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The Elastase-PK101 structure: Mechanism of an ultrasensitive activity-based probe revealed

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Abstract

Human neutrophil elastase (HNE) plays a central role in neutrophil host defense, but its broad specificity makes HNE a difficult target for both inhibitor and probe development. Recently we identified the unnatural amino acid containing activity-based probe PK101, which exhibits astounding sensitivity and selectivity for HNE, yet completely lacks mechanistic explanation for its unique characteristics. Here, we present the crystal structure of the HNE-PK101 complex, which not only reveals the basis for PK101 ultrasensitivity but also uncovers so far unrecognized HNE features. Strikingly, the Nle(*O*-Bzl) function in the P4 position of PK101 reveals and leverages an "exo-pocket" on HNE as critical factor for selectivity. Furthermore, the PK101 P3 position harbors a methionine dioxide function, which mimics a posttranslationally oxidized methionine residue and forms a critical hydrogen bond to the backbone amide of Gly219 of HNE. Gly219 resides in a Gly-Gly motif that is unique to HNE, yet compulsory for this interaction. Consequently this feature enables HNE to accommodate substrates, which have undergone methionine oxidation, which constitutes a hallmark posttranslational modification of neutrophil signaling.

Introduction

Proteases are central mediators of a large variety of physiological processes. Proteolytic cleavage events are at the basis of protein degradation, enzyme activation and protein maturation and regulate a wide range of pathways from cell death, migration and proliferation, inflammation and immune response to blood coagulation (*1*). Aberrant proteolysis on the other hand is frequently linked to serious disorders. Furthermore, proteases are usually expressed in the cell or secreted as inactive zymogens that need activation via processes like proteolytic cleavage or dimerization. Activation of proteases underlies tight temporal and spatial regulation and thus generally protease location is not a good marker for protease function. Instead, spatial-temporal location of the active form of a given protease is necessary for understanding its function. For this purpose, activity-based probes have been developed for a variety of proteases (*2*). These probes are designed like active site-reacting protease inhibitors, specifically label an active protease and are thus powerful tools for research and diagnostics. Furthermore, these probes additionally pave the way for the development of potent inhibitors for select proteases for potential therapeutic use (*2*). Activity-based probes are often developed using sequence selectivity profiles obtained through library scanning methods with the goal of achieving the most selectivity possible with a limited amount of natural and/or unnatural amino acids. Most commonly these probes are designed to utilize the substrate binding pockets of the active site of proteases. The central challenge in probe design is the limited number of selectivity determining pockets. Usually, probes are limited to a strong P1 interaction with 2–3 additional sites and are limited by size requirements. Thus, the probes are commonly short peptides and cannot take advantage of distal exosites, which could confer additional selectivity.

A prime example for a desirable, yet difficult target for activity-based probe design is human neutrophil elastase (HNE), a serine protease of the chymotrypsin family (EC 3.4.21.37). HNE is expressed in neutrophil granulocytes and is stored in intracellular granules (*3*). During infection, these granules fuse with the phagosome and HNE plays a crucial role in pathogen clearance (*4*). More recently HNE has also been identified as a component of neutrophil extracellular traps (NETs), a defense mechanism in which neutrophils expel their DNA and anti-pathogenic factors to trap and eliminate pathogens (*5*). A central question however is, while HNE is present in NETs if the protease is in fact active. This emphasizes the need for an ultrasensitive and selective HNE activity-based probe.

Mechanistically, HNE has a very shallow active site cleft resulting in a very broad specificity. The specificity profile is in essence limited to preferred cleavage after small aliphatic residues in P1 like alanine, valine or isoleucine (*6-8*). Only minor selectivity preferences are proposed for the P2 – P4 residues (*6-9*). These attributes make the design of selective probes for HNE activity a very challenging task.

However, despite these challenges we were able to develop a nearly ideal synthetic substrate using the Hybrid Combinatorial Substrate Library (HyCoSuL) strategy (*10*). This substrate, Ac-Nle(*O*-Bzl)-Met(O)$_2$-Oic-Abu-ACC, includes unnatural amino acids to enhance both the rate of substrate hydrolysis as well as specificity. Indeed, it surpassed the substrate with the commonly used peptide sequence AAPV by more than 7,000-fold with a k_{cat}/K_m of 4.79×10^7 $s^{-1}mol^{-1}$ and showed high selectivity (900 fold) to HNE over the closely related protease PR3 (Myeloblastin) (*10*). Based on this substrate, an activity-based probe was designed, constituting of the recognition epitope (Nle(*O*-Bzl)-Met(O)$_2$-Oic-Abu) with a phosphonate warhead, a PEG(4) linker and a biotin group for detection (biotin-PEG(4)-Nle(*O*-Bzl)-Met(O)$_2$-Oic-Abu-PO$_3$Ph$_2$, Figure 1A). This probe, termed PK101, proved to be an ultrasensitive agent for the detection of HNE activity. Our experiments showed that PK101 is able to identify HNE activity both in *in vitro* experiments and in cell lysates, whereas no active HNE was detected in NETs in our initial experiments (*10*). However, the mechanistic base for this extraordinary labeling of this challenging protease remained elusive.

Results and discussion

Overall structure of the PK101-Elastase complex

To gain insight into the ultra-sensitivity of PK101 (Figure 1A) we solved its structure in complex with HNE at 1.63Å resolution (Table 1). Analogous to previous HNE structures, the asymmetric unit contains one HNE molecule, which is bound by one PK101 molecule (Figure 1B), with HNE tightly packed around a 3-fold crystallographic rotation axis in space group P6$_3$ (Supplemental Figure 1). HNE is fully defined from Ile16 to Gln243 (the chymotrypsin-numbering scheme is used throughout (*11*); a conversion chart is given in Supplemental Table 1) with the loop at Gly145-Asn147 showing greater flexibility as seen by weak electron density. Additionally, two sugar units typical for HNE are visible at the N-linked glycosylation sites at Asn109 and Asn159, respectively (Supplemental Figure 2). Thus, the HNE part of the structure is overall very similar to the one observed for the HNE-AAPV-CMK (MeO-Suc-Ala-Ala-Pro-Val chloromethyl ketone inhibitor) complex (Figure 1C) previously solved (*11*) with RMSD values of 0.582Å for all HNE Cα residues (Supplemental Figure 3).

The ultrasensitive probe inhibitor PK101 is covalently linked to the active site serine via its phosphonate warhead (Figure 1). Both the warhead and the recognition epitope (Nle(*O*-Bzl)-Met(O_2)-Oic-Abu) of PK101 are well defined by the 1.63Å resolution electron density (Figure 1A). The end of the PEG linker and the biotin group show enhanced flexibility as indicated by weaker or missing electron density and higher B-factors (Figure 1A) and thus do not stably interact with the protease (Figure 1B). PK101 overall binds HNE in the canonical substrate manner forming an antiparallel β-strand with the protease. Comparison with the structure of the HNE-AAPV-CMK (1PPG (*11*)) complex allows for definition of the HNE S1 to S4 pockets (Figure 1C) and reveals the intriguing binding mode of PK101 as detailed in the following.

The PK101 phosphonate warhead

The phosphorus atom of the phosphonate warhead (Figure 2A) is covalently linked to Oγ of the active site serine Ser195 (Figure 2B). Analogous to observations in previous structures of cathepsin G (1CGH, (*12*)) or trypsin (1MAX, (*13*)) in complex with phosphonate inhibitors the PK101 phosphonate warhead is completely reacted and both phenyl groups are eliminated (Figure 2B) (*14*). The phosphonate group exhibits tetrahedral geometry, mimicking the substrate transition state (Figure 2B). Accordingly, one of the phosphonate oxygen atoms is coordinated by the oxyanion hole comprised of the backbone amides of Gly193 and Ser195, whereas the other oxygen atom interacts with the Nε of the active site His57 (Figure 2B). The high-resolution data further reveals a symmetric electron density for the two phosphonate oxygen atoms indicating a delocalized electron system (Figure 2B).

S1 interaction

In the P1 position PK101 contains L-2-Aminobutyric acid (Abu), with similar activity to Val, but much lower hydrophobicity, which was crucial in design of optimal structure of PK101 for biological experiments. In line with this, our structure shows that Abu occupies the S1 pocket of HNE in similar fashion than valine (Figure 1C), whereby Cβ and Cγ of Abu exactly mimic the Cβ and Cγ1 of the valine counterpart (Figure 2C). Thus, it ideally occupies the pocket formed by HNE Val190, Val 216, Phe192 and the Cys191-Cys220 disulfide bond, explaining the nearly equal proficiency of Abu and Val in the P1 position (*10*). Furthermore, PK101 binds with a strict stereo-selectivity for the L-isoform, while, due to the synthesis strategy, both D- and L-isoforms of the P1 residue where present in PK101 applied to HNE prior to crystallization. This is in line with previous studies reporting stringent selectivity of serine proteases for the L-isoform for phosphonate inhibitors (*12, 13, 15*).

S2 interaction – increase in probe activity

In the classic example of AAPV-CMK the HNE S2 pocket is occupied by a proline, which is largely mimicked by the Oic (L-Octahydroindole-2-carboxylic acid) moiety in PK101 identified by HyCoSuL (*10*). The Oic residue maintains the same rigid backbone conformation as proline in the AAPV-CMK inhibitor. However, its additional cyclohexane ring provides improved hydrophobic contacts with Leu99 of the 90s loop and His57 in the S2 pocket of HNE (Figure 2D). This explains the about 2.5-fold higher rate of substrate hydrolysis for Oic over Pro for the P2 residue in the substrate-based HyCoSuL approach

(*10*). Additionally, an overlay of the PK101-HNE complex with a structure of PR3 (1FUJ(*16*)) shows that the S2 pocket in PR3 is more open and thus does not ideally interact with the cyclohexane ring of Oic (Figure 2D). This emphasizes the specificity advantage conferred by Oic in the P2 position of the probe for HNE over PR3 (*10*).

Neutrophil-characteristic methionine oxidation is mimicked by PK101 – the unique HNE S3 pocket

An important factor of neutrophil function is the presence of reactive oxygen species, which lead to the oxidative posttranslational modification of amino acids. Amongst these, one of the most prevalent modifications is the oxidation of methionine residues that constitute a hallmark of neutrophil function with up to 50% of methionines of bacterial pathogens showing this modification upon engagement by neutrophils (*17, 18*). A consequence of this process is the occurrence of oxidized protein substrates and a possible adaption of proteases to accommodate such modifications in their selectivity profile. Intriguingly, the best P3 residue identified in the HyCoSuL approach, and thus the P3 residue of PK101 is methionine dioxide ($Met(O)_2$, also termed methionine sulfone), a representative of an oxidized methionine (*10*). The structure of the PK101-HNE complex reveals a specific adaption of HNE to accommodate an oxidized methionine residue in P3 (Figure 3A). The Oδ2 of $Met(O)_2$ forms a hydrogen bond with the main chain amide of Gly219 (Figure 3B). This binding adds to hydrophobic interactions of the stem of $Met(O)_2$ with residues Phe192 and Val216 of HNE, which are absent in an inhibitor with the short side-chain alanine in P3 (Supplemental Figure 4). Thus, the accumulative effect of the methionine stem and the coordination of the Oδ2 of $Met(O)_2$ makes binding of an oxidized methionine in this extended P3/S3 interaction more favorable. This may not only be of relevance for probe design, but may also hint to a specific adaption of HNE towards natural substrates containing an oxidized methionine in P3. Importantly, the coordination of the Oδ2 is strictly dependent on the backbone confirmation adopted by Gly218 and Gly219, which is critical for correctly positioning the amide of Gly219. Stunningly, mining all human serine proteases using Scansite (*19*) revealed that a Gly-Gly motif in this position is absolutely unique to HNE with even the close relative PR3 exhibiting a Trp-Gly sequence incapable of adopting this conformation (Figure 3D). Together, these observations indicate that HNE has uniquely adapted to bind oxidized methionine residues, as are likely to be present in the oxidative environment of neutrophils during pathogen clearance.

PK101 reveals an 'exo-pocket' endemic to HNE adjacent to the S4 pocket

As outlined before, generally HNE is characterized by a very shallow active site and thus design of specific and sensitive substrates or probes proved to be challenging. PK101, however, has the very long 6-Benzyloxy-L-norleucine (Nle(*O*-Bzl)) side-chain in position P4. The crystal structure shows that this side-chain significantly adds both binding strength and specificity in an unexpected manner to the activity-based probe. Expectantly, the backbone and Cβ of Nle(*O*-Bzl) bind the S4 pocket in the same manner as Ala in the P4 position of the AAPV-CMK inhibitor (Figure 1C). However, the long hydrophobic stem of Nle(*O*-Bzl) extends the hydrophobic interactions and thus provides additional binding strength. Surprisingly, the *O*-Bzl group of Nle(*O*-Bzl) further extends into a hydrophobic pocket formed beyond the S4 pocket. This "exo-pocket" is formed by Phe215, Ala227, the disulfide bond between Cys168 and Cys182 and the guanidinium groups of Arg177 and Arg217 of HNE (Figure 3A). Investigation of all human serine proteases show that all other proteases as for example cathepsin G, which is also expressed by neutrophils or the family head chymotrypsin do have a full 170s loop with an

extra α-helix that is missing in HNE (Figure 3C/D). These eight extra residues block the exo-pocket and clash with the P4 Nle(O-Bzl) when overlaid on the HNE-PK101 structure (Figure 3C). The only exception is the close HNE relative PR3, which also has a shortened 170s loop/helix resulting in a similar exo-pocket (Figure 3C/D). This pocket is however far shallower than that observed for HNE (Figure 3C). Thus, the difference in pocket shape between HNE and PR3 could be further exploited by using even bulkier unnatural amino acids in P4, for example benzoylphenylalanine (Bpa) which in the HyCuSoL has the same rate as Nle(O-Bzl) in HNE, but is about 50% less active in PR3 (7). This clearly shows the power of using unnatural amino acids in substrate or probe design because no side-chain of any natural amino acid would be able to extend into this S4 'exo-pocket', whose potential only now became revealed through the unique binding mode of PK101.

In general, the example of this unexpected S4 exo-pocket shows how using unnatural amino acids in the HyCoSuL approach can utilize unexpected and extended binding pockets that are not easily accessible with natural amino acids. In this regard, a structure-based PDB-mining approach that takes into account extended binding pockets that can be exploited by unnatural amino acids may be helpful in future probe design for other proteases.

In summary, our structure shows how the activity-based probe PK101 achieves its potency and specificity for HNE by not only extending the interface surface, but also by exploiting and revealing unique characteristics of HNE. This is especially eminent for the S3 pocket interaction where the structure highlights how HNE can preferably recognize methionine-oxidized substrates that are a hallmark of the oxidative environment of neutrophils during infection. Additionally, an unexpected exo-pocket adjacent to the S4 pocket of the protease was revealed by the unnatural amino acid-based PK101 and may serve as an initiator for *in silico* approaches to mine for exo-pockets adjacent to existing substrate binding sites in other proteases and aid the design of probes and inhibitors as tools and initial agents for potential therapeutic drug development.

Material and Methods

Complex formation and crystallization

Human neutrophil elastase was purchased from Preparatis (Poland). PK101 was synthesized as reported previously (*10*) and a 10mM stock was prepared in DMSO. For crystallization, HNE was dissolved in HNE buffer (10mM HEPES pH 7.9, 100mM NaCl) to ~9mg/ml, added to a 3-fold molar excess of PK101 and incubated at 37°C for 30min, leading to >95% inhibition of HNE activity (Supplemental Figure 4). To remove DMSO and unreacted PK101, HNE-PK101 was buffer exchanged to HNE buffer using a Vivaspin 500 10,000 MWCO centrifugal concentrator. Final protein concentration for crystallization was about 8mg/ml. Small hexagonal rod-shaped crystals grew in setting drops at room temperature with 0.1M Tris pH8, 0.8M ammonium sulfate as precipitant (*11*). Crystals were harvested after 10 days, transferred to 0.1M Tris pH8, 0.9M ammonium sulfate and 20% ethylene glycol and cryo-cooled in liquid nitrogen.

Data recording and processing

Diffraction data was collected National Synchrotron Light Source beamline X29 at 100K, processed in iMosflm (*20*) and aimless (*21*) from the CCP4 suite (*22*). Phases were obtained using molecular

replacement with the protein chain of 1PPG (*11*) as search model in Phaser (*23*). Model building and refinement were performed in Coot (*24*) and Refmac (*25*). Library files for PK101 building blocks and links not defined in the standard CCP4 libraries were generated with JLigand (*26*). Validation and optimization were done with MolProbity (*27*) and pdb_redo (*28*). Data collection and refinement statistics are reported in Table 1. Coordinates and structure factors are deposited in the PDB under accession number xxxx.

Inhibition assay

To confirm full inhibition of HNE by PK101 a fluorogenic activity assay was performed. After complex formation as described in crystallography methods, HNE and HNE-PK101 were diluted to 1nM in HNE assay buffer (50mM HEPES pH7.4, 100mM NaCl, 0.1% igepal CA-630). A fluorogenic substrate was added (Ac-Nle(O-Bzl)-Met$(O)_2$-Oic-Abu-ACC) to a final concentration of 10µM and fluorescence release was measured at 37°C on a fMax fluorimeter (Molecular Devices). The excitation wavelength was 355nm and the emission wavelength was 460nm with a cutoff of 455nm. Data were analyzed in GraphPad Prism.

Database search

Scansite 3 beta (*19*) was used to search all human proteins in the SwissProt database with keywords 'serine protease' using the regular expression 'GDSG.{10,25}GG' to search for the chymotrypsin-like active site motive 'GDSG' followed by the 'GG' motive within 10 to 25 residues. The search resulted in 6 hits that were manually checked. Only one of the hits, human elastase, had the GG motive in the 220s loop.

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FIGURE LEGENDS

Figure 1: Structure of the PK101-HNE complex. **A)** Schematic depiction of the functional groups of PK101 (top) and stick representation of PK101 with 2FoFc electron density contoured at 1σ. **B)** Ribbon representation of HNE with PK101 (sticks, colored as in A) covalently attached to the active site serine Ser195 (spheres). **C)** Comparison of AAPV-CMK (left, green, from 1PPG(*11*)) and PK101 (right, magenta) recognition epitopes in complex with HNE (grey surface). The amino acids binding to each specificity pocket are indicated underneath the structures.

Figure 2: Interactions of the PK101 warhead and P1/P2 residues with HNE. **A)** Schematic of the PK101 recognition epitope and warhead. **B)** Detailed interactions between the PK101 phosphonate warhead and the active site residues of HNE. The 2FoFc electron density is shown in cyan mesh and contoured at 1σ. **C)** Interactions of the PK101 P1 Abu residue (magenta) with HNE and comparison with Val P1 of the AAPV-CMK inhibitor (green). **D)** Comparison of P2 interactions of HNE (grey surface) binding to P2 Oic of PK101 (purple) or P2 Pro of AAPV-CMK (green) and overlay of PR3 (wheat surface) with PK101.

Figure 3: Interactions of the P3 and P4 residues and details of specificity and selectivity. **A)** Binding of PK101 P3 and P4 residues (magenta) to HNE (grey surface). **B)** Details of the P3 Met$(O)_2$ Gly-Gly motif interaction. The 2FoFc electron density is shown in cyan contoured at 1σ. The hydrogen bond is indicated by a black dash with distance shown. **C)** A model of the PK101 P4 residue Nle(O-Bzl) with PR3 (eggshell, 1FUJ(*16*)), bovine chymotrypsin (blue, 2CHA (*29*)) and cathepsin G (light orange, 1CGH (*12*)) shows that only PR3 forms a similar exo-pocket as HNE whereas chymotrypsin and cathepsin G have a full 170s helix/loop (pink color in ribbon representation) that clashes with the side-chain of PK101 (clashes indicated by green surface). **D)** Sequence alignment between HNE and its closest homologues PR3 and cathepsin G and the S1 family prototype bovine chymotrypsin. The secondary structure for HNE is indicated above the alignment. The 170s helix/loop (orange box), clashing residues as in Figure 3C (green background) and the position corresponding to the HNE Gly-Gly motive (blue background) are indicated.

Table 1: Crystal, data collection and refinement statistics.

Supplemental Table 1: Conversion between HNE and chymotrypsin numbering schemes. Deletions in HNE are colored in red, insertions in green.

Supplemental Figure 1: Overview of the crystallographic HNE-PK101 trimer. The trimer is constituted by three asymmetric units colored in green, cyan and magenta, respectively. The crystallographic three-fold axis is indicated with a triangle.

Supplemental Figure 2: Overview of the asymmetric unit of HNE-PK101 including glycosylation. HNE is shown in grey ribbon representation with the active site residues shown as yellow sticks. PK101 is rendered as magenta sticks. The N-linked glycosylation sites are labeled and shown as magenta sticks, attached sugar chains are shown as orange sticks.

Supplemental Figure 3: Comparison of HNE from the HNE-PK101 complex and HNE-AAPV-CMK complex (1PPG) structures. The HNE chain of the HNE-PK101 structure is colored from blue to red from the N-terminus to the C-terminus. The HNE moiety from the AAPV-CMK-bound structure (1PPG(*11*)) is colored in grey.

Supplemental Figure 4: Comparison of P3/S3 interactions in PK101 and AAPV-CMK. Surface representation of the S3 pocket from HNE-PK101 structure. Residues interacting with the PK101 P3 Met$(O)_2$ residue (magenta) are shown as yellow sticks. An overlay of the P3 alanine residue (cyan) from the AAPV-CMK-bound structure (1PPG(*11*)) is also shown.

Supplemental Figure 5: HNE and HNE-PK101 activity assay. HNE and HNE-PK101 activity were measured by fluorescence release by hydrolysis of the HNE substrate Ac-Nle(O-Bzl)-Met$(O)_2$-Oic-Abu-

ACC (top). Comparison of the relative activity of HNE and PK101-HNE shows more than 95% inhibition of the HNE used for crystallography by PK101 (bottom).

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