Staphostatins are the endogenous inhibitors of the major secreted cysteine proteases of *Staphylococcus aureus*, the staphopains. Our recent crystal structure of staphostatin B has shown that this inhibitor forms a mixed, eight-stranded β-barrel with statistically significant similarity to lipocalins, but not to cystatins. We now present the 1.8-Å crystal structure of staphostatin B in complex with an inactive mutant of its target protease. The complex is held together through extensive interactions and buries a total surface area of 2300 Å².

Unexpectedly for a cysteine protease inhibitor, staphostatin B binds to staphopain in an almost substrate-like manner. The inhibitor polypeptide chain runs through the protease active site cleft in the forward direction, with residues IG-TS in P2 to P2’ positions. Both in the free and complexed forms, the P1 glycine residue of the inhibitor is in a main chain conformation only accessible to glycines. Mutations in this residue lead to a loss of affinity of the inhibitor for protease and convert the inhibitor into a substrate.

Staphopains A and B are the major secreted cysteine proteases of *S. aureus*. The literature contains evidence both for and against their role as virulence factors. Random mutagenesis has shown that transposon insertion into the V8-protease gene attenuates *S. aureus* virulence in three separate animal models (mouse abscess, bacteraemia, and wound infection models) (17). More recent work suggests that loss of virulence could be because of a polar effect of the insertion event on the downstream *sspB* gene encoding staphopain B (18). Consistent with this, inactivation of the *sspB* gene attenuates *S. aureus* virulence in the skin abscess model. In contrast, staphopain A, a close homologue of staphopain B, appears to be dispensable for virulence at least in this model.¹

Staphopains A and B are remote members of the papain superfamily of enzymes and are encoded on the genome as preproenzymes. The emergence of multiple antibiotic resistance in *Staphylococci*, in particular *Staphylococcus aureus*, has become a major medical concern. With the prevalence of methicillin-resistant *S. aureus* in hospitals throughout the world (1, 2) and with the appearance of strains that are resistant to “last resort” glycopeptide antibiotics (3, 4), treatment of *S. aureus* infections in difficult cases could soon depend on a few new compounds that have either been recently introduced to the clinic or are still in clinical trials (5–9). A possible strategy for the development of new antibacterials is to target bacterial virulence factors. The *S. aureus* arsenal of virulence factors includes secreted toxins (10, 11), immune modulatory molecules (12), adhesion molecules (13), signaling factors (14, 15), and possibly secreted proteases (16).

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The Staphostatin-Staphopain Complex

A FORWARD BINDING INHIBITOR IN COMPLEX WITH ITS TARGET CYSTEINE PROTEASE

The Staphostatin-Staphopain Complex

This article must therefore be hereby marked "i na c -"
EXPERIMENTAL PROCEDURES

Cloning, Expression, and Protein Purification—Staphopain B was recombinantly expressed and purified as described before (20). Mutants of staphopain B were generated according to the QuickChange (Stratagene) site-directed mutagenesis protocol and could be purified like the wild-type with the exception of the C-terminal deletion mutant (A99–109) that was insoluble.

For purification of staphopain B from the native source, culture supernatants were brought to 80% ammonium sulfate saturation. Precipitated proteins were collected by centrifugation (10,000 rpm, 30 min) and the pellet was dissolved in 50 mM Tris, pH 7.6. After extensive dialysis against the same buffer, the sample was clarified by centrifugation (10,000 rpm, 1 h) and loaded on a DE-52 column (Whatman pre-equilibrated with 50 mM Tris, pH 7.6). After extensive washing, bound protein was eluted with a NaCl gradient (0–500 mM). Fractions were checked by SDS-PAGE and assayed for proteolytic activity by zymography or by the azocoll (Calbiochem) assay (23). Fractions containing staphopain B (typically 50–100 mM NaCl) were pooled, dialyzed against 50 mM sodium phosphate buffer, pH 7.0, supplemented with 2 mM ammonium sulfate, and subjected to hydrophobic chromatography on phenyl-Sepharose (Amersham Biosciences). In a final step, the eluate was dialyzed against 50 mM Tris, pH 7.6, to remove salt.

Alternatively, proenzyme staphopain B was cloned into pGEX-5T (24) and expressed in E. coli as a glutathione S-transferase fusion construct with the N-terminal glutathione S-transferase moiety linked to proenzyme via a threonyl cleavable linker. The hybrid protein was purified by affinity chromatography on glutathione-Sepharose 4B (Amersham Biosciences) in the presence of 2 M pro tease at room temperature (5 h, 1:1000 weight ratio of the V8 proteinase to staphopain B). Eluted protein was then bound to thiopropyl-Sepharose 6B (Amersham Biosciences) and washed with 200 mM NaCl in 100 mM Tris, pH 7.8. Staphopain B was recovered from the column with 20 mM dithiothreitol in 50 mM Tris, pH 8.0. In some purifications, this affinity purification step was replaced with hydrophobic chromatography on phenyl-Sepharose (Amersham Biosciences) running a gradient from 2 to 0 M ammonium sulfate in 50 mM Tris, pH 7.5. The protein eluted close to the end of the gradient.

Both procedures yielded sufficient amounts of enzyme for analytical work, but not enough protein for crystallization. Therefore, we settled for an inactive mutant of mature staphopain B with alanine replacing the active site cysteine. A variant of overlap PCR was used to generate the mutant open reading frame, which was subsequently cloned via EcoRI and XhoI into a derivative of pET15b (Novagen) that lacks the original EcoRI site of the vector and carries six histidines and an NcoI site. Thus, the N-terminal sequence of our construct was MGGHHHHHHHFQDV . . . , where DQV are the first residues of the native staphopain B sequence. E. coli BL21(DE3) were grown at 37 °C and, after induction with 0.5 mM isopropyl-1-thio-D-galactopyranoside, were shifted to 30 °C for 5 h.

Cells were harvested, resuspended in 5 mM Tris, pH 7.5, with lysozyme and DNase I, and opened by sonication as usual. After centrifugation (40,000 rpm, 30 min) to remove insoluble debris, supernatant was applied to a nickel-nitrilotriacetic acid-agarose (Qiagen) column. The column was washed with 10 mM imidazole in buffer A (20 mM Tris, pH 7.5, 50 mM NaCl) and eluted with 30 mM imidazole also in buffer A. The eluate was concentrated, mixed with a slight stoichiometric excess of inhibitor, and the mixture applied to a Sephacryl S-500 (Amersham Biosciences) gel filtration column in buffer B (5 mM Tris, pH 7.5, 1 mM EDTA). The eluate was concentrated on YM-10 Centricons to a final OD (280 nm) between 30 and 50 and used for crystallization.

Analysis of Inhibitor Affinity—Analytical gel filtration runs were performed with a Superose 6 HR 10/30 (Amersham Biosciences) gel filtration column in buffer B (5 mM Tris, pH 7.5, 1 mM EDTA) and washed with 200 mM NaCl in 100 mM Tris, pH 7.8. Staphopain B and inhibitor each in the asymmetric unit and belonged to the P212121 space group. Detailed characteristics of our synchrotron dataset are presented in Table I.
The N-terminal part of the sequence with the exception of the active site cleft in the “standard view” for this superfamily of domain according to their orientation left and right of the domains, which are generally referred to as the L- and R-papain clan of cysteine proteases, staphopain B consists of two structure (21). Briefly, like all proteases that belong to the model that could be produced on the basis of the staphopain A of staphopain B is in excellent agreement with the homology presented in Table I.

The Structure of Staphopain B—As expected from the high sequence identity between staphopains A and B, the structure of staphopain B is in excellent agreement with the homology model that could be produced on the basis of the staphopain A structure (21). Briefly, like all proteases that belong to the papain clan of cysteine proteases, staphopain B consists of two domains, which are generally referred to as the L- and R-domain according to their orientation left and right of the active site cleft in the “standard view” for this superfamily of proteases (29) (see Fig. 1).

As in other superfAMILY members, the L-domain is built from the N-terminal half of the sequence with the exception of the most N-terminal residues, and contains the active site helix that carries the nucleophilic cysteine at its amino terminus. In staphopains, the L-domain is more compact than in other papain-like enzymes. In essence, it only contains one additional helix and the linker that connects it to the R-domain.

The R-domain contributes the catalytic histidine and asparagine and is built around a six-stranded, antiparallel pseudo-barrel. We consider the barrel “pseudo,” because the two strands formed from residues Ser-367 to Asp-370 and from residues Leu-375 to Leu-378 are too far apart to be linked by main chain hydrogen bonds. The barrel is significantly different from the barrel in all other papain-like proteases, including cathepsin B, by far the closest staphopain relative (see Fig. 1). One hairpin that contributes two strands to the staphopain barrel is so far unique to staphopains, and a second hairpin loop is significantly shorter and does not bend backwards as in all other papain-like enzymes.

The Staphostatin B-Staphopain B Interface—In standard orientation staphostatin B binds to the “top” of staphopain (see Fig. 2A), roughly interacting with the analogous segments of protease that are also used for contacts with either cystatins (30) (see Fig. 2B) or the globular domains of profragments (31, 32) (not shown). Nevertheless, because the staphostatin, cystatin, and profragment folds are unrelated, the details of the staphostatin B-staphopain B interaction are without precedence in protease-inhibitor complexes so far. The interaction of staphopain B with staphostatin B buries a total surface area of about 2300 Å² and involves contacts of the inhibitor with the L- and R-domains and the active site cleft of protease.

Excluding contacts of the active site spanning residues with protease, there are few specific contacts of the inhibitor with the L-domain. An array of ordered water molecules is located at the interface between protease and inhibitor in both copies of the complex in the asymmetric unit of our crystals. In at least one copy of the complex, there is some additional, as yet unidentified density possibly representing a component of the buffer, and in one copy of the complex, Asp-239 of protease forms a salt bridge with Lys-38 of inhibitor.

The R-domain of protease makes only contacts with residues in the C-terminal half of the inhibitor sequence. Once more excluding contacts to the active site spanning residues of inhibitor, all contacts are contributed by residues that are located in two loops of the six-stranded pseudo-barrel of protease. Interestingly, these are the two loops that are specific for staphopains in the papain superfamily (see Fig. 2). We therefore expect that the contacts of inhibitor with R-domain contribute significantly to the specificity of staphostatins for staphopains. In particular, the salt bridge of Lys-66 of inhibitor with Asp-335 of protease and the salt bridge between Asp-80 of inhibitor and Arg-382 of protease appear favorable.

Staphostatins Bind Similar to Substrates—The most prominent interactions between staphostatin B and staphopain B are mediated through loop residues 97 to 101 of inhibitor that span the active site of protease in the forward direction (see Fig. 2A). In analogy to “standard mechanism” inhibitors of serine proteases, we call this loop of inhibitor “the binding loop.” The binding loop is very well ordered in the staphostatin B-staphopain B complex, and its conformation is essentially identical in both copies of the complex in the asymmetric unit. Interestingly, this conformation does not appear to be preserved in free staphostatin B, because both copies of inhibitor in crystals of staphostatin B alone (22) are significantly different (see Fig. 3A). Although the binding loop is of course not cleaved by the inactive alanine mutant of protease in our crystals, the interaction with protease is best described in terms of

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### Table 1

**Data collection and refinement statistics**

| Parameter                                      | Value         |
|------------------------------------------------|---------------|
| Space group                                    | P212121       |
| a (Å)                                         | 73.49         |
| b (Å)                                         | 94.97         |
| c (Å)                                         | 110.93        |
| Independent reflections                       | 64.796        |
| Resolution (Å)                                | 1.8           |
| Completeness (%)                              | 92%           |
| \( R_{	ext{mol}} \) (% last shell in brackets) | 5.2 (18.2)    |
| \( I/\sigma \) (% last shell in brackets)     | 9.8 (3.5)     |
| Refinement statistics                         |               |
| \( R_{	ext{factor}} \) (%)                  | 19.2          |
| \( R_{	ext{free}} \) (%)                    | 22.1          |
| Root mean square deviation bond distance (Å)  | 0.02          |
| Root mean square deviation angles (°)         | 2             |
| Ramachandran core (%)                        | 88.6          |
| Ramachandran additionally all (%)            | 11.0          |
| Ramachandran generously all (%)              | 0.4           |
| Ramachandran disallowed (%)                  | 0             |

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The most prominent interactions between staphostatin B and staphopain B are mediated through loop residues 97 to 101 of inhibitor that span the active site of protease in the forward direction (see Fig. 2A). In analogy to “standard mechanism” inhibitors of serine proteases, we call this loop of inhibitor “the binding loop.” The binding loop is very well ordered in the staphostatin B-staphopain B complex, and its conformation is essentially identical in both copies of the complex in the asymmetric unit. Interestingly, this conformation does not appear to be preserved in free staphostatin B, because both copies of inhibitor in crystals of staphostatin B alone (22) are significantly different (see Fig. 3A). Although the binding loop is of course not cleaved by the inactive alanine mutant of protease in our crystals, the interaction with protease is best described in terms of
Berger and Schechter’s nomenclature (33) for the interaction between substrates and their target proteases. Residues P2 to P3/H11032 of the binding loop are in contact with protease (see Fig. 3B). The P2 residue in staphostatin B is Ile-97. Its carbonyl oxygen and amide are engaged in hydrogen bonds with Thr-284 of protease, and the side chain inserts into a hydrophobic pocket lined by residues Pro-286, Met-289, Leu-325, Ala-341, and Tyr-387 (S2 pocket not shown in Fig. 3B). The isoleucine side chain does not seem to fill the pocket entirely, and leucine could fit as well. As the P2 residue in staphostatin A is leucine, we suspect that the extraordinary specificity of staphostatins for the proteases in their own operon is probably not because of S2-P2 interactions.

The P1 residue is Gly-98, a conserved glycine in all known staphostatin sequences. With Ramachandran angles (ψ,φ) ~ (145°, 120°), this residue has a strained backbone conformation that would be sterically forbidden for any other residue (and lead to a close contact of the Cβ with Ser-241O of protease). It appears that this conformation is mainly fixed through hydrogen bonds, although in part indirectly. Gly-98NH donates a hydrogen bond to Gly-339O and may receive hydrogen bonds from either Ser-92O/H11032 or Ser-93NH. Whereas the hydrogen bond to Gly-339O is convincing, atomic distances suggest that Ser-92O/H11032 is more likely to donate its hydrogen bond to Thr-99O/H11032, which in turn donates a hydrogen bond to Gly-339O, and the relatively long bond distance and poor geometry argue against a hydrogen bond from Ser-93NH to Gly-98O. Thus, it seems more likely that the conformation of the P1 glycine is indirectly fixed through the amide bond to the P1 residue.

The P1 residue Thr-99 contributes major interactions with protease. In addition to the above described interactions of its side chain O/H11032, it accepts a hydrogen bond from Trp-362N to its carbonyl oxygen and its main chain NH would be ideally positioned to donate a hydrogen bond to His-340N/H11032, the histidine of the catalytic triad. Assignment of this hydrogen bond implies that His-340 is uncharged and in the tautomeric state with the proton on N-ε. This is unproven for the pH of our crystals (around 6.3), and unusual at least for wild-type papain-like cysteine proteases that would be expected to contain a thiolate-imidazolium ion pair in their active sites (34).

The P2 residue Ser-100 is again fixed through hydrogen bonds, but in contrast to the P1 residue, all its hydrogen bonding partners are residues of inhibitor and not of protease.
The side chain of the P3' residue Arg-101 faces protease again, hydrogen bonds with Trp-3620, and fills a cleft lined on one side by Trp-362 and Phe-380 and on the other side by Gln-236 and Phe-238.

The staphostatin B-staphopain B binding mode results in a remarkable placement of the scissile peptide bond relative to the catalytic residues of protease. Although our crystals contain an inactive variant of protease with alanine replacing the active site cysteine, the location of the nucleophilic sulfur can be tentatively inferred from its location in the staphopain A structure (21). According to this model, it would be placed almost in the plane of the P1-P1' peptide bond behind the carbonyl carbon so that the O-C-S angle would be close to 180°, a far too high value for efficient nucleophilic attack (35).

**Mutations of the P1 Glycine of Inhibitor Reduce the Affinity for Protease**—The most conspicuous feature of the binding mode of staphopain B to staphostatin B is the strained backbone conformation of the P1 glycine that would be Ramachandran forbidden for any other amino acid. To test the importance of this conformation, we generated staphostatin B mutants with alanine and arginine replacing the P1 glycine. Both mutations resulted in a loss of affinity for staphopain B. In native gel electrophoresis, only wild-type inhibitor, but neither of the inhibitor mutants comigrate with protease. This is true both for wild-type protease and for the inactive staphopain B mutant. In gel filtration experiments, the inactive mutant of protease did not lead to the generation of the minor band, even prolonged incubation of protease with inhibitor led to either no degradation of inhibitor at all, or to the generation of a band that ran just slightly faster than the full-length inhibitor on denaturing SDS-PAGE gels (after boiling samples in reducing sample buffer). The time course of the generation of this band was somewhat peculiar: with protease preparations that did lead to the generation of the minor band, an initial amount of truncated inhibitor appeared very rapidly (on the subminute time scale) on mixing. No further processing was observed (data not shown).

Additional evidence indicates that the minor band on our gels represents processed staphostatin B, which has been cleaved by staphopain B exactly in the position predicted from the crystallographic studies. First, N-terminal sequencing confirmed that the N-terminal sequence of the minor band matches the sequence of the N terminus of the inhibitor. Second, and more conclusively, the predicted and experimentally determined masses for the processed inhibitor agree to within ±2 Da, well within the error margin of the MALDI-TOF setup (see Fig. 6A).

**Mutations to the P1 Glycine Convert Staphostatins to Substrates**—Interestingly, mutations in the P1 glycine of staphostatin B make the inhibitor more susceptible to proteolysis by staphopain B: incubation of the P1 alanine mutant of inhibitor with protease for 30 min yields two peaks in MALDI-TOF, one for uncleaved inhibitor and a second, very substantial peak
with a mass of 11,761, matching the molecular mass of the N-terminal processing fragment to within 2 Da (see Fig. 6B).

Further incubation of mutant inhibitor with protease for 3 h leads to complete degradation of the mutant inhibitor. A similar conversion of the inhibitor into a substrate is observed for the more disruptive glycine to arginine mutation in the P1 position. Again, we observe a processing fragment of the correct molecular weight, and as expected for substrate, the amount of processed fragment grows with the time of incubation (see Fig. 6C). Although the arginine mutant is a less efficient substrate for protease than the alanine variant (see Fig. 6C), both mutants are more efficiently processed than the wild-type inhibitor, for which the processed fragment is barely detectable in our setup (compare the three panels in Fig. 6).

As in the case of the minor processing of wild-type inhibitor, we repeated the experiment with protease from native and recombinant sources to exclude the effect of contaminating proteases. In particular, we can exclude the effect of V8, a protease that is present in culture supernatants of S. aureus and was also used for in vitro processing of recombinant pro-staphopain B to the mature enzyme. Although large amounts of V8 protease (stoichiometric ratio 1:1 and more) can degrade staphostatin B, no degradation fragment corresponding to the molecular weight of the mature staphostatin B can be observed in MALDI-TOF, consistent with the strict preference of V8 for cleavage after glutamic or aspartic acid residues (36).

**DISCUSSION**

**Could the Structure of the Staphopain-Staphostatin Complex Have Been Predicted?**—At the outset of this work, experimental structures for staphopain A (21) and staphostatin B (22) were available. With 47% sequence identity between staphopain A and staphopain B, a homology model for staphopain B could be built with reasonable confidence.

Nevertheless, standard three-dimensional dock protocols to dock staphostatin B to the homology model of staphopain B failed to identify the crystallographic complex. In retrospect, this is easy to understand. Superposition of mature staphopain A on the staphopain B model in the complex leads to significant protease-inhibitor overlap. In the absence of a structure of free staphopain B, we cannot distinguish whether the backbone differences between free staphopain A and staphopain B in the complex are because of differences in the structure of the two proteins, or whether they represent structural adaptations of staphopain B on inhibitor binding.

In retrospect, a different computational strategy to identify the binding mode between staphopain B and staphostatin B would have been more successful. In the course of structure verification checks with VERIFY-3D (37, 38) we noted that the binding loop upstream of the most C-terminal β-strand scores very poorly for free inhibitor. In contrast, the very same strand gets excellent scores in the staphopain-staphostatin complex (data not shown). We plan to test on a larger sample of ligand-
receptor complexes whether this observation is accidental or whether it applies on a wider scale and could be used for the prediction of ligand-receptor binding modes. For the time being, we caution that the termini of proteins often score poorly in VERIFY3D, and that the method would have also suggested two false positive regions in staphostatin B, the N terminus, and the only helix in the molecule.

A Novel Mechanistic Strategy for Protease Inhibition—Several mechanistic strategies for protease inhibition have emerged from the study of complexes of papain-like cysteine proteases with their inhibitors: cystatins avoid cleavage by meandering around the active site (see Fig. 2B); profragments of papain-like proteases span the active site in opposite directions as natural substrates; the same is true of the inhibitors of apoptosis that target caspases (39); in SpeB, the profragment distorts the active site (40); this is also seen in a serine protease-serpin complex (41) and is likely to carry over to complexes of cysteine proteases with serpins; finally, distortion of the active site and covalent inhibition have been reported for the complex of p35 with caspase-8 (42). None of these paradigms fits the staphopain-staphostatin complex. Perhaps the closest analogy to our work is found in standard mechanism inhibitors of serine proteases (43). These inhibitors remain soluble, intact molecules after cleavage, held together through extensive contacts between the cleaved parts that are often reinforced through disulfide bridges. Unfortunately, we could not determine whether cleaved staphostatin B is soluble or inhibitory. The limited supply of wild-type protease precluded preparative staphostatin B cleavage, and attempts to produce cleavage products separately failed because the large N-terminal portion of the inhibitor could not be expressed in soluble form.

A Comparison of Binding Modes for Staphostatin B and Substrates—A full understanding of the staphopain-staphostatin complex should explain why staphostatins behave as inhibitors and not as substrates. A comparison of the staphostatin B-staphopain B binding mode with the postulated binding mode (29) of substrates to papain-like proteases is instructive. For substrates, the P2 residue should insert into the S2 pocket and form two main chain hydrogen bonds with protease, as observed for staphostatin B (see Fig. 3). The NH of the P1 glycine should donate a hydrogen bond to Gly-3390, again as observed for staphostatin B. Modeling studies of a papain-substrate complex before nucleophilic attack (44) suggest that the carbonyl oxygen of the scissile peptide bond should point toward Cys-243NH and Gln-237N (in staphopain B numbering), the two residues that according to most authors (29, 44) form the oxyanion hole in papain-like proteases. Our results for the staphostatin B-staphopain B complex are incompatible with this model. As shown in Fig. 3B, the unusual main chain conformation of the P1 residue places the P1 carbonyl oxygen far away from its expected location for substrates, so that both nucleophilic attack and stabilization of the tetrahedral intermediate should be compromised, implying a block of the reaction early in the mechanistic cycle.

A Block Early or Late in the Mechanistic Cycle?—Consistent with the structural results, our biochemical data also suggest an early block of the proteolysis reaction. If a tetrahedral intermediate was indeed formed, it should be detectable on denaturing SDS gels, or, because the thioester linkage could be unstable in reducing conditions, especially after boiling of samples in loading buffer, it should manifest itself as two bands with molecular weights corresponding to the free protease and cleaved inhibitor. This was never observed, neither with wild-type nor with recombinant protease preparations. Consistent with the failure to observe a thioester intermediate, we were not able to increase the yield of processed staphostatin B with the addition of hydroxylamine, a common reagent to cleave thioester linkages (data not shown).

The superposition of free and bound inhibitor structures shows that the critical conformation of the P1 glycine of inhibitor is not perforemed in the inhibitor structure and results from binding of inhibitor to protease. Our observation that some inhibitor molecules are cleaved immediately on mixing points to imperfections in this process. Once the proper complex is formed, the inhibitor is resistant to cleavage, consistent with no further proteolysis of inhibitor after the initial mixing phase in our experiments.

Interestingly, the peptide Abz-Gln-Gly-Ile-Gly-Thr-Ser-Arg-Pro-Lys(Dnp)-Asp-OH that was designed as a mimic of the binding loop of inhibitor behaves as a slow substrate. Ther-
fore, it is likely that in the wild-type protease-inhibitor complex, interactions of the binding loop with other parts of the inhibitor in addition to contacts with protease are required for the inhibitory binding mode.

Clearly, more work will be required to elucidate the details of staphostatin-mediated inhibition of staphopains. Nevertheless, it is already clear that the staphopain B-staphostatin B complex shows a new way for a proteinaceous inhibitor to inhibit a papain-like cysteine protease. Three key features of this new mode of inhibition stand out: the unusual conformation of the conserved P1 glycin of inhibitor, interactions of the binding loop of inhibitor with both primed and non-primed subsites of protease, and orientation of the binding loop polypeptide chain as in substrates rather than opposite to it as usual in cysteine protease inhibitor complexes. It remains to be seen whether these features characterize a mechanistic outlier among cysteine protease inhibitors, or whether they represent the first example of a more widespread, new “style” of cysteine protease inhibition.

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