG, *N*-acetyl-L-aspartyl-L-glutamate; NLD, Naaladase; PepT, peptidase T; PepV, peptidase V; Sgap, *S. griseus* aminopeptidase; TfR, transferrin receptor.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 1Z8L).

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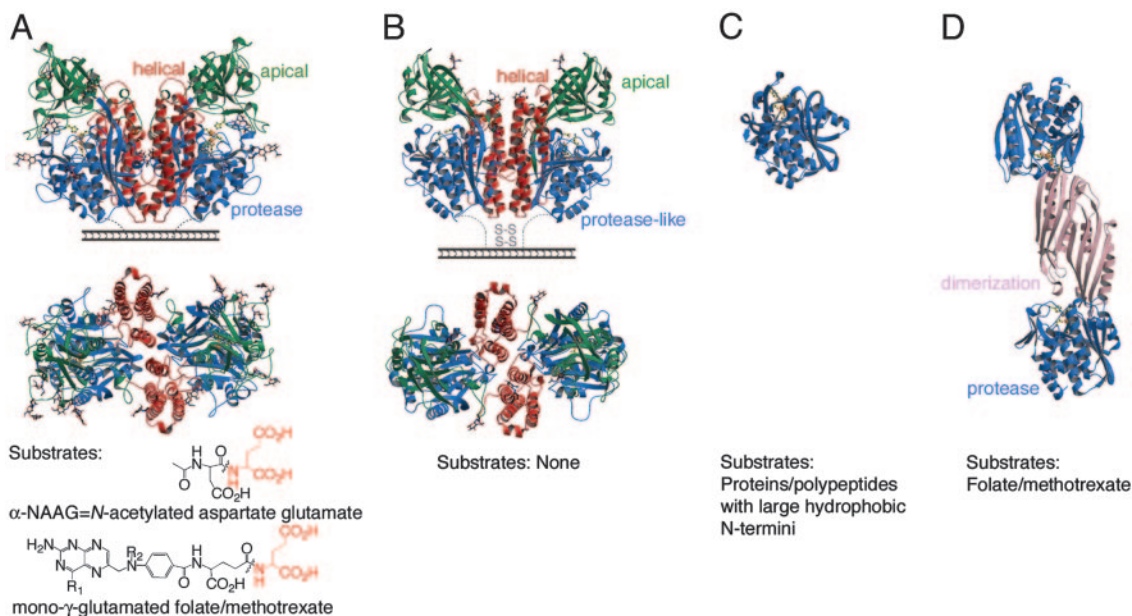


Fig. 1. Structural comparison of PSMA and related proteins. The common fold of the protease or protease-like domain consists of a central seven- to eight-stranded mixed β -sheet and 6–11 flanking α -helices. Ribbon diagrams of side and top view of PSMA (A), side and top view of TfR1 (PDB ID code 1CX8) (B), AAP (PDB ID code 1RTQ) (C), CPG₂ (PDB ID code 1CG2) (D). The protease (or protease-like) domains are shown in blue, the apical domains of PSMA and TfR1 in green, the helical domains in red, and the CPG₂ dimerization domain in pink. Water molecules are red, zinc ions are orange, zinc-binding residues are yellow sticks, and carbohydrate residues are shown in a color-coded ball-and-stick representation. Representative substrates are shown below the appropriate molecule. R₁ = OH or NH₂, and R₂ = H or CH₃ for folate and methotrexate, respectively. Note: A mono- γ -glutamated folate is shown for clarity; in poly- γ -glutamated folate/methotrexate, the additional glutamates (up to seven) are attached to the glutamate shown via a γ -peptide linkage.

AAP, PepV, PepT, and CPG₂. An arginine-rich patch in PSMA replaces the hydrophobic substrate-binding site of the aminopeptidases, consistent with their different substrate preferences. Substrate docking and sequence alignments suggest that the arginine patch stabilizes the binding of a C-terminal glutamate-containing substrate.

Materials and Methods

For additional experimental details, see the supporting information, which is published on the PNAS web site.

Expression and Purification of Soluble Human PSMA. The cDNA encoding full-length human PSMA was kindly provided by Joseph Neale and Tomas Bzdega (Georgetown University, Washington, DC). The extracellular portion of PSMA (residues 44–750) was subcloned into a baculovirus transfer vector (pAcGP67A; BD Biosciences) containing a hydrophobic secretion signal, Factor Xa cleavage site, and N-terminal 6x-His affinity tag. Supernatants from High 5 cells infected with recombinant virus were concentrated and exchanged into 50 mM Tris, pH 7.4/150 mM NaCl. PSMA was purified at yields of \approx 10 milligrams of protein per liter of infected cell supernatant by using Ni-NTA affinity (Qiagen, Chatsworth, CA) and Superdex 200 16/60 (Amersham Pharmacia Biosciences) size-exclusion chromatography. The enzymatic activity of the purified PSMA ectodomain was within the previously reported range for cleavage of α -NAAG by blood plasma glutamate carboxypeptidase II (2.5–4.5 nanomoles glutamate produced per minute per milligram of enzyme) (30).

Crystallization, Data Collection, and Processing. Crystals of PSMA were obtained at 4°C in 2 days by vapor diffusion from hanging drops containing 0.8 μ l of protein (at \approx 10 mg/ml in 20 mM Tris, pH 7.5) and 0.8 μ l of reservoir solution [18% polyethylene glycol (PEG) 3350/0.2 M sodium thiocyanate]. Crystals were trans-

ferred serially through increasing concentrations of glycerol and PEG 3350 to a final concentration of 0.2 M sodium thiocyanate/28% PEG 3350/20% glycerol for cryopreservation. Data were

Table 1. Data collection and refinement statistics for PSMA

Space group	P2 ₁
Unit cell (<i>a</i> , <i>b</i> , <i>c</i> , β)	(74.9 Å, 157.8 Å, 133.8 Å, 93.2°)
Molecules per asymmetric unit	Two dimers
Resolution (outer shell)	30–3.5 Å (3.62–3.5)
Theoretical reflections in resolution range	77,001
Working set	70,431 (91.5%)
Test set	3,671 (4.8%)
Completeness (outer shell)*	99% (98.1)
R_{merge} (outer shell) [†]	14% (43.2)
I/σ (outer shell)	15.4 (5.9)
R_{cryst} [‡] , R_{free} [§]	25.2%, 28.4%
Average B factor (main chain, side chain)	66 Å ² (54, 72)
non-H atoms per asymmetric unit	22,876
Protein, Zn(II), carbohydrate, water	22,184; 8; 672; 12
rms deviation from ideality: bond, angle	0.009 Å, 1.47°
Non-Gly residues in Ramachandran plot	
Most-favored	74.2%
Additionally allowed	22.9%
Generously allowed	2%
Disallowed	0.8%

*Completeness indicates number of independent reflections/total theoretical number.

[†] $R_{\text{merge}}(I) = 100 \times \Sigma(|I - \langle I \rangle|) / \Sigma I$, where I is the integrated intensity of a given reflection.

[‡] $R_{\text{cryst}}(F) = \Sigma ||F_{\text{obs}}| - |F_{\text{calc}}|| / \Sigma |F_{\text{obs}}|$, where $|F_{\text{obs}}|$ and $|F_{\text{calc}}|$ are the observed and calculated structure-factor amplitudes for the HKL reflection.

[§] R_{free} is calculated from reflections in a test set not included in the atomic refinement.

protease-like domains of bacterial binuclear zinc exopeptidases (Sgap, AAP, PepV, PepT, and CPG₂) and TfR1, respectively (Fig. 1), and contains 11 α -helices, an eight-stranded β -sheet, and insertions (residues 485–487 and 502–510), which extend a loop joining the sixth and seventh β -strands and include a short α -helix. As in TfR1, the apical domain is inserted between strands 1 and 2 of the protease domain β -sheet. PepV, PepT, and CPG₂ (16–18) (Fig. 1D) also contain an inserted domain, although for these proteins the insertions are between strands 6 and 7 of the central β -sheet, and each serves as a dimerization domain.

The apical domain of PSMA (also called the protease-associated domain) consists of two β -sheets, three α -helices, and one helical turn. This domain is also found in subtilases and trafficking receptors (37). In PSMA, it forms one side of the substrate-binding cavity. In contrast, Sgap and AAP lack an apical domain counterpart and have more open active sites (19, 20).

The helical domain of PSMA contains six α -helices and associated connecting regions, one of which (residues 687–704) is involved in forming the substrate-binding cavity. Another important function of the helical domain is in forming the dimer interface, which involves three helices (Fig. 1A). Although PSMA can convert between a dimeric and monomeric form *in vitro* by addition of metal chelators or changes in salt concentration (38), only the dimeric form of PSMA has folate hydrolase and naaladase activity and is expressed on the surface of prostate cells (38). The dimer interface of PSMA is large, burying a total of $\approx 4,600 \text{ \AA}^2$, making it unlikely that an intact monomer would dissociate from PSMA without at least partial unfolding, perhaps rationalizing why PSMA monomers are inactive (38). Consistent with experiments demonstrating that deletion of the 15 C-terminal residues in PSMA abolishes enzymatic activity (39), the C-terminal residues 721–747 of PSMA form a helix at the dimer interface, suggesting that C-terminal truncation of the ectodomain disrupts PSMA dimerization.

Comparison with TfR1. Although the overall structure and domain organization of PSMA and TfR1 are similar (Fig. 1) (23, 40) (rms deviation of 2.1 \AA for 847 of 1,270 C α atoms in the homodimers), specific structural differences are relevant to their different functions. First, two large insertions in the apical domain of PSMA (residues 145–158 and 238–248) contribute to a large increase in buried surface area between the apical and protease domains (a total of $\approx 4,170 \text{ \AA}^2$), as compared with TfR1 ($\approx 2,630 \text{ \AA}^2$). There is also a modest increase in buried surface area between the PSMA apical and helical domains ($\approx 2,830 \text{ \AA}^2$), as compared with TfR1 ($\approx 2,540 \text{ \AA}^2$). These additional interdomain contacts are involved in positioning the PSMA apical domain to form one side of the substrate-binding cavity. In addition, insertions in the PSMA helical and protease domains (residues 698–703 and 543–548) narrow its substrate-binding cavity, as compared with the analogous region of TfR1. Finally, PSMA does not bind HFE or transferrin (data not shown), and the residues involved in TfR1 binding to HFE or transferrin (40–42) are nonconservatively substituted in PSMA (supporting information).

Glycosylation of PSMA. Glycosylation of PSMA is required for secretion of the recombinant extracellular domain, membrane expression of full-length PSMA, and enzymatic activity (43, 44). The PSMA ectodomain contains nine potential N-linked carbohydrate sites, all of which have been shown to be glycosylated (43, 44). Seven of the glycosylation sites show carbohydrate density in $2F_{\text{obs}} - F_{\text{calc}}$ maps (Asn-76, -121, -140, -153, -459, -476, and -638), but none of the glycosylation sites are close enough to the PSMA active site to directly contact a bound substrate. Therefore, loss of enzymatic activity resulting from prevention or removal of glycosylation (43, 44) likely results from indirect

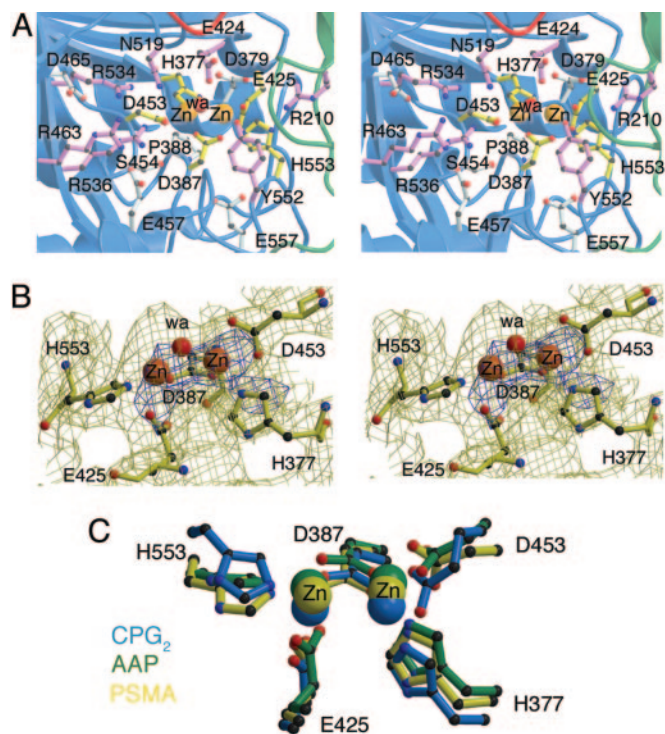


Fig. 3. The PSMA active site. (A) Stereoview of the PSMA active site. Zinc ions are orange spheres, and a water molecule is shown as a red sphere. Zinc-binding residues are yellow sticks, water- or substrate-binding ligands are purple sticks, and residues with structural roles are light blue sticks. (B) Stereoview of the PSMA model in the region of the PSMA active site superimposed on a 3.5-\AA $2F_{\text{obs}} - F_{\text{calc}}$ annealed omit electron density map contoured at 1σ (yellow) and 4σ (blue). The zinc ions and zinc-binding residues were omitted from the model before generation of the map. Some of the extra density corresponds to nonzinc-binding residues that were omitted from the figure for clarity. (C) Overlay of the active sites of PSMA (yellow), aminopeptidase AAP (PDB ID code 1RTQ), and carboxypeptidase CPG₂ (PDB ID code 1CG2) with the zinc ions as spheres and the zinc-binding residues in ball-and-stick representation.

effects, such as misfolding or inhibition of dimerization. We note that the N-linked carbohydrates attached to Asn-638 are positioned such that they could be involved in intersubunit interactions, suggesting that their removal may affect the dimerization of PSMA, thereby resulting in the reduced enzymatic activity associated with PSMA monomers (38).

Antibody Epitopes. The structure of PSMA clarifies the epitopes recognized by monoclonal antibodies being studied for applications in cancer imaging and therapeutics (reviewed in ref. 4). The epitope for the J591 antibody that is in clinical trials has been mapped by using truncated PSMA constructs to residues 153–347 (7). The structure reveals that this region corresponds to the majority of the apical domain, which is distant from the membrane surface (Fig. 1A) and hence generally exposed and accessible. It was previously suggested that the epitope for J591 is near Asn-476 or the carbohydrate normally attached to this residue, because a mutant at this site (N476A) was not detected by J591 (43). However, the PSMA structure shows that Asn-476 is distant from the apical domain and is instead located in the membrane-proximal region of the molecule, where it is less accessible to antibodies.

Active Site of PSMA. The structure of PSMA also provides insight into its catalytic mechanism and substrate specificity. Although zinc was not added to PSMA during purification or crystalliza-

ortholog in plant development and a potential role for its substrate as a signaling compound (53).

Mechanistic Implications. Taken together with prior crystallographic and mechanistic studies of members of the zinc-dependent exopeptidase superfamily (16–19, 36, 47–49, 54, 55), we can use the structure and substrate docking results (supporting information) to propose a catalytic mechanism for the cleavage of α -NAAG by PSMA (Fig. 4) that is consistent with mutagenesis studies. In this mechanism, α -NAAG binds such that the glutamate side chain forms electrostatic interactions with the arginine patch of PSMA, and the oxygen from the carbonyl to be attacked and the C terminus bind to the zinc ions. The remainder of the substrate (e.g., *N*-acetyl-aspartate in α -NAAG) is accommodated by the substrate-binding cavity with specific interactions with Arg-210. Larger substrates [e.g., poly- γ -glutamated folates with up to four γ -linked glutamates (9)] require some structural rearrangements to avoid steric clashes with the pocket and could have an alternate mode of binding. Nevertheless, the proposed reaction mechanism is the same: binding of one water molecule to the zinc ions and deprotonation by Glu-424 to activate the water molecule for nucleophilic attack of the peptide bond of the bound substrate, leading to a tetrahedral intermediate, bond cleavage, and product dissociation. This mechanism is consistent with catalysis by other

members of the zinc-dependent exopeptidase superfamily, in which each active site includes one water molecule that is activated for nucleophilic attack of the peptide bond through deprotonation by a nearby carboxylate (Glu-424 for PSMA) (16–19, 36, 47–49, 54). The active site architecture of PSMA and its implications for substrate binding and catalysis provide insight into the function of this important enzyme and a rationale for designing reagents for detection and treatment of cancer and/or neurological disorders.

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