Three-dimensional Structure of a Mutant HIV-1 Protease Displaying Cross-resistance to All Protease Inhibitors in Clinical Trials*

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Analysis of mutational effects in the human immunodeficiency virus type-1 (HIV-1) provirus has revealed that as few as four amino acid side-chain substitutions in the HIV-1 protease (M46I/L63P/V82T/I84V) suffice to yield viral variants cross-resistant to a panel of protease inhibitors either in or being considered for clinical trials (Condra, J. H., Schleif, W. A., Blahy, O. M., Gadryelski, L. J., Graham, D. J., Quintero, J. C., Rhodes, A., Robbins, H. L., Roth, E., Shiva, P. L., Titus, D., Yang, T., Teppler, H., Squires, K. E., Deutsch, P. J., and Emin, E. A. (1995) Nature 374, 569–571). As an initial effort toward elucidation of the molecular mechanism of drug resistance in AIDS therapies, the three-dimensional structure of the HIV-1 protease mutant containing the four substitutions has been determined to 2.4Å resolution (Condra, J. H., Schleif, W. A., Blahy, O. M., Gadryelski, L. J., Graham, D. J., Quintero, J. C., Rhodes, A., Robbins, H. L., Roth, E., Shiva, P. L., Titus, D., Yang, T., Teppler, H., Squires, K. E., Deutsch, P. J., and Emin, E. A. (1995) Nature 374, 569–571). As an initial effort toward elucidation of the molecular mechanism of drug resistance in AIDS therapies, the three-dimensional structure of the HIV-1 protease mutant containing the four substitutions has been determined to 2.4Å resolution (Condra, J. H., Schleif, W. A., Blahy, O. M., Gadryelski, L. J., Graham, D. J., Quintero, J. C., Rhodes, A., Robbins, H. L., Roth, E., Shiva, P. L., Titus, D., Yang, T., Teppler, H., Squires, K. E., Deutsch, P. J., and Emin, E. A. (1995) Nature 374, 569–571). As an initial effort toward elucidation of the molecular mechanism of drug resistance in AIDS therapies, the three-dimensional structure of the HIV-1 protease mutant containing the four substitutions has been determined to 2.4Å resolution (Condra, J. H., Schleif, W. A., Blahy, O. M., Gadryelski, L. J., Graham, D. J., Quintero, J. C., Rhodes, A., Robbins, H. L., Roth, E., Shiva, P. L., Titus, D., Yang, T., Teppler, H., Squires, K. E., Deutsch, P. J., and Emin, E. A. (1995) Nature 374, 569–571). As an initial effort toward elucidation of the molecular mechanism of drug resistance in AIDS therapies, the three-dimensional structure of the HIV-1 protease mutant containing the four substitutions has been determined to 2.4Å resolution (Condra, J. H., Schleif, W. A., Blahy, O. M., Gadryelski, L. J., Graham, D. J., Quintero, J. C., Rhodes, A., Robbins, H. L., Roth, E., Shiva, P. L., Titus, D., Yang, T., Teppler, H., Squires, K. E., Deutsch, P. J., and Emin, E. A. (1995) Nature 374, 569–571).

In this work, the native and bound structures of a mutant HIV-1 protease, with a 70-fold change in affinity (Kᵢ = 26 nM) toward MK639, have been determined. The observed structural features of the MK639-bound mutant and wild-type proteases are consistent with the extent of resistance raised against MK639.

MATERIALS AND METHODS

The pET-3b-HIVPR plasmid containing the synthetic gene for HIV-1 protease in the high expression vector pET3b was provided by Dr. Jordan Tang, Oklahoma Medical Research Foundation (7). A 354 base pair cassette was designed based on the wild type sequence of pET-3b-HIVPR with appropriate nucleotide changes at positions 171 (G → C), 221 (T → C), 277 (T → A), 278 (T → C), 283 (A → G), and 285 (C → T) within the 833-base pair gene to yield the quadruple (4X) mutant protease (M46I/L63P/V82T/I84V). The 4X cassette was subcloned into the unique restriction sites Bpu1102I and BstEII within pET-3b-HIVPR to produce pET-3b-HIVPR-4X. Sequence-verified clones were transformed into competent Escherichia coli BL21(DE3) pLysS (I do and Tang cured) host cells (8). Cells were cultured, induced, and lysed as described (7). Enzyme purification, activity and inhibition assays were conducted as described previously (7, 9). Crystallization of the mutant protease was accomplished at 4°C with use of the vapor diffusion method. The enzyme was prepared at 15 mg/ml in a pH 5 solution containing 10 mM MES, 1 mM EDTA, 1 mM DTT. The reservoir solution contained 0.6 M NaCl, 1 mM DTT, 3 mM sodium threonate; MES, 2-(N-morpholino)ethanesulfonic acid.

The abbreviations used are: HIV, human immunodeficiency virus; 4X, the quadruple variant of HIV-1 protease containing the mutations M46I, L63P, V82T, and I84V; r.m.s., root mean square; DTT, dithiothreitol; MES, 2-(N-morpholino)ethanesulfonic acid.

The emergence of drug resistance remains a major bottleneck in the pursuit of a long lasting, antiviral treatment against AIDS (1). When faced with selective pressure of an inhibitor, some 20 of the 99 amino acid residues of the HIV protease undergo mutations (2–4). Sequence analyses of virus isolates from patients participating in clinical trials have revealed that various amino acid substitutions in the HIV protease, in combination with as many as 10 or more residues, are associated with the decrease in antiviral efficacy of the protease inhibitor MK639 upon its prolonged usage in the course of 1 year (2) (Structure 1).
and not P4122, encompassed 29,001 measurements with 8,651 unique reflections (82.3% completeness) and an Rmerge of \( (\sum(I - \langle I \rangle) / \sum I) \) of 3.3%. The coordinates of the wild-type HIV-1 protease were used as the molecular model. The initial R factor, defined as \( \sum(F_o - F_c)^2 / \sum F_o^2 \), was 0.308. Using the X-PLOR program package (11), rigid body refinements of the two monomers alternately of the protease reduced the R value to 0.239 using the data in the 8-Å to 3.5-Å resolution range. Another cycle of X-PLOR refinement further reduced the R value to 0.226 at 2.4-Å resolution. A 2Fo – Fc map was calculated, and a mutant protease model was built by employing the program CHAIN on a Silicon Graphics system. The model was further refined; the final model included 82 solvent molecules with an R factor of 0.171 for a total of 8,101 reflections. The Rs was 0.312, and the r.m.s. deviations, from ideal values of bond and angles, were 0.016 Å and 1.9°, respectively.

Crystals of the 4X protease complexed with MK639 were obtained at ambient temperature in hanging drops under vapor diffusion conditions against a solution of 0.1 M NaAc, pH 5.4, 0.5 M NaCl, 1 mM DTT, and 3 mM NaN3. The cell constants of the crystals were \( a = 60.06 \) Å, \( b = 86.90 \) Å, and \( c = 46.68 \) Å in the space group P2_1212. There was one protease dimer per asymmetric unit. The diffraction data, collected as described for the native enzyme and extending to 2.0-Å resolution, included 41,085 measurements with 13,590 independent reflections (86% completeness) and an Rmerge of 5.02%. The structure of the inhibitor-bound HIV-1 mutant protease was determined with the difference Fourier method employing the structure of the HIV-1 protease MK639 as the model. The final model included 109 solvent molecules and an R factor of 0.170 for reflectionsin the 6-Å to 2.0-Å resolution. The Rs was 0.255. The r.m.s. deviations of atoms from ideal values of bond length and angle were 0.015 Å and 1.8°, respectively. The inhibitor MK639 was found to bind the mutant HIV-1 protease in a single orientation.

**RESULTS AND DISCUSSION**

Fig. 1 depicts in stereoview the Cα tracings of the wild-type HIV-1 protease and its quadruple (4X) mutant. The r.m.s. deviation for all 198 Cα atoms between the 4X and the wild-type protease is 0.5 Å.3 The largest deviations in the Cα position of these two molecules occur at the tip and in the hinge of the flap loops.4 The substituted side chains, Ile-46, Pro-63, Thr-82, and Val-84, are highlighted for both subunits of the enzyme as dictated by the 2Fo – Fc electron density map. Residues 82 and 84 are located in the active site of the enzyme. Residue 46 is in the flexible flap loop of the enzyme, while residue 63 is near the hinge region of the flap; both side chains point toward the bulk solvent.

Unlike the wild-type HIV-1 proteases, the two subunits of the 4X mutant are not related by a 2-fold crystallographic axis in the asymmetric unit. Nonetheless, the r.m.s. deviation for the Cα atoms of the two subunits is relatively small (0.37 Å). The greatest difference between the subunits lies mainly in the flap loop and in the peptide chains containing residues 15–20 and 64–72.5 By excluding residues 15–20, 34–40, 48–52 and

3 This value is well within the average r.m.s. found for the protease x-ray structure determined independently in different laboratories (12).  
4 The displacements in the position of Cα atoms in the flap loops of the 4X mutant from those of the wild-type protease are: Pro-39 (0.98 Å), Pro-39' (0.93 Å), Gly-40 (0.54 Å), Gly-40' (1.19 Å), Gly-49 (1.50 Å), and Gly-49' (1.27 Å). In contrast, the deviations for the four substituted residues are less drastic: Met-46 (0.66 Å), Met-46' (0.51 Å), Leu-63 (0.74 Å), Leu-63' (0.47 Å), Val-82 (0.62 Å), Val-82' (0.78 Å), Ile-84 (0.31 Å), and Ile-84' (0.21 Å).

5 For example, the largest displacements in the Cα positions between the two subunits in these regions are: Pro-39 (0.99 Å) and Gly-50 (1.11 Å) in the flap loop, Gly-17 (0.68 Å) in the 15–20 peptide segment, and Gly-68 (0.89 Å) in the 64–72 peptide segment.
64–72, the r.m.s. deviation for the Cα atoms in the two subunits is further reduced to 0.25 Å. For the four substituted residues between the subunits, the displacements of the Cα atoms are 0.2 Å for Met-46, 0.31 Å for Leu-63, 0.2 Å for Val-82, and 0.18 Å for Ile-84.

Fig. 2 illustrates the electron density of the quadruple mutant HIV-1 protease surrounding residues 46 and 63 as defined by its electron density seen at 2.0 Å resolution and by superposing the Cα backbone of the MK639-bound mutant enzyme onto that of the wild type HIV-1 protease. The wild-type CG1 atom of Val-82 is now the mutant OG1 atom of Thr-82 introducing an unfavorable interaction with the inhibitor. The mutant OG1 atom of Thr-82 points away from the plane of the stereoview and is shielded in this diagram; but its CG2 atom is still within van der Waals distance (3.58–3.72 Å) from the phenyl group of MK639 as opposed to a distance of 3.54–3.70 Å seen for the corresponding wild-type atom.

Fig. 3. Stereo diagrams of the active site of HIV-1 protease complexed with MK639 (previously L-725,524) as defined by x-ray diffraction data at 2.0 Å resolution. The inhibitor is depicted in green and the protease in thin purple wire. Residues of Ile-50, Ile-50', Val-82, Val-82', Ile-84, and Ile-84' are highlighted in thick pink lines. The dash lines mark the distances within van der Waals contacts between atoms of the inhibitor and the CD1 atoms of Ile-84/Ile-84', the CG1 atom of Val-82, and the CG2 atom of Val-82. Stick colored in yellow represents the positions of the side chains of Thr-82, Thr-82', Val-84, and Val-84' of the mutant protease in the MK639 complex as defined by its electron density seen at 2.0 Å resolution and by superposing the Cα backbone of the MK639-bound mutant enzyme onto that of the wild type HIV-1 protease. The wild-type CG1 atom of Val-82 is now the mutant OG1 atom of Thr-82 introducing an unfavorable interaction with the inhibitor. The mutant OG1 atom of Thr-82 points away from the plane of the stereoview and is shielded in this diagram; but its CG2 atom is still within van der Waals distance (3.58–3.72 Å) from the phenyl group of MK639 as opposed to a distance of 3.54–3.70 Å seen for the corresponding wild-type atom.

Fig. 4. Comparison of the binding conformation of MK639 as seen in the active sites of (A) the HIV-1 (green) and HIV-2 (yellow) proteases (see Ref. 6 for details) and (B) the HIV-1 protease (green) and the 4X mutant (purple) as defined by x-ray diffraction data at 2-Å resolution. The inhibitors were superimposed with the matrices derived by the least square fitting of all Cα atoms of each pair of the proteases. For clarity, the proteins are omitted in the stereoviews.

The bound mode is now only 0.2 Å. For clarity, the flaps of the enzyme are only partially included in this figure. Residues Val-82 and Ile-84 are highlighted in thick pink lines. The Ile-84 and Ile-84' are symmetry-related by a two-fold rotation but Val-82 and Val-82' are only pseudo symmetrically related because their propyl side chains are oriented differently about the Cα-Cβ bond. The dashed lines mark the distances, within van der Waals radii, between atoms of the inhibitor and the CD1 atoms of Ile-84/Ile-84', as well as the CG2 and CG1 atoms of Val-82 and Val-82', respectively. Related by a pseudo two-fold rotation, the t-butyl and indanyl groups of MK639 are bound in the S2 and S2' pockets of the enzyme. Correspondingly, the pyridyl methyl piperidine and benzyl rings of the inhibitor are situated in the S3/S1 and S1' pockets. A bound water molecule
is seen to be cushioned tetrahedrally between the tips of the flap loops (Ile-50 and Ile-50') and the 2 amide oxygen atoms of the inhibitor. (A second bridging water molecule is found between the N2 amide nitrogen of the inhibitor and the carbonyl oxygen of Asp-29.) Not seen in Fig. 3 are interactions of the hydroxyethylene group of the inhibitor hydrogen bonded to Asp-25 and Asp-25' (beneath the inhibitor structure). Altogether, seven atoms of MK639 are within hydrogen bonding distances from atoms of the enzyme, either directly or indirectly. These interactions have been reported in detail (6) for the HIV-2 protease. For comparison, the conformation of MK639 (as seen in the active sites of HIV-1 and HIV-2 protease) are shown in Fig. 4A.

In Fig. 3 are also shown thick lines colored in yellow representing the positions of the side chains of Thr-82, Thr-82', Val-84, and Val-84' in the MK639-complexed 4X mutant protease, as dictated by its electron density and by super-positioning its C\textsubscript{\alpha} backbone onto that of the MK639-complexed wild-type HIV-1 protease. Both mutations in the active site introduce small alterations (~0.3 Å) in their side chain positions. Most importantly, the V82T substitution, while isosteric, introduces an unfavorable hydroxyl moiety (OG1) at what was previously (wild-type) the CG1 position of Val-82' in the S1 pocket, within van der Waals distance (3.39 Å) to the pyridyl piperidine group of MK639. The corresponding substitution (OG1 of V82T) in the S1' site points away from the inhibitor and thus does not directly impact on binding because its CG2 atom is still within van der Waals distance (3.58–3.72 Å) from the phenyl moiety. The 184V and 184' substitutions, on the other hand, symmetrically create in both the S1 and S1' pockets a small void in bulk (~25 Å\textsuperscript{3}) that can be expected to lead to a decrease in van der Waals interactions with the piperidine group and with the benzyl moiety of the inhibitor, respectively.\textsuperscript{6} In an apparently unsuccessful attempt to fill this void, the CD1 atom of Ile-50' from the tip of the flap domain relocates by 1.29 Å toward this pocket by rotating ~35° about the C\textsubscript{\gamma}-C\textsubscript{\gamma} bond and 170° about the C\textsubscript{\gamma}-C\textsubscript{\gamma} bond; however, a similar change is not seen for Ile-50. The structures of the bound MK639 in the active sites of the wild-type and 4X mutant proteases are shown in Fig. 4B, revealing that the binding mode of MK639 is essentially unchanged in the active site of the two enzymes. Together, these results suggest that resistance against inhibitor binding, in the case of MK639, is caused by subtle changes of the substituted side chains (the introduction of an isosteric but unfavorable hydrophilic group and the introduction of a smaller side chain to decrease van der Waals contacts) rather than repositioning the bound inhibitor (due to the introduction of a spatially hindering, larger side chain). The side-chain positions of Thr-82 and Val-84 are also not different in the open and closed forms of the mutant protease (data not shown), indicating that these residues are not perturbed, within a r.m.s. deviation of ~0.2 Å, upon binding of MK639. The observed changes in binding interactions of MK639 in the active sites of the 4X protease are consistent with an attenuated affinity of this inhibitor by a factor 70 (~2.5 kcal/mol),\textsuperscript{5} a value sufficient to render a potent protease inhibitory ineffective as an antiviral agent against the HIV (2).

Whereas structural contributions of V82T and 184V toward resistance against MK639 are accountable with the diffraction data shown here, the role of the M46I and L63P substitutions is not obvious. These mutations away from the immediate vicinity of the active site induce significant changes in the C\textsubscript{\alpha} positions of residues in the flap domain of the enzyme (as seen text above related to Fig. 1 and Footnote 4), but the significance of these changes is unclear. However, preliminary kinetic results from our laboratory suggest that the combination of M46I and L63P mutations affords the protease greater catalytic efficiency than the native enzyme, and the V82T/I84V double mutations render the HIV-1 protease a very poor enzyme.\textsuperscript{8} Thus, it may be that the M46I and L63P modifications, by introducing fine adjustments to the protease conformation, compensate dynamically for deleterious effects of the mutations (V82T and 184V) in the active site.

Our observations are in contrast to those reported by Baldwin et al. (15). These authors have observed rearrangements of the HIV-1 protease C\textsubscript{\alpha} backbone around residues 81-84 by up to 0.6 Å on binding of a symmetrical inhibitor, A-77003, when Val-82 is substituted by an alanine. These changes lead to a repacking of enzyme and inhibitor atoms in the S1 but not S1' subsite in a manner that would diminish the potential loss of binding affinity. The remainder of the mutant protease complex is little altered from that of the wild-type enzyme (supporting our interpretation that the changes seen in the flap domain of the 4X mutant are due to the M46I and L63P substitutions). It is difficult to generalize at this time the effect of resistance mutations of the protease based on limited available data thus far. To further extend our understanding of the structural basis of drug resistance in the HIV-1 protease, we are pursuing determination of additional inhibitor-bound structures of the 4X HIV-1 protease.

REFERENCES

1. Richman, D. D. (1995) Nature 374, 494
2. Condra, J. H., Schleif, W. A., Blahy, O. M., Gadarwsky, L. J., Graham, D. J., Quintero, J. C., Rhodes, A., Robbins, H. L., Roth, E., Shivaparaksh, M., Titus, D. W., Yang, T., Teppler, H., Squires, K. E., Deutsch, P. J., and Eminn, E. A. (1995) Nature 374, 569–571
3. Hussien, R. N., Shirasaki, T., Butler, K. M., Pizzo, P. A., and Mitsu, H. (1993) J. Pediatr. 123, 9–12
4. Richman, D. D. (1994) in Textbook of AIDS Medicine (Broder, S., Merigan, T. C., and Bolognesi, D., eds) pp. 295–295, Williams and Wilkins, Baltimore
5. Varca, J. P., Dorsey, D. B., Welsh, W. A., Levin, R. B., McDaniel, S. L., Darke, P. L., Zavag, J., Quintero, J. C., Blahy, O. M., Roth, E., Sardana, V. V., Schlabach, A. J., Graham, P. I., Condra, J. H., Gotlib, L., Holloway, M. K., Chen, J. C., Chen, I.-W., Vistad, K., Ostovic, D., Anderson, P. S., Eminn, E. A., and Huij, J. R. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 4086–4100
6. Chen, Z. L., Y. C., Chen, E., Hall, D. L., Darke, P. L., Culberson, C., Shafer, J. W., and Kuo, L. C. (1994) J. Biol. Chem. 269, 26334–26348
7. Ido, E., Han, H., Keady, F. F., and Tang, J. (1991) J. Biol. Chem. 266, 24359–24366
8. Chen, E. (1994) Methods Enzymol. 241, 29–46
9. Darke, P. L., Jordan, S. P., Hall, D. L., Zavag, J. A., Shafer, J. A., and Kuo, L. C. (1994) Biochemistry 33, 98–105
10. Spinelli, S., Liu, Q. Z., Alzari, P. M., Hired, P. H., and Poljak, R. J. (1991) Biochemistry 30, 1991–1996
11. Brunger, A. T., Kuriyan, K., and Karplus, M. (1987) Biochemistry 26, 458–460
12. Wlodawer, A., and Erickson, J. W. (1993) Annu. Rev. Biochem. 62, 543–585
13. Hansch, C., and Coates, E. (1970) J. Pharmocol. Sci. 59, 731–743
14. Andrews, P. R., Craik, D. J., and Martin, J. L. (1984) J. Med. Chem. 27, 1648–1657
15. Baldwin, E. T., Bhat, T. N., Liu, B., Pattabiraman, N., and Erickson, J. W. (1995) Struct. Biol. 2, 244–249

\textsuperscript{5} The energetic contribution of a methyl moiety to binding has been estimated to be ~2–3 kcal/mol (13). However, the combined contribution of the V82T (but not V82T', see text), 184V, and 184' substitutions should account for a total loss of ~3 kcal/mol. This rough estimate is in reasonable agreement with the 70-fold drop in binding affinity (i.e. 1\textsuperscript{\textminus}RT ln K\textsubscript{i} = 2.55 kcal/mol). Also, repositioning of the Ile-50' side chain may partially alleviate loss in hydrophobic interactions due to the I84V replacement.

\textsuperscript{6} H. B. Schock and L. C. Kuo, unpublished data.