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Targeting structural flexibility in HIV-1 protease inhibitor binding

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Summary

HIV-1 protease remains an important AIDS drug target. Even though it has been known that ligand binding induces large conformational changes in the protease, the dynamic aspects of binding have been largely ignored. Several computational models describing protease dynamics have been reported recently. These have reproduced experimental observations, and also explained how ligands gain access to the binding site through dynamic behavior of the protease. Specifically, the transitions between three different conformations of the protein have been modeled in atomic detail. Two of these forms were determined by crystallography, the third one was implied by NMR experiments. Based on these computational models, it has been suggested that binding of inhibitors in allosteric sites may affect protease flexibility and disrupt its function.

Keywords

HIV-1 protease; molecular dynamics; flexibility; HIV protease inhibitors; HIV protease flaps; resistance; HIV protease allosteric inhibitors

It has been more than 20 years since the human immunodeficiency virus (HIV) was first discovered to be the causative agent of acquired immunodeficiency syndrome (AIDS) [1,2]. There are currently three main avenues for preventing virus replication. First, blocking attachment of virus to host cell surface by inhibitors of binding to co-receptors, such as CCR5 [3,4]. Second, blocking the process of reverse transcription [5], an approach taken by a major class of anti-AIDS drugs including, for example, AZT, delavirdine, nevirapin, etc. Third, blocking the function of the viral protease (HIV PR), which is described in more detail in this review.

The necessity of HIV PR for virus replication was demonstrated through several experiments. Deletion mutagenesis of the gene encoding HIV PR resulted in the production of virus particles that had an immature morphology and were noninfectious [6]. This was confirmed by mutation of the active site aspartic acids [7] and later, by chemical inhibition with protease inhibitors [8]. These seminal experiments provided conclusive proof that viral

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protease is essential for the life cycle of HIV and highlighted this enzyme as an important target for the design of specific antiviral agents.

These findings stimulated further interest in HIV protease, leading to solution of high-resolution three dimensional structures of HIV PR using X-ray crystallography [9]. Current FDA-approved HIV PR drugs function as active site inhibitors and serve as a prime example of structure-based rational design (examples of some clinically approved inhibitors are ritonavir, nelfinavir and amprenavir) [10–12]. Although many successful drugs have been developed, their effectiveness has been hampered by the emergence of drug-resistant variants [13].

A greater understanding of the mechanistic events associated with binding of HIV PR substrates and inhibitors is critically important for the design of novel inhibitors of the enzyme. There is evidence that flexibility of many enzymes plays an important role in inhibitor binding and resistance [14,15]. This review will focus on recent advances and challenges in understanding protease dynamic behavior and its potential for revealing new approaches to HIV PR inhibitor/drug design. A particular focus is the emergence of computational techniques that can provide detailed insight into the dynamic aspects of HIV PR behavior. In order to understand how substrate gains access to the protease binding site and how resistance due to protease mutations arises, many computational studies on this enzyme have been carried out. Since recent molecular dynamics simulations of HIV PR suggested that the dynamics of this enzyme is crucial for its function, affecting flexibility of the protease by, for example allosteric inhibitors, could open new opportunities for inhibitor design.

Experimental data on structure of HIV-1 protease: large structural rearrangement on binding

An extensive set of X-ray crystal structures of HIV-1 protease in both bound and unbound forms has been solved [9], revealing a C2 symmetric homodimer with a large substrate binding pocket covered by two glycine rich β -hairpins, or flaps [16–18]. Consistent structural differences are present between the bound and free states of the protein (Figure 1). In all of the liganded forms, the flaps are pulled in towards the bottom of the active site ('closed' form), while the structures for the unbound enzyme all adopt a 'semi-open' conformation with the flaps shifted away from the dual Asp25-Thr26-Gly27 catalytic triads, but still substantially closed over the active site and in contact with each other. The non-flap residues show only slight variation. A more striking difference between the two forms is that the relative orientation (the "handedness") of the β -hairpin flaps is reversed (Figure 1a,b). Although large scale flap opening is presumably required for normal substrate access to the active site (Figure 1c), no crystallographic structures representing such an open configuration have been reported. Despite the four available crystal structures of unbound HIV PR [16–19] all show the semi-open conformation, it was not entirely clear whether this reflects the preferred flap conformation or crystal packing effects [10,20,21]. However it is known from NMR experiments [22,23] that the flap region has a high degree of flexibility. Based on solution NMR data for the free protease, Torchia et al. have suggested [23–25] that the ensemble of unbound structures is dominated by the semi-open family with sub-

nanosecond timescale fluctuation in the flap tips, and with closed structures possibly being a minor component of the ensemble. The semi-open form is in slow equilibrium (~100 μ s) with a less structured, open form that exposes the binding site cavity.

Simulations of HIV-1 protease: exploring flap flexibility

While X-ray crystallography provides invaluable high resolution structures, they primarily reflect an average structure of a single low energy conformation with little information on dynamics. NMR experiments can provide a more realistic view of the dynamic behavior in solution and at more biologically relevant temperatures. While several HIV PR structures with bound inhibitors were fully solved by solution NMR, thus far this technique has not provided structural data for the unbound protease in solution, due to difficulties related to protease auto-cleavage, as well as its high flexibility in solution.

Computational methods such as molecular dynamics simulations can provide a detailed, atomic resolution model for time-dependent structural variation. In addition, these simulations can provide estimates of the energetics associated with different HIV PR states, thus providing insight into thermodynamic aspects involved not only in binding but also in conversion between different protease conformations. Unfortunately, up until recently, realistic simulations were hampered by limitations in the model description and time scales that could be reached.

Numerous prior computational studies have aimed at understanding flap opening dynamics. Collins et al. [26] reported flap opening resulting from molecular dynamics (MD) simulations in the gas phase which involved forcing the atomic coordinates for non-flap regions of a closed structure to the semi-open state. Scott and Schiffer [27] also observed irreversible flap opening, but the extent of flap opening was not quantitatively described. Instead the authors focused on the flap tip regions, which “curled” back into the protein structure during the opening event, burying several hydrophobic residues. This flap curling was hypothesized to provide a key conformational trigger necessary for subsequent large-scale flap opening and therefore HIV-PR function. A more recent study [28] highlighted the challenges in obtaining accurate simulation data by demonstrating that similar irreversible flap opening could arise from insufficient equilibration during system setup; these artifacts were not observed when more extensive solvent equilibration was performed. More recently, Hamelberg and McCammon [29] used activated dynamics to produce flap opening in HIV-PR. In this case, a *trans* $\rightarrow$ *cis* isomerization of the Gly-Gly peptide bond was hypothesized to trigger the flap opening. Perryman et al. [30,31] reported dynamics of unbound wild type and V82F/I84V mutant in which the closed form opened somewhat, but the authors did not report whether the flaps in these unbound protease simulations actually adopted the semi-open flap handedness observed in crystal structures. Nevertheless, the high flexibility of the flaps, particularly for the mutant, was demonstrated and used for active site inhibitor design for the drug-resistant mutant [32]. Importantly, none of these prior computational studies of the free protease reported that the flaps were able to adopt the semi-open conformation from either the open structures that were sampled or in other cases from the initial closed conformation. Therefore it is unclear if such opening events are relevant to the true

dynamics of the HIV protease or simply represent an inability of the simulation models to reproduce experimental observations.

Very recently, several reports were published where multiple and most importantly, reversible opening of the protease flaps was observed. These works serve as a testimony that simulation methods have finally reached a state where they can provide valuable insights into enzyme function on biologically relevant timescales.

McCammon and coworkers [33,34] developed a coarse grain model of HIV protease in which each residue is modeled using a single bead at the position of the C α carbon. This treatment substantially reduces the complexity of the system, permitting the simulations to model behavior on the microsecond timescale. Numerous opening and closing events were seen; these were realized primarily by large lateral movements of the flaps that exposed the binding cavity. With the current coarse grain model, however, the long timescales are enabled through neglect of atomic detail, which comes at the cost of not being able to describe more subtle differences, such as those observed between closed and semi-open crystal structures. There is also no straightforward way to determine how flap behavior is influenced by dynamics on the atomic level in terms of specific sidechain interactions, or to gain an understanding of how solvation is coupled to dynamics.

Recently, a multi-scale model was applied to HIV PR dynamics in which full atomic detail was maintained for the protease, and aqueous solvent was modeled using a continuum approach [35]. These simulations showed spontaneous conversion between the bound and unbound crystal forms upon removal of an inhibitor, and reversible opening of the flaps. While the simulations of inhibitor bound form were very stable with no substantial conformational changes (Figure 2a/f), the behavior of the system changed dramatically if the ligand was not present. Removal of the ligand from the protease resulted in spontaneous conversion of the closed flap conformation to semi-open form (Figure 2b), similar to that observed in ligand-free protease crystal structures. Notably, this conversion in the simulations is accompanied by the change in flap handedness that is in agreement with crystal structures (Figure 1). These simulations provide further evidence that the semi-open form is the true conformation of unbound HIV PR in solution and that a rearrangement of the binding pocket indeed occurs upon ligand binding.

Another characteristic feature of the semi-open ensemble generated in these molecular dynamics simulations was that it exhibited much higher flexibility, particularly in the flaps region (Figure 2b/g), as compared to the closed ensemble. Simulations initiated from the free protease crystal structures showed the same behavior. When these simulations were extended to longer times, flexibility of the flaps produced transient openings with large scale rearrangements of the flaps and flap tip distances over 20Å (Figure 2c/d). These fully open forms can easily accommodate entry of substrates. Importantly, these fully open conformations were only transiently populated, and reproducibly returned to the semi-open state (Figure 2e), indicating that the opening events were not artifacts caused by instability of the system or a poor quality model. Full flap opening in the simulations occurred through a concerted downward rotation around the center in the vicinity of the dimer interface and resulted in noticeable mutual rotation of the two monomers accompanied by large upward

motion of the flaps. Opening via lateral movements of the flaps as reported in the coarse grain model [34] was also observed.

Even though the direct observation of the fully open structure and of conversions between three different flap conformations in atomic detail simulations was very encouraging, the question of how relevant this open state was for ligand binding remained open. A subsequent study [36] addressed these questions by performing unrestrained, all-atom MD simulations following manual placement of a cyclic urea inhibitor into the substrate binding site of the open protease (Figure 2h). In those simulations, the inhibitor reproducibly induced the protease to undergo spontaneous conversion to the closed form (Figure 2j), as seen in all inhibitor bound HIV PR crystal structures. In a typical trajectory, the inhibitor formed specific hydrogen bonds with one of the catalytic aspartic acids and one flap (Figure 2i), accelerating the closing process. Subsequently, the other flap closed and helped to pack bulky groups of the inhibitor into the binding pocket (Figure 2j). These simulations reproduced not only the greater degree of flap closure, but also the striking difference in flap handedness between bound and free protease. The transitions between the three forms are summarized in Figure 3.

Another report [37] used molecular dynamics constrained to dihedral angle space to speed up the sampling. The authors observed transitions between semi-open and open conformations, although the semi-open structure following the opening event did not show close agreement with the crystallographic form, which might have resulted from simplifications used in internal coordinate space dynamics. The same authors followed this report with another study [38] reporting protease flap closing induced by substrate binding. Once again, flap closing was observed to follow an asymmetric path.

In summary, the recent molecular dynamics simulations coming from different groups and using different simulation methodologies and force-fields provide compelling evidence that the major features of protease dynamics are generally reproduced and are independent of specific system setup details. These studies also serve as a clear indication that the flexibility and the dynamic behavior of HIV-1 protease are amenable to computational analysis, and the resulting data may form the foundation of a flexibility-based drug design process.

Proposed molecular mechanisms of resistance

Classical and *ab-initio* MD simulations reveal [39,40] that protease flexibility modulates the activation free energy barrier of the enzymatic cleavage reaction. In drug-resistant mutants, the active site mutations are often associated with mutations that partially restore the enzymatic function (“compensatory mutations”) and frequently occur in regions distant from the active site. The mutations in these positions may enhance the catalytic rate of the protease mutants by affecting the flexibility of the protein. While the authors provide a plausible explanation of how compensatory mutations work, they do not suggest how this understanding could be extended to the design of drugs that escape protease mutations.

Schiffer in her earlier MD simulation study [27] proposes a model for overcoming resistance based on an observation of HIV-1 protease conformation with flaps “curled” such that they allow substrate access to the active site. In this conformation the hydrophobic tips of the

flaps (G⁴⁸GIGG⁵²) curl in and pack against the hydrophobic inside wall of the active site groove. The authors suggest that this 'open' conformation is crucial and the inhibitors should be designed to lock the flaps in their 'open' conformation, and believe that such inhibitors would be less susceptible to the development of drug-resistant variants.

Along this line, a crystal structure of the unbound HIV-1 PR for the multiple drug resistant (MDR) 769 isolate (PDB code 1TW7) in a 'wide-open' conformation was reported recently [41]. Unlike all previous experimental structures, the binding pocket is more exposed due to open conformation of the flaps. Even though the structure differs from the 'open' structure proposed by Schiffer, the idea of resistance remains roughly the same. The authors indicate that drug resistance in this strain arises at least partly from the changes in the flap conformation, and presumably the inability of current inhibitors to induce flap closing. In a recent report, molecular dynamics simulations were performed for this MDR isolate, starting from the open crystal structure. While simulations that included crystal packing contacts reproduced the crystal structure, simulations in solution reproducibly reverted to the semi-open form observed in crystal structures of the wild-type protease [42]. The results suggest that although the mutations may have slightly shifted the balance between these forms, the dominant structure in solution at room temperature remains the semi-open form.

An appealing explanation of resistance was provided by Freire based on microcalorimetric measurements of protease binding thermodynamics [43,44]. In solution the peptide substrate has a higher flexibility than the synthetic inhibitors and therefore suffers a higher conformational entropy loss upon binding. On the other hand, due to its higher flexibility, the peptide substrate is more amenable to adapt to backbone rearrangements or subtle conformational changes induced by mutations in the protease. The synthetic inhibitors are less flexible, and their capacity to adapt to changes in the geometry of the binding pocket is more restricted.

Both computational and experimental studies showed that, in case of HIV-1 protease, there are differences in thermodynamic stability among the alternate protease forms that should be included when considering ligand binding affinity. Since the structure of the transient open form was only suggested in recent molecular dynamics studies, the description thus far has focused on thermodynamic differences between closed and semi-open forms. For example, the free energy change G calculated by reaction path method estimated that the semi-open form is more favorable than closed, with stabilization contribution coming primarily from the entropic term [45]. This analysis is consistent with NMR relaxation data and is very reasonable given the high glycine content of the flap tips. As was shown by calorimetric experiments, a large favorable entropy change is also the major driving force for high binding affinity of current HIV-1 PR inhibitors [46,47]. However, in this case it is the favorable solvation entropy associated with the burial of a large hydrophobic surface upon inhibitor binding. Detailed thermodynamic analysis [46] of wild-type and active site resistant mutant (V82F/I84V) suggests that the mutation lowers the binding affinity in two ways: first, by directly altering the interaction between inhibitors and the protease (binding enthalpy and entropy), and second, indirectly by altering the relative stability of free (semi-open) and bound (closed) form upon inhibitor binding. Importantly, mutations that stabilize the semi-open flap conformation will lower binding affinity due to the increased energy

penalty required for closing the flaps upon substrate/inhibitor binding. Therefore, the free energy change associated with the conformational change of the protein has to be included in any accurate calculation of binding affinity.

These few examples of proposed mechanism of resistance raise several important points. To preserve the function of the mutant protease (i.e. still efficiently cleave the viral polyprotein) the enzyme can introduce alterations in the active site but the correct dynamics or flexibility must be preserved, and/or the active site changes must be compensated by the flexibility of the substrate. Since the competitive advantage of the synthetic inhibitors' strong binding likely arises from their rigidity, it has been rather challenging to design flexible inhibitors that bind stronger than the natural substrate while retaining the ability to adapt to a binding pocket that varies in shape. An example of such flexible inhibitor is KNI-764 (also known as JE-2147) which was shown to remain potent against MDR protease strains [48]. It was again demonstrated by calorimetric measurements [49,50] that, in contrast to previous inhibitors, these second generation inhibitors bind strongly mainly due to favorable enthalpy change.

Another strategy to evade mutations in the active site is to design inhibitors that primarily form interactions with the backbone rather than sidechains of the active site [51,52], such that mutations in the binding site may not effect the inhibitor binding. A conceptually different strategy arises from consideration of HIV PR flexibility. Rather than accommodating changes in shape or affinity of the binding site, one may try to interfere with the dynamics of the protein. Assuming that enzyme dynamics must remain conserved, the virus may have a more difficult time evolving mutations that would restore functional dynamics of the protease.

Drug design targeting protein flexibility – new allosteric inhibitors

In light of the discussion above, an attractive alternative approach to designing protease inhibitors would target the thermodynamic balance of the closed, semi-open and open ensembles. This might be achieved by designing allosteric inhibitors, which do not directly compete with substrate for the same binding site, but indirectly change the flexibility of the protease such that the balance of the three states is shifted.

The possibility of allosteric inhibitors of HIV protease was suggested previously [30,31]. Based on molecular dynamics simulations that showed anticorrelated behavior between flap opening and compression of the allosteric site in the elbow region, the authors suggested targeting the protease elbow regions (see Figure 1b) as an allosteric site. However, no experimental evidence to support this hypothesis is yet available. It is interesting to note, however, that the only experimentally determined structure with an open binding pocket [41] (the crystal structure of the MDR isolate discussed above) indeed has a crystal packing contact involving insertion of residues from a symmetry-related neighbor into the elbow region [42]. This observation provides further evidence that this site may be a promising candidate for allosteric inhibition.

Another potential target for allosteric inhibition is the protease dimer interface (see Figure 1b). NMR experiments measuring backbone amide chemical exchange transverse relaxation rates [24] indicated that the flexibility in the four-stranded β -sheet dimer interface increases

upon inhibitor binding. This points out a coupling between the binding site and the dimer interface. This coupling (even though in the opposite direction) is also observed in the crystal structure of a free HIV-1 protease in which the N- and C-termini of the two protease monomers were tethered [53]. Unlike all other crystal structures of the free protease, this 'monomeric' protease exhibits the closed flap conformation. Last but not least, there has been a continuing effort to design inhibitors of protease dimerization [54,55]. An interesting recent report [56] demonstrated that some of the inhibitors initially designed to prevent dimerization actually did not disrupt the dimer interface and yet showed substantial protease inhibition. The authors thus concluded that these compounds acted as allosteric inhibitors binding at the dimer interface, indirectly reducing the binding affinity of the substrate.

Further evidence that these sites may provide useful targets for allosteric inhibitors has been shown by, Rezacova et al [57], who developed monoclonal antibodies with potent inhibition of the protease function. These targeted two non-binding site regions of the enzyme: one corresponds to residues 36–46 (flap elbow) and the other to residues 1–6 at the dimer interface. The mechanism of inhibition is not clear but the authors suggest that disruption of the native fold or even dimerization interface is a possibility. Yet another example of potentially exploitable allosteric inhibition was reported for beta-lactam compounds [58]. The authors demonstrated that the inhibitors are noncompetitive and that they only interact with ligand-bound enzyme, and suggested the mechanism of inhibition through interaction of beta-lactam compounds with the closed flap region of the enzyme-substrate complex.

Even though the existence of HIV PR allosteric sites has not yet been shown experimentally, it has been argued that their presence is very likely for all dynamic proteins [59]. The allosteric inhibitors do not compete with natural substrate and thus their effect is not decreased by higher concentration of the substrate. They also have a potential for better selectivity [59]. Moreover, the hydrophobic character of HIV PR active site leads to hydrophobic protease inhibitors and therefore results in their undesirable poor water solubility [60,61]. Thus the additional advantage of the two allosteric sites discussed before stems from their polar character, which could avoid difficulties with inhibitor solubility.

Conclusions

The pronounced differences in bound and unbound protease crystal structures and NMR studies provide experimental evidence that HIV-1 protease flexibility plays a crucial role in its function. Numerous computational studies reviewed here emphasized the importance of protease dynamics in substrate and inhibitor binding. Taken together, this opens new opportunities for developing protease inhibitors in which protease dynamics and flexibility, as determined through computer simulation, are explicitly targeted in the inhibitor design process. Specifically, influencing the thermodynamics of the three protease states (closed, semi-open and open) might disrupt its function. It remains to be seen whether this approach makes it more difficult for the enzyme to evolve resistant mutations.

Computer simulations aimed at accurate quantitative description of protease dynamics still face challenges, even though atomic level and coarse grain simulations complement each other in their predictive abilities. All-atom simulations provide a model which is able to

reproduce experimentally observed structural changes, predict a new open structure inferred from experiments and show transitions between all these forms. On the other hand, coarse grain models could provide a more statistically valid thermodynamic description of the flap opening and thereby provide quantitative estimates for the shift in equilibrium arising from protease mutations. Thus far, the accuracy of the coarse grain model does not appear to be sufficient to reproduce the detailed conformational changes that accompany binding, i.e. changes between closed and semi-open forms. Full atomistic models may therefore serve as an important reference for calibrating and improving the coarse grain models.

In summary, due to improved quality of the computational models and the ability to extend simulations to biologically relevant timescales, computational techniques have finally reached the stage where they can reproduce experimental observations. Perhaps more important, however, is that simulations can now complement experiments by providing valuable insights into dynamic events as well as energetic aspects of ligand binding and drug resistance.

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Figure 1.

Top and side views of the three important conformations of HIV-1 protease. **(a)** The ‘closed’ form is observed in crystal structures with ligands bound (structure with pdb code 1HVR is shown). **(b)** The flaps of the free protease assume a ‘semi-open’ conformation in crystal structures (1HHP is shown). The top views highlight the change in flap handedness between ‘closed’ and ‘semi-open’ structures. Proposed allosteric inhibition sites are labeled as (1) and (2). **(c)** The fully ‘open’ form in which the active site becomes accessible to substrate or inhibitors was not observed in crystal structures but was implied from NMR experiments. The structure shown is from molecular dynamics simulations [35].

Figure 2.

Snapshots from molecular dynamics simulations of inhibitor bound and free protease, and from simulations following the manual docking of the inhibitor into the binding site. The ‘closed’ conformation (a) is represented by ensemble of closed structures with high similarity (f). In contrast, the ‘semi-open’ conformation (b) represents a much more flexible ensemble (g) with larger fluctuations of the flaps. Those eventually lead to full opening of flaps (c, d); the open form is transient and returns to semi-open conformation (e). When the inhibitor is manually placed into a binding site (h), it induces an asymmetric flap closure with initial closing of one of the flaps (i) and finally converting to fully closed form (j) with flaps pulled into the binding site and flap handedness appropriate for the closed state.

Figure 3.

Schematic representation of simulated transitions between the three protease forms. The closed flap conformation converts to semi-open upon removal of ligand. Ligand induces the closure of the open form. Free protease exists primarily in semi-open form but transiently changes to fully open and, occasionally, even to the closed form that is only weakly populated in the absence of a ligand.