Published in final edited form as:

J Mol Biol. 2011 July 22; 410(4): 756–760. doi:10.1016/j.jmb.2011.03.038.

Accessory Mutations Maintain Stability in Drug Resistant HIV-1 Protease

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Abstract

The underlying mechanisms driving the evolution of drug resistance in HIV are only partially understood. We investigated the evolutionary cost of the major resistance mutations in HIV-1 protease in terms of protein stability. The accumulation of resistance mutations destabilizes the protease, limiting further adaptation. From an analysis of clinical isolates, we identified specific accessory mutations that were able to restore the stability of the protease or even increase it beyond the wild-type baseline. Resistance mutations were also found to decrease the activity of HIV protease near neutral pH values, while incorporating stabilizing mutations improved the enzyme's pH tolerance. These findings help to explain the prevalence of mutations located far from the active site and underscore the importance of protein stability during the evolution of drug resistance in HIV.

Introduction

The evolution of drug resistance in response to antiretroviral therapies continues to be a major problem in the treatment of HIV/AIDS. HIV protease inhibitors have been an important component of these therapies since the 1990s, and the viral protease has been studied extensively in order to elucidate the mechanisms of resistance. Typically, the development of resistance involves multiple mutations in the enzyme's active site and periphery.¹ Mutations near the active site decrease inhibitor binding, often at the cost of substrate processing, which can be restored by distal mutations.^{2–6}

Changes in an enzyme's active site are associated with protein destabilization, and successive mutations may destabilize the protein sufficiently to completely disrupt function.⁷ The important role of “permissive” mutations that improve protein stability has been shown in the development of bacterial antibiotic resistance,⁷ and a similar phenomenon has been noted with oseltamivir resistance in influenza.⁸

In protein engineering contexts, incorporation of stabilizing mutations has been used to promote the development of new enzyme functionality. The directed evolution of an enzyme may be accompanied by decreases in stability that require compensation by other mutations to proceed. To complement such studies, the application of neutral drift, evolution without

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external selection, has been used to increase protein stability and “evolvability.”¹¹ Similarly, viral replication in drug-naïve patients occurs without antiviral drug selection, and the polymorphisms that arise could similarly have consequences for protein stability.

Only a relatively small number of mutations in HIV protease are associated with major resistance against nearly all current protease inhibitors (positions shown in Figure 1), and their consequences for protein stability are largely unknown. Some studies have noted the increased stability of protease after the introduction of multiple mutations, but have not quantified their individual effects. One group found that the drug resistance mutation I84V lowered the melting temperature (T_m) of HIV protease, while the presence of 10 other mutations raised the T_m above the wild-type baseline.¹³ These findings are consistent with the hypothesis that changes in the active site caused by resistance mutations negatively impact protease stability, leading to the development of other mutations that re-stabilize the enzyme. To further investigate this phenomenon, we studied the contributions of individual resistance mutations to HIV protease stability and identified compensatory mutations that were able to restore stability.

Major drug resistance mutations destabilize HIV protease

The V82A, I84V, and L90M mutations in protease are each capable of providing major resistance against several clinically-approved inhibitors.¹⁴ Positions 82 and 84 lie inside the active site, and mutations at these points directly affects the binding of substrate and inhibitors. Changes at position 90 affect the dimer interface, consequently altering the binding site. Using the NL4-3 strain as a template, protease mutants containing substitutions at these positions were constructed, then expressed and purified as described previously.¹⁵

Subsequently, the melting temperature of these mutants was determined using differential scanning calorimetry (DSC). As shown in Figures 2 and 3a, the T_m of each mutants was at least 2.8°C lower than the wild-type NL4-3 protease. Additionally, a double mutant containing both I84V and L90M mutations showed a large T_m decrease roughly equivalent to the sum of the individual mutations. In absolute terms, the measured T_m values for the wild-type and I84V proteases were roughly 10°C higher than reported by Muzzamil et al.,¹³ an inconsistency likely due to differences in experimental pH. However, the relative T_m change between the two proteases was fairly consistent, approximately 4°C in the previous study versus 2.8°C in our study.

Accessory mutations can re-stabilize drug resistant HIV protease

Additional mutations can compensate for the deleterious effects of major drug resistance mutations.¹⁶ In HIV protease, the effects of such compensatory mutations are frequently characterized in terms of enzymatic activity. However, these mutations may also have some effect on protein stability. To investigate the role of specific mutations in compensating for destabilized drug resistant mutants, we identified common polymorphisms in viral isolates from drug-naïve patients that were also enriched within treated patient isolates.¹⁷ L10I, L63P, A71V, and V77I fit our criteria and the proportions of viral isolates containing these mutations is shown in Figure 3b. Interestingly, these mutations have been significantly associated with the V82A, I84V, and L90M mutations in a covariation study of sequences taken from individuals treated with protease inhibitors.¹⁸

The L10I, L63P, A71V, and V77I mutations were individually combined with I84V, and the stability of the resulting mutants was measured using DSC (Figure 3a). The L10I and A71V mutations were able to restore stability to within 1°C of wild-type, while the L63P-I84V mutant exhibited stability greater than wild-type. The V77I mutation had only a marginal

effect, and this may be reflected in the low level of enrichment when comparing drug-naïve and treated patient isolates.

Mutant HIV proteases have increased pH sensitivity

Changes in the thermal stability of HIV protease mutants indicate a parallel with other enzyme systems, but the consequences of these changes for enzymatic function or viral fitness is not obvious. Since mutations such as V82A can simultaneously affect substrate binding and protein stability, it is difficult to directly relate a change in T_m to changes in the protease's function. However, stability is often associated with a protein's pH tolerance, which is crucial for HIV protease, as its optimal pH is considerably more acidic than that of human cytosol.

The relative rates of substrate processing over a range of physiologically relevant pH values were determined using a fluorogenic hexapeptide substrate. Maximum rates were found near pH 5, and the processing declined with increased pH in all cases (Figure 4). This decline was more marked in the mutants, with all falling below 50% of their maximum activity at neutral pH. The I84V and I84V-L90M mutants were inactive at pH 7 and above, but the addition of stabilizing mutations was able to improve their function in this range.

Discussion

The ability of particular mutations to restore stability may explain their presence in many drug resistant patient isolates despite their distance from inhibitor binding sites. Specific structural mechanisms contributing to stability changes, whether an increase or decrease, have been addressed in other work. The consequences for viral fitness could relate to environmental pH constraints, as described above. In addition, the mutations displaying decreased protein stability have been associated with decreased dimer stability,²⁰ which is critical for the cleavage of substrates and autoprocessing.

The overall pattern of mutations in HIV protease is similar to the directed evolution of a cytochrome P450 enzyme,⁹ where changes in inhibitor affinity or substrate specificity result in a destabilization that can be counterbalanced by additional mutations. Without the stabilizing mutations, the enzymes would reach a point where further adaptations would be deleterious. In HIV protease, the stabilizing mutations detailed above appear to have little fitness penalty alone, as they appear to be common polymorphisms in clinical isolates. However, in a clinical setting, the presence of these stabilizing mutations may indicate a greater potential to develop resistance mutations. Also, while this study has centered on subtype B HIV, the prevalence of subtypes varies in different geographical regions. The distinctive sequence features that are characteristic of each subtype, such as greater frequency of specific polymorphisms, may result in varying levels of protein stability and evolvability. The consensus sequence of the F2 subtype,²¹ for instance, contains a proline at position 63, a substitution that confers increased stability. As the use of protease inhibitors becomes more widespread around the world, certain subtypes may exhibit alternative resistance pathways,²² potentially leading to more rapid resistance development.

Considerations of mutant stability may also play a role in the design of future antiviral agents. Current HIV protease inhibitors all target the enzyme's active site, and resistance mutations often affect multiple inhibitors. A recent screen uncovered chemical fragments that bind to two potential allosteric sites in protease that could lead to a new class of inhibitors.²³ While the evolution of resistance to allosteric inhibitors would be less constrained by considerations of substrate binding, destabilizing mutations would have a cumulative effect on the enzyme. Specifically designing an inhibitor for high affinity toward

specific stabilizing mutations, such as L63P, could also provide a way to directly attack the virus's ability to evolve.

Acknowledgments

We thank Meaghan Happer and John H. Elder for providing materials and Sebastian Breuer and David Goodsell for helpful discussions. M.W.C. was supported by 5T32NS0412119. This work was supported by NIH grants GM083658, GM48870 and AI4081585 and CFAR grant number 3 P30 AI036214-13S1. This is manuscript number MEM-20983 from the Scripps Research Institute.

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Fig. 1.
The structure of HIV protease, a symmetric homodimer, with inhibitor (green) bound. Major resistance positions are shown in red on one subunit, and positions of candidate stabilizing mutations are shown in blue on the other.

Fig. 2. Structural stability of wild-type, I84V, and L10I-I84V HIV proteases, as determined by differential scanning calorimetry. Protease samples were measured using a Nano II DSC (Calorimetry Sciences Corporation) in a buffer containing 20 mM NaCl and 20 mM sodium acetate at pH 5.0. The protease (dimer) concentration for all samples was 30 μM except for I84V-L90M, which was 24 μM .

Fig. 3.

Effect of drug resistance and accessory mutations on the stability of HIV protease. **(a)** The melting temperatures of HIV protease and mutants, as determined by differential scanning calorimetry. **(b)** The prevalence of specific mutations in drug-naïve and treated patient isolates. Data from the Stanford HIV Drug Resistance Database.

Fig. 4.

Drug resistance and accessory mutations affect the pH tolerance of HIV protease. Enzyme velocity was measured at 37°C in an FLx800 fluorescence microplate reader (Bio-Tek) using the fluorescently labeled substrate aminobenzoyl-Thr-Ile-Nle-p-nitro-Phe-Gln-Arg-NH₂ (H-2992; Bachem). The final concentrations of protease and substrate were 25 nM and 30 μM, respectively. The reaction solution contained 200 mM NaCl and a pH buffer consisting of 200 mM citric acid and 100 mM dibasic sodium phosphate. **(a)** The pH tolerance of HIV protease is diminished by drug resistance mutations. The values shown indicate mean ± SEM from duplicate experiments. **(b)** The pH tolerance of the I84V mutant is increased by the addition of peripheral mutations. **(c)** The normalized mean activity of wild-type and mutant HIV proteases at pH 5.5 and 7.0.