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Two inhibitor molecules bound in the active site of *Pseudomonas* sedolisin: a model for the bi-product complex following cleavage of a peptide substrate

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This paper is dedicated to Professor David Eisenberg on the occasion of his 65th birthday.

Abstract

High-resolution crystallographic analysis of a complex of the serine-carboxyl proteinase sedolisin with pseudo-iodotyrostatin revealed two molecules of this inhibitor bound in the active site of the enzyme, marking subsites from S3 to S3′. The mode of binding represents two products of the proteolytic reaction. Substrate specificity of sedolisin was investigated using peptide libraries and a new peptide substrate for sedolisin, MCA–Lys–Pro–Pro–Leu–Glu#Tyr–Arg–Leu–Gly–Lys(DNP)–Gly, was synthesized based on the results of the enzymatic and crystallographic studies and was shown to be efficiently cleaved by the enzyme. The kinetic parameters for the substrate, measured by the increase in fluorescence upon relief of quenching, were: $k_{cat} = 73 \pm 5\,s^{-1}$, $K_m = 0.12 \pm 0.011\,\mu M$, and $k_{cat}/K_m = 608 \pm 85\,s^{-1}\,\mu M^{-1}$.
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Keywords: Serine-carboxyl proteinases; Substrate specificity; Inhibitor binding; Reaction products

Sedolisins (serine-carboxyl proteinases) form the family S53 of clan SB of serine proteinases, as annotated in the MEROPS database (http://merops.sanger.ac.uk). These enzymes have been known for a number of years as pepstatin-insensitive carboxyl proteinases [1]. Their placement in the same clan as subtilisin followed the solution of crystal structures of two representative members of this family, sedolisin (also known as *Pseudomonas* serine-carboxyl proteinase or PSCP) [2,3] and kumamolisin (kumamolysin, KSCP) [4]. The nomenclature describing these enzymes was changed several times in the last several years; the recently introduced terms "sedolisin" and "kumamolisin" [5] will be used throughout this paper.

Sedolisins share a number of common properties such as maximum activity at comparatively low pH, the presence of conserved acidic residues (aspartate and glutamate) required for the activity, stabilization by a Ca^{2+} cation, and the lack of inhibition by pepstatin. *Pseudomonas* sp. 101 sedolisin was the first member of the family to be isolated and described, and its enzymatic properties have been studied by biochemical and mutagenesis approaches [1,6–8]. Biochemical data are also available for several other related bacterial enzymes, such as sedolisin-B from *Xanthomonas* sp. T-22 (XSCP) [1]; kumamolisin, an enzyme isolated from a thermophilic bacterium *Bacillus* novo sp. MN-32 [9]; kumamolisin-B, an alcohol-resistant proteinase J-4 isolated from bacterium *Bacillus coagulans* [10]; and kumamolisin-As (ScpA), a peptidase with collagenolytic activity isolated from *Alicyclobacillus sendaiensis* [11]. Although neither the biological role of these enzymes in bacteria

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nor their natural substrates are known, similar enzymes appear to play a very important role in mammals. Peptidase CLN2, an enzyme which, when mutated, leads to a fatal human neurodegenerative disease, classical late-infantile neuronal ceroid lipofuscinosis [12], was identified as a tripeptidyl-peptidase I [13,14]. The disease caused by defects in the gene encoding CLN2 manifests itself by lysosomal accumulation of proteinaceous autofluorescent storage material in the neurons and other cells. In addition to humans, CLN2 orthologs have been identified in macaque, mouse, rat, dog, and cow, with sequence identity exceeding 80% [15], making it likely that these enzymes may be universally important for the other mammals as well. A mouse knockout of the CLN2 gene is being developed [16].

With the natural substrates for the bacterial serine-carboxyl proteinases not known, substrate specificity of these enzymes can be gleaned from their interactions with the inhibitors or by their ability to process peptide libraries. Tyrostatin (*N*-isovaleryl-tyrosyl-leucyl-tyrosinal) (Fig. 1A) is a potent inhibitor of sedolisin and sedolisin-B, but not of kumamolisin [4] or kumamolisin-As [11]. Two inhibitors utilizing the same chemistry, AcIPF and AcIAF, originally designed to inhibit kumamolisin [4], have also been shown to be submicromolar inhibitors of sedolisin [3]. All of these inhibitors bind covalently to the active site serine (Ser287 in sedolisin) and their side chains fill enzyme subsites extending from S1 to S3 or S4 [17], providing clues to the specificity of the enzymes for the side chains of residues preceding the cleavage site. However, these covalently bound inhibitors do not yield any information about the shape and specificity (if any) of the subsites following the scissile peptide bond of a substrate. Surprisingly, it was previously reported that parts of the inhibitor occupying putative S′ sites could be found in some crystals of the complexes of sedolisin and pseudo-iodotyrostatin (Fig. 1B) even in the absence of the covalently bound inhibitor [3,18]. We also found that by manipulation of the pH and solvent composition it was possible to replace the covalently bound inhibitors and to assure their full occupancy [3]. As shown below, an unexpected result of these studies was the discovery of two complete molecules of pseudo-iodotyrostatin (Fig. 1B) bound in the active site of sedolisin, one of them attached covalently on the S side of the active site, and the other making non-covalent interactions on the S′ side. This fortuitous occurrence provides a much more detailed description of the binding of inhibitors, and, by extension, substrates and products. Further elaboration of the subsite preferences of sedolisin, obtained by analysis of peptide libraries, led to synthesis of a new synthetic peptide substrate of this enzyme.

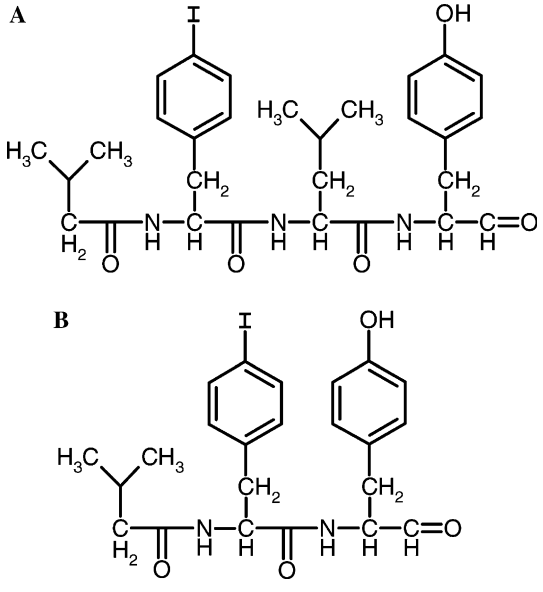

Fig. 1. Chemical formula of the inhibitors iodotyrostatin (A) and pseudo-iodotyrostatin (B) used to generate the complex with sedolisin. These inhibitors are modifications of a naturally occurring compound tyrostatin and synthetic pseudo-tyrostatin, respectively, with iodine substituting for a hydroxyl of a tyrosine.

Materials and methods

Preparation of the inhibitor complex. Protein used in these studies was cloned, overexpressed in *Escherichia coli*, and purified as described elsewhere [6]. All crystals were grown using a complex of sedolisin with an inhibitor sample that nominally contained iodotyrostatin (for details, see [3]). Crystals were grown at room temperature as described before [2,18]. To exchange inhibitors, crystals were transferred from their original mother liquor to a new one containing 1.6 M Li_2SO_4 at pH 7.5 and kept in that solution for approximately 30 min. This procedure was based on our previous observation indicating that the hemiacetal linkage of the covalently bound inhibitor reversibly dissociates at such pH, as monitored by the disappearance of any density that could be attributed to the inhibitor in the Fourier maps (data not shown). The crystals were subsequently transferred to another mother liquor containing 1.2 M ammonium sulfate, 10% glycerol, 5% guanidine hydrochloride, and 5% methanol, at pH 3.32. Stock solution of the inhibitor was prepared by mixing solutions of 10 mg/ml pseudo-iodotyrostatin in DMSO, and added to the mother liquor at the ratio of 1:10 (v/v). Crystals were soaked in the resulting solution for 3 h and then used for data collection.

X-ray data collection and structure refinement. X-ray diffraction data were collected on beamline X9B, NSLS, Brookhaven National Laboratory, using an ADSC Quantum4 CCD detector. The reflections were integrated and merged using the HKL2000 suite [19], with the results summarized in Table 1. The atomic model of the inhibitor complex of sedolisin was refined using the program SHELXL [20], performed against *F*-squared in the conjugate gradient (CGLS) mode. After each round of refinement the model was compared with the respective electron density maps and modified using the interactive graphics display program O [21]. The default SHELXL restraints were used for the geometrical [22] and displacement parameters. At first, the model was refined without any inhibitors. After the first run of the refinement, two molecules of pseudo-iodotyrostatin were built into the difference electron density maps. Water molecules were added in the subsequent refinement runs. The model was refined isotropically to convergence and then the refinement was continued anisotropically.

Table 1
Details of X-ray data collection and refinement

Ligand	Pseudo-iodotyrostatin
Space group	$P6_2$
Unit cell dimensions (Å)	
$a = b$	97.84
c	82.68
Wavelength (Å)	0.979
Resolution (Å)	1.3
Measured reflections	848,476
Unique reflections	110,018
R_{merge} (%)	8.2 (77.1)[a]
$I/\sigma(I)$	20.25 (2.3)
Completeness (%)	99.9 (99.5)
Refinement	
R—no σ cutoff (%)	16.37
R_{free} (%)	19.44
Rms bond lengths (Å)	0.013
Rms angle distances (Å)	0.029
Protein atoms	2701
Inhibitor atoms	60
Metals	1
Water sites	475
Disordered side chains	2
PDB Accession code	1NLU

[a] Values in parentheses are for the highest resolution shell.

The introduction of anisotropic displacement parameters in high-resolution refinement was validated by the drop in both R_{cryst} and R_{free}. Water oxygen atoms were refined with unit occupancies, although some of the sites are probably only partially occupied. The refinement parameters are presented in Table 1. The coordinates and structure factors have been deposited at the Protein Data Bank under an accession code 1NLU.

Substrate specificity analysis by synthetic combinatorial libraries. Analysis of the substrate specificity of sedolisin was studied by using two combinatorial libraries. Details of the synthesis and analysis of these libraries will be described elsewhere (Beyer, Dunn et al., in preparation). Briefly, the two libraries had the following sequences:

P1 library: Lys–Pro–(Xaa)–Glu–P1#Nph–(Xaa)–Leu
P1′ library: Lys–Pro–Ile–(Xaa)–Nph#P1′–Gln–(Xaa)

For each library, 19 different pools were synthesized where a different amino acid was substituted into either the P1 (P1 library) or the P1′ (P1′ library) position. They included all common amino acids found in proteins except Cys and Met, to avoid problems with oxidation. The latter amino acid was, however, substituted by norleucine (Nle). Nph is *p*-NO_2Phe and (Xaa) indicates that, at these points of the sequences, a mixture of the same 19 amino acids was added. Thus, each pool contained 361 peptides and the 19 pools of the P1 or the P1′ library totaled 6859 peptides each, or 13,718 in all.

An aliquot of each pool of the P1 and P1′ libraries to give approximately 100 μM total peptide concentration was used to test for cleavage by sedolisin in a spectrophotometric assay. For the P1 library, cleavage between the P1 and Nph yields a decrease in absorbance between 284 and 324 nm. For the P1′ library, cleavage between Nph and the P1′ residue yields an increase in absorbance in the same range. Thus, the assays of the 38 P1 and P1′ pools yielded an evaluation of the preferences of sedolisin for the P1 and P1′ amino acids. The better the fit within the active site cleft to optimize catalysis, the faster the observed rate of cleavage.

In the second step of the analyses, the products of cleavage for the Phe(P1) pool were separated by RP-HPLC and the masses of the penta- and tri-peptide products were determined. As several of the amino acids yield isobaric penta- and tri-peptides, appropriate single peptides were synthesized as controls. In this way we could differentiate the products containing Ile, Leu, or Nle on the one hand and Gln and Lys on the other hand. The amount of each product was determined by integrating the curves produced by continuous mass analysis. Appropriate serial dilutions of control peptides were done to insure that there was a linear response for the penta- and tri-peptide products.

Synthesis of new substrates for sedolisin. Based on the results of the specificity analyses, a peptide was designed that had the best or nearly the best amino acid for each of the six positions, S3–S3′, with sequence, Pro–Leu–Glu#Tyr–Arg–Leu. This peptide was not a good substrate and was attacked at several positions, as shown by HPLC and mass spectrometry of the digestion mixture. Furthermore, it cannot be studied by spectroscopic methods. Therefore, we prepared a fluorescent substrate by adding the MCA group to one end and the DNP group to the ε-amino group of a Lys on the other end. This peptide, MCA–Lys–Pro–Pro–Leu–Glu#Tyr–Arg–Leu–Gly–Lys(DNP)–Gly, was an excellent substrate for sedolisin. Product analysis for cleavage of the fluorescent substrate was carried out by separating an aliquot of a digestion mixture by RP-HPLC and analyzing the resulting peaks by MALDI-TOF mass spectrometry.

Determination of the kinetic parameters for cleavage of the fluorescent substrate by sedolisin. Concentration of the fluorescent substrate was determined by quantitative amino acid analysis of a measured aliquot of the stock solution. Upon cleavage, the fluorescence intensity increased due to relief of quenching of the MCA group by the DNP group. The rate of fluorescence change at 10 different substrate concentrations was determined and converted to micromolar per second by dividing by a standard value for the total cleavage of a measured amount of substrate. These values were plotted versus substrate concentration and V_{max} and K_m were determined. The V_{max} value for the fluorescent substrate was converted to k_{cat} by dividing by the concentration of enzyme, which was determined from V_{max} for cleavage of Lys–Pro–Ile–Glu–Phe#Nph–Arg–Leu and the published value of the k_{cat} for that substrate [23].

Results and discussion

Binding of the inhibitors

Two molecules of pseudo-iodotyrostatin are bound in the active site of sedolisin. The electron density is very clear for both of them (Fig. 2). One of these molecules (molecule 1) is covalently bound through its aldehyde functional group to Ser287 of the enzyme and its conformation is identical to the one reported before for this and other similar inhibitors [2,3]. The side chains of molecule 1 occupy the S1–S3 pockets of the enzyme (Fig. 3). The side chain of the P1 Tyr is wedged into a pocket created by the side chain of Arg179, the main chain of residues 133–136, and the main and side chains of residues 167–170. The Oε atom of P1 Tyr makes an excellent hydrogen bond with the side chain of Ser190 and a slightly longer bond with Oε2 of Glu175. The side chain of tyrosine is completely buried and its sole polar group makes excellent hydrogen-bonded interactions, thus the presence of such a residue in the substrate could be beneficial.

The P2 iodoPhe residue of pseudo-iodotyrostatin occupies a rather open area bounded on one side by the side chains of Ile35, Asp74, Trp81, and Glu80, while exposed to the solvent on the other side. The carboxylate of Asp74 is located ~3.75 Å from the iodine atom of iodoPhe, beyond the range of direct interactions, and no solvent molecules can be seen in its close vicinity. The P3

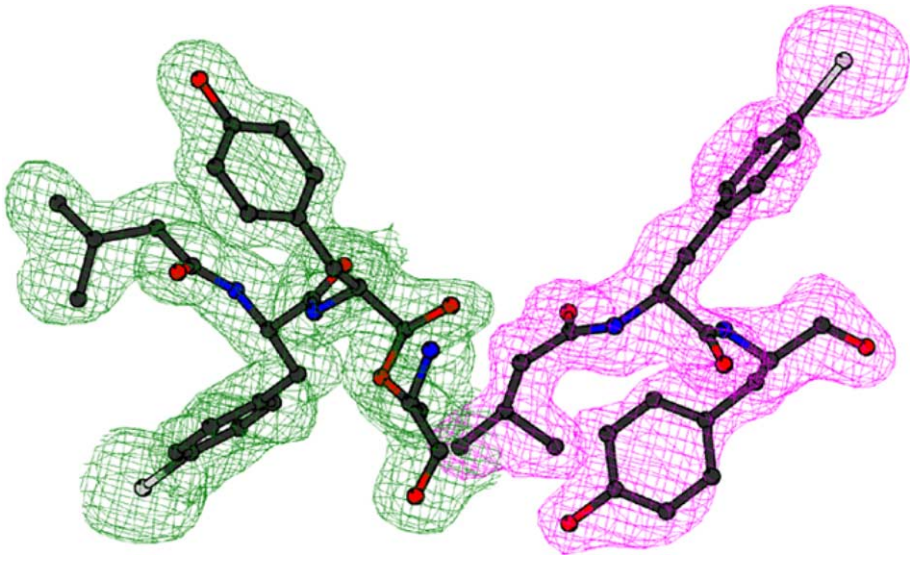

Fig. 2. Electron density map showing two molecules of pseudo-iodotyrostatin bound in the active site of sedolisin. The map covering the molecule covalently bound to the S1–S3 subsites of sedolisin is blue, whereas it is red for the non-covalently bound subsites S1′–S3′. This is a $2F_o - F_c$ map contoured at 1σ level.

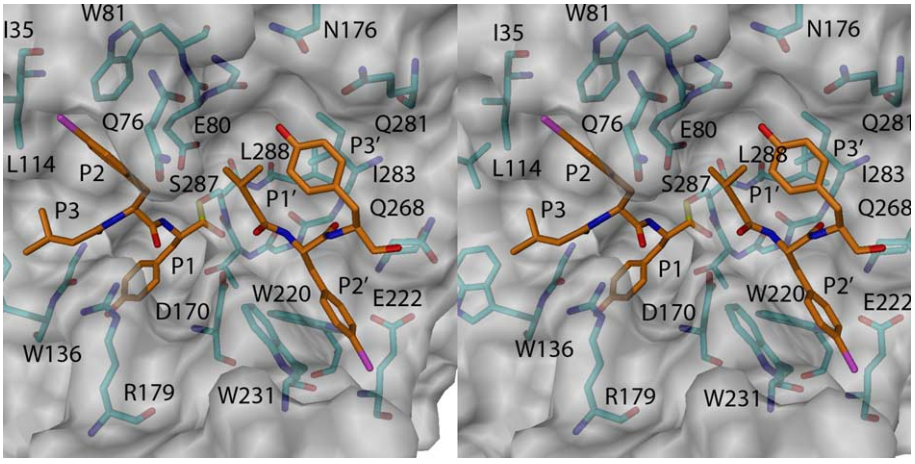

Fig. 3. Interactions of the inhibitors in the active site of sedolisin. The enzyme is represented as a blue stick model superimposed with a gray semi-transparent Connolly surface. The two inhibitor molecules are displayed as gold stick models. All residues of the inhibitors and selected side chains of the protein are labeled. Figure produced with DINO (http://www.bioz.unibas.ch/~xray/dino).

isovaleryl group of the inhibitor does not make clear contacts with the enzyme, but is oriented only through a hydrogen bond between the carbonyl of its main chain peptide and the amide nitrogen of Gly135. It is clear that a variety of residues could occupy this position.

Although some indications of the binding of a second inhibitor molecule (molecule 2) have been reported before [2], this is the first time that the complete molecule has been seen. This molecule of pseudo-iodotyrostatin is bound non-covalently (Fig. 3) and the direction of its peptide chain is the same as for molecule 1 discussed above. These two molecules, taken together, seem to mimic the binding of a six-residue substrate. However, it must be stressed that the distance between the P1 carbonyl C atom and the (modeled) P1′ N is about 1 Å too long to

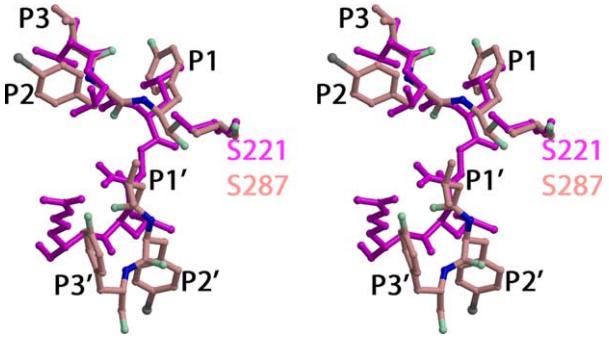

Fig. 4. A comparison of the conformation of the inhibitors that represent the products and the substrate of sedolisin and subtilisin. Two molecules of pseudo-iodotyrostatin bound to sedolisin are shown in ball-and-stick representation and are colored pink. A corresponding part of eglin-C bound to subtilisin [24] is shown in magenta.

form a covalent bond, thus suggesting that the two inhibitors are more indicative of the mode of binding of a product of proteolysis. Some rearrangements are needed to simulate the productive binding of a substrate, but such a model can be made quite easily. The side chain of the P1′ isovaleryl group makes good hydrophobic interactions with the side chain of Ile283 and slightly weaker interactions with Leu83. The side chain of the P2′ iodoPhe is perfectly stacked with Trp231, with the parallel faces of these aromatic residues separated by less than 3.5 Å. Other neighboring residues providing hydrophobic contacts are Trp220, Gln268, and Glu222. One face of the P2′ residue is, however, largely exposed to solvent.

The only hydrophobic interactions of the P3′ tyrosine are with the side chain of Ile283 and its hydroxyl moiety does not make any direct contacts with the enzyme, although it does become involved in water-mediated interactions. It is likely that this residue could be easily replaced by other ones. The terminal aldehyde moiety (corresponding to the C terminus of the peptide) points away from the protein, making it easy to model the extension of the chain of a true substrate.

Comparison with subtilisin inhibited by eglin-C

While all available structures of the members of both the subtilisin and sedolisin families complexed with either small-molecule inhibitors or with short substrates show only the interactions on the side preceding the cleavage site (S side), more extensive interactions can be gleaned by analyzing complexes of subtilisin with protein inhibitors. The structure of a complex of subtilisin Carlsberg and leech eglin-C, a small (70 amino acids) protein inhibitor of serine peptidases, was solved at high resolution (1.2 Å; PDB code 1cse; [24]) and offers a particularly good system for such a comparison. Six amino acids of eglin-C that occupy subsites S3–S3′ of subtilisin are ValI43–ThrI44–LeuI45–AspI46–LeuI47–ArgI48. Superposition of the two pseudo-iodotyrostatin molecules on this part of eglin-C is shown in Fig. 4. This superposition was accomplished by comparing the whole enzymes rather than just their active sites or inhibitors.

It is clear that the agreement between molecule 1 of pseudo-iodotyrostatin and the corresponding part of eglin-C is better than the agreement of molecule 2. The P1 Tyr of pseudo-iodotyrostatin 1 is almost perfectly superimposable on LeuI45, although the environment is considerably different in the two enzymes. Some of the main chain interactions (such as with residue 169 in sedolisin and the corresponding E154 in subtilisin, as well as 135/E154) are similar in the two enzymes, but the presence of Arg179 in sedolisin makes its P1 pocket quite unique. The direction of the P2 side chains (iodoPhe in pseudo-iodotyrostatin and ThrI44 in eglin-C) is also identical, with that pocket being much smaller in subtilisin due to the presence of residues E99–E100 that do not have

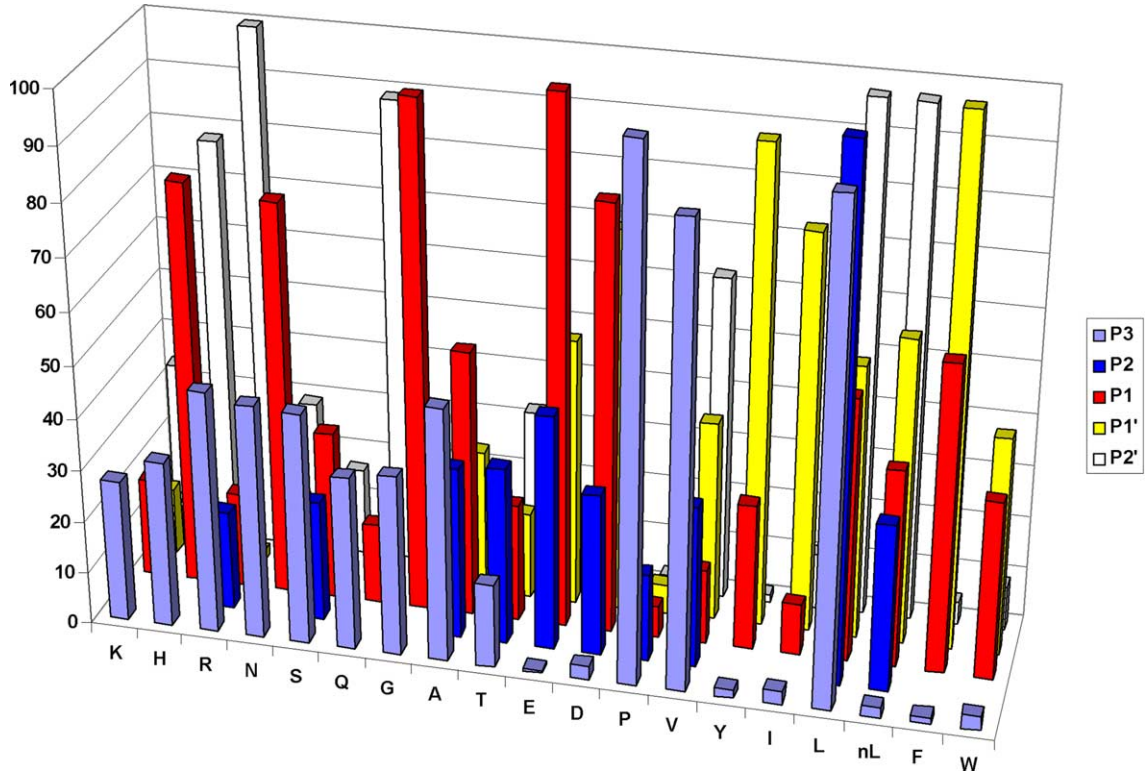

Fig. 5. Preferences of sedolisin from S3 to S2′. All values in each position are normalized to a maximum of 100% and plotted versus the amino acid in that position.

equivalents in sedolisin. The P3 isovaleryl group in pseudo-iodotyrostatin follows the direction of the main chain of eglin-C, rather than being superimposed on the equivalent ValI43 in eglin-C; again, the main difference between the corresponding subsites in the two enzymes is due to the presence of Arg179 in sedolisin that defines both the S1 and the S3 pockets.

Although the interactions made by the second pseudo-iodotyrostatin molecule on the S′ side of the active site of sedolisin are distinct from those in the subtilisin/eglin-C complex, the similarity is sufficiently high to suggest that the mode of binding of molecule 2 to sedolisin is meaningful. The position of the isovaleryl P1′ side chain of the sedolisin inhibitor is very close to that of AspI46 in eglin, with the directions of both the main chains and the side chain being quite similar. A shift between these residues (~1.25 Å for their Cα atoms) must be related to the fact discussed above that the two molecules of pseudo-iodotyrostatin mimic a product rather than a substrate. Even the directions of the corresponding carbonyl groups are very similar in the two complexes. The residues providing hydrophobic character to the S1′ pocket are superimposable Ile283 in sedolisin and LeuE217 in subtilisin. The direction of the main chain of pseudo-iodotyrostatin still follows that of eglin-C for the P2′ site, with the divergence between the Cα atoms becoming larger (~2.3 Å). The defining feature of this subsite in sedolisin is the presence of Trp231, providing aromatic environment for the side chain of P2 iodoPhe; no corresponding residues are found in subtilisin. However, hydrophobic contacts are provided on the opposite side of the pocket by parts of Glu268 in sedolisin and AsnE218 in subtilisin. The overall directions of the residues occupying the S3 pockets (Tyr in pseudo-iodotyrostatin/sedolisin and ArgI48 in eglin-C/subtilisin) are almost identical, although the distance between their Cα atoms has reached 4.75 Å. The preference for a hydrophilic residue in subtilisin is enhanced by the interactions of the guanidinium group with the carbonyl oxygen and the side-chain amide of AsnE62; no equivalent interactions can be made in sedolisin and thus this pocket is more hydrophobic.

The overall similarity of the binding of the two inhibitors of sedolisin to its target enzyme compared to the subtilisin/eglin-C complex reinforces the suggestion that the mode of binding in the former may represent the mode of product binding. The similarity of binding of the inhibitors of sedolisin and subtilisin on the S side has been remarked before [2,3]; the results for the S′ side presented here are new and provide a direction for a search for novel substrates or inhibitors of this enzyme.

Specificity of sedolisin for six positions in synthetic substrates

Substrate specificity of sedolisin was investigated by analysis of the cleavage of peptides belonging to two separate libraries (see Materials and methods). The results from the LC-MS analyses of the processed peptide libraries are presented in Fig. 5 for the P3, P1, P1′, and P2′ analyses normalized to 100%. The higher the peak for a given amino acid, the higher the preference the enzyme exhibited for that substitution in the octapeptide substrate. While the 13,718 peptides does not exhaust the total number of possible hexapeptide sequences from P3–P3′ (almost 5 million), the targeted approach does provide a strategy to examine specificity within the context of a successful substrate. For many of the ~5 million possible peptides, no cleavage would be observed. In addition, data from the analysis of sedolisin specificity in the P2 position from the series Lys–Pro–Ile–(Xaa)–Phe#Nph–Arg–Leu and P3′ data [23] from the series Lys–Pro–Ile–Glu–Phe#Nph–Arg–(Xaa) are plotted in Fig. 5. For the P2 and P3′ positions, we have an incomplete data set.

Kinetic parameters for cleavage of the fluorescent substrate

The fluorescent substrate, MCA–Lys–Pro–Pro–Leu–Glu#Tyr–Arg–Leu–Gly–Lys(DNP)–Gly, was cleaved at the Glu#Tyr bond as shown by RP-HPLC separation of the cleavage products. The N-terminal product, MCA–Lys–Pro–Pro–Glu, eluted at 6.9 min and the C-terminal product, Tyr–Arg–Leu–Gly–Lys(DNP)–Gly, eluted at 13.5 min. The masses of the two products agreed with their structures. The K_m, k_{cat}, and k_{cat}/K_m values for the cleavage of the fluorescent substrate by sedolisin were 0.12 ± 0.011 μM, $73 \pm 5\ s^{-1}$, and $608 \pm 85\ s^{-1}\ \mu M^{-1}$.

The complex of two pseudo-iodotyrostatin molecules with sedolisin can also be viewed as representing the product complex following enzymatic cleavage of a hexapeptide substrate. The hemiacetal involving Ser287 would represent the acyl enzyme formed following nucleophilic attack of the Ser Oγ, while the S′ pseudo-iodotyrostatin would represent the amino product from cleavage. We can speculate that this complex is stable while the normal amino product would quickly dissociate from the enzyme because the former is neutral whereas the latter would have a positive charge on the amino group.

Binding of substrates and products

As mentioned above, structural data presented here describe the experimentally determined mode of binding of inhibitors that most likely mimic the binding of products of a hydrolysis reaction, whereas the kinetic data were obtained using substrate libraries and the final "best" substrate. While pseudo-iodotyrostatin has been shown to be a nanomolar inhibitor of sedolisin [3], it was never optimized, thus it is not surprising that substrates based on its sequence are not the ones that are

cleaved most efficiently. The residues of the most efficient substrate of sedolisin that occupy the S3–S3′ pockets are –Pro–Leu–Glu#Tyr–Arg–Leu–, while the inhibitors are Iva–iodoPhe–Tyr. Careful comparison of the subsites indicates no conflicts between these results. Either the isovaleryl or Pro side chains can be easily fitted into the S3 pocket and the presence of Pro might be beneficial to guiding the other N-terminal amino acids of the substrate into conformations that would provide more optimal binding to the enzyme. Leu fits very well into the large S2 pocket that could in principle accommodate any type of hydrophobic residue. The preference for Glu in P1 may indicate that the side chain of Arg179 might rearrange in order to make an ion pair. Such a rearrangement is quite likely as long as the residue occupying this pocket is smaller than the iodoPhe observed in the crystal structure reported here (or indeed, in all other structures of inhibitor complexes of sedolisin and kumamolisin that contain only large aromatics in this position). Tyr appears to be a good choice for a P1′ residue, since it can be easily positioned to make a hydrogen between its hydroxyl and the side chain amide of Gln76. The hydrophobic part of the side chain of the P2′ Arg could interact with the indole of Trp231, while the guanidinium group might make a salt bridge to the carboxylate of Glu222, providing overall better interactions than those of the iodoPhe of the inhibitor. Finally, with the S3′ pocket being completely open on one side, the Leu side chain could easily interact with Ile283. While the details of the mode of binding of the most efficient substrate developed in this study may require further structural confirmation, the overall fit to the active site of the enzyme appears to be acceptable. The results present here emphasize that some care should be applied when interpreting the results of binding of even quite efficient inhibitors to the design of substrates.

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