1.9 Å X-Ray Study Shows Closed Flap Conformation in Crystals of Tethered HIV-1 PR

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ABSTRACT **Three-dimensional structure of an asymmetrically mutated (C95M) tethered human immunodeficiency virus type 1 protease enzyme (HIV-1 PR) has been determined in an unliganded form using X-ray diffraction data to 1.9 Å resolution. The structure, refined using X-PLOR to an *R* factor of 19.5%, is unexpectedly similar to the ligand-bound native enzyme, rather than to the ligand-free native enzyme. In particular, the two flaps in the tethered dimer are in a closed configuration. The environments around M95 and C1095 are identical, showing no structural effect of this asymmetric mutation at position 95. Oxidation of Cys1095 has been observed for the first time. There is one well-defined water molecule that hydrogen bonds to both carboxyl groups of the essential aspartic acids in the active site. Proteins 2001;43:57–64.**

Key words: HIV-1 protease; X-ray; crystal; structure; regulation

INTRODUCTION

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), encodes its own protease (PR) enzyme (HIV PR), which is absolutely essential for the production and propagation of infectious virions.[1–3] Therefore, HIV PR has become an attractive target for the design of antiviral compounds against AIDS,[4] especially after the discovery of viruses resistant against reverse transcriptase (RT) inhibitors.[5] X-ray structures of HIV PR at moderate resolutions of 2.4–2.7 Å have established HIV PR as a homodimeric aspartyl protease, with each polypeptide chain providing one aspartyl residue at the active site.[6–8] The coming together of the two monomers to form the active dimer in polyprotein precursors might well be a rate-limiting factor in viral processing and assembly. In the structure of HIV-1 PR, the N-terminus of one subunit is very close to the C-terminus of the second subunit, facilitating covalent linking of the two subunits to produce a single-chain tethered dimmer. Tethered dimers with linking peptides of different sequence and length have been produced by genetic engineering techniques.[9–11] Such tethered dimers have been shown to have an overall fold similar to the wild-type enzyme, and are more stable at slightly basic pH values.[11] The optimal pH value for maximum activity is approximately one unit higher for tethered dimers than for wild-type enzyme.[10,11] Tethered dimers also provide a unique opportunity to study the effects of introducing asymmetric mutations in the two subunits of HIV PR.

Cysteine modification is known to affect the activities of various cellular proteins.[12–14] HIV-1 PR monomer contains two cysteine residues—Cys67 located at the surface and Cys95 located at the dimer interface,[6–8]—both of which are highly conserved among viral isolates found in HIV infected patients.[15] These cysteines do not appear to affect catalysis as site specific mutants (C67A and C95A) have the same activity as the wild-type enzyme.[16] The suggestion that these cysteines probably regulate enzyme activity,[16,17] is supported by recent reports on reversible inactivation of the enzyme through chemical modification of the cysteines.[18,19]

We report the 1.9-Å native structure of tethered HIV-1 PR carrying the asymmetric mutation C95M in one subunit of the dimer. The flaps in the present structure assume a closed conformation, in contrast to their open conformation in all earlier studies of unliganded HIV PR. This result is functionally significant because the flaps control access to the active site. The C95M mutation in only one subunit has not caused any major changes in the positions of the main chain atoms around residue 95.

MATERIALS AND METHODS

Purification of C95M Tethered HIV-1 PR

An HIV-1 PR tethered dimer gene construct (a kind gift from Dr. John Erickson) encoded a 57-codon N-terminal flanking peptide that is a part of the N-terminal polyprotein of the *pol* gene. cDNA sequencing of the gene insert established that the amino acid residue at position 95 was Met, whereas at 1095 it was a Cys. Thus, this tethered dimer has a C-to-M mutation in only one subunit at position 95. BL21(DE3) cells were induced for the expression of the protease gene. The gene product accumulated in the inclusion bodies was extracted in a denatured form and then refolded using the following protocol.[11] Cells were harvested and lysed using a sonicator to prepare inclusion bodies. Inclusion bodies were extracted with 67% acetic

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Atomic coordinates have been deposited with Protein Data Bank and the PDBID is 1G6L.

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acid. Extracts were diluted 33-fold with water, dialyzed overnight against water, and then dialyzed for 2 h at 4°C against a refolding buffer of pH 6.5, consisting of 20 mM piperazine-N,N′-bis diethane sulfonic acid (PIPES), 100 mM NaCl, 1 mM dithiothreitol (DTT), 10% glycerol. The 57-residue N-terminal flanking peptide autocleaved from the 29-kDa precursor protein to yield the 22-kDa tethered protease during refolding, showing that the C95M mutant is enzymatically active. Further purification was achieved using a Mono S fast protein liquid chromatography (FPLC) column. A yield of 10 mg of pure protein was obtained from 1 L of cells.

Enzyme Activity of C95M Tethered HIV-1 PR

The heptapeptide SQNYPIV, which bears a sequence similar to the known p17–p24 junction in the gag polyprotein, is used efficiently as a substrate by HIV-1 protease.[20] We synthesized this heptapeptide by solid-phase peptide synthesis on a Semi-automatic NovaSyn Peptide Synthesizer, using FMOC chemistry. Peptide purification was done on a PepRPC FPLC column, using a trifluoroacetic acid (TFA)/water–acetonitrile gradient system. The peptide sequence was confirmed by two-dimensional nuclear magnetic resonance (2D NMR) (S.C. Panchal and R.V. Hosur, personal communication). This substrate at a concentration of 1 mM was incubated with 4 ng/μl concentration of purified C95M tethered dimer in 50 mM mesylate (MES) buffer pH 6.0 containing 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM DTT, and 10% glycerol at 37°C. Proteolysis was determined by FPLC on a PepRPC column, using a linear gradient of 0–30% acetonitrile in 0.1% TFA over 30 min. The two product peaks gained in intensity (data not shown) at the expense of the substrate peak with passage of time, establishing that the asymmetric mutant C95M was enzymatically active.

Crystallization of C95M Tethered HIV-1 PR

The hanging drop vapor diffusion method was used to grow crystals at room temperature. In this investigation, 5 μl of the enzyme at 6 mg/ml in 50 mM sodium acetate pH 4.5 was mixed with an equal volume of 198 mM sodium phosphate, 99 mM sodium citrate at pH 6.2, and 10% saturated ammonium sulfate (well buffer) and sealed over the well. The crystals appeared as hexagonal rods within 2–3 days and reached a size of 0.3 mm × 0.1 mm × 0.1 mm in 2 weeks. Interestingly, once formed, the crystals were not soluble in water, even after soaking for several hours.

X-Ray Data Collection and Refinement

The crystals belonged to the space group $P6_1$ with unit cell dimensions: $a = b = 63.15$ Å and $c = 83.61$ Å. One C95M tethered dimer occupies the asymmetric unit. Diffraction data were collected on the BL41XU beam line at Spring8, as well as on an inhouse RAXIS IIC imaging plate detector system mounted on a Rigaku RU200 HB rotating anode X-ray generator operating at 50 kV and 100 mA and fitted with double focusing mirror optics. The data were processed, scaled, and merged using the DENZO[21] package of programs on a Silicon Graphics workstation. The dataset of 14,318 independent reflections and an R-merge of 5.5%, was 95.8% complete to 1.9-Å resolution. The structure was solved using the molecular replacement technique as implemented in the AmoRe program.[22] The protein part of the HIV-1/A77003 complex[23] was used as the search model after excluding the flap residues (42–58) from the calculations. Crystallographic refinement using XPLOR[24] resulted in an initial R-factor of 32% after one cycle of positional and slow-cooling refinement. The model was built into the $(2F_o–F_c)$ and $(F_o–F_c)$ electron-density maps using O,[25] followed by simulated annealing refinement with XPLOR. Finally 96 water molecules were added and the structure refined to a R-factor of 19.5%. A test set containing 5% of randomly chosen diffraction data was reserved for the determination of R-free to monitor the progress of the refinement. The final R-free is 28.4%. All molecular superpositions were done using O.

TABLE I. Crystallographic Data and Refinement Statistics

Statistic	Unit
Space group	$P6_1$
Cell parameters	$a = b = 63.15$ Å; $c = 83.61$ Å; $\gamma = 120$ degrees
Molecules in the asymmetric unit	One tethered dimer
Resolution	1.9 Å
Measured reflections	43,302
Unique reflections	14,318
R-merge	5.5%
Completeness	95.8%
No of scattering atoms	2,147
No of solvent molecules	96
Resolution range used in refinement	8–1.9 Å
R-factor	19.5%
R-free	28.4%
RMSD bonds (Å)	0.013
RMSD angles	1.8 degrees
⟨B⟩ factor (Å^2) main chain	25.33
⟨B⟩ factor (Å^2) side-chain	31.68
⟨B⟩ factor (Å^2) solvent	41.0

RESULTS

HIV-1 PR tethered dimer is shown to have activity very similar to that of the native enzyme.[26] Two subunits within the dimer are covalently linked through a five-residue linker of amino acid sequence GGSSG. We have numbered the residues in the first subunit 1–99, while those in subunit 2 are numbered 1001–1099. The residues in the linker region are numbered 100–104.

Quality of the Model

The electron-density map for the entire molecule is of good quality (Fig. 1). There are no breaks in the main chain, when the $(2F_o–F_c)$ electron-density map is contoured at the 1σ level, except for the linker region, which is not very well defined. The refinement statistics has been summarized in Table I. The error in atomic positions is of the order of 0.23 Å, as deduced from a Luzzati plot. The average of the *B*-values of protein atoms is 28.3 Å^2, while

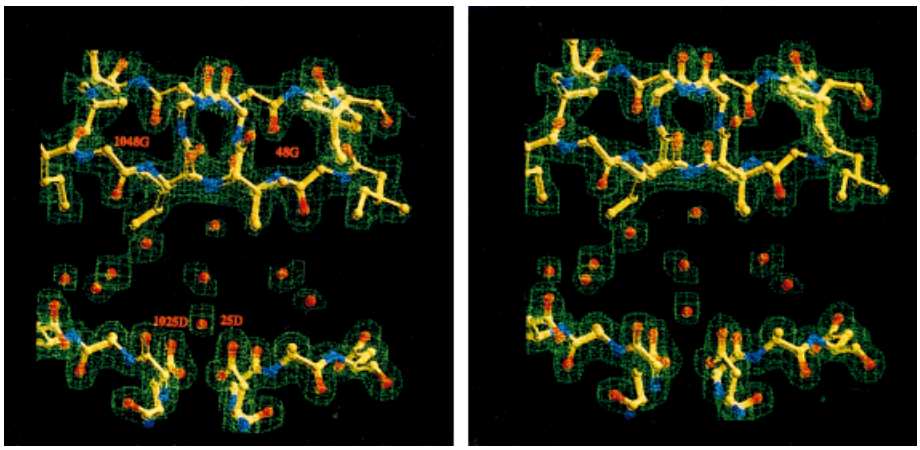

Fig. 1. Stereo view of ($2F_o$–F_c) electron-density maps contoured at the 1σ level, covering the active site and the flap region of the tethered dimer of HIV-1 PR. The flaps are very well defined in electron density. The active site cavity is filled with loosely held waters indicated by red spheres.

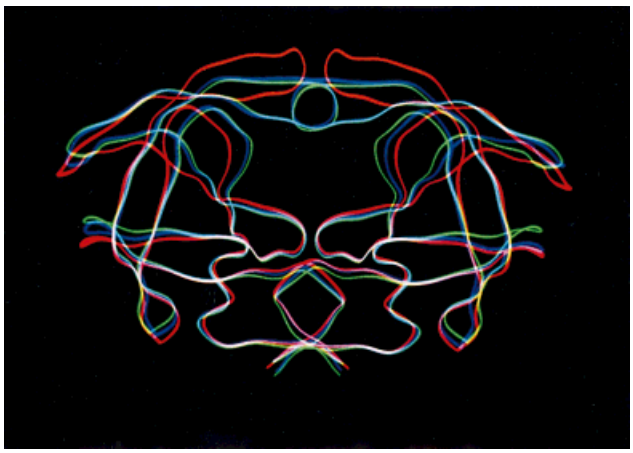

Fig. 2. Cα tracings for superposed models of HIV-1 PR native type enzyme (red), HIV-1 PR complexed to A77003 (blue) and the asymmetric mutant C95M of tethered HIV-1 PR (green). The overall folds are very similar except in the region of the flaps. The flaps (top region) adopt a closed conformation in the present structure as compared to the open flaps in the wild-type enzyme. The positions of the main chain of the 80s loop (residues 78–85) in the active site are significantly different from the native HIV-1 PR.

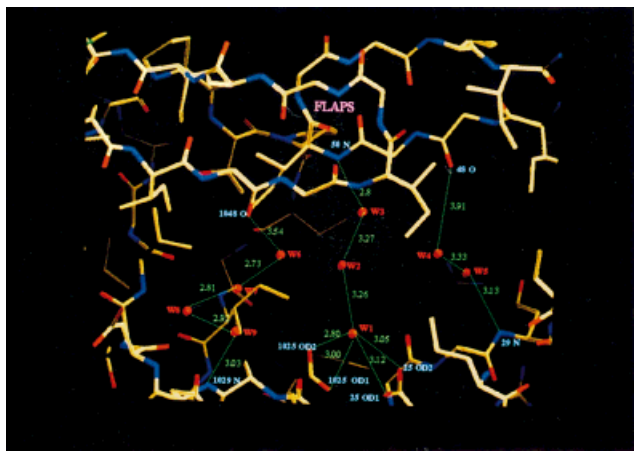

Fig. 3. Hydrogen bonding networks involving water molecules W1–W9 (red spheres) located in the active site, linking the flaps to the core of the enzyme. W1 is within hydrogen bonding distances from carboxyl groups of both catalytic aspartic acids: D25 and D1025. Such a water molecule is found in all native structures of HIV PR and is implicated in nucleophilic attack on the scissile peptide bond.

the average for the solvent atoms is 41.0 Å^2. The model stereochemistry is very good, with more than 95% residues occupying the most favored region of the Ramachandran map. The side-chain conformations belong to the allowed rotamer states. The root-mean-square deviations (RMSD) from ideal values of bond lengths and bond angles are 0.013 Å and 1.8°, respectively. The two monomeric domains in the asymmetric unit are very similar with the RMSD for 99 Cα pairs being 0.16 Å. There are differences in the conformations of few equivalent residues in the two subunits. The flap residues I50 and G51 have different backbone dihedrals enabling the formation of the interflap hydrogen bond 50CO · 1051NH. The rotamer conformations of F53 and F1053 are different. While the six-member ring of F1053 stacks on top of the β-ribbon structure of the flap, the ring of F53 is pointing away. There is a difference in the side-chain conformations of R8 and R1008.

The electron-density map indicates that the molecular model is locally disordered in two regions: (1) the linker (residues 101–104) and (2) the side-chains of residues 53 and 1046. The disorder of the linker suggests that this pentapeptide loop is very flexible and has no fixed conformation. The side-chain disorder is attributable to clashes of two crystallographically related molecules in hexagonal crystals and has also been observed before in structures of HIV-1 protease inhibitor complexes.[27,28] The main chain of these residues is not disordered, however. There was no indication of disorder in any other region of the molecule. We conclude that at this resolution the two subunits occupy unique positions because of different crystal contacts.

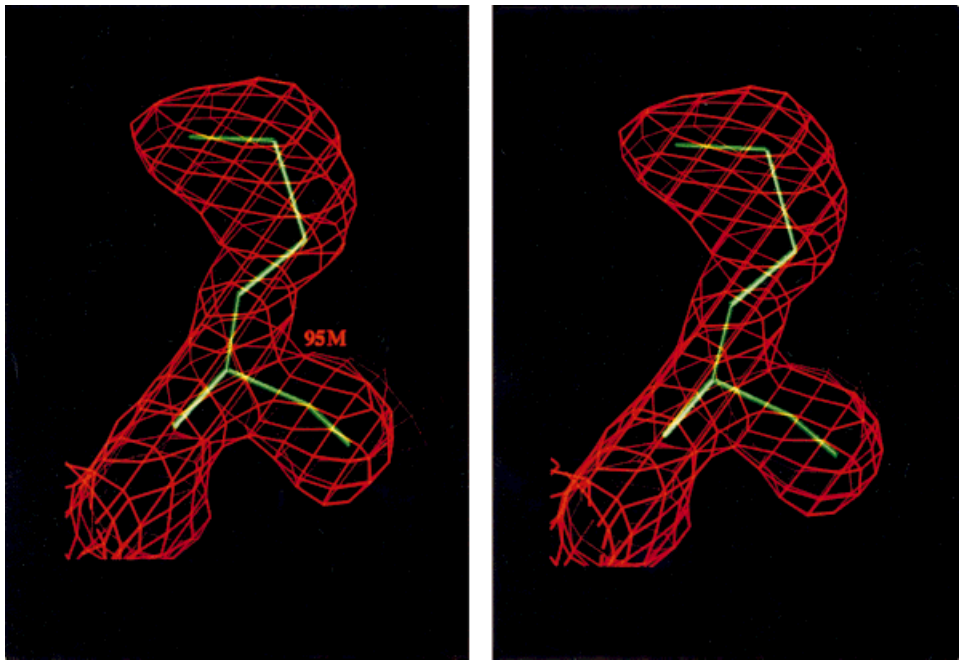

Fig. 4. A $2F_o–F_c$ omit map around the site of mutation C95M. The electron density clearly shows the presence of a methionine at position 95 in the tethered dimer.

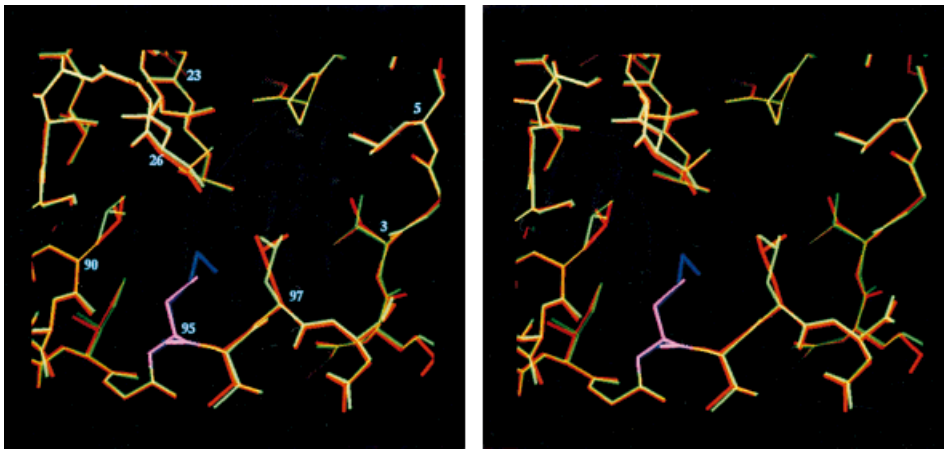

Fig. 5. Stereo view of the comparison of the environments around residue 95 and 1095 in the two subunits of the tethered dimer.

Molecular Conformation

We have determined the structure of HIV-1 PR tethered dimer mutant in a ligand-free form. The RMSD between the HIV-1 PR native enzyme and the present structure is 1.37 Å when the Cα atoms of all but the flap residues (42–58 and 1042–1058) are included in the superposition (Fig. 2). It is very interesting, however, that a smaller RMSD, within the range of 0.4–0.8 Å is obtained when all 198 residues from the ligand-bound HIV-1 PR structures[4] are used in the comparison, implying that the present structure resembles more closely the ligand-bound form of the enzyme, rather than the ligand-free native form. In particular, the configuration of the flap in the present structure is that of a closed type (Fig. 2). This is the first report of a closed flap configuration when the tethered HIV-1 PR is not complexed to any inhibitor molecule. Closed flaps were also observed, however, in one crystal form of native simian immunodeficiency virus protease (SIV PR)[29] and native HIV-1 PR (T.N. Bhat and J.W. Erickson, personal communication). The RMSD between 198 Cα atom pairs of SIV protease and the present structure is 1.04 Å . An unliganded SIV protease structure with the flaps in an open conformation was recently reported.[30] The RMSD between the open flap SIV PR and the present structure is 1.81 Å when the Cα atoms of all but the flap residues are included in the superposition.

TABLE II. List of Nonbonded Contacts <4 Å From Residue 95 in the Two Subunits

Atom from subunit 1	Atom from subunit 2	Distance (Å)
95 Met CA	1099 Phe CB	3.89
95 Met CA	1098 Asn O	3.13
95 Met CB	1098 Asn O	3.92
95 Met CG	1098 Asn O	3.98
95 Met CG	1005 Leu CD1	3.50
95 Met SD	1097 Leu CG	3.63
95 Met SD	1097 Leu CD1	3.81
95 Met CE	1099 Phe CB	3.32
95 Met CE	1099 Phe CG	3.51
95 Met CE	1099 Phe CD1	3.15
95 Met CE	1098 Asn O	3.92
95 Met CE	1097 Leu CG	3.87
95 Met C	1098 Asn O	3.44
95 Met O	1005 Leu CD1	3.59
98 Asn O	1095 Cys CA	3.35
99 Phe CB	1095 Cys CA	3.91
5 Leu CD1	1095 Cys CB	3.91
97 Leu CD2	1095 Cys SG	3.56
98 Asn O	1095 Cys C	3.51
5 Leu CG	1095 Cys O	3.98
5 Leu CD1	1095 Cys O	3.69

Water Molecules in the Active Site

A few water molecules with reasonably low temperature factors and good electron densities have been found in the active site cavity. One among these water molecules, hydrogen bonds to side-chain carboxyls of catalytic aspartic acid residues (Fig. 3). This water molecule has been observed only in native structures of aspartyl proteases and is implicated in the mechanism of action of these enzymes.[31] The remaining water molecules hydrogen bond to residues D29, D30, D1029, D1030, and I50 in the active site.

Cys-to-Met Mutation at Position 95

Figure 4 shows the $(2F_o–F_c)$ omit electron density for the mutated residue 95. Figure 5 is a stereoview of the comparison of the environment around residues 95 and 1095. Table II lists the nonbonded contacts shorter than 4 Å involving residues 95 and 1095 of the C95M tethered dimer.

Difference Densities Near Sulfur Atoms of Cysteine Residues

Figure 6 is a stereo view of $(F_o–F_c)$ difference electron-density map contoured at the 3σ level, near the sulfur atom of Cys1095. Similar density was also observed near Cys67 and Cys1067. This extra density is at a distance of 1.6–1.8 Å from the sulfur atom, suggesting oxidation of all three cysteines to sulfenic acids.

DISCUSSION

Flap Conformation

In retroviral proteases, the conformation of the flaps is functionally very important as the flaps control the access of the active site to the polyprotein substrates.[32,33] If the flaps were to assume a widely open conformation, the active site cavity would be accessible from outside, whereas the access would be blocked if the flaps assume a closed conformation. Therefore, the conformation and dynamics of the flaps has been probed by theoretical[34,35] and experimental[36] methods. More recent theoretical calculations estimate that both the open and closed flap conformations have similar potential energy and should therefore be equally accessible for native HIV-1 PR.[37] In another study, the difference in potential energies of the open and closed flap structures has been estimated at 3.0 kcal/mole,[38] with an enthalpically and entropically unfavorable closed conformation. It is generally believed that in aspartyl proteases the flaps occupy a very shallow energy minimum, and their conformation can be easily affected by relatively small perturbations. In all the published crystallographic reports of the structures of ligand-free HIV-1 PR[6–8,39] and HIV-2 PR,[40] the flaps are suggested to be trapped in an open conformation because of crystal contacts.[4,41]

The structure presented in this study is the first report of unliganded tethered HIV-1 PR crystallized in a hexagonal space group. The present structure is at a resolution of 1.9 Å, which is higher than that described in all previous reports. In the present structure, the flaps assume a closed configuration similar to that found in structures of HIV PR bound by a ligand. This result contradicts the general belief that interactions with the ligand in the active site cavity drive the flaps to assume closed conformation. The closed flap configuration in the present structure is stabilized by the formation of the following intersubunit interactions: (1) one hydrogen bond between main chain atoms of residues at the tip of the flaps (50 CO · 1051 NH = 2.8 Å); (2) a water bridged hydrogen bond between main chain atoms of residues 51 and 1079 (51 NH · 266Wat = 2.8 Å and 266Wat · 1079 CO = 2.9 Å; (3) weak water-mediated hydrogen bonds linking the closed flaps to the core (Fig. 3); and (4) hydrophobic crystal contacts between flap residues F53 and M46. Since the flaps in SIV PR are closed even in the absence of these hydrogen bonds, it is unlikely that these intersubunit interactions are the cause for the closed flap. Some correlation between crystallization conditions and flap conformation has also been suggested.[30] While SIV PR has crystallized in the closed flap configuration at a lower pH, it adopts an open flap configuration at higher pH values. Similarly, the open flap structures of native HIV-2 PR, HIV-1 PR and quadruple mutant HIV-1 PR were seen in crystals grown at pH 7.0–7.5. In the present case, the crystals of identical morphology (hexagonal rods) and unit cell dimensions were grown under a wide range of pH values of 5.5–7.0.

Conformation of the 80s Loop and Sizes of the Subsites

Functional analysis of HIV-1 and SIV (or HIV-2) proteases has demonstrated significant differences in their substrate specificity. Stebbins et al.[42] have shown that the conformation of the 80s loop comprising of residues 78–85 influences significantly substrate specificities of this enzyme. In SIV PR the main chain in this region extends

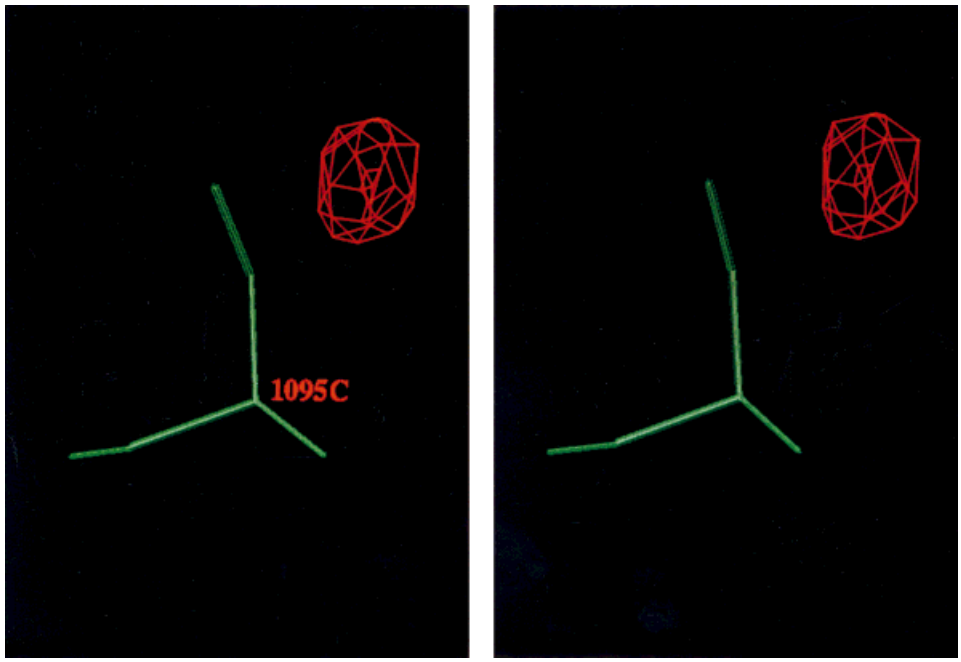

Fig. 6. Stereo view of the F_o–F_c electron-density map around Cys1095. Extra electron density clearly seen in the maps contoured at the 3σ level. Similar density also observed around the sulfur atom of Cys67 and Cys1067.

toward the active site by more than 1 Å, producing a change in the shape and size of the S1/S1′ subsite in the active site cavity. Interestingly, the conformation of the 80s loop in the present structure is very similar to that of SIV PR. The RMSD for the 80s loop residues (78–85 and 1078–1085) is 0.36 Å as compared with SIV PR. This region deviates substantially from the ligand-free structures reported earlier, with an RMSD of 2.1 Å for the 80s loop, as compared with native HIV-1 PR. As a consequence of this movement of the 80s loop, the distance between the Cα atoms of residues 82 and 1082 are 19.34 Å and 18.66 Å in the C95M tethered dimer and SIV PR, respectively, while the corresponding distance is 21.89 Å in native HIV-1PR. Since the activity of the tethered dimer is very similar to that of the native HIV-1 PR, the above result suggests that the position of the main chain atoms in this loop alone does not alter ligand specificity of this enzyme. Since none of the residues in this loop is involved in crystal contacts, it is highly unlikely that the loop movement in the present hexagonal crystals could have been caused by crystal packing. The average B-factor of the backbone atoms for residues 78–85 and 1078–1085 is 24.5 $Å^2$ as compared with 25.3 $Å^2$ for the rest of the protein backbone. Hoog et al.[43] have attributed the observed smaller S1/S1′ subsites in SIV PR to the absence of a hydrogen bond that is present in the native enzyme between the carboxylate of Glu34 and the backbone carbonyl of Thr 80. In SIV PR, the residue 34 is a Thr and is therefore unable to form that bond. In the present structure, the side-chain orientation of Glu 34 is such that it is unable to hydrogen bond with backbone atoms of any residue from the 80s loop. The observed smaller S1/S1′ subsite could therefore be a consequence of the absence of these hydrogen bonding interactions. Alternatively, the position of the 80s loop may be dependent on the configuration of the flaps. In two separate crystallographic studies of SIV PR, the flaps were found to adopt two different conformations, with the flaps closed in one study[29] and open in the other.[30] In SIV PR with closed flaps, the 80s loop moves toward the active site exactly as in the present structure, with an RMSD of 0.36 Å. By contrast, the RMSD for the 80s loop is 1.67 Å as compared with the open flap SIV PR structure. In this position of the 80s loop and the flaps, there are seven hydrophobic contacts <4 Å involving residues I50, T80, P81, I84, I1050, and T1080, permitting a favorable van der Waals interaction. The intersubunit hydrogen bond linkage between carbonyl of 1079 and the flap tips (51NH) through a water molecule further stabilizes the positions of the flap and 80s loop.

Oxidation of Cysteines

The ($2F_o$–F_c) and (F_o–F_c) electron-density maps contoured at the 1σ and 3σ levels, respectively, displayed unexplained density adjacent to the sulfur atoms of Cys67, Cys1067, and Cys1095 (Fig. 6). The distances from the sulfur to the center of these densities were within the range of 1.6–1.8 Å, suggesting that the sulfur atoms of these cysteines may have been oxidized. Similar density observed by Thanki et al.[44] near only Cys67, was interpreted as being indication of oxidation of Cys67. Although it has been known for quite some time that cysteine sulfenic acid derivatives can be introduced into proteins by mild oxidation, crystallographic evidence for Cys–SOH has been presented only recently.[12] The reason for this

cysteine oxidation in the present structure is unclear. There is always a possibility that it could be attributed to a radiation effect during data collection or that it could possibly have occurred during crystallization, even though it was carried out in the presence of DTT.

Role of Cysteine at Position 95

The position 95 has a Cys residue in all 22 strains of HIV-1 protease isolated so far.[15] Although this residue is not directly involved in catalysis, its mutation seems to influence enzyme activity in different ways. Mutation to polar residues resulted in low levels of activity, while mutation to hydrophobic residues such as Leu and Phe gave nearly wild-type activity, stressing the hydrophobic environment of this side-chain.[45] The region 94–99 is one of the most rigid main chain regions of HIV-1 PR and contributes substantially to the stability of the dimer.[36,46] It was suggested that the integrity of this region is very important for the proper folding of the enzyme. In the present structure, even though the residue at position 95 (subunit 1) is a Met and that at position 1095 (subunit 2) is a Cys, the main chain environments around these two residues are identical (Fig. 5). The presence of the covalent linkage between residues 99 and 1001 in the present structure, might, in principle, influence the conservation of the environment around residue 95. Since the linker peptide has been found to be very flexible in the present structure, it is possible that this influence is negligible. The longer side-chain of methionine compared with cysteine appears to have been easily accommodated without any major backbone alterations. Most of the interactions from additional atoms present in Met are hydrophobic in nature (Table II). Therefore, replacement of these additional atoms by polar atoms N and O, as would occur in the case of nitrosation of the Cys residue[18] in the wild-type sequence, may be energetically unfavorable. In remedying this situation, some atomic rearrangement might take place in a way that would affect dimerization in the wild-type enzyme, affecting its activity. In this context it is interesting that covalent binding of NO to cysteines forming S-nitrosothiols in HIV PR has been reported to result in reversible inactivation of HIV-1 PR.[18]

CONCLUSIONS

The structure of C95M tethered HIV-1 protease determined to 1.9-Å resolution, has revealed that the flaps in the enzyme can assume a closed configuration even in the absence of any inhibitor bound in the active site. The active site cavity is reduced in size due to movement of the 80s loop. The asymmetric mutation C95M, which is a part of the dimer interface, has not altered the main chain environment around residue 95. The addition of a hydrophilic group at residue 95, which is in a strongly hydrophobic environment, might affect the dimer stability and molecular conformation of HIV-1 PR. Our studies provide a possible explanation to the recent reports on reversible inactivation of HIV-1 PR through cysteine modification.

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