High-Resolution Crystal Structure of Endoplasmic Reticulum Aminopeptidase 1 with Bound Phosphinic Transition-State Analogue Inhibitor

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Supporting Information

ABSTRACT: Endoplasmic reticulum aminopeptidase 1 (ERAP1) is an intracellular enzyme that helps generate peptides presented by Major Histocompatibility Complex Class I (MHC class I) molecules and is an emerging target for immunotherapy applications. Despite almost two decades of research on ERAP1, lack of high-resolution crystal structures has hampered drug-development efforts. By optimizing the protein construct, we obtained a high-resolution (1.60 Å) crystal structure of the closed-conformation of ERAP1 with a potent phosphinic pseudopeptide inhibitor bound in its active site. The structure provides key insight on the mechanism of inhibition as well as selectivity toward homologous enzymes and allows detailed mapping of the internal cavity of the enzyme that accommodates peptide-substrates. Bis-tris propane and malic acid molecules, found bound in pockets in the internal cavity, reveal potential druggable secondary binding sites. The ability to obtain high-resolution crystal structures of ERAP1 removes a major bottleneck in the development of compounds that regulate its activity and will greatly accelerate drug-discovery efforts.

KEYWORDS: Aminopeptidase, enzyme, inhibitor, structure, X-ray, mechanism, antigen, peptide, immune system

Endoplasmic reticulum aminopeptidase 1 (ERAP1) is an intracellular enzyme that helps generate antigenic peptides that are presented to the immune system by Major Histocompatibility Complex Class I (MHC class I) molecules and is an emerging target for immunotherapy applications. Despite almost two decades of research on ERAP1, lack of high-resolution crystal structures has hampered drug-development efforts. By optimizing the protein construct, we obtained a high-resolution (1.60 Å) crystal structure of the closed-conformation of ERAP1 with a potent phosphinic pseudopeptide inhibitor bound in its active site. The structure provides key insight on the mechanism of inhibition as well as selectivity toward homologous enzymes and allows detailed mapping of the internal cavity of the enzyme that accommodates peptide-substrates. Bis-tris propane and malic acid molecules, found bound in pockets in the internal cavity, reveal potential druggable secondary binding sites. The ability to obtain high-resolution crystal structures of ERAP1 removes a major bottleneck in the development of compounds that regulate its activity and will greatly accelerate drug-discovery efforts.

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as mapping of the internal peptide binding site of ERAP1. Two buffer components, bis-tris propane and malic acid molecules, were found bound in pockets of the internal cavity that might constitute opportunities for modulating peptide selectivity.

ERAP1 was found in the closed conformation, adopting an overall architecture considerably similar to the previously solved structure of ERAP1 bound with bestatin and to a lesser extent similar to the closed structures of ERAP2 and IRAP (50% sequence identity), the other members of the oxytocinase subfamily of M1 aminopeptidases, when co-crystallized with other phosphinic pseudotripeptides. However, the present structure being in remarkably high resolution for a protein molecule of that size, allows an unprecedentedly detailed structural analysis of an enzyme of the family. This high resolution was achieved by using an optimized ERAP1 construct that is described in detail in the Supporting Information. This construct features a removable C-terminal purification tag and has a 28-amino acid sequence (amino acids 486–513), encoded by exon 10 and part of exon 11 of the ERAP1 gene, substituted by a short loop consisting of a GSG sequence. While this short sequence has been reported to be ligand-induced rearrangements, unlike in the homologous IRAP. The phenyl moiety (B) of the phenylalanine residue of DG046 interacts via T-shaped aromatic interactions both with Tyr438 of ERAP1 and intramolecularly with the phenyl group (A) of the homophenylalanine. The later interaction is probably causing the altered conformation of homophenylalanine when bound into the S1 cavity. It appears that the lack of an aromatic residue at S2’ of ERAP1, contrary to ERAP2 and IRAP (Ser869 in ERAP1 vs Tyr892 in ERAP2 and Tyr961 in IRAP), releases the P2’ site of the ligand from being stacked in a parallel mode with any aromatic group from domain IV, adopting a totally different rotamer (Figure 2). This altered conformation results in a more distant placement of its C-terminal carbonyl group, which in DG046 is located by ~3 Å farther from the metal ion, compared to the corresponding group of DG013A when bound on ERAP2. This network of π–π interactions greatly limits the conformational freedom of the ligand and appears optimized for the active site of ERAP1 (see Figure S2 for a depiction of the B-factors of the atoms of the inhibitor). This probably contributes to the potency of this inhibitor, which is comparable to the larger compound DG013A.

Figure 1. (A) Chemical structure of the phosphinic pseudopeptide DG046. (B) DG046 bound in the ERAP1 active site. The inhibitor is shown in yellow sticks and the main residues in ERAP1 that make interactions are shown in green. Oxygen atoms are in red, nitrogen in blue, phosphorus in orange. The active site zinc(II) atom is shown as a gray sphere. The approximate location of the active site specificity pockets S1, S1’, and S2’ are indicated.

Figure 2. Superposition of active-site residues of ERAP1 (in green) to homologous ERAP2 (PDB code SAB0, in cyan) and IRAP (PDB code 5MJ6, in magenta). Catalytic zinc(II) atom is shown as a gray sphere. Numbering of each residue is indicated in different colors depending on the enzyme. Phosphinic inhibitor carbon atoms are shown in yellow, oxygen atoms are in red, nitrogen in blue, phosphorus in orange. Only residues that come within 4 Å of the inhibitor are shown. The active site zinc(II) is shown as a gray sphere. The approximate location of the active site specificity pockets S1, S1’, and S2’ are indicated.
To address selectivity of the inhibitor for the three enzymes of the oxytocinase subfamily, we superimposed the structures of ERAP2 and IRAP on the structure of ERAP1 and examined the locations of active-site residues that could impact inhibitor binding (Figure 2). DG046 has been characterized to be a potent (nanomolar) inhibitor of all three enzymes, with similar potency for ERAP1 and ERAP2 and a greater potency for IRAP (43 ± 4 nM for ERAP1, 37 ± 4 nM for ERAP2, and 2 ± 1 nM for IRAP). In ERAP1, the inhibitor’s phenyl group B comes in close proximity with Ser689. In both ERAP2 and IRAP, the equivalent position has a Tyr residue (892 and 961, respectively), which could provide favorable stacking interactions. Given the network of π-stacking interactions between Phe433, phenyl A, and phenyl B, such interactions can be highly stabilizing in ERAP2 and IRAP. However, both ERAP2 and IRAP lack the favorable H-bonding interaction of Ser316, present in ERAP1, which may in part ameliorate the increase in potency due to Tyr892 and Tyr961. ERAP2 furthermore has a bulky Trp373 that is proximal to the C-terminal end of the inhibitor. The interplay between additional positive interactions and negative ones may explain the similar levels of potency for ERAP2. IRAP however has a hydrophobic Ile461 that could make van der Waals interactions with the propargyl group in the inhibitor, helping in properly orienting it to stack with His357, leading to increased potency. Regardless of small differences in potency, however, most of the inhibitor–protein interactions are with residues that are conserved in all of the three enzyme’s active sites, leading to the limited selectivity of this compound.

Additional electron density within the internal cavity of the enzyme but away from the catalytic site was interpreted to belong to other ligands present during the crystallization process (Figures S3 and S4). Based on the constituents of the crystallization conditions and the high quality of the electron density maps we identified bis-tris propane and malic acid bound on distinct clefts inside the enzyme. The average B-factors of the bis-tris propane and malic acid atoms were 23.2 Å² and 36.2 Å², respectively, which are similar to the values of the main chain atoms of their immediate protein environment. Figure 3 illustrates how bis-tris propane is wedged between domains II and IV. Trp921 of the C-terminal domain α-helix shields the side walls of this novel binding cleft, which we term “side pocket” and interacts extensively via van der Waals interactions with the hydroxymethyl groups of bis-tris propane. The buffer molecule is being stabilized further by a number of H-bonds with residues Asp406, Glu409, Asn641, and Gln675 from the three neighboring α-helices (two from domain IV and one from domain II) and also via an H-bond with the domain III lysine 551, which protrudes toward the side pocket. The side pocket is also present in the previously reported structure of the closed ERAP1, albeit empty from any ligand. Herein, the high resolution of the structure and the presence of a bound ligand allowed us to trace the side chains of residues that previously were not detected. Comparing the current ERAP1 structure with the previous “closed” ERAP1 (PDB ID 2YD0) shows that the binding of bis-tris propane to the side pocket is not accompanied by conformational changes in any of the stabilizing α-helices, indicating probably that bis-tris propane is uninvolved in the closure of domain IV. However, as the side pocket is located between the functionally important domains II and IV and close to the hinge domain III, its possible role as an allosterically modulating site cannot be excluded. This pocket is framed by four α-helices and is widely accessible for ligands from the direction of the catalytic site. The total volume of the side pocket, which apart from the space occupied by bis-tris propane extends beyond the limiting α-helices of domains II and IV, was calculated at 2216 Å³.

Malic acid was found bound close to the nozzle of the domain IV channel sealing the internal cavity from the outer environment (Figure 3). Its binding occurs via strong H-bonds of one carboxyl group with the side chains of the domain IV residues Lys685 and Tyr684 as well as with water-mediated H-bonds of its other carboxyl group with Gln730. This binding pocket is occupied on the one side by polar or charged residues (mostly basic), which adopt an extended conformation and converge toward the spatial position of one of the malic acid carboxylates. Gln730, which stabilizes the second carbonyl of malic acid via the water-mediated interaction, is one of
ERAP1’s polymorphic residues (Q730E) that has been consistently shown to associate with disease predisposition and antigen presentation. Our crystal structure shows that Gln730 lies deep in the internal cavity of ERAP1 and conceptually can interact with residues of a bound peptide. Thus, the other common variant for this position, glutamic acid, would alter such interactions.

This local difference of the surface electrostatic potential of the internal cavity could contribute to changes in substrate specificity and/or to the conformational dynamics of the enzyme, especially since this residue lies among extended hydrophobic patches (Figure 3C). Recently, the crystal structure of a single chain bimodular protein comprising the C-terminal domain of ERAP1 (residues 529–944) fused with part of a well-known ovalbumin-derived epitope (IINFEKL) was published, showing strong interactions of the IINFEKL peptide with the same pocket of domain IV of a symmetry related ERAP1 molecule. Interestingly, malic acid in the present structure adopts a binding pose that partially overlaps with the carboxyl group of the IINFEKL peptide and forms a similar pattern of interactions. Enzymatic studies have shown ERAP1’s allosteric activation for the hydrolysis of both peptides and small pseudosubstrates, by small peptides such as IINFEKL, and is believed that part of the elaborate activation mechanism comprises binding of the C-terminus of the modulating peptides at the site where malic acid was bound. Although malic acid was part of the crystallization condition and is not a known binder or regulator of ERAP1’s enzymatic activity, its spatial coincidence with the C-terminus carboxyl group of the IINFEKL peptide indicates a noticeable affinity of that pocket for carboxyl-containing species. Intriguingly, in the previously reported closed structure of ERAP1 this region is occupied by strong electron density that was not attributed to any compound. Our attempt to fit the cacodylate ion, which was a constituent of the crystallization buffer, was successful in eliminating the residual electron density from the particular site and improving the refinement statistics (Figure S5).

Examination of the internal cavity of the enzyme provides insight on function and substrate recognition. Compared to the open conformation of ERAP1, in the closed conformation, domain IV moves toward domain II forming a large internal cavity of ∼10 974 Å³ that fully occludes the catalytic site from the external environment (Figure 4). The volume and dimensions of the internal cavity are sufficient for the accommodation of peptides even as long as 16-residues long, a hallmark of ERAP1 catalytic properties. Using the 3 V Web server and an oxygen atom as probe, three narrow channels connecting the internal cavity with the outer environment were detected. Two are framed by domains I, II, and IV, while the third one threads among α-helices of domain IV (H14, H15, H16, and H20). Notably, one of the former channels is located above the S1 pocket and in our crystal structure is occupied by several water molecules and one ethylene glycol, which was a component of the cryoprotecting solution (Figure 4). The position of this channel is such that could facilitate postcleavage amino acid release to allow progressive peptide trimming. Although its width in the crystal structure is small and is thus unlikely, it can accommodate larger amino acids, its size may be larger when the enzyme is in solution, and crystal packing restraints are not present.

Overall, the three ligands detected in our crystal structure occupy three distal edges of the cavity (Figure 4). Tracing from the catalytic site where DG046 is located toward MLT or B3P suggests possible trajectories for long peptide substrate binding. Thus, it is very likely that the MLT and B3P ligands reveal secondary binding pockets for long peptides. Interestingly, along that path lie four ERAP1 SNPs that have been associated with disease predisposition and have been shown to affect peptide processing, suggesting that direct interactions between these SNPs and peptides trapped in the cavity may underlie their functional effects. Furthermore, optimization of compounds that bind into one of those secondary sites may affect binding of some peptidic substrates, altering the specificity of ERAP1, something that may translate to changes in the cellular immunopeptidome, an exciting prospect for immunotherapy approaches that rely on modulating antigen presentation.

In summary, we present the first high-resolution crystal structure of ERAP1, in complex with a potent inhibitor. The inhibitor is found in the active site making key interactions that explain both potency and selectivity. We also found additional ligands bound within the enzyme’s large internal cavity that suggest possible trajectories for the binding of large peptides and potential druggable pockets that can regulate the specificity of the enzyme.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.9b00002.

Detailed description of the protein construct, purification, crystallization conditions, and structure determination; table showing data collection and refinement statistics; supporting figures showing the inhibition curve of the crystallized ERAP1 construct from DG046, the B-factors of DG046 atoms, the models of malic acid and bis-tris propane built into 2Fᵦ⁻Fᵦ, electron density; and the cacodylate ion built in residual electron density of the ERAP1 crystal structure with PDB code 2YD0 (PDF).
The pathway of intracellular antigen processing and its role in regulating adaptive immune responses in human disease.

**Author Contributions**

P.G. crystallized the complex, performed diffraction experiments, and solved and interpreted the structure. M.N. and P.R. designed the construct and screened crystallization conditions. E.S. supervised the project, helped design the protein construct, interpreted the data, and wrote the paper in collaboration with P.G. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

Atomic coordinates and structure factors have been deposited in the Protein Data Bank (www.pdb.org), with PDB code 6Q4R.

**Biography**

Efstathios Stratikos studied chemistry at the University of Athens in Greece and then moved on to receive a Ph.D. in biochemistry from the University of Illinois at Chicago in 1999. After postdoctoral training in structural and molecular biology at Harvard University, he returned to Greece in 2005 to establish the Protein Chemistry Laboratory at the National Centre for Scientific Research “Demokritos” in Athens, where he is still employed at the rank of research director. During the last decade his research has focused on the collaboration with P.G. All authors have given approval to constructing, interpreting the data, and writing the paper in its current form.

**ABBREVIATIONS**

ERAP1, endoplasmic reticulum aminopeptidase 1; MHC-I, Major Histocompatibility Complex Class I; ER, endoplasmic reticulum; HLA, human leukocyte antigen; MW, molecular weight; ERAP2, endoplasmic reticulum aminopeptidase 2; IRAP, insulin-regulated aminopeptidase; SNPs, single nucleotide polymorphisms; L-AMC, leucine-7-amino-4-methylcoumaryl-7-amido-4-methylcoumarin.

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