Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

A distinct binding mode of a hydroxyethylamine isostere inhibitor of HIV-1 protease

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Received 15 September 2000
Accepted 27 November 2000

PDB Reference: HIV-1–peptidomimetic inhibitor complex, 1fqx.

Crystallization conditions for an HIV-1 protease–inhibitor complex were optimized to produce crystals suitable for X-ray diffraction experiments. The X-ray structure of the HIV-1 protease complex was solved and refined at 3.1 Å resolution. In contrast to Saquinavir, the mimetic hydroxy group of the inhibitor Boc-Phe-Ψ[(*S*)-CH(OH)-CH_2NH]-Phe-Glu-Phe-NH_2 is placed asymmetrically with respect to the non-crystallographic twofold axis of the protease dimer so that hydrogen bonds between the amino group of the inhibitor and the catalytic aspartates can be formed. The inhibitor binds in the centre of the active site by a compact network of hydrogen bonds to Gly27, Gly127, Asp25, Asp125 and *via* the buried water molecule W301 to Ile50 and Ile150.

1. Introduction

In recent years, HIV protease has become an attractive target for anti-AIDS treatment. Results from the clinical use of HIV protease inhibitors show the resistance the virus develops after inhibitors are applied. This resistance is caused by either individual or grouped mutations of the virus protease sequence (Deeks & Kahn, 1997). This study is a part of a project focused on characterization of a group of hydroxyethylamine peptidomimetic inhibitors with native and mutated protease (Hašek *et al.*, 1998). In this project, the HIV-1 protease mutants and inhibitors are prepared and biochemically characterized by the group of J. Konvalinka at the Institute of Organic Chemistry and Biochemistry, the native HIV-1 protease is prepared by the group of J. Sedláček at the Institute of Molecular Genetics and the crystallization and structure determination are carried out at the Institute of Macromolecular Chemistry of the Academy of Sciences of the Czech Republic. Since structurally similar compounds exhibit a broad specificity profile towards various resistant HIV strains (Rinnová *et al.*, 2000; Weber *et al.*, manuscript in preparation), it is important to analyze the molecular interactions of the inhibitor–enzyme complex in this structural class of compounds. This paper deals with the structure of HIV-1 protease complexed with the inhibitor Boc-Phe-Ψ[(*S*)-CH(OH)CH_2NH]-Phe-Glu-Phe-NH_2 (SE).

2. Materials

Boc-Phe-Ψ[(*S*)-CH(OH)CH_2NH]-Phe-Glu-Phe-NH_2 (where Boc is a *tert*-butyloxycarbonyl group) belongs to the series of substances that replace the cleaved peptide bond of the substrate with a hydroxyethylamino group. The inhibitor was prepared by alkylation of the N-terminal amino group of the tripeptide H-Phe-Glu(OBzl)-Phe-NH_2 with the pure diastereoisomer (2*R*,3*S*)-3-[(*tert*-butyloxycarbonyl)amino]-1,2-epoxy-4-phenylbutane at elevated temperature in a protic solvent followed by hydrogenolysis of the benzyl protecting group (Konvalinka *et al.*, 1997). The inhibition constant measured for the BRU isolate of HIV-1 protease was K_i = 0.15 n*M* (Konvalinka *et al.*, 1997).

Native HIV-1 protease (Sedláček *et al.*, 1993) at a concentration of 3 mg ml^{-1} was inhibited with a fourfold molar excess of the inhibitor dissolved in dimethyl sulfoxide to a concentration of 11 m*M*. For crystallization experiments, the protease–inhibitor complex was kept in sodium acetate buffer, 0.05% 2-mercaptoethanol, 1 m*M* ethylenediaminetetraacetic acid pH 5.8. The complex crystallized in hanging drops with Hampton Research Crystal Screen 9 solution [0.2 *M* NH_4 acetate, 0.1 *M* sodium citrate, 30%(*w*/*v*) PEG 4000] as precipitant. Needle-like crystals with dimensions 0.8 × 0.04 × 0.04 mm were used for diffraction experiments. Other details of the crystallization of the series of complexes have been published elsewhere (Dohnálek *et al.*, 1998; Buchtelová *et al.*, 1999).

3. X-ray data collection

Diffraction data were collected on crystals mounted in a capillary at room temperature (298 K) using a 300 mm MAR Research image-plate detector and a Rigaku R-200 rotating-anode generator (the in-house

Table 1
Diffraction data statistics and structure parameters.

Diffraction data	
Space group	$P6_1$
Unit-cell parameters (Å,°)	$a = b = 63.13$, $c = 83.12$, $\alpha = \beta = 90$, $\gamma = 120$
Diffraction limits (Å)	26.0–3.1
No. of measured diffraction maxima	14926
No. of unique reflections	3263
R_{sym}	0.177
Data completeness (%)	96.8
Structure parameters	
No. of non-H atoms	1646
No. of non-H atoms in disordered inhibitor	2 × 51
No. of refined water molecules	28
Average temperature factor B (Å^2),	
Main-chain atoms	19.4
Side-chain atoms	22.6
R.m.s.d. bond distances from ideal (Å)	0.008
R.m.s.d. angles from ideal (°)	1.37
Crystallographic residual R factor	0.18

diffractometer of the Oxford Laboratory of Molecular Biophysics), $\lambda_{Cu\,K\alpha}$ = 1.5418 Å. One data set of 24 images covered a 51° φ-angle rotation. The images were processed and scaled using the *HKL* software package (Otwinowski, 1993) to give a set of 3263 experimental structure-factor amplitudes. Tables 1 and 2 show the data and refinement statistics.

4. Refinement

Hexagonal symmetry allows several space groups in the case of protein crystals; the systematic absences observed in the reflections on the 00l axis point to the following possible space groups: $P6_1$, $P6_5$, $P6_122$ or $P6_522$. Space group $P6_122$ together with the size of one asymmetric unit of the crystal lead to one half of the protease dimer (one polypeptide chain) forming one asymmetric unit. The twofold symmetry axis through the active site of the protease excludes the presence of an asymmetric (peptidomimetic) inhibitor bound in this site and can be explained only by a disordered structure with two types of asymmetric units equally statistically spread through the crystal. These two types of asymmetric units then differ in the orientation of the inhibitor chain. Refinement in the higher symmetry space group $P6_122$ did not lead to satisfactory results and so further refinement proceeded in $P6_1$ with non-crystallographic restraints between the subunits of the protease dimer. The structure does not refine against the diffraction data in space groups $P6_5$ and $P6_522$.

The initial model in $P6_1$ yielded a crystallographic R factor of 0.37 for data in the resolution range 26.0–3.1 Å.

The R factor dropped rapidly after the first cycles of rigid-body fitting and initial positional refinement of atomic positions to R = 0.25. In two other cycles of refinement, the specific conformations of side chains were settled, leading to R = 0.22. At this point, the inhibitor was built in the active site according to the difference electron-density map maxima ($|F_o| - |F_c|$ coefficients).

Non-crystallographic symmetry restraints were applied to the protein and inhibitor chains, correlating both the positions and individual B factors of the dimer subunits and the two inhibitor positions. This resulted in an R-factor value of 0.195. As the difference electron-density maps proved to be very close to symmetrical, with a twofold axis in the centre of the active site, a second molecule of the inhibitor was built by rotation around this axis by 180°. The occupancy for all inhibitor atoms was set to 0.5 and two possible orientations of the inhibitor were modelled and refined. Water molecules (O atoms) were built abiding by following rules:

(i) presence of a difference electron-density map maximum above 3.2σ;

(ii) at least one potential hydrogen-bond contact within 3.5 Å;

(iii) presence of a $2F_o - F_c$ electron-density maximum above the 1.0σ level after refinement;

(iv) water oxygen temperature factor $B < 30.0$ Å^2 after refinement.

At this stage, the value of the crystallographic R factor was 0.18. The R_{free} factor was not applied, as the data-to-parameter ratio would decrease significantly. 91% of the protein residues lie within the most favoured regions of the Ramachandran plot (Fig. 1).

For data manipulation, refinement and model building, the computer programs in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994), *X-PLOR* (Brünger, 1992), *CNS* (Brunger *et al.*, 1998) and *O* (Jones & Kjeldgaard, 1993) were utilized.

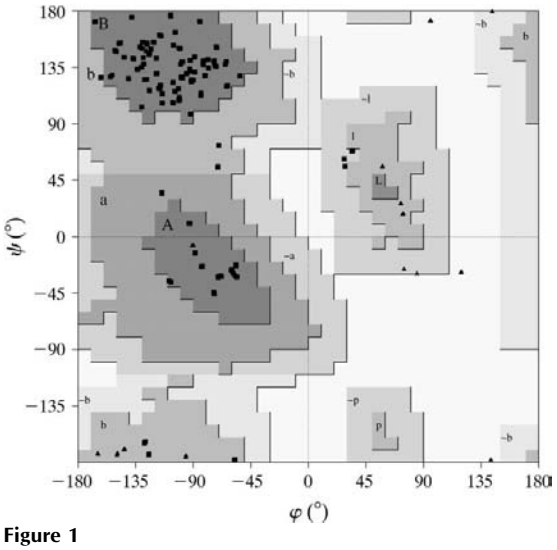

Figure 1
Ramachandran plot of HIV-1 protease in complex with the inhibitor SE as calculated and drawn with the program *PROCHECK* (Laskowski *et al.*, 1993). 91% of residues lie in the most favoured regions, four residues (Cys$A$67, Cys$B$67, Gln$A$61 and Gln$B$61) are found in the generously allowed region ~l. All four residues are involved in intermolecular crystal contacts and Cys67 forms a tight hairpin turn of the protease flap.

5. Discussion

The structure of the complex HIV protease–SE inhibitor is formed by two chemically identical subunits, numbered residues 1–99 and 101–199. Each unit consists of seven β-strands, one α-helix and seven loops.

5.1. Inhibitor binding

The inhibitor binds in the substrate-binding site of the dimer, with its hydroxyethylamino group making contacts to Asp25 and Asp125. The position of the disordered inhibitor in the cleft is shown in Fig. 2 and the averaged intermolecular contacts are shown in Fig. 3.

All hydrophobic side chains of the inhibitor are involved in protein–inhibitor contacts. Hydrogen-bond donors and acceptors of the inhibitor (N, O atoms), except the Boc urethane O atom and the C-terminal amide NH_2, form hydrogen bonds to the protease dimer (Fig. 3). The carbonyl O atoms of Boc and $O_{P1'}$ form hydrogen bonds to protein Ile50 and Ile150 *via* the structurally important water W301. The class of inhibitors under investigation offers a sufficient number of inhibitor main-chain interactions and instead of one hydroxy O atom of the central group (common with other hydroxyethylene and hydroxyethylamine peptidomimetics), offers both the hydroxy and amino groups. Thus, both OH and NH are separately involved in hydrogen bonds to the active-site Asp25 $O^{\delta1}$ and Asp125 $O^{\delta2}$. The high flexibility of the inhibitor molecule, the two independent hydrogen bonds at the active-site residues and the convenient position of the hydro-

Table 2
Crystallographic *R* factor as a function of resolution.

Shell	Resolution limits (Å)	No. of reflections	*R*
1	6.18–26.00	413	0.157
2	4.91–6.18	416	0.144
3	4.29–4.91	432	0.123
4	3.90–4.29	416	0.165
5	3.62–3.90	423	0.180
6	3.41–3.62	424	0.223
7	3.24–3.41	428	0.259
8	3.10–3.24	311	0.286
All	3.10–26.00	3263	0.180

phobic phenyl groups in the hydrophobic pockets of the protease result together in the high affinity of the inhibitor molecule. The configuration of the chiral C atom and the character of the P_2' side chain also play important roles in the inhibitor binding.

Interatomic distances show that the OH at the chiral C atom and the amino-group NH of the hydroxyethylamine of the inhibitor can each participate in three hydrogen bonds to the carboxyl O atoms of Asp25 and Asp125 of the protease. The OH and NH groups lie each within hydrogen-bonding distances of three acceptors. The hydroxy group of the transition-state analogue is not centred above Asp25 and Asp125 as is usual with other hydroxyethylamine peptidomimetics; its position is adjusted so that both hydroxy and amino groups bind to two different carboxyl O atoms of aspartic acids. This shift also places both phenylalanine side chains of the inhibitor P_1 and P_1' symmetrically with respect to the non-crystallographic symmetry twofold axis of the protease to the hydrophobic pockets of the protease S_1 and S_1'.

The carboxy group of inhibitor side chain P_2' – the glutamic acid residue – is not well localized in the electron-density maps and therefore its potential hydrogen bonds as shown in Fig. 3 are less reliable compared with other atoms of the inhibitor.

5.2. Comparative analysis

5.2.1. Complexes of other hydroxyethylamine inhibitors. The structure of the complex was compared with the complexes of Saquinavir (Krohn *et al.*, 1991) and Amprenavir (Kim *et al.*, 1995), PDB codes 1hxb and 1hpv, respectively (Berman *et al.*, 2000). A least-squares fit of C^{α} atoms of protease residues 25–30 and 50–51 leads to the following results.

(i) Hydroxyethylamine contacts. Saquinavir, Amprenavir and SE bear a hydroxyethylamino group replacing the scissile peptide bond of a substrate; however, in the case of the former two inhibitors only the hydroxy group can participate in hydrogen bonding. In the case of SE, the NH group becomes an important agent in hydrogen bonding to Asp25 and Asp125 of the protease dimer. The hydroxy group of Saquinavir and Amprenavir forms four hydrogen bonds to all Asp25 O and Asp125 O atoms and is approximately centred above these hydrogen-bond acceptors. SE offers two donors, OH and NH, making hydrogen bonds to the O atoms of Asp25 and Asp125 (Fig. 3). The hydroxy group of SE displays a relative shift from the dimer axis with respect to those of Amprenavir and Saquinavir of about 1.6 Å, which equals approximately half the OH—NH distance in the SE complex. As the OH of SE is placed between Asp25 O and Gly127 O, the main-chain O atoms of Gly27 and Gly127 in the SE complex are pushed away from the dimer axis to a Gly27 O—Gly127 O distance of 9.6 Å compared with the Saquinavir complex value of 7.8 Å, whereas the Gly27 C^{α}—Gly127 C^{α} distance remains unchanged at 6.6 Å.

(ii) Asp25 and Asp125. Both aspartic acid $O^{\delta1}$ atoms in the Saquinavir and Amprenavir complexes participate in three hydrogen bonds, two of them sharing one H atom of $OH_{inhibitor}$ or the $O^{\delta1}$—H—$O^{\delta1}$ bond. Similarly, in the SE complex, $O^{\delta1}$ atoms participate in three hydrogen bonds but bonds to the inhibitor are mediated by two independent H atoms of the OH and NH groups of SE. The $O^{\delta2}$ atoms in the compared complexes are involved in one hydrogen bond each in the case of Amprenavir or Saquinavir, whereas in the case of SE they become the acceptors of two H atoms from the OH and NH groups. These differences in the hydrogen-bonding pattern are reflected in the different relative positions of the Asp25 and Asp125 side chains in these structures. The $O^{\delta1}$—$O^{\delta1}$ distance of 2.7 Å in the cases of Saquinavir and Amprenavir shortens in the SE complex to 2.4 Å, showing a much stronger $O^{\delta1}$—H—$O^{\delta1}$ interaction. The Asp25 C^{α}—Asp125 C^{α} distance remains unchanged at approximately 6.5 Å in all these structures. As the unliganded HIV-1 protease (PDB code 3phv; Lapatto *et al.*, 1989) displays the longest contact distances (Asp25 $O^{\delta1}$—Asp125 $O^{\delta1}$, 3.0 Å; Asp25 C^{α}—Asp125 C^{α}, 6.8 Å) in the compared set of structures, it can be stated that Asp25 and Asp125 are brought closer on inhibitor binding.

(iii) Protease carbonyl O atoms Gly27 O and Gly127 O form hydrogen bonds to the inhibitor more effectively in the SE complex (NH_{P1}, $NH_{P2'}$) than in Saquinavir (only NH_{P1}) or Amprenavir (no bonds).

5.2.2. Complexes with an amino group binding to the catalytic aspartates. An amino group binding to the active-site aspartic acids was also observed in the following classes of structures of HIV-1 or SIV protease with inhibitors or products.

(i) Peptidomimetic inhibitors with NH in the isostere. These include the reduced amide isostere, hydroxyethylamine (*R*′-acyclic) isostere, hydroxyethylamide isostere and aminodiol isostere. The *C*2 symmetrical aminodiol isostere inhibitor BMS-182193, Boc-NH-CH(CH_2Ph)-CH(OH)-CH_2-NH-CH_2-CH(OH)-CH(CH_2Ph)-NH-Boc (Kervinen *et al.*, 1996; PDB code 1odw) and the SE inhibitor share an identical Boc-NH-CH(CH_2Ph)-CH(OH)-CH_2-NH moiety. The SE complex and 1odw structure were superimposed on all C^{α} atoms with the following results.

(*a*) The overall position and conformation of Boc-Phe remains almost unchanged.

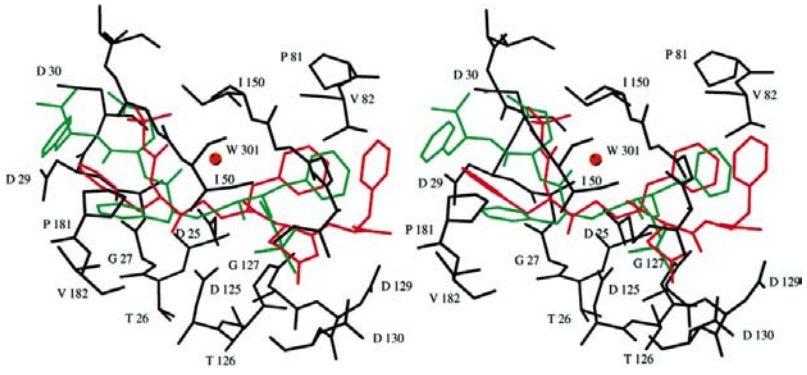

Figure 2
A stereoview of the disordered position of the inhibitor bound in the substrate-binding site of the protease: two positions of the SE inhibitor (atoms in green and red) and the surrounding protease chains are shown. This figure was drawn with the program *MOLSCRIPT* (Kraulis, 1991).

(*b*) The hydroxy group on the chiral C of the isostere is in the *R* configuration in BMS-182193 and in the *S* configuration in the SE inhibitor. In the SE complex, it points towards the main chain of residues 25–27 between the side chain of residue 25 and the carbonyl of Gly27 and can form hydrogen bonds to both Asp25 carboxy O atoms. On the other hand, the BMS-182193 hydroxy group binds to the Asp25 $O^{\delta2}$, accessing it from the side of the flaps and therefore its bond to $O^{\delta1}$ is disabled. The carboxy groups of Asp*A*25 and Asp*B*25 in the SE complex are rotated with respect to the BMS-182193 complex by approximately 30° around the $C_\beta-C_\gamma$ bond. This rotation brings Asp25 $O^{\delta1}$ into closer contact with the SE hydroxy group.

(*c*) The isostere amine of BMS-182193 lies approximately centred between residues *A*25 and *B*25 in the plane defined by their side chains and makes hydrogen bonds to both of them (distances of 2.9 and 3.2 Å, respectively). Similarly, the SE isostere amine binds symmetrically to both aspartates (distances of 2.8 and 2.9 Å), but it is shifted away from the dimer symmetry axis by approximately 0.5 Å (Fig. 3). The *C*2 symmetry of BMS-182193 causes the central position of its isostere amino group compared with the asymmetrical SE inhibitor.

(ii) Peptides and analogues of catalytic products binding by the N-terminus to the active-site aspartic acids. Inhibitor GR123976 (Jhoti *et al.*, 1994; PDB code 1hte) was observed to bind to the protease active-site cleft together with a tetrapeptide H-Leu-Gln-Glu-Ser-OH so that the peptide NH_2-terminus forms one hydrogen bond to Asp*A*25 $O^{\delta2}$ (N—O distance of 3.4 Å); it also hydrogen bonds to the carboxy-terminal group of the inhibitor GR123976. In structural studies of the interaction of catalytic products with HIV-1 and SIV protease (Rose *et al.*, 1996; PDB codes 1ytg, 1yti, 1ytj), the terminal NH_2 (NH_3^+) group interacts with the catalytic aspartates *A*25 and *B*25 in two different arrangements.

(*a*) The amino-group hydrogen bonds only to one of the aspartic acid residues with N—$O^{\delta1}$ and N—$O^{\delta2}$ distances of 2.9 Å and 3.1 Å, respectively.

(*b*) The amino-group hydrogen bonds to both $O^{\delta2}$ atoms of Asp*A*25 and Asp*B*25 symmetrically with N—$O^{\delta2}$ distances of 3.1 Å. The latter example of a terminal amino-group binding to the catalytic aspartates closely resembles the way in which the amine in SE inhibitor shares its hydrogen in two hydrogen bonds to the O atoms of both aspartic acid residues *A*25 and *B*25.

5.3. Biomedical implications

In spite of the low resolution and relatively poor $R_{\rm merge}$ of the crystallographic data of the reported structure, important qualitative conclusions can be drawn. It was shown that the inhibitor molecule binds in the active site of HIV-1 protease, creating 20 hydrogen bonds to the active-site Asp25 and Asp125, to the S_1, S_2, S_1', S_2' and S_3' binding sites of the protease dimer and to four water molecules. Two sets of hydrogen bonds between the hydroxyethylamine group and Asp25 and Asp125, together with the favourable hydrophobic interactions of P_1 and P_1' phenyl groups with hydrophobic pockets S_1 and S_1' and strong hydrogen bonds between the protease and P_2' side chain (Glu), result in the high affinity of this molecule to the protein. These interactions also determine the high binding specificity of this molecule, as the position in the protease cleft depends on the distances between individual groups of the inhibitor interacting with their protease counterparts.

A higher number of SE–protease hydrogen bonds (13 bonds) compared with the Saquinavir complex (11 bonds) is compensated by the fixed conformation of the decahydroisoquinoline group of Saquinavir, which is favourable for hydrophobic interactions; this results in a similar affinity of the inhibitors for the protease dimer, represented by K_i values of 0.12 n*M* for Saquinavir and 0.15 n*M* for SE.

A comparative analysis with a similar *C*2 symmetrical inhibitor BMS-182193 (Kervinen *et al.*, 1996) confirms the different binding modes of the hydroxyethylamine isostere in *S* and *R* configurations that can lead to a quantitative explanation of the structure–activity relationship of the hydroxyethylamine isostere-containing inhibitors. Also, the central role of the amino group in the hydroxyethylamine (*R*′-acyclic) isostere, owing to its bonding to both catalytic aspartates, diminishes the previously acknowledged key role of an isostere hydroxy group binding to the aspartic acid residues *A*25 and *B*25 in HIV-1 protease.

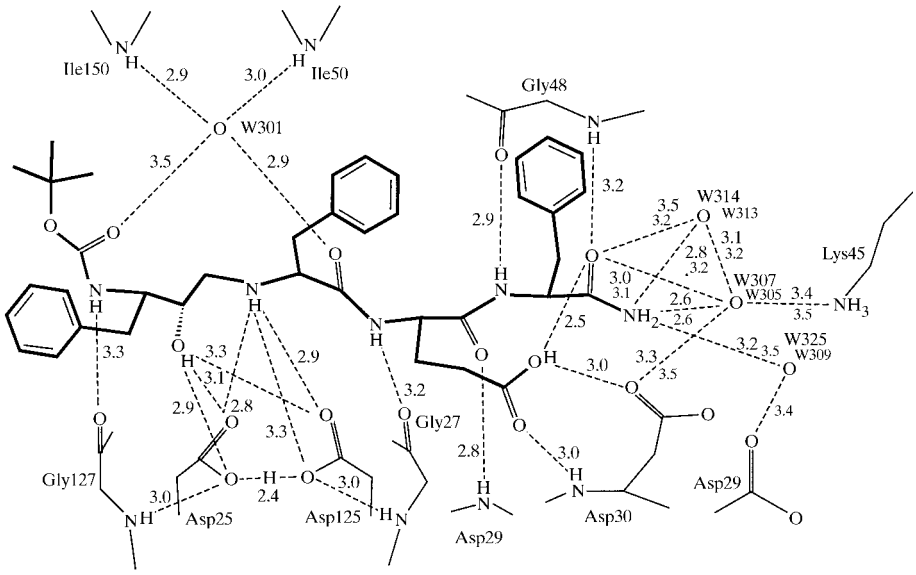

Figure 3
A schematic view of inhibitor SE–HIV protease contacts representing potential hydrogen bonds. Distances are given in Å. Two positions of the disordered inhibitor produce two sets of slightly different intermolecular distances. Average values of the distances are displayed, except solvent contacts to $CONH_2$ group of the inhibitor, where distances and water molecules on the opposite sites of the protease dimer are marked by different type sizes.

The work was supported by the Czech Technical University (grant No. 30497487), the Grant Agency of the Academy of Sciences of the Czech Republic (project A4050811), the Grant Agency of the Czech Republic (projects No. 203/97/P031 and No. 203/98/K023) and by an International Research Scholar's Award from the Howard Hughes Medical Institute (HHMI 75195-54081) to JK. The authors wish to thank L. N. Johnson, M. J. Adams and C. E. Naylor of Oxford University LMB for support and for providing the diffractometer time.

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