Targeting structural flexibility in HIV-1 protease inhibitor binding

Viktor Hornak[1] and Carlos Simmerling[1,2]

[1] Stony Brook University, Center for Structural Biology, Stony Brook, NY 11794-5155, USA
[2] Stony Brook University, Department of Chemistry, Stony Brook, NY 11794-3400, USA

HIV-1 protease remains an important anti-AIDS drug target. Although 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 have 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, and the third was implied by NMR experiments. Based on these computational models, it has been suggested that binding of inhibitors in allosteric sites might affect protease flexibility and disrupt its function.

Introduction

It has been >20 years since HIV was first identified as the causative agent of AIDS [1,2]. There are currently three main avenues for preventing HIV replication. First, blocking the attachment of virus particles to host-cell surfaces using 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 HIV-1 protease (HIV-1 PR), a process that is described in more detail in this review.

The necessity of HIV-1 PR for virus replication was demonstrated through several experiments. Deletion mutagenesis of the gene encoding HIV-1 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 the viral 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-1 PR, leading to solution of high-resolution 3D structures of HIV-1 PR using X-ray crystallography [9]. Current FDA-approved HIV-1 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 the binding of HIV-1 PR substrates and inhibitors is crucially important for the design of novel inhibitors of the enzyme. There is evidence that flexibility of many enzymes plays an important part 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-1 PR inhibitor and drug design. A particular focus is the emergence of computational techniques that can provide a detailed insight into the dynamic aspects of HIV-1 PR behavior. To understand how the substrate gains access to the protease-binding site and how resistance caused by protease mutations arises, many computational studies have been carried out on this enzyme. Recent molecular dynamics (MD) simulations of HIV-1 PR suggested that the dynamics of this enzyme is crucial for its function. Therefore, affecting flexibility of the protease by, for example, allosteric inhibitors, could create new opportunities for inhibitor design.

Corresponding author: Simmerling, C. (carlos.simmerling@stonybrook.edu)

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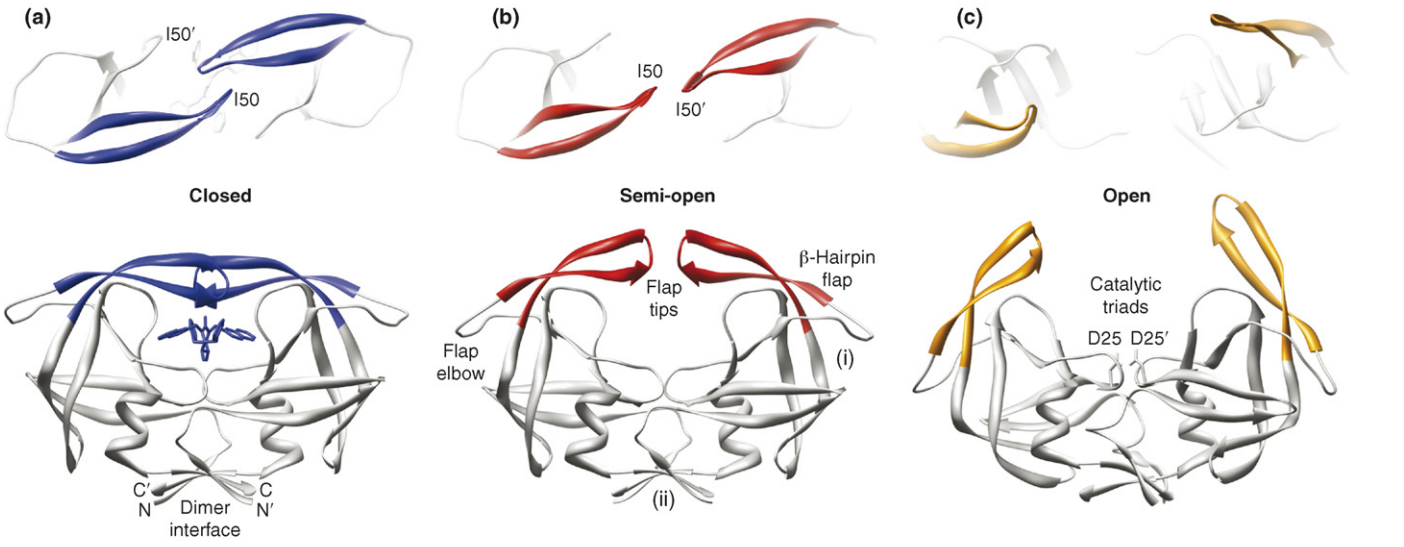

FIGURE 1

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 protein databank (PDB) code 1HVR is shown]. **(b)** The flaps of the free protease assume a 'semi-open' conformation in crystal structures (PDB 1HHP is shown). The three top views (a–c) highlight the change in flap handedness between closed and semi-open structures. Proposed allosteric inhibition sites are labeled as (i) and (ii). **(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].

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

An extensive set of X-ray crystal structures of HIV-1 PR, in 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 (i.e. the 'closed' form), whereas 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 orientation (i.e. 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. Although the four available crystal structures of unbound HIV-1 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 and colleagues [23–25] suggested that the ensemble of unbound structures is dominated by the semi-open family with subnanosecond 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 μs) with a less-structured, 'open' form that exposes the binding-site cavity.

Simulations of HIV-1 protease: exploring flap flexibility

Although X-ray crystallography provides invaluable high-resolution protein 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 of proteins in solution and at more biologically relevant temperatures. Although several HIV-1 PR structures with bound inhibitors were fully solved by solution NMR, so far this technique has not provided structural data for the unbound protease in solution, because of difficulties related to protease autocleavage, as well as its high flexibility in solution.

Computational methods such as MD 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-1 PR states, thus providing insight into thermodynamic aspects involved not only in binding but also in conversion between different protease conformations. Unfortunately, until recently, realistic simulations were hampered by limitations in the model description and timescales that could be reached.

Numerous earlier computational studies have aimed at understanding flap opening dynamics. Collins *et al.* [26] reported flap opening that results from MD simulations in the gas phase, which involved forcing the atomic coordinates for non-flap regions of a closed structure toward 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

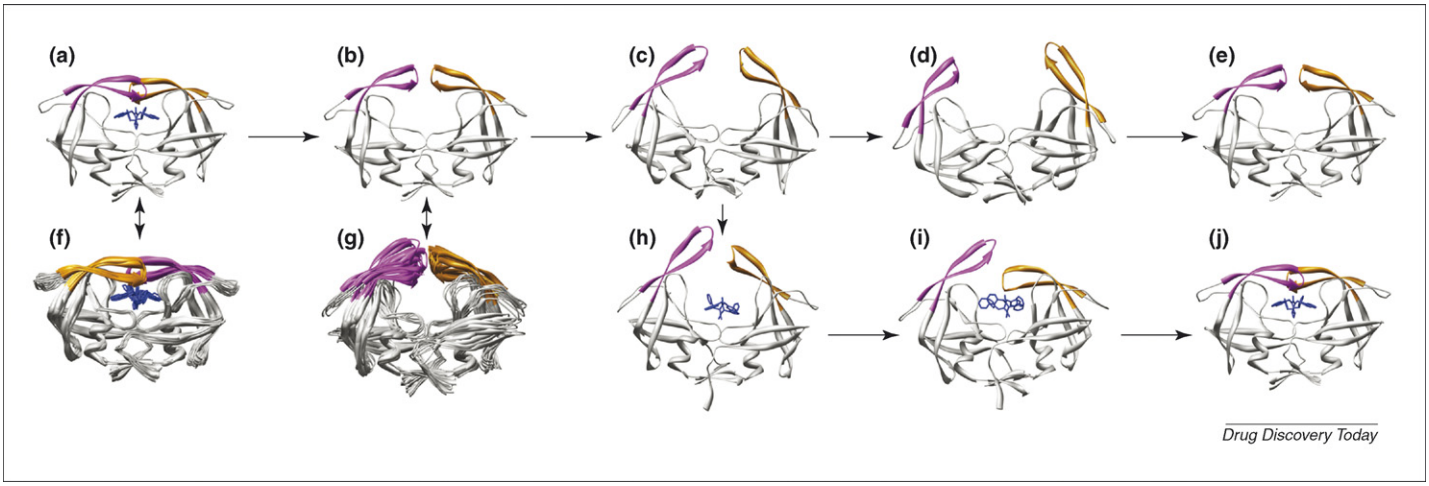

FIGURE 2

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 an ensemble of closed structures with high similarity **(f)**. By contrast, the 'semi-open' conformation **(b)** represents a much more flexible ensemble **(g)** with larger fluctuations of the flaps. These eventually lead to full opening of flaps **(c,d)**; the 'open' form is transient and returns to the 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)**, finally converting to the fully closed form **(j)** with flaps pulled into the binding site and flap handedness appropriate for the closed state.

opening and, therefore, HIV-1 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-1 PR. In this case, a *trans* → *cis* isomerization of the Gly–Gly peptide bond was hypothesized to trigger the flap opening. Perryman *et al.* [30,31] reported dynamics of the unbound wild-type and a 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 to investigate active-site inhibitors for the drug-resistant mutant [32]. Importantly, none of these previous 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-1 PR or simply represent an inability of the simulation models to reproduce experimental observations.

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 co-workers [33,34] developed a coarse grain model of HIV-1 PR 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, enabling 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. However, with the current coarse grain model, the long timescales can only occur 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 multiscale model was applied to HIV-1 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. Although the simulations of closed inhibitor-bound HIV-1 PR were 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 the 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-1 PR in solution and that a rearrangement of the binding pocket occurs upon ligand binding.

Another characteristic feature of the semi-open ensemble generated in these MD simulations was that it exhibited much higher flexibility, particularly in the flap region (Figure 2b,g), compared with 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 20 Å (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 located in the vicinity of the dimer interface, and resulted in noticeable mutual rotation of the two monomers accompanied by a large upward motion of the flaps. Opening via lateral movements of the flaps, as reported in the coarse grain model [34], was also observed.

Although the direct observation of the fully open structure and of conversions between three different flap conformations in atomic detail simulations was encouraging, the question of how relevant this open state was for ligand binding remained unanswered. 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 these simulations, the inhibitor reproducibly induced the protease to undergo spontaneous conversion to the closed form (Figure 2j), as seen in all inhibitor-bound HIV-1 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 MD 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 as induced by substrate binding. Once again, flap closing was observed to follow an asymmetric path.

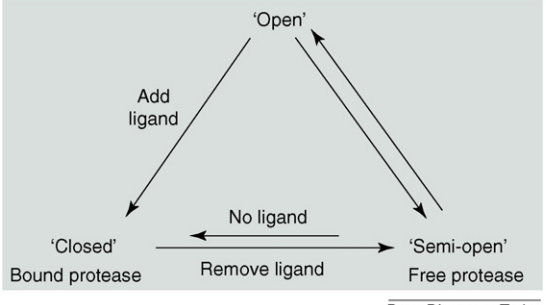

FIGURE 3

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

In summary, the recent MD simulations, coming from different groups and using different simulation methodologies and force-fields, provide compelling evidence that the major features of protease dynamics are 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 PR are amenable to computational analysis, and the resulting data can form the foundation of a flexibility-based drug-design process.

Proposed molecular mechanisms of resistance

Classical and *ab initio* MD simulations [39,40] reveal 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 (i.e. 'compensatory mutations') and frequently occur in regions that are distant from the active site. The mutations in these positions can enhance the catalytic rate of the protease mutants by affecting the flexibility of the protein. Although 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.

In their earlier MD simulation study, Scott and Schiffer [27] propose a model for overcoming resistance based on an observation of HIV-1 PR conformation with flaps curled such that they enable substrate access to the active site. In this conformation, the hydrophobic tips of the flaps ($G^{48}GIGG^{52}$) curl in and pack against the hydrophobic inside wall of the active-site groove. These authors suggest that this open conformation is crucial and the inhibitors should be designed to lock the flaps in their open conformation, and they also 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 [protein databank (PDB) code 1TW7] in a 'wide-open' conformation was recently reported [41]. Unlike all previous experimental structures, the binding pocket is more exposed because of the open conformation of the flaps. Although the structure differs from the open structure proposed by Scott and Schiffer [27], the idea of resistance remains roughly the same. The authors of MDR protease structure indicate that drug resistance in this strain arises at least partly from the changes in the flap conformation, and presumably from the inability of current inhibitors to induce flap closing. In a recent report, MD simulations were performed for this MDR isolate, starting from the open crystal structure. Although simulations that included crystal-packing contacts reproduced the crystal structure, simulations in solution reproducibly reverted to the semi-open form that is observed in crystal structures of the wild-type protease [42]. The results suggest that, although the mutations might 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 and colleagues 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. By contrast, because of 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.

Computational and experimental studies showed that, in the case of HIV-1 PR, there are differences in thermodynamic stability among the alternate protease forms that should be included when considering ligand-binding affinity. Because the structure of the transient open form was only suggested in recent MD studies, the description to date has focused on thermodynamic differences between closed and semi-open forms. For example, the free energy change calculated by the reaction path method estimated that the semi-open form is more favorable than the closed form, with stabilization contribution primarily coming from the entropic term [45]. This analysis is consistent with NMR relaxation data and is reasonable given the high glycine content of the flap tips. As 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 the 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 (i.e. the binding enthalpy and entropy); and, second, indirectly by altering the relative stability of free (i.e. semi-open) and bound (i.e. closed) forms upon inhibitor binding. Importantly, mutations that stabilize the semi-open flap conformation will lower binding affinity because of the increased energy penalty required for rearranging the flaps upon substrate or inhibitor binding. Therefore, the free-energy change associated with the conformational change of the protein has to be included in every accurate calculation of binding affinity.

These few examples of the proposed mechanism of resistance raise several important points: to preserve the function of the mutant protease (i.e. efficiently cleaving 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. Because the competitive advantage of the synthetic inhibitors' strong binding probably arises from their rigidity, it has been challenging to design flexible inhibitors that bind with higher affinity than the natural substrate, at the same time retaining the ability to adapt to a binding pocket that varies in shape. An example of such a flexible inhibitor is KNI-764 (also known as JE-2147), which was shown to remain potent against MDR protease strains [48]. It was demonstrated by calorimetric measurements [49,50] that, in contrast to previous inhibitors, these second-generation inhibitors bind strongly because of a 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 might not affect the inhibitor binding. A conceptually different strategy arises from the consideration of HIV-1 PR flexibility. Rather than accommodating changes in shape or affinity of the binding site, one could try to interfere with the dynamics of the protein. Assuming that enzyme dynamics must remain conserved, the virus could have a harder time evolving mutations that would restore the functional dynamics of HIV-1 PR.

Drug design targeting protein flexibility: new allosteric inhibitors

In light of the discussion in this review, an attractive alternative approach to designing protease inhibitors would target the thermodynamic balance of the closed, semi-open and open ensembles. This could be achieved by designing allosteric inhibitors that 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-1 PR was suggested previously [30,31]. Based on MD simulations that showed anticorrelated behavior between flap opening and compression of the elbow region [31], targeting of the protease elbow regions as an allosteric site was suggested (see Figure 1b). 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] (i.e. the discussed crystal structure of the MDR isolate) 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 might 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] indicate that the flexibility in the four-stranded β-sheet dimer interface increases upon inhibitor binding. This illustrates coupling between the binding site and the dimer interface. A coupling between active site flaps and the dimmer interface is also observed in the crystal structure of a free HIV-1 PR 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 that were initially designed to prevent dimerization did not actually disrupt the dimer interface and yet showed substantial protease inhibition. These authors thus concluded that these compounds acted as allosteric inhibitors binding at the dimer interface and indirectly reducing the binding affinity of the substrate.

Further evidence that these sites can provide useful targets for allosteric inhibitors has been shown by Rezacova *et al.* [57]; they developed monoclonal antibodies (mAbs) with potent inhibition of HIV-1 PR function. These mAbs targeted two non-binding-site regions of the enzyme: one corresponds to residues 36–46 (i.e. the 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 the dimerization interface, is a possibility. Yet another example of potentially exploitable allosteric inhibition was reported for β-lactam compounds [58]. The authors of that study demonstrated that the inhibitors are noncompetitive and that they only interact with ligand-bound

enzyme, and suggested a mechanism of inhibition through interaction of β-lactam compounds with the closed flap region of the enzyme–substrate complex.

Although the existence of HIV-1 PR allosteric sites has not yet been shown experimentally, it has been argued that their presence is likely for all dynamic proteins [59]. The allosteric inhibitors do not compete with natural substrate and, thus, their effect is not decreased by a higher substrate concentration. They also have a potential for better selectivity [59]. Moreover, the hydrophobic character of the HIV-1 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 previously in this review stems from their polar character, which could avoid difficulties with inhibitor solubility.

Concluding remarks

The pronounced differences in bound and unbound protease crystal structures and NMR studies provide experimental evidence that HIV-1 PR flexibility plays a crucial part in its function. Numerous computational studies reviewed here emphasize the importance of protease dynamics in substrate and inhibitor binding. Altogether, this provides 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 (i.e. closed, semi-open and open) might disrupt HIV-1 PR function. It remains to be seen whether this approach makes it harder for the enzyme to evolve resistant mutations.

Computer simulations aimed at accurate quantitative description of protease dynamics still face challenges, although atomic level and coarse grain simulations complement each other in their predictive abilities. All-atom simulations provide a model that can experimentally reproduce observed structural changes, predict a new open structure inferred from experiments and show transitions between all three forms. By contrast, 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. To date, the accuracy of the coarse grain model does not seem to be sufficient to reproduce the detailed conformational changes that accompany binding (i.e. changes between closed and semi-open forms). Full atomistic models can therefore serve as an important reference for calibrating and improving the coarse grain models.

In summary, because of 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 and energetic aspects of ligand binding and drug resistance.

Appendix A. Supplementary information

An animation illustrating the full flap opening (i.e. the transition from semi-open to fully open and back to the semi-open form, as depicted in Figure 2b–e) is available online as supplementary information, at doi:10.1016/j.drudis.2006.12.011

References

1 Barresinoussi, F. *et al.* (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune-deficiency syndrome (AIDS). *Science* 220, 868–871
2 Gallo, R.C. *et al.* (1983) Isolation of human T-cell leukemia-virus in acquired immune-deficiency syndrome (AIDS). *Science* 220, 865–867
3 Blair, W.S. *et al.* (2000) HIV-1 entry - an expanding portal for drug discovery. *Drug Discov. Today* 5, 183–194
4 Moore, J.P. and Stevenson, M. (2000) New targets for inhibitors of HIV-1 replication. *Nat. Rev. Mol. Cell Biol.* 1, 40–49
5 Ren, J. *et al.* (1995) High-resolution structures of HIV-1 Rt from 4 Rt-inhibitor complexes. *Nat. Struct. Biol.* 2, 293–302
6 Kohl, N.E. *et al.* (1988) Active human immunodeficiency virus protease is required for viral infectivity. *Proc. Natl. Acad. Sci. U. S. A.* 85, 4686–4690
7 Seelmeier, S. *et al.* (1988) Human immunodeficiency virus has an aspartic-type protease that can be inhibited by pepstatin-A. *Proc. Natl. Acad. Sci. U. S. A.* 85, 6612–6616
8 Mcquade, T.J. *et al.* (1990) A synthetic HIV-1 protease inhibitor with antiviral activity arrests HIV-like particle maturation. *Science* 247, 454–456
9 Vondrasek, J. and Wlodawer, A. (2002) HIVdb: a database of the structures of human immunodeficiency virus protease. *Proteins Struct. Funct. Genet.* 49, 429–431
10 Wlodawer, A. and Vondrasek, J. (1998) Inhibitors of HIV-1 protease: A major success of structure-assisted drug design. *Annu. Rev. Biophys. Biomol. Struct.* 27, 249–284
11 Wlodawer, A. and Erickson, J.W. (1993) Structure-based inhibitors of HIV-1 protease. *Annu. Rev. Biochem.* 62, 543–585
12 Vacca, J.P. and Condra, J.H. (1997) Clinically effective HIV-1 protease inhibitors. *Drug Discov. Today* 2, 261–272
13 Condra, J.H. *et al.* (1995) *In-vivo* emergence of HIV-1 variants resistant to multiple protease inhibitors. *Nature* 374, 569–571
14 Teague, S.J. (2003) Implications of protein flexibility for drug discovery. *Nat. Rev. Drug Discov.* 2, 527–541
15 McCammon, J.A. (2005) Target flexibility in molecular recognition. *Biochim. Biophys. Acta* 1754, 221–224
16 Navia, M.A. *et al.* (1989) 3-Dimensional structure of aspartyl protease from human immunodeficiency virus HIV-1. *Nature* 337, 615–620
17 Wlodawer, A. *et al.* (1989) Conserved folding in retroviral proteases - crystal-structure of a synthetic HIV-1 protease. *Science* 245, 616–621
18 Lapatto, R. *et al.* (1989) X-Ray-analysis of HIV-1 proteinase at 2.7 Å resolution confirms structural homology among retroviral enzymes. *Nature* 342, 299–302
19 Spinelli, S. *et al.* (1991) The 3-dimensional structure of the aspartyl protease from the HIV-1 isolate bru. *Biochimie* 73, 1391–1396
20 Lange-Savage, G. *et al.* (1997) Structure of HOE/BAY 793 complexed to human immunodeficiency virus (HIV-1) protease in two different crystal forms - structure/function relationship and influence of crystal packing. *Eur. J. Biochem.* 248, 313–322
21 Miller, M. *et al.* (1989) Structure of complex of synthetic HIV-1 protease with a substrate-based inhibitor at 2.3-Å resolution. *Science* 246, 1149–1152
22 Nicholson, L.K. *et al.* (1995) Flexibility and Function in HIV-1 protease. *Nat. Struct. Biol.* 2, 274–280
23 Freedberg, D.I. *et al.* (2002) Rapid structural fluctuations of the free HIV protease flaps in solution: relationship to crystal structures and comparison with predictions of dynamics calculations. *Protein Sci.* 11, 221–232
24 Ishima, R. *et al.* (1999) Flap opening and dimer-interface flexibility in the free and inhibitor-bound HIV protease, and their implications for function. *Structure* 7, 1047–1055
25 Katoh, E. *et al.* (2003) A solution NMR study of the binding kinetics and the internal dynamics of an HIV-1 protease-substrate complex. *Protein Sci.* 12, 1376–1385
26 Collins, J.R. *et al.* (1995) Flap opening in HIV-1 protease simulated by activated molecular-dynamics. *Nat. Struct. Biol.* 2, 334–338
27 Scott, W.R.P. and Schiffer, C.A. (2000) Curling of flap tips in HIV-1 protease as a mechanism for substrate entry and tolerance of drug resistance. *Structure* 8, 1259–1265
28 Meagher, K.L. and Carlson, H.A. (2005) Solvation influences flap collapse in HIV-1 protease. *Proteins* 58, 119–125

29 Hamelberg, D. and McCammon, J.A. (2005) Fast peptidyl *cis-trans* isomerization within the flexible Gly-rich flaps of HIV-1 protease. *J. Am. Chem. Soc.* 127, 13778–13779

30 Perryman, A.L. *et al.* (2004) HIV-1 protease molecular dynamics of a wild-type and of the V82F/I84V mutant: possible contributions to drug resistance and a potential new target site for drugs. *Protein Sci.* 13, 1108–1123

31 Perryman, A.L. *et al.* (2006) Restrained molecular dynamics simulations of HIV-1 protease: the first step in validating a new target for drug design. *Biopolymers* 82, 272–284

32 Perryman, A.L. *et al.* (2006) Optimization and computational evaluation of a series of potential active site inhibitors of the V82F/I84V drug-resistant mutant of HIV-1 protease: an application of the relaxed complex method of structure-based drug design. *Chemical Biology & Drug Design* 67, 336–345

33 Chang, C.E. *et al.* (2006) Gated binding of ligands to HIV-1 protease: Brownian dynamics simulations in a coarse-grained model. *Biophys. J.* 90, 3880–3885

34 Tozzini, V. and McCammon, J.A. (2005) A coarse grained model for the dynamics of flap opening in HIV-1 protease. *Chem. Phys. Lett.* 413, 123–128

35 Hornak, V. *et al.* (2006) HIV-1 protease flaps spontaneously open and reclose in molecular dynamics simulations. *Proc. Natl. Acad. Sci. U. S. A.* 103, 915–920

36 Hornak, V. *et al.* (2006) HIV-1 protease flaps spontaneously close to the correct structure in simulations following manual placement of an inhibitor into the open state. *J. Am. Chem. Soc.* 128, 2812–2813

37 Toth, G. and Borics, A. (2006) Flap opening mechanism of HIV-1 protease. *J. Mol. Graph. Model.* 24, 465–474

38 Toth, G. and Borics, A. (2006) Closing of the flaps of HIV-1 protease induced by substrate binding: a model of a flap closing mechanism in retroviral aspartic proteases. *Biochemistry* 45, 6606–6614

39 Piana, S. *et al.* (2002) Drug resistance in HIV-1 protease: flexibility-assisted mechanism of compensatory mutations. *Protein Sci.* 11, 2393–2402

40 Piana, S. *et al.* (2002) Role of conformational fluctuations in the enzymatic reaction of HIV-1 protease. *J. Mol. Biol.* 319, 567–583

41 Martin, P. *et al.* (2005) Wide-open 1.3 Å structure of a multidrug-resistant HIV-1 protease as a drug target. *Structure* 13, 1887–1895

42 Layten, M. *et al.* (2006) The open structure of a multi-drug-resistant HIV-1 protease is stabilized by crystal packing contacts. *J. Am. Chem. Soc.* 128, 13360–13361

43 Luque, I. *et al.* (1998) Molecular basis of resistance to HIV-1 protease inhibition: a plausible hypothesis. *Biochemistry* 37, 5791–5797

44 Vega, S. *et al.* (2004) A structural and thermodynamic escape mechanism from a drug resistant mutation of the HIV-1 protease. *Proteins* 55, 594–602

45 Rick, S.W. *et al.* (1998) Reaction path and free energy calculations of the transition between alternate conformations of HIV-1 protease. *Proteins* 32, 7–16

46 Todd, M.J. *et al.* (2000) Thermodynamic basis of resistance to HIV-1 protease inhibition: calorimetric analysis of the V82F/I84V active site resistant mutant. *Biochemistry* 39, 11876–11883

47 Ohtaka, H. *et al.* (2004) Thermodynamic rules for the design of high affinity HIV-1 protease inhibitors with adaptability to mutations and high selectivity towards unwanted targets. *Int. J. Biochem. Cell Biol.* 36, 1787–1799

48 Yoshimura, K. *et al.* (1999) JE-2147: a dipeptide protease inhibitor (PI) that potently inhibits multi-PI-resistant HIV-1. *Proc. Natl. Acad. Sci. U. S. A.* 96, 8675–8680

49 Velazquez-Campoy, A. and Freire, E. (2001) Incorporating target heterogeneity in drug design. *J. Cell. Biochem. (Suppl.)* 37, 82–88

50 Velazquez-Campoy, A. *et al.* (2001) The binding energetics of first- and second-generation HIV-1 protease inhibitors: implications for drug design. *Arch. Biochem. Biophys.* 390, 169–175

51 Ghosh, A.K. *et al.* (2006) Structure-based design of novel HIV-1 protease inhibitors to combat drug resistance. *J. Med. Chem.* 49, 5252–5261

52 Tie, Y.F. *et al.* (2004) High resolution crystal structures of HIV-1 protease with a potent non-peptide inhibitor (UIC-94017) active against multi-drug-resistant clinical strains. *J. Mol. Biol.* 338, 341–352

53 Pillai, B. *et al.* (2001) 1.9 Å X-ray study shows closed flap conformation in crystals of tethered HIV-1PR. *Proteins Struct. Funct. Genet.* 43, 57–64

54 Hwang, Y.S. and Chmielewski, J. (2005) Development of low molecular weight HIV-1 protease dimerization inhibitors. *J. Med. Chem.* 48, 2239–2242

55 Shultz, M.D. *et al.* (2004) Small-molecule dimerization inhibitors of wild-type and mutant HIV protease: a focused library approach. *J. Am. Chem. Soc.* 126, 9886–9887

56 Bowman, M.J. *et al.* (2005) Switching between allosteric and dimerization inhibition of HIV-1 protease. *Chem. Biol.* 12, 439–444

57 Rezacova, P. *et al.* (2005) Crystal structure of a cross-reaction complex between an anti-HIV-1 protease antibody and an HIV-2 protease peptide. *J. Struct. Biol.* 149, 332–337

58 Sperka, T. *et al.* (2005) β-lactam compounds as apparently uncompetitive inhibitors of HIV-1 protease. *Bioorg. Med. Chem. Lett.* 15, 3086–3090

59 Gunasekaran, K. *et al.* (2004) Is allostery an intrinsic property of all dynamic proteins? *Proteins* 57, 433–443

60 Fleisher, D. *et al.* (1996) Improved oral drug delivery: solubility limitations overcome by the use of prodrugs. *Adv. Drug Deliver. Rev.* 19, 115–130

61 Sohma, Y. *et al.* (2003) Development of water-soluble prodrugs of the HIV-1 protease inhibitor KNI-727: importance of the conversion time for higher gastrointestinal absorption of prodrugs based on spontaneous chemical cleavage. *J. Med. Chem.* 46, 4124–4135