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Rapid X-ray diffraction analysis of HIV-1 protease–inhibitor complexes: inhibitor exchange in single crystals of the bound enzyme

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Abstract

The ability to replace an inhibitor bound to the HIV-1 protease in single crystals with other potent inhibitors offers the possibility of investigating a series of protease inhibitors rapidly and conveniently with the use of X-ray crystallography. This approach affords a fast turnaround of structural information for iterative rational drug designs and obviates the need for studying the complex structures by co-crystallization. The replacement approach has been successfully used with single crystals of the HIV-1 protease complexed with a weak inhibitor. The structures of the complexes obtained by the replacement method are similar to those determined by co-crystallization.

1. Introduction

Knowledge of the bioactive conformation and detailed interactions of a ligand with its cognate receptor molecule has been very useful in modern rational drug designs. Three-dimensional structures of complexes of the receptor and various ligands offer insights at the atomic level and are important for specific and systematic modifications of ligands to achieve full complementary and tight binding. This process emphasizes the need for a rapid turnaround of structural information.

The crystal structure of the complex of a receptor molecule and a bound ligand is determined by either soaking crystals of the receptor in a solution containing the ligand or by co-crystallizing the receptor with the ligand. The more convenient method is that of soaking: the receptor crystals are soaked in the ligand solution for a sufficient time to allow penetration and binding of the ligand. The conformation of the ligand bound to the receptor is then determined with the use of molecular replacement and difference Fourier methods. This approach offers the advantage of limited sample usage, little or no alterations in crystal packing and rapid structure determination. However, not all receptor molecules crystallize in forms that are amenable to the soaking approach, due to either crystal packing that leaves the binding site inaccessible to the ligand or significant conformational changes that occur upon ligand binding. These limitations necessitate co-crystallization of the receptor and ligand. This alternate route is often an unfavorable one: hydrodynamic properties of each receptor–ligand complex can vary, thus demanding an exhaustive search of new crystal growth conditions, variations in crystallization conditions may lead to significantly different crystal forms and hence the need of *de novo* structure determination, and at times it may not be possible to obtain usable single crystals.

HIV-1 protease has been extensively studied as a therapeutic target for the treatment of AIDS. Several low molecular weight inhibitors of the protease have been approved by the Food and Drug Administration for the treatment of AIDS (Kohl *et al.*, 1988; Debouck, 1992; Darke & Huff, 1994; Carr & Cooper, 1996; McDonald & Kuritzkes, 1997; Vacca & Condra, 1997). Structural information about the specific interactions of these inhibitors with the protease has been invaluable for the design of these potent anti-retroviral agents and a large number of crystal structures of HIV-1 protease with a bound inhibitor have been determined to high resolution (Wlodawer & Erickson, 1993; Appelt, 1993; Ringe, 1994; Chen *et al.*, 1994; Vondrasek & Wlodawer, 1997). Native HIV-1 protease is a homodimeric molecule, and it crystallizes as a symmetric dimer with the monomers related to each other by a crystallographic dyad symmetry (Navia *et al.*, 1989; Wlodawer *et al.*, 1989; Lippatto *et al.*, 1989). The active site lies at the interface between monomers and harbors two catalytically active aspartate residues, one from each monomer. Each monomer also donates a solvent-exposed β-hairpin structure that form the so-called flaps. In the native enzyme, the flaps are in the 'open' conformation. Concurrent with binding, the flaps clamp down on the inhibitor, resulting in a 7 Å shift at the tips of the flaps as well as a change in the 'handedness' (Miller *et al.*, 1989). As a result, crystals of the native protease are unsuitable for the soaking method. To date, all the X-ray structures of HIV-1 protease complexes have been determined *via* co-crystallization.

We report here a 'replacement' method for studying HIV-1 protease–inhibitor complexes thus obviating the need for co-crystallization. Crystals of the complex of HIV-1 protease and a weak inhibitor are placed in a solution containing an inhibitor with a higher affinity for a sufficient time to allow replacement of the weak inhibitor by the stronger inhibitor. This process does not significantly alter crystal packing, offers an easy means to obtain single crystals of HIV-1 protease bound with a different inhibitor, and affords rapid structure determination using the direct refinement approach. A similar approach has previously been described for studying inhibitor-bound complex structures of trypsin (Walter & Bode, 1983). In this study, crystals of the complex of trypsin and benzamidine were soaked in the solution containing 5 m*M* *p*-amidinophenylpyruvate, a relatively better binding inhibitor of trypsin. Structure of *p*-amidinophenylpyruvate bound to trypsin was determined at 1.4 Å resolution by analysing the difference Fourier map between *p*-amidinophenylpyruvate–trypsin and free trypsin, using phases from free trypsin.

2. Methods and results

2.1. *Crystallization and structure determination*

Crystals of the complexes of HIV-1 protease and various inhibitors were obtained at room temperature *via* vapor

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diffusion using the hanging-drop method. The enzyme concentration was 6 mg ml^{-1} in a 10 mM MES pH 5.0, 1 mM DTT, 1 mM EDTA and 3 mM NaN_3. The reservoir solution contained 0.6 M NaCl and 0.1 M Na OAc, pH 5.2. All inhibitors were dissolved in 100% DMSO and mixed with the protein containing buffer in a molar ratio of 1:3–1:5, with a final DMSO concentration of 2–5%. The inhibitor–protein mixture and the reservoir solution were mixed in a 1:1(v/v) ratio to set up hanging drops of 8–10 µl. All crystals obtained had symmetry consistent with space group $P2_12_12$, with one dimer per asymmetric unit. Diffraction data were collected at room temperature on an RAXIS-II imaging-plate system using Cu $K\alpha$ radiation. The data were indexed and integrated using *DENZO* and scaled using *SCALEPACK* (Otwinowski & Minor, 1997). The structures of the complexes were determined by direct refinement using only the coordinates of the HIV-1 protease previously refined as a complex with inhibitor II (Chen *et al.*, 1994). Structures were refined with the application of *X-PLOR* (Brünger, 1993), using Engh and Huber parameters (Engh & Huber, 1991). The program *CHAIN* (Sack, 1988) was used for model building. Inhibitor potency data was determined as described previously (Heimbach *et al.*, 1989).

2.2. *Inhibitor exchange by soaking*

Crystals of the complex of HIV-1 protease and inhibitor I were transferred into the mother liquor used for crystallization. Inhibitors II, III, IV or V dissolved in 100% DMSO were individually added to the mother liquor to a final inhibitor concentration of ~1 mM. Crystals were soaked for at least 48 h, prior to data collection.

2.3. *Structure analysis*

Crystals of the complex of HIV-1 protease and I, a racemic mixture of two diastereomers with an apparent K_i value of 0.85 µM, were prepared for the present study (Fig. 1). The crystals diffracted to 2.0 Å resolution. When the structure was

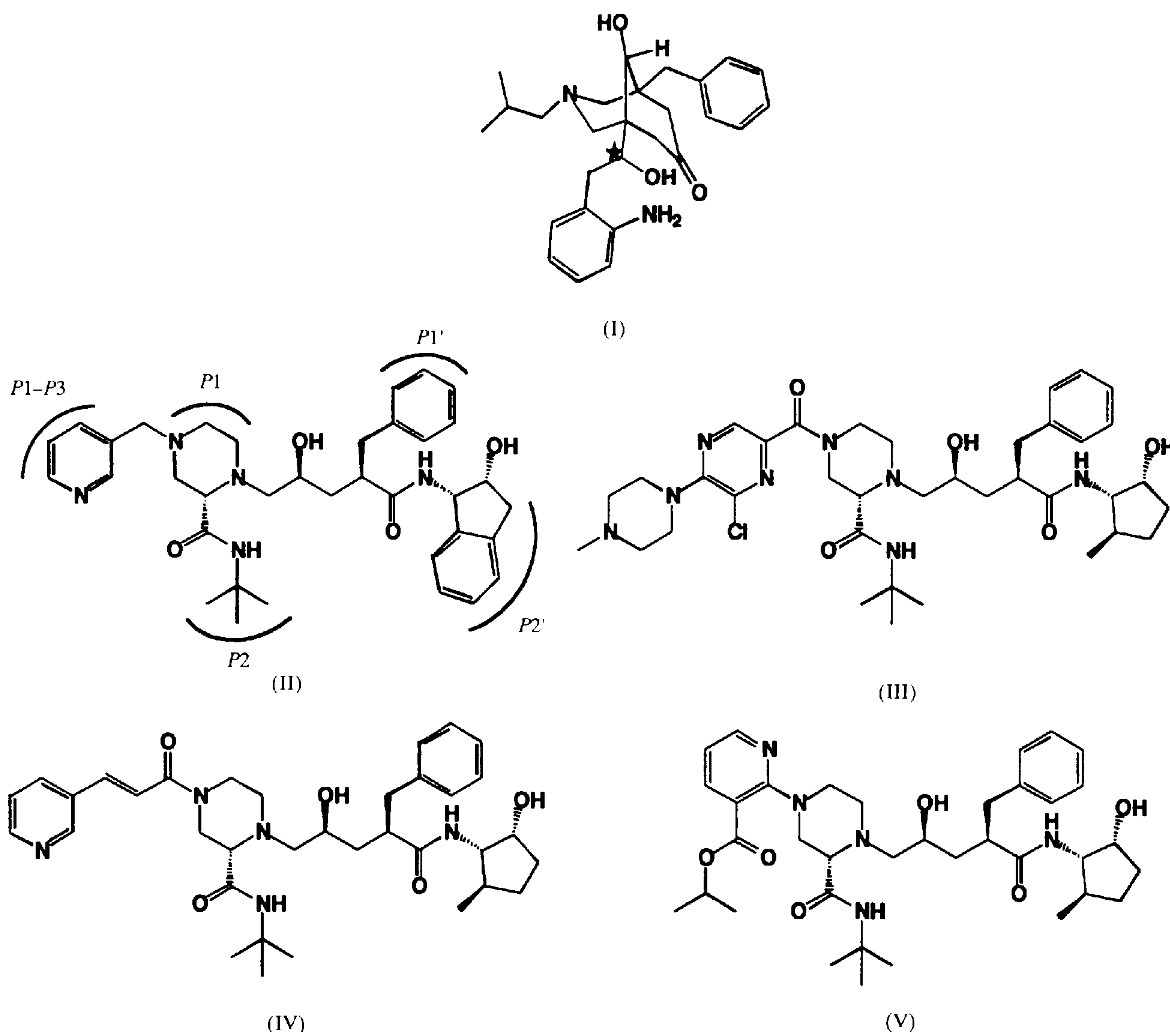

Fig. 1. Chemical structures of HIV-1 protease inhibitors employed in this studies. For I, the unresolved chiral center of the diastereomers is marked by *.

Table 1. *Cell parameters, data-reduction statistics, and structure-refinement statistics for the inhibitor complexes of the HIV-1 protease*

Cell parameters and data statistics for co-crystals and soaked crystals (in parentheses)	I	II	III	IV	V
Cell constants (Å)					
a	59.22	59.57 (58.60)	58.64 (58.47)	58.80 (58.91)	(58.9)
b	87.29	87.07 (87.37)	87.28 (87.33)	87.38 (87.44)	(87.46)
c	46.50	46.71 (46.64)	46.83 (46.66)	46.79 (46.81)	(47.0)
Resolution limits (Å)	2.0	2.0 (2.8)	1.9 (2.5)	1.9 (2.8)	(2.5)
Observations $I/\sigma(I) \geq 0.0$	48362	42709 (25963)	95383 (28610)	90150 (26268)	(35192 to 2.4 Å)
Unique reflections	13205	12434 (6385)	16008 (7334)	16262 (4953)	(6799 to 2.5 Å)
Completeness (%)	84.3	78.0 (87.7)	81.8 (83.9)	83.2 (78.5)	(77.7)
R_{merge} (%)	8.0	6.2 (7.0)	4.8 (7.8)	5.9 (11.5)	(8.9)
Mosaicity	0.3	0.29 (0.41)	0.26 (0.35)	0.2 (0.55)	(0.47)
Highest resolution bin					
Resolution limits (Å)	2.25–2.05	2.25–2.05 (2.9–2.8)	1.97–1.9 (2.59–2.5)	1.97–1.9 (2.9–2.8)	(2.59–2.5)
Completeness (%)	71.8	59.8 (70.5)	40.0 (83.0)	31.4 (77.3)	(74.6)
$I/\sigma(I)$	2.1	2.2 (2.0)	3.1 (3.95)	2.1 (2.8)	(2.3)
Observations		(1013)	1963 (1708)	1066 (1262)	(1536)
Unique reflections	669	2314 (428)	757 (697)	601 (476)	632
R_{merge}(%)		(37)	21.3 (22.4)	25.9 (27.1)	(27)

Refinement statistics	II	III	IV
Resolution limits (Å)	6.0–2.0 (8–2.8)	8.0–1.9 (8.0–2.5)	8.0–1.9 (8.0–2.8)
R factor (%)	16.6 (17.5)	19.7 (17.1)	20.7 (16.7)
R.m.s bond angle (°)	1.9 (2.0)	2.1 (2.0)	2.0 (1.8)
R.m.s. bond length (Å)	0.017 (0.001)	0.014 (0.013)	0.010 (0.007)
R.m.s. deviation between co-crystal and soaked complex structures (Å)			
198 Cα pairs	0.41	0.14	0.21
Cα of residues 44–57 of both subunits (flaps)	0.43	0.12	0.20
Inhibitors alone	0.47	0.25	0.20

determined by direct refinement, models of either diastereomer of I could be built easily into the electron-density map at the active site of the protease. Apart from the central bicyclic ring structure, the orientation and position of the three side chains were uniquely defined (Fig. 2*a*) and they are the same for either diastereomer. Within van der Waals contacts of the phenylalanine ring of I were Pro81, Val82 and Gly27, of the benzyl amine group were Ile50′, Asp29′, Ile47′ and Gly48′, and of the valine moiety were Ile50, Val32 and Ala28. Primed residues correspond to the second monomer of the dimer in the asymmetric unit.

Crystals of HIV-1 protease bound with I were soaked in a ~1 m*M* solution of II (Indinavir, K_i = 400 p*M*) (Vacca *et al.*, 1994). After 48 h the crystals developed superficial striations with slightly jagged edges. In the X-ray beam, these soaked crystals diffracted to 2.8 Å resolution as opposed to a 2.0 Å resolution observed prior to soaking. The structure of the soaked complex was determined by direct refinement. The resultant electron density for the inhibitor was well defined in the difference Fourier map ($F_o - F_c$) at 1.5σ and corresponded unambiguously to the structure of II (Fig. 2*b*) and not that of I (Fig. 2*c*).

The quality of the crystal structure of II obtained by the replacement approach was evaluated by comparing this structure to that obtained with co-crystallization. The main difference was the diffracting strength of the co-crystals to 2.0 Å resolution as reported previously (Chen *et al.*, 1994). The overall bound conformations of II were essentially the same in the soaked and co-crystal forms (Table 1). For both, the electron density of II was very well defined other than for the pyridyl group at the *P*1–*P*3 site. Density for the conserved water molecule between the flaps and the inhibitor was weakly defined. The inhibitor molecules were superimposable with an r.m.s. deviation of 0.47 Å. A maximum shift of ~0.8 Å was observed for some atoms of the *P*1–*P*3 of II. The overall conformation of the protease was also essentially the same in the co-crystal and soaked forms, with the exception of the loop containing residues 80′, 81′ and 82′ that faces the *P*1–*P*3 site of the inhibitor. The Pro81′ was shifted by ~1.5 Å in position, consistent with the shift observed for the *P*1–*P*3 subsite. The corresponding loop in the second monomer was only marginally shifted with Pro81 being shifted by ~0.5 Å. Tips of the flaps displayed some slight conformational changes. The main-chain amide N atom of Gly52 was shifted by 1.2 Å and the residue Gly52′ was shifted by 0.87 Å. Despite these subtle conformational alterations, the interactions of the inhibitor with different subsites of the protease were conserved in the two structures.

To further evaluate the general applicability of the 'replacement' method, crystals of the HIV-1 protease complex of I were soaked in solutions of three other inhibitors whose binding conformations to the HIV-1 protease were previously

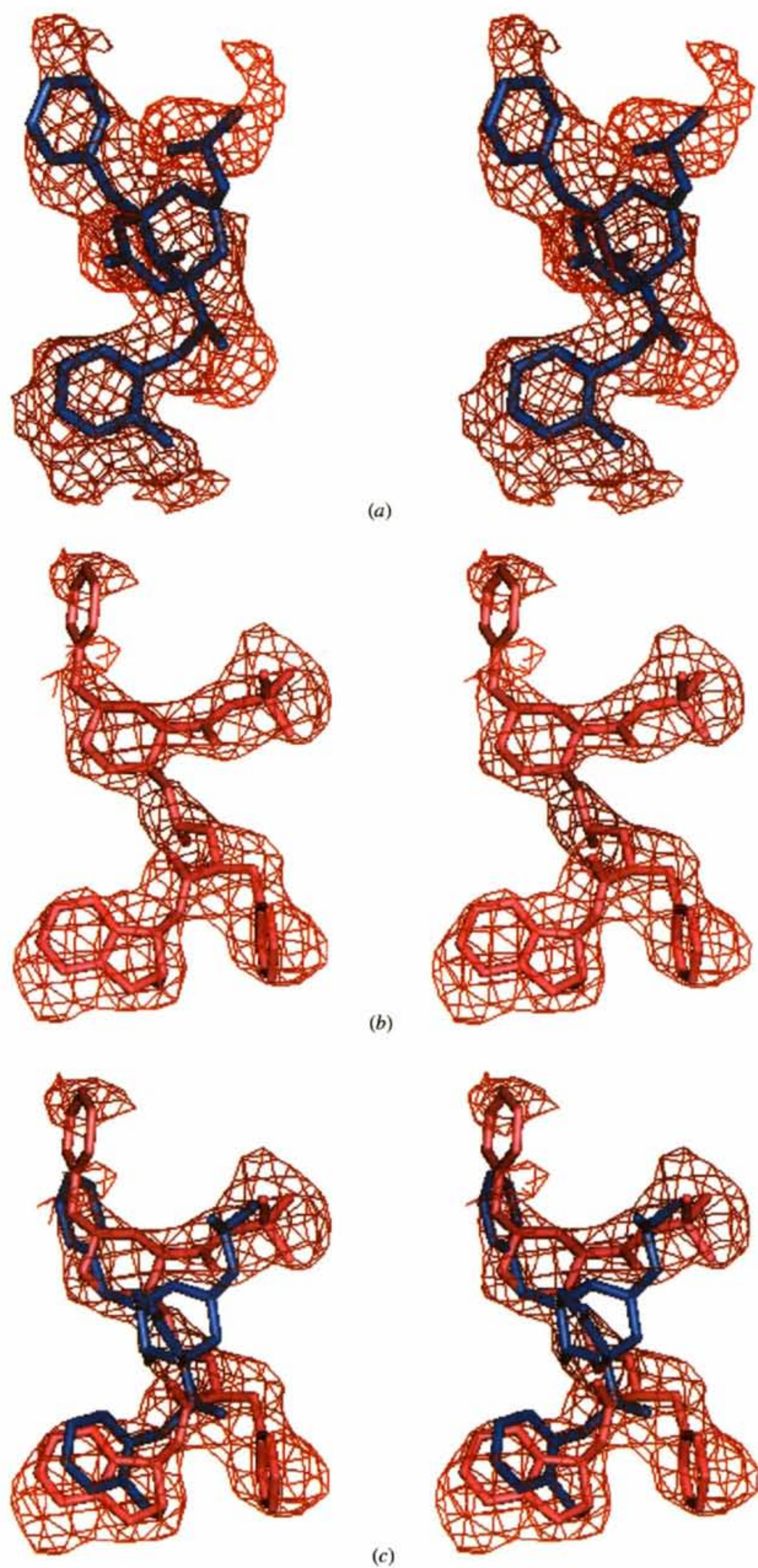

Fig. 2. (*a*) $F_o - F_c$ map contoured at 1.5σ for the complex of HIV-1 protease and I. The map was computed with data between 20.0 and 2.0 Å resolution. The structure was determined with a co-crystal of the HIV-1 protease and I. The atomic model for one of the two diastereomers is shown in the density. (*b*) $F_o - F_c$ map contoured at 1.5σ for the complex of HIV-1 protease and II. A crystal of the complex of HIV-1 protease and I was soaked in a solution containing II, in absence of the protease, for 48 h prior to data collection. The map was computed with data between 20.0 and 2.8 Å resolution. (*c*) Atomic model of I (blue) obtained from a co-crystal was superposed with the atomic model of II (pink) obtained from a soaked crystal. Electron density in the $F_o - F_c$ map, contoured at 1.5σ, corresponds to the soaked complex structure of II.

unknown. Diffraction data were collected for three compounds, III–V, with K_i values of 39.8, 21.2 and 36.5 nM, respectively. After soaking the crystals for 48 h, the structures of the complexes were determined. For all structures, the protease molecule needed almost no adjustment and the inhibitors could be modeled easily into the difference ($F_o - F_c$) electron-density maps at 1.5σ level. For the inhibitors in the complexes, the electron density of III (Fig. 3*a*) was more ordered than that for IV (Fig. 3*b*), whereas V was very poorly defined at the *P*1–*P*3 site (data not shown). None could be interpreted with the structure of I. Overall, the structures of III and IV were unambiguous and the quality of the maps as well as the refinement did not pose any concern in their use to interpret protein–inhibitor interactions. The structure of V was lacking in one part of the inhibitor and could not be used without ambiguity. Thus, the replacement method was not universally applicable.

To validate the bound structures of III and IV, these compounds were also co-crystallized separately with HIV-1 protease. The resultant structures at 1.9 Å resolution were very similar to those obtained with the soaked crystals. For both, the *P*1–*P*3 groups were better defined in the electron-density maps of the co-crystal structures, although not completely ordered. The *P'* groups of both inhibitors were as cleanly defined in the co-crystal as they were in the soaked forms. The comparisons of the bound structures of II–IV determined with the soaked and co-crystals are shown in Fig. 4 and Table 1.

Attempts were made to completely diffuse out inhibitor I from the crystals of its complex with HIV-1 protease. This was performed by first washing and then soaking the complex

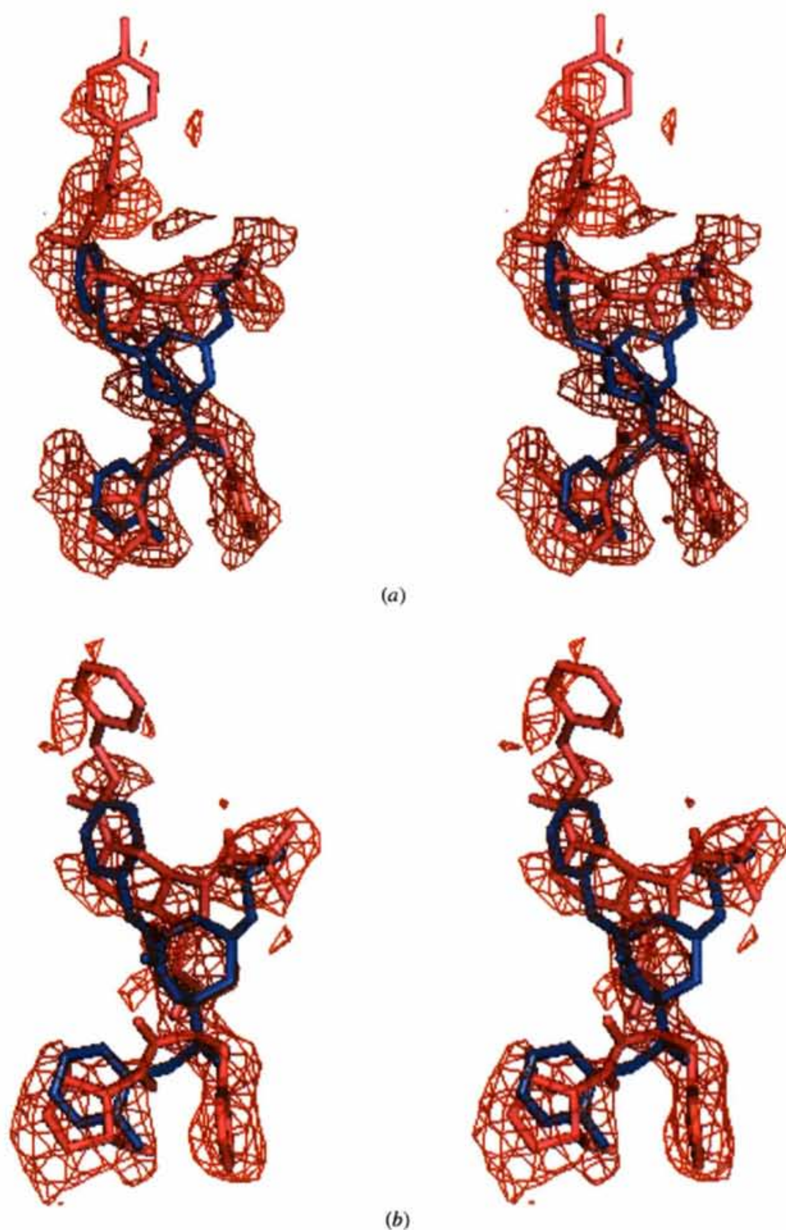

Fig. 3. (*a*) $F_o - F_c$ map contoured at 1.5σ for the complex of HIV-1 protease and III. A soaked crystal of the complex of the HIV-1 protease and I was soaked for 48 h in a solution containing III, in absence of the protease, prior to data collection. Map was computed for data between 20.0 and 2.5 Å resolution. The atomic model of III (pink) built in to the density is superimposed with the atomic model of I (blue). (*b*) $F_o - F_c$ map contoured at 1.5σ for the complex of HIV-1 protease and IV. The structure was determined with a soaked crystal of the complex of HIV-1 protease and I in a solution containing IV. Map was computed with data between 22.0 and 2.8 Å resolution. The atomic model of IV (pink) built in to the density is superimposed with the atomic model of I (blue).

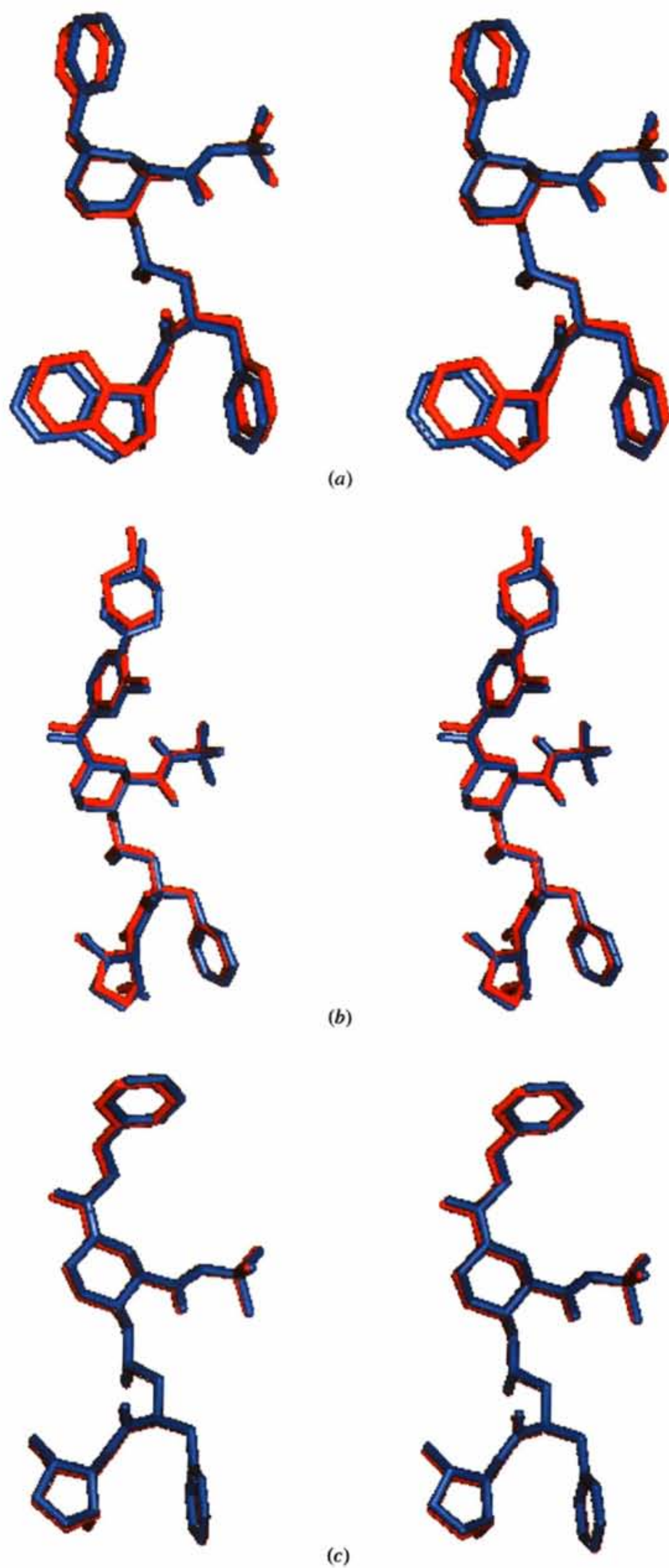

Fig. 4. Superposition of the bound structures of II (*a*), III (*b*) and IV (*c*) in the active site of the HIV-1 protease as determined with soaked crystals (red) and co-crystal (blue). Best fits between the molecules was achieved by least-squares superposition of 198 Cα pairs of the protease molecules.

crystals in crystallization buffer (without inhibitor) for one week. X-ray diffraction data were collected from the washed crystals, and the structure determined. A well defined electron density of inhibitor I was found in the difference Fourier ($F_o - F_c$) map at the 2.0σ level. Similar results were obtained with the crystals of complexes of HIV-1 protease with III and IV. In separate experiments, the possibility to replace a tight-binding inhibitor with a weak inhibitor in the crystals of an HIV-1 protease complex was attempted. Crystals of the complexes of HIV-1 protease with III as well as with IV were soaked separately in their respective mother liquor solutions containing 2–5 mM of I for a period up to a week prior to data collection. The difference Fourier ($F_o - F_c$) maps obtained from these soaked crystals contained density that only accommodated the structure of the original inhibitor and not I. Together these observations suggested that inhibitor exchange in the orthorhombic crystal form used in the present study apparently was a one-way process, allowing only weakly bound inhibitors to be replaced by more potent inhibitors. The competition between two inhibitors for binding was dictated by their affinity towards the protease. Additionally, it is not possible to create an empty active-site pocket with flaps in the closed conformation. On the other hand, it was unclear as to the extent to which the flaps of the protease inter-converted between the 'closed' and 'open' conformations during inhibitor exchange. This inter-conversion has been judged to be necessary for inhibitor binding in solution. Movements of the flaps, if it occurred at all during soaking of crystals of the bound protease, might be a reason for the decrease in resolution observed for the soaked crystals. This is partly reflected in the increased mosaic spread observed for all the soaked crystals, compared to those of co-crystals (Table 1). The racemic nature of I contributed to the low apparent affinity of this inhibitor preparation, and both diastereomers could bind the HIV-1 protease as judged with the resolved electron density at 2.0 Å resolution. These phenomena might have presented a heterogeneity (a high ground-state energetic) in the crystal of the bound protease to afford exchange of inhibitors.

3. Concluding remarks

In conclusion, it is possible to study the bound conformation of newly developed inhibitors of HIV-1 protease by replacing a weakly bound inhibitor in the crystals with a more potent compound. Despite the successful exchange of a limited number of inhibitors, there are several important caveats in our approach. Following the exchange, the crystals of the HIV-1 protease–inhibitor complex diffract to a lower resolution. In comparison, the structure of the complex of trypsin and p-amidinophenylpyruvate was determined at 1.4 Å resolution. In the present study, the extent of the decrease in resolution and the quality of the resultant electron-density vary from crystal to crystal and depends upon the inhibitor being exchanged in. For example, the electron density for the water molecule between the bound inhibitor and the tip of the flaps, an essential structural feature recognized in the structures of the HIV protease–inhibitor complexes is weak for the soaked complex of II as compared to that found for the co-crystal. In contrast, the density of this water molecule is well defined for the soaked complexes of III and IV. Also, when compared to the co-crystal form, residues 45–55 of the flaps show greater conformational mobility in the soaked form of II but not of III and IV. For the inhibitors with well defined electron densities, the conclusions regarding details of the bound conformation of the inhibitors and the protease are identical between the soaked crystals and co-crystals within an r.m.s. error of ~0.12–0.47 Å (Table 1). The results presented, therefore, provide the proof of the concept of exchanging inhibitors at the active site of the HIV-1 protease in the crystalline state. This approach, with the aforementioned limitations, should be useful for obtaining rapid crystallographic information for the development of novel tight binding inhibitors of HIV protease.

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† Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (References: 1HSG, 2BPY, 2BPV, 2BPX, 2BPZ, 2BPW for II, III and IV co-crystal and soaked structures, respectively). At the request of the authors, the atomic coordinates and structure factors will remain privileged until 22 January 1999.

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