Interaction Characteristics of Viral Protease Targets and Inhibitors

*Perspectives for Drug Discovery and Development of Model Systems*

BY

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### Abbreviations

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>RU</td>
<td>Resonance units</td>
</tr>
<tr>
<td>$K_M$</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>$k_{cat}$</td>
<td>Turnover number, rate of catalysis</td>
</tr>
<tr>
<td>$K_i$</td>
<td>Inhibition rate constant</td>
</tr>
<tr>
<td>$K_D$</td>
<td>Affinity rate constant</td>
</tr>
<tr>
<td>$k_{on}$</td>
<td>Association rate constant</td>
</tr>
<tr>
<td>$k_{off}$</td>
<td>Dissociation rate constant</td>
</tr>
<tr>
<td>$k_t$</td>
<td>Mass transport rate constant</td>
</tr>
<tr>
<td>$k_B$</td>
<td>Boltzmann constant, $1.381 \times 10^{-23} \text{ J} \cdot \text{K}^{-1}$</td>
</tr>
<tr>
<td>$h$</td>
<td>Planck’s constant, $6.626 \times 10^{-34} \text{ J} \cdot \text{s}$</td>
</tr>
<tr>
<td>$R$</td>
<td>Gas constant, $8.3145 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$</td>
</tr>
<tr>
<td>$T$</td>
<td>Temperature in °K</td>
</tr>
<tr>
<td>$\Delta G$</td>
<td>Free energy change</td>
</tr>
<tr>
<td>$\Delta H$</td>
<td>Enthalpy change</td>
</tr>
<tr>
<td>$\Delta S$</td>
<td>Entropy change</td>
</tr>
</tbody>
</table>
INTRODUCTION

The quest for knowledge and a desire to comprehend “how things work” has been a driving force behind scientific development. Within the field of biochemistry and other life sciences, an inevitable pursuit has been to increase our understanding of molecular interactions. It is well established that interactions among molecules are essential for many biological processes, although the details of many specific cases are still poorly understood. Exploring and characterizing these events is therefore important for increasing the general scientific knowledge of molecular interactions and for application of such knowledge to specific biochemical and medical issues. The work presented in this thesis contributes to the pursuit of a greater understanding of molecular interactions, particularly those between viral proteases and inhibitors.

Protein-ligand interactions

Model systems
Biological systems are complex and therefore determination of specific interaction characteristics usually requires a simplified, controlled environment. A well-defined and standardized environment, intended to represent some part of the more complex biological system, can be referred to as a model system. The model system will preferably be suitable for addressing the scientific questions of interest, and close enough to the ‘natural’ environment to provide correct information. In addition, the methods and conditions chosen for a model system should be compatible with the components being studied.

Targets and ligands
The molecular interactions addressed in the present investigation involve the binding of a low molecular weight compound (the ligand) to a protein molecule (the target). Characterization of the interaction between a ligand
and a target becomes especially pertinent when the target is of physiological or medical importance. The target may be, among other things, an essential step in a metabolic pathway or an important component in processing and infectivity of a microorganism. For example, the proteases of human immunodeficiency virus (HIV), cytomegalovirus (CMV) and hepatitis C virus (HCV) are responsible for the processing of viral proteins. A ligand capable of inhibiting such a protease, and thus obstructing the production of infective virus, will be quite interesting. A ligand may bind to additional targets, such as related proteases or plasma proteins present in the host. If the ligand interacts with host proteins, it may result in side effects that would prohibit its use as a drug candidate for inhibition of the viral protease. Nevertheless, such ligands may still provide clues for designing a ligand that can specifically inhibit the viral protease target. In addition, the nature of the protein-ligand interactions may also be elucidated by analyzing closely related targets or ligands.

Non-covalent interactions

Biomolecular interactions, such as those between viral proteases and reversible inhibitors, are generally based on non-covalent forces [11, 18]. The weak nature of these forces, as compared to covalent bonds, allows for reversibility of interactions, whereas the additive influence of non-covalent forces can lead to considerable strengths of binding. The strongest of these forces, and most long-range, occurs when two oppositely charged ions interact to form salt bridges (Table 1). Additional electrostatic interactions include those between charged ions and dipoles and between dipoles. Hydrogen bonds, dispersion forces and hydrophobic interactions also have electrostatic qualities but, by convention, are not referred to as electrostatic interactions. Hydrogen bonds form when a proton is shared by two electronegative atoms; in proteins these are most often nitrogen or oxygen. At close range, atoms are attracted through dispersion forces (van der Waals interactions) due to the weak dipoles formed in atoms with no net charge. However, atoms that are close enough to have overlapping electron clouds experience strong repulsive forces. Finally, hydrophobic interactions have a strong influence on ligand binding. This entropy-driven effect is not due to attraction of the ligand to the target, but rather to the ligand being pushed out of solution (and into the binding site of the protein). The hydrophobic effect is correlated to the polarity of the solvent and is stronger in more polar environments. The strength of electrostatic interactions, such as salt bridges, also depends on the polarity (or the dielectric constant) of the environment, and is therefore greater in the hydrophobic core of a protein than in a hydrophilic solvent.
Table 1 Non-covalent bonds of importance for protein-ligand interactions

<table>
<thead>
<tr>
<th>Type</th>
<th>Relation of strength to distance (r) &amp;1</th>
<th>Typical energy (kJ/mol) &amp;1,2</th>
<th>Notable characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion-ion</td>
<td>( \frac{1}{r} )</td>
<td>60</td>
<td>- The strength of the electrostatic interactions depends upon the dielectric constant, (D).</td>
</tr>
<tr>
<td>Ion-dipole</td>
<td>( \frac{1}{r^2} )</td>
<td>-8 to 8</td>
<td>- These forces are greater in the center proteins ((D \sim 2 \text{ to } 4)) than in hydrophilic solvent ((D \sim 80 \text{ in water})).</td>
</tr>
<tr>
<td>Dipole-dipole</td>
<td>( \frac{1}{r^3} )</td>
<td>-2 to 2</td>
<td></td>
</tr>
<tr>
<td>Ion-induced dipole</td>
<td>( \frac{1}{r^4} )</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Hydrogen bond</td>
<td>( r \sim 0.3 \text{ nm} ) &amp;3</td>
<td>12-38</td>
<td>- Influenced by polarizability, electron charge, mass and number of outer sphere electrons of the interacting atoms</td>
</tr>
<tr>
<td>van der Waals (dispersion)</td>
<td>( \frac{1}{r^6} )</td>
<td>0.1-1.0</td>
<td></td>
</tr>
<tr>
<td>Hydrophobic &quot;effect&quot;</td>
<td></td>
<td></td>
<td>- Tendency of hydrophobic molecules to aggregate, or associate, in a polar environment</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Strength of hydrophobic interactions is determined by the hydrophobicity of the solvent</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Increased entropy of the solvent due to binding of a hydrophobic ligand is an important factor for protein-ligand interactions</td>
</tr>
</tbody>
</table>

&1 Reference [83], tables 1.1, 1.3 & 2.1; &2 Reference [18], chapter 11; &3 for hydrogen bonds between oxygens \((O \sim O)\), nitrogens \((N \sim N)\) or \((O \sim N)\)

The strength and nature of the non-covalent forces contributing to protein-inhibitor interactions are influenced by alterations of the ligand (inhibitor) or target (protease), especially if accompanied by changes at the protein-ligand interface. For example, if one of the ions involved in a salt bridge reverses charge, the attractive force between two opposite charges will become a repulsive one between two similar charges. If the charged ions or dipoles involved in electrostatic interactions move away from each other, these interactions become weaker. The same is true of hydrogen bonds and van der Waals interactions. If the interacting groups in ligand and target move closer (or are replaced by larger groups) this may result in stronger interactions, but may also result in a repulsive force if the groups come too close to each other. Lastly, changing the polarity of a ligand will alter the influence of the hydrophobic effect on binding.
Characterizing interactions

A number of methods are available for the characterization of molecular interactions. The specific molecules being studied, the inquiries being made, and the availability of instrumentation will dictate which methods are used. Structural, thermodynamic and kinetic information is generally helpful for understanding protein-ligand interactions. Structural information can be obtained using nuclear magnetic resonance (NMR) or X-ray crystallography. Analysis of interaction energetics can be performed directly by measuring the enthalpy of binding using isothermal calorimetry (ITC) or indirectly by determining the equilibrium dissociation constant ($K_D$) at different temperatures and performing a van’t Hoff analysis.

The equilibrium dissociation constant for protein-ligand interactions may be determined using a number of different methods, some of which are mentioned here. For reversible, competitive inhibitors, kinetic analysis may be performed in the presence of inhibitor and the equilibrium dissociation constant of inhibition ($K_i$) may be determined. The limitations of this type of analysis are similar to the limitations inherent in the kinetic assay. Determination of kinetic activity requires a measurable change, usually in ultraviolet (UV) absorption or fluorescence, as a reaction proceeds and may therefore require labeling of the assay components. In some cases, stopped flow spectroscopy may be employed in the determination of the kinetic rate constants. Generally, the applicability of spectroscopic methods is dependent upon the specific assay and components being used.

Direct determination of the $K_D$ in solution may be performed using, for example, analytical ultracentrifugation, equilibrium dialysis or equilibrium gel filtration. Drawbacks include the requirement of stable components for equilibrium dialysis, given the time needed to reach equilibrium, and relatively large volumes for equilibrium gel filtration. Microcalorimetry may be used to determine $K_D$ by measuring the heat of binding (enthalpy) using ITC, or by calculating the effect of ligand binding on the thermal denaturation of a protein using differential scanning calorimetry. Recent advances in ITC instrumentation allow determination of subnanomolar $K_D$ values, provided the minimum concentration of protein is similar to or lower than the dissociation constant.

Surface plasmon resonance biosensor-based technology (SPR) can be used to determine the equilibrium dissociation constant and the kinetic rate constants for association and dissociation. A drawback of this method is that attachment of one of the interacting molecules to a surface is required. Benefits include the ability to characterize interactions without the need to label components, the possibility to analyze many interactions under identical conditions (screening) and the relative ease of changing assay conditions.
The HIV-1 protease

HIV-1 protease was the primary target used in the current studies (Figure 1). This enzyme is well established as an aspartic protease based on structural similarity to other aspartic proteases [87], a conserved Asp-Thr-Gly catalytic triad [91] and inhibition by known aspartic protease inhibitors [69]. HIV protease is inactivated by substitution of the catalytic aspartic acid residues (Asp 25 and 25’) [14, 41] and has a mechanism similar to that of other aspartic proteases [71]. In brief, the catalytic aspartates achieve hydrolysis of the peptide bond by catalyzing a nucleophilic attack by a water molecule on the carbonyl of the scissile amide bond (Figure 2). The natural substrates are diverse and not easily classified, but generally have hydrophobic residues on either side of the scissile bond or an aromatic residue/proline combination [81]. Hydrolysis of peptide substrates requires a length of 7-8 amino acids and the substrate specificity of HIV-1 protease is not easily defined (recently reviewed [6]). Most importantly, cleavage of structural proteins and enzymes from the viral Gag and Gag/Pol precursor polyproteins by HIV protease is

Figure 1 The HIV-1 protease with inhibitor bound
The catalytic aspartate residues, Asp 25 and Asp 25’, are shown; (a) the flexible flap regions; (b), the binding pocket of the active site; and (c), the dimer interface
essential for maturation and infectivity of HIV [41], making the protease an important target for anti-AIDS drugs.

Figure 2 Schematic presentation of substrate binding in the active site of an aspartic protease (a) and the transition-state formed upon nucleophilic attack (b)
Structural characteristics

The protease of HIV is a dimer consisting of two identical monomers, associated through non-covalent interactions (Figure 1). Several general structural characteristics of the HIV protease are important to consider when studying ligand binding to the enzyme. Dimer association is crucial for activity and the dimer is stabilized by ligand binding [42, 89]. The substrate-binding site (Figure 1b) is formed at the hydrophobic interface between the two monomers, and is enclosed by the flap regions of the protease (Figure 1a) upon substrate or ligand binding. The flap regions of the enzyme are flexible, and have been reported to move at least 7 Å, adding an extra “dynamic” aspect to the study of ligand binding [73, 82]. In fact, most of the protease, including some residues in the active site, has been shown to have inherent flexibility [94], contributing to the enzyme’s ability to accommodate diverse substrates. Most inhibitors of HIV-1 protease are competitive and bind at the active site (for review see [81, 88]). Alternative approaches that exist, such as disruption of the dimer interface [93] (Figure 1c) and allosteric inhibition [13], have not been pursued as anti-viral drug leads. Finally, the HIV-1 protease exhibits impressive tolerance towards changes in the primary structure, with variation reported at over 50 % of the residues [75]. About 20 % of the residues have been reported to vary under selection pressure of inhibitors [7, 68], contributing to the seriously growing problem of resistance to currently available inhibitors.

HIV-1 protease inhibitors

The specific characteristics of the active site of HIV protease and the native substrates can be utilized in the design of inhibitors, and structure-based drug design has proven to be successful for the development of anti-HIV drugs (for review see [88]). Interacting groups of the inhibitor correspond to side chains of the substrate and are designated P4, P3, P2, P1, P1’, P2’, P3 and P4’, according to the nomenclature of Schecter and Berger [72], where P4 to P1 are on the amino-terminal side of the scissile bond and P1’ to P4’ on the carboxy-terminal side. These correspond to S4 to S4’, the subsites of the binding site of the protease (Figure 2a). The design of inhibitor molecules is generally based on the peptide substrates, resulting in peptidomimetic inhibitors. However, the final optimized inhibitor may bear little resemblance to the original peptide prototype. The central region of the inhibitor, P1 to P1’, corresponds to the cleavage site of the substrate. Although it mimics the transition state (Figure 2b), it is not hydrolyzed by the protease. A number of chemical moieties are utilized as transition state analogues, some of which are shown in Figure 3. All of the clinical HIV-1 protease inhibitors currently in use are peptidomimetic and contain a
hydroxyethylamine moiety (Figure 4). Information about the 3-D structure of HIV-1 protease, together with computer-aided modeling techniques, contributed considerably to the development of some inhibitors, such as nelfinavir [32] and amprenavir [39]. Another approach to inhibitor design involves the incorporation of a structural water molecule into inhibitors, which has lead to the development of cyclic compounds as HIV-1 inhibitors [24]. The replaced water is involved in forming important hydrogen bonds between the amide groups of Ile 50 and Ile 50’ in the protease flaps and the substrate [43].

![Common amide bond isosteres used in design of protease inhibitors](image)

**Figure 3** Common amide bond isosteres used in design of protease inhibitors
Figure 4 Clinically relevant HIV-1 protease inhibitors
At present, there are six HIV-1 protease inhibitors approved by the FDA, amprenavir [39, 77], indinavir [15], nelfinavir [32], ritonavir [38], saquinavir [70] and lopinavir [76] (used in combination with ritonavir), and others that are in clinical trials, such as atazanavir [21]. Since the approval of saquinavir in 1995, inhibitors of HIV protease have contributed to the tremendous success of anti-AIDS highly active antiviral therapy (HAART) [81]. However, drug resistance has emerged as a major cause for failure of these therapies and the need for a new generation of protease inhibitors is great.

Development of resistance

HIV is highly mutable due to lack of proofreading of the reverse transcriptase, an enzyme unique to the retroviruses, and tolerates considerable variation of the primary amino acid sequence without loss of function. The development of resistance towards anti-AIDS drugs is complex, however, and depends on more than just the occurrence of mutations in the gene encoding the HIV-protease [17]. Additional aspects include the physiological responses of the host to drug treatment, leading to reduction of the plasma level of the drugs (due to, for example, increased drug metabolism or protein binding) [58]. Reduced plasma drug levels, although not actually constituting viral resistance, may allow for low-level replication of virus and therefore increase the chance that a resistant variant of HIV will emerge. Mutations constantly occur in the HIV genome, including the gene encoding the protease of HIV-1, which results in a pool of viruses with disparate genetic sequences. The fittest variant of the virus will dominate, and consequently, drug resistant variants will become more predominant during treatment due to the selective pressure of anti-viral drugs. Substitutions of amino acid residues in the protease as a response to drug treatment, first reported for indinavir by Condra et al. [9], have been reported for all of the clinically relevant protease inhibitors (Figure 4) [reviewed in 58, 7].

To understand the influence of variation of the enzyme on the interaction between protease and inhibitor, it is helpful to consider both the location of the substituted residue in the protease and the proposed contribution of the residue to the binding process. Least ambiguous is the occurrence of substitutions in the binding pocket of the active site, as these residues have direct contact with substrates and inhibitors. Resistance due to substitutions in the active site has been attributed to decreased affinity of protease-inhibitor interactions. Of the many residues forming the binding pocket, some are conserved, such as residues in and close to the catalytic triad (Asp 25-Thr 26-Gly 27), whereas others show considerable variation, such as Val 82 and Ile 84. The residues Val 82 and Ile 84 both contribute to the S1/S1’
subsites of the protease, as well as S3/S3’ and S2/S2’, respectively [86], and variation of these residues has been reported for many protease inhibitors (Table 2) [10, 47, 55, 59, 75]. Reduction of the size of residue 84, from Ile to Val, may reduce van der Waals interactions and decrease the strength of the protease-inhibitor interactions [7]. A similar decrease in the size of residue 82, from Val to Ala, may have the same effect, whereas increasing the size of this residue could lead to steric hindrance. Structural analysis of variants of the HIV protease, with substitutions at residue 82 and inhibitor bound, showed unexpected adjustments in the backbone of the protease in response to the altered residues [4, 25]. Alteration of residue 30 from aspartic acid to asparagine (D30N), located in the S2 subsite of the protease, has been reported during treatment with nelfinavir [66]. This change removes an electrostatic charge from the residue and therefore can contribute to weakening of electrostatic interactions between the protease and the P2 phenylhydroxyl group of nelfinavir [32, 48, 66].

Table 2 Substitutions reported with resistance to HIV-1 protease inhibitors

<table>
<thead>
<tr>
<th>Protease inhibitor</th>
<th>Primary mutations</th>
<th>Secondary mutations</th>
</tr>
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<tbody>
<tr>
<td>Saquinavirb</td>
<td>G48V, L90M</td>
<td>I84V</td>
</tr>
<tr>
<td>Ritonavirc</td>
<td>V82A/F/T/(S), I84V</td>
<td>L90M</td>
</tr>
<tr>
<td>Indinavird</td>
<td>V82A/F/T, M46L/I</td>
<td>I84V, L90M</td>
</tr>
<tr>
<td>Nelfinavire</td>
<td>D30N, N88D/S, I84V, L90M</td>
<td>I84V, L90M</td>
</tr>
<tr>
<td>Amprenavirf</td>
<td>150V, M46L</td>
<td>I84V</td>
</tr>
<tr>
<td>Lopinavir-ritonavirg</td>
<td>K20M/R, F53L</td>
<td>V82A/F/T, I84V, L90M</td>
</tr>
<tr>
<td>Atazanavirh</td>
<td>150L, M46I</td>
<td></td>
</tr>
</tbody>
</table>

*Many additional mutations occur and only those included in the present study are included here.*

Variation of residues located in the flexible flaps forming the top of the active site of the protease (given the orientation in Figure 1) also emerges in association with resistance to protease inhibitors. Gly 48 contributes to the formation of the S2/S2’ and S3/S3’ binding site, and can interact with P1/P1’ groups in inhibitors [86]. Substitution to G48V, associated almost exclusively with resistance to saquinavir (Table 2) [29], increases this residue’s spatial occupancy of the P3/P3’ binding site and leads to steric
hindrance with the P3 group of saquinavir [54]. Consequently, there is a shift in the inhibitor position in the active site and a reduction of van der Waals interactions [26]. Altered van der Waals interactions are also proposed to occur upon substitution of Ile 50 to Val, a variant of the protease that emerges during treatment with amprenavir [54]. In addition, substitutions have also been reported at residues 46, 47, 53 and 54 of the flaps, indicating that this region is tolerant to variation provided that the flexible nature of the flaps is preserved [73].

Substitutions at more distal residues affect the protease through modification of dimer stability, catalytic efficiency, active site geometry and inhibitor-binding properties [17]. Substitution of residue Leu 90, located at the dimer interface, by methionine has been implicated in resistance or cross-resistance to many of the clinical inhibitors [23, 29, 33, 37, 68]. Methionine at residue 90 may influence the conformation of the active site and lead to destabilization of the dimer [26, 48, 49, 89]. Residue Asn 88 has been found to mutate to N88D in association with a D30N substitution during nelfinavir treatment [66], and is proposed to influence inhibitor binding through altered stabilization of residue 30 in the active site [48]. Additional variations at polymorphic sites [75], such as residue 10, 63 and 71, are thought to compensate for loss of catalytic activity due to mutations in the active site [33, 50, 56]. The accrual of amino acid substitutions in the protease progressively decreases the inhibitory capabilities of the inhibitors, which cumulates in viral resistance to a protease inhibitor.

The discovery of effective anti-AIDS drugs is complicated by the development of resistance. There is a need for new inhibitors, as well as better descriptors of the interactions, to be able to keep up with this quickly changing virus. In addition, the HIV-1 protease has been extensively studied and is an excellent protein for the development of model systems and methods that can be applied to other related problems.
PRESENT INVESTIGATION

AIMS
The aim of the current project has been to characterize viral protease-inhibitor interactions, with focus on applications to drug discovery. This included:

1. Obtaining relevant targets for characterization of protein-ligand interactions
2. Exploring resistance development by studying the effects of varying the target on inhibition
3. Resolving kinetic and thermodynamic contributions to inhibitor binding to gain insight into protein-ligand interactions, resistance development and inhibitor discovery
4. Identifying protein-ligand interaction characteristics that improve the description of anti-viral drug efficacy

According to estimates from the world health organization, 42 million people are currently infected with HIV worldwide, and about 170 million are infected with HCV [1, 2]. The development and therapeutic use of viral protease inhibitors has contributed to improved treatment of patients infected with HIV, and has increased our knowledge of the targets and ligands involved [67]. However, the need for improved inhibitors is great. For these reasons, viral protease targets were chosen for the present investigation. Although the HCV proteases (NS2-3 and NS3/4a) were cloned and established as targets (paper I and unpublished data), the HIV-1 protease has been the focus of the current study.

An enzymatic assay was initially used for the characterization of resistant variants of HIV-1 protease and inhibitors (paper II). Due to the complexity of the interactions and to limitations of the inhibition assay, important questions remained unanswered. For example, 1) how do changes in inhibitor or target affect inhibition and binding, and are there structural features that correlate with increased or decreased inhibition? And 2) what
are the important in vitro characteristics of an inhibitor that correspond to an effective inhibitor in vivo? Interaction studies had previously laid the foundation for a new model system for the study of HIV-1 protease-inhibitor interactions [51, 52], which was successfully used to kinetically characterize a set of inhibitors [53]. The present investigations built upon this foundation and further characterized the interactions between HIV-1 protease and clinical inhibitors. The goals were to increase our general understanding of the protease-inhibitor interactions and to investigate applications to improving model systems for drug discovery. Analysis of interactions between variants of HIV-1 protease and clinical inhibitors were performed to address the issue of resistance (paper III). In addition, the thermodynamic characteristics of the interactions were determined to investigate whether the energetics of the interactions would yield valuable information (papers IV). Results obtained from the current drug discovery project, including previous work by co-workers, were used to investigate the correlation between the protease-inhibitor interaction characteristics and inhibition of viral replication in cell culture (paper V).

Experimental methodology

Obtaining the target of interest

Direct purification of viral proteins from virus is often an unrealistic endeavor due to the small amounts of the target protein and to the desire to minimize the handling of live, infectious virus. Therefore, the techniques of molecular biology were invaluable for obtaining target protein for this study. By extracting and cloning the genetic information encoding for the protein of interest, these techniques were employed to produce sufficient quantities of target protein to work with. In addition, modifications incorporating specific changes in the final protein product were introduced using site-directed mutagenesis. The application and relevance of the techniques used in this study are briefly introduced here. General descriptions of the techniques are available in a number of laboratory manuals [3] and general textbooks, and more specific details have been published elsewhere [52, 63] (papers I and II).

The information flow between DNA, RNA and proteins, known as the central dogma, is essential for life as we know it. Genetic information, stored in the form of DNA, can be replicated as new copies of DNA or transcribed into RNA. Through RNA, the genetic information can be translated into proteins, which perform many vital functions in living organisms. An
important exception to the central dogma occurs in retroviruses, such as HIV, where the genetic information is stored as RNA and incorporated into host DNA by an enzyme, reverse transcriptase. Knowledge of the processes involved in the cellular information flow has been utilized for the production of DNA and proteins in the laboratory. If genetic information encoding for the target protein of interest is present as DNA, it may be directly amplified using PCR, the polymerase chain reaction. However, if the information is present as RNA in the virus, as is the case with the HCV virus, the RNA must first be extracted and translated into DNA. The reverse-transcription of RNA to cDNA can be accomplished using a reverse transcriptase, and the cDNA obtained may be used as a template in a PCR reaction. In this way, genetic information encoding the HCV proteases was obtained.

Once the desired DNA has been amplified using PCR, it is ligated into circular pieces of DNA, called vectors, and inserted into host cells, i.e. bacteria, for cloning or expression purposes. Mutations were introduced into the genes encoding the HIV-1 and the HCV NS2-3 genes using a site-directed mutagenesis technique based upon PCR technology [63] (paper II and unpublished data). The wild-type HIV-1 protease from strain HXB and mutants Q7K, G48V, V82A, I84V, L90M, G48V/L90M, I84V/L90M and G48V/V82A/I84V/L90M were cloned, expressed and purified. For expression of the HIV-1 protease, a pET-vector system (Novagen) containing an IPTG-inducible T7 promoter for use in E. coli bacteria, strain BL 21, was utilized. Once expressed, the protein of interest was present in a mix of bacterial proteins, and had to be separated and purified from this mix. Expression and purification of the full length NS3 protein of HCV is thoroughly described in paper I.

The purification procedure used for the HIV-1 protease exploits the net charge of the protein in two chromatographic steps [13, 63]. When expressed, the protein formed insoluble aggregates, inclusion bodies, which were harvested and washed in a non-denaturing buffer. The protein was then solubilized in a denaturing buffer and refolded to the active form by dialysis. Production of protein in inclusion bodies facilitated the removal of bacterial proteins early in the purification and helped protect the protease from autocatalysis, as it is inactive until after refolding. A disadvantage was that the mutant variants did not necessarily refold as easily as the wild type. The purity and identity of the protein was analyzed by SDS-PAGE, with detection by silver staining and Western blotting. The concentration of
purified HIV-1 protease was estimated by UV absorption at 280 nm and the enzyme was stored at –80 °C until just before use.

Enzymatic activity and inhibition

With the aim of characterizing the interactions between protease and inhibitors, the kinetic properties of HIV-1 protease were investigated and inhibition studies were performed with a series of compounds (paper II). The fluorescence assay for determining the activity of the protease was based on cleavage of an internally quenched peptide DABCYL-γ-Abu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS (Bachem, Bubendorf, Switzerland), designed to mimic the p17/p24 (MA/CA) cleavage site of HIV-1 (Val-Ser-Gln-Asn-Tyr-/-Pro-Ile-Val-Gln-Asn) [63]. Kinetic characterization of HIV-1 protease, described in Nillroth et al. [63], yielded the kinetic parameters, $k_{cat}$ and $K_M$, and the catalytic efficiency ($k_{cat}/K_M$). The dissociation constants for the enzyme-inhibitor complex ($K_i$ values, eq K1) were determined by assessing the effect of inhibitors on the catalytic activity of the protease (paper II).

$$K_i = \frac{[E]_{free} \times [I]_{free}}{[EI]}$$  \hspace{1cm} K1

In classical inhibition studies, it is assumed that the concentration of free inhibitor ($[I]_{free}$) is the same as the total inhibitor concentration ($[I]$). However, when the concentrations of enzyme and inhibitor required for inhibition are similar, a considerable portion of the added inhibitor forms a complex with the enzyme, and the free inhibitor concentration is no longer the same as the total inhibitor concentration. Inhibition under these conditions is described as tight-binding inhibition [11], which is the case for clinically relevant inhibitors of HIV-1 protease. Therefore, discovery and characterization of such inhibitors requires assay conditions and analysis procedures appropriate for tight-binding inhibitors. The assay for determining $K_i$-values was performed with constant enzyme and substrate concentrations and varying inhibitor concentration (up to about 90% inhibition). Rapid equilibrium between enzyme and inhibitor was assumed and pre-incubation of inhibitor and substrate was performed to ensure solvation of the components and stabilization at the assay temperature. An equation describing tight-binding inhibition (eq K2), and assuming $[S] << K_M$, was fit to the data using non-linear regression analysis [44, 63, 74].
Additional components of equation K2 are the total enzyme concentration, $[E]_t$, the substrate concentration, $[S]$, the initial velocity, $v_0$, the turnover number, $k_{cat}$, and the Michaelis-Menten constant, $K_M$.

Biosensor-based binding studies

Further characterization of inhibitor binding to the HIV-1 protease was performed by resolving the dissociation constant of the inhibitor-enzyme complex, affinity ($K_D$), into association and dissociation rate constants, $k_{on}$ and $k_{off}$, using biosensor technology (papers III and IV). The biosensor method can be described briefly as an analytical variant of affinity chromatography visualized in real-time, with the protease covalently attached to the solid support, to form the biosensor surface, and with the inhibitor as the ligand in the mobile buffer phase. Because this method is relatively new and predominates in the current studies, the principles and background important for using this technology are addressed.

Basic experimental principles

The biosensor technology used in these studies utilizes surface plasmon resonance (SPR), for which the SPR response is a measure of the refractive index of the solution at the sensor surface. The SPR response is related to the mass of the analytes at the surface and is expressed in resonance units (RU). The surface consists of a thin (about 100 nm) layer of carboxymethylated dextran to which molecules can be immobilized. The nature of the immobilized molecule determines the specificity and binding capacity of the sensor surface. The HIV-1 protease was immobilized on the surface of a sensor chip using amine coupling, in which uncharged amino groups on the protease were covalently linked to an activated dextran matrix (Sensorchip CM5, Biacore AB, Uppsala, Sweden). Cross-linking was performed to stabilize the enzyme on the surface [52]. A control surface was prepared on each sensor chip by activating and deactivating one of the sensor surfaces without immobilizing protein.
Figure 5 A typical sensorgram
The injection start and stop, maximum binding response (max) baseline (B) association phase (A) and dissociation phase (D) are indicated.

During the analysis, a continuous flow of buffer passes over the sensor surface. A baseline is obtained as the response when only buffer is flowing over the surface (Figure 5, B). Ligand is injected over the surface as a section of defined volume in the continuous buffer flow. As the ligand is transferred from the buffer to the sensor surface, association of ligand (inhibitor) to the immobilized molecule (protease) elicits a change in the SPR response (Figure 5, A). After injection, dissociation of the ligand from the protease into the flowing buffer can be observed (Figure 5, D). Analysis of interactions between inhibitors and resistant mutants of HIV-1 protease were performed on a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden) based on a previously reported method [52]. Analysis of the temperature dependence of binding of inhibitors was performed on a Biacore S51 instrument (Biacore AB, Uppsala, Sweden) and a running buffer recommended for the S51 instrument series was used (Table 3).

Regeneration
Before a new binding event can be studied, all ligand must be dissociated from the immobilized molecule, as indicated by a return of the response to the baseline level. To facilitate this, the surface is often regenerated by injection of a solution that will remove the bound ligand without destroying the activity of the immobilized protein. This is especially important for inhibitors with slow dissociation. If the surface is not properly regenerated, it will become blocked, preventing binding of new ligand. The composition of
the regeneration solution was determined by the sensitivity and characteristics of the immobilized molecule. For the most part, 100% ethylene glycol provided satisfactory regeneration of HIV-1 protease without reducing the binding capacity of the surface, which would indicate the loss of enzyme activity. However, at lower temperatures the dissociation rate for the inhibitors studied were slower and alternative regeneration solutions were required. The high viscosity of ethylene glycol may have contributed to its decreased effectiveness at lower temperatures. A number of regeneration solutions, differing in pH, ionic strength or detergent (SDS) content, were tested for the different inhibitors at 5-15°C. Interestingly, the solutions that were effective differed among inhibitors. For example, low pH regenerated surfaces after injection of saquinavir, but not lopinavir, for which high pH was required. In addition, the solutions were rather harsh, indicating that disruption of the protease may have contributed to the release of inhibitor. Solutions that destroyed the surface were identified by permanent loss of binding capacity.

Table 3 Assay conditions for present investigations

<table>
<thead>
<tr>
<th></th>
<th>Inhibition assay</th>
<th>Binding assay, paper III</th>
<th>Binding assay, paper IV</th>
<th>Cell Culture assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionic strength</td>
<td>1 M NaCl</td>
<td>0.15 M NaCl</td>
<td>0.137 M NaCl</td>
<td>0.14 M NaCl a</td>
</tr>
<tr>
<td>pH</td>
<td>5.0</td>
<td>7.4</td>
<td>7.2-7.4</td>
<td>7.0-7.4</td>
</tr>
<tr>
<td>Temperature °C</td>
<td>30</td>
<td>20-25</td>
<td>5-35</td>
<td>37</td>
</tr>
<tr>
<td>Solvent (DMSO)</td>
<td>5 %</td>
<td>3 %</td>
<td>3 %</td>
<td>1 %</td>
</tr>
<tr>
<td>Buffer</td>
<td>100 mM acetic acid</td>
<td>0.01 M Hepes</td>
<td>0.01 M PBS</td>
<td>Culture medium b</td>
</tr>
<tr>
<td>Additional components</td>
<td>Substrate 3 mM EDTA</td>
<td>0.0027 M KCl, 3 mM EDTA</td>
<td>0.005 % surfactant p-20</td>
<td>Amino acids, vitamins, glucose, etc.</td>
</tr>
</tbody>
</table>

DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; a NaCl and other organic salts; b RPMI Medium 1640 Glutamax I

Generating reliable data: sample preparation and quality controls

To ensure reliable, reproducible results from the biosensor analysis, careful sample preparation and performance of quality controls was essential [60, 61]. The SPR biosensor can detect very small changes in refractive index, and is consequently sensitive to artifacts. Because the refractive index of the injected sample solution may differ from that of the running buffer, there are often changes in the response level that do not depend upon a biospecific
interaction. These differences in bulk refractive index can be corrected for by using a control sensor surface (lacking immobilized protein but otherwise treated identically). The sensorgram obtained from the control surface can then be subtracted from the sensorgram obtained from the analyzed surface. Additional differences in refractive index and small systematic fluctuations in the sensorgrams are corrected for by subtracting an average blank (prepared by summing all of the blank injections where buffer was injected in the same fashion as the ligand, and taking an average value). Careful matching of running buffer and sample buffer also minimized differences in bulk refractive index. This was especially important, because a large bulk refractive index change was caused by very small differences in the final concentration of DMSO, the solvent used for storage and solubilization of inhibitors.

In addition to using a reference surface and blank injections, a number of controls of the surface were prepared. The binding capacity of the surface was checked regularly using positive and negative controls. To control for and minimize carry-over between injections, a short blank injection was included after regeneration of the sensor surface. In addition, the sensorgrams were analyzed for 1) indications of unspecific binding, indicated by trends in the reference channel during injection of ligand; 2) blockage, due to insufficient regeneration; and 3) bulk refractive index errors, visualized as discontinuities in the sensorgram at the start and stop of the injection phase. Instabilities in analysis temperature were revealed by a drift in baseline levels over a number of cycles, demonstrating the importance of maintaining constant temperature.

Proper experimental procedures clearly increase the quality of the kinetic data. In addition, experimental values obtained from biosensor analysis are obviously more reliable if based upon replicate analysis at multiple concentrations. For each enzyme/inhibitor/temperature combination studied, a series of different inhibitor concentrations was therefore measured and experiments were performed at least four times. The concentration series for each set of sensorgrams was checked for dilution errors by including extra samples of inhibitor at a concentration in the middle of the measurement range. These samples were prepared separately from the concentration series. Performance of quality controls and proper experimental procedures ensured that the data obtained in the present studies reflected the characteristics of the protease-ligand interactions.

**Affinity and interaction rate constants**

For the interactions between HIV-1 protease and inhibitors, one inhibitor (I) binds to one enzyme dimer (E) to form the enzyme-inhibitor complex (EI),
as described by equation K3, where $k_{on}$ (M$^{-1}$s$^{-1}$) is the association rate constant and $k_{off}$ (s$^{-1}$) is the dissociation rate constant.

$$
\begin{align*}
E + I & \xrightarrow{k_{on}} EI \\
& \xleftarrow{k_{off}} 
\end{align*}
$$

The equilibrium dissociation constant ($K_D$) is a measure of the ratio of free components to bound complex at equilibrium.

$$
K_D = \frac{[E][I]}{[EI]} \tag{K4}
$$

It represents the affinity between the inhibitor and the enzyme and can also be expressed in terms of the individual rate constants, $k_{on}$ and $k_{off}$, as:

$$
K_D = \frac{k_{off}}{k_{on}} \tag{K5}
$$

The rates of complex formation ([E]+[I] → [EI]) and liberation of the free components ([EI] → [E]+ [I]) are related to $k_{on}$ and $k_{off}$, respectively, and to the concentrations of interacting components (Table 4, 1a-b). Because association and dissociation occur simultaneously, the observed rates will be the sum of the forward and reverse steps (Table 4, 2a).

Certain assumptions can be made during analysis. First, because the protease (E) was immobilized, the total enzyme concentration was assumed to be constant throughout both the association and dissociation phases. Secondly, because a constant flow of inhibitor (I) was passed over the surface during injection, the concentration of free inhibitor ([I]) was considered to be constant during the association phase. Similarly, due to the constant flow of buffer over the surface during the dissociation phase, the concentration of free inhibitor ([I]) was considered to be zero. Given these assumptions, the association and dissociation can be described by the equation system for 1:1 binding in Table 4, 2a-b. Non-linear regression analysis of the binding response over time for the association and the dissociation phases, based on the integrated form of equation 2 in Table 4, allowed determination of the individual rate constants. This did not describe interactions with very fast association rates (> $1*10^7$ M$^{-1}$s$^{-1}$). By including a mass transport coefficient [19, 35], $k_t$, in the equation system describing 1:1 binding, the data could be described for the majority of the analyses (Table 4, 3a-c), as discussed below. Preliminary analysis of the inhibitor-protease
interactions using a model accounting for conformational changes did not indicate an improved description of the data (data not shown). The equations describing the data were simultaneously fit to a set of sensorgrams from a series of different inhibitor concentrations (global fitting) using non-linear regression analysis with BIAevaluation 3.1.0 or Biacore S51 Evaluation Software (Biacore AB, Uppsala, Sweden).

Table 4

Kinetic equations used for interaction analysis

<table>
<thead>
<tr>
<th>Rates of forward and reverse steps</th>
</tr>
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| 1a \( \left( \frac{d[E,I]}{dt} \right)_{\text{forward}} = k_{on} [E][I] \) | Rate of complex formation  
| 1b \( -\left( \frac{d[E,I]}{dt} \right)_{\text{backward}} = k_{off} [EI] \) | Rate of return to free components  

Equation system for 1:1 binding

| 2a \( \frac{d[E,I]}{dt} = k_{on} [I][E] - k_{off} [EI] \) | Association rate  
| 2b \( \frac{d[E]}{dt} = -(k_{on} [I][E] - k_{off} [EI]) \) | Dissociation rate  

Equation system for 1:1 binding and limited mass transfer

| 3a \( \frac{d[I]}{dt} = k_i ([I]_0 - [I]) - (k_{on} [I][E] - k_{off} [EI]) \) | Change of inhibitor concentration at the surface  
| 3b \( \frac{d[E,I]}{dt} = k_{on} [I][E] - k_{off} [EI] \) | Association rate  
| 3c \( \frac{d[E]}{dt} = -(k_{on} [I][E] - k_{off} [EI]) \) | Dissociation rate  

Concentration of the enzyme-inhibitor complex, [EI]; concentration of free enzyme, [E]; concentration of free inhibitor, [I]; concentration of inhibitor at the sensor surface, [I]; concentration of free inhibitor at time zero, [I]_0; the association rate constant, \( k_{on} (\text{M}^{-1}\text{s}^{-1}) \); the dissociation rate constant, \( k_{off} (\text{s}^{-1}) \); and the mass transport coefficient, \( k_i \).

**Mass transport**

A problem encountered during the binding experiments that merits attention was that of mass transport, the theory and phenomenon of which is addressed more comprehensively elsewhere [20, 34]. As the buffer solution containing inhibitor passes over the biosensor surface, the inhibitor must be
transported from solution into the dextran matrix in order to interact with the protease. When the rate of association \( (k_{on}) \) is slower than the rate of mass transport \( (k_t) \), the observed response depends solely upon the association rate and corresponds to characteristic 1:1 Langmuir binding (Table 4, eq 2). However, some of the inhibitors had relatively fast association rates. Resolution of the association rate for interactions with association rates close to or faster than the rate of transport will be problematic. Consider the following mechanism where \( I_S \) represents the free inhibitor at the sensor surface.

\[
\begin{align*}
\text{(observed association)} & \Rightarrow \\
E + I & \xrightleftharpoons[k_{\text{on}}][k_t] E + I_S & \xrightleftharpoons[k_{\text{off}}][k_t] EI
\end{align*}
\]

The observed association depends upon both \( k_{on} \) and \( k_t \) and the observed dissociation depends upon both \( k_{off} \) and \( k_t \). If the influence of mass transport dominates, the observed association and dissociation rates will not be equivalent to \( k_{on} \) and \( k_{off} \), as defined above. The rate of mass transport may also influence the dissociation phase of the sensorgram. For interactions with fast association rates, some of the ligand may re-associate with the immobilized protein before it can be transported away from the surface. For interactions with fast dissociation rates, the ligand may dissociate much faster than it can be transported away. In these cases, free ligand will possibly re-bind to the immobilized protein, resulting in slower apparent \( k_{off} \)-values.

Fortunately, the effect of limited mass transport can be accounted for in the analysis by including the mass transport coefficient (Table 4, 3a-c) [19, 35]. The soundness of the returned \( k_{on} \) value can be estimated by calculating the ratio, \( k_{on} \ast R_{\text{max}}/k_t \), where \( R_{\text{max}} \) is the maximum binding level and a returned value of five or more indicates that \( k_{on} \) was too fast to be reliably determined given the analysis conditions [34]. Because \( K_D \) values are not affected by mass transport, simulations using identical \( K_D \) values, but different values of \( k_{on} \) and \( k_{off} \), were used to estimate the reliability of the calculated rate constants (BIAsimulation and Biacore S51 evaluation software, Biacore AB).

The influence of the rate of mass transport on the determination of rate constants can be reduced by increasing the flow rate and decreasing the binding capacity of the surface. The binding capacity is related to the concentration of molecules immobilized on the surface and is reflected in the
binding response. Although decreasing the amount of immobilized molecules on the surface will reduce the influence of mass transport, sufficient binding responses must be maintained for extraction of kinetic data. For the resistance studies performed on the Biacore 3000 instrument (paper III), immobilization levels of 2000 to 4500 RU were used, depending on the enzyme variant. The increased sensitivity of the Biacore S51 instrument allowed a reduction of immobilization levels to about 800 RU (paper IV). Increasing the flow rate of the buffer solution will also minimize the influence of mass transport, as faster flow facilitates faster transport.

It should be noted that increasing the temperature also increases the rates of the studied interaction. If the interaction rates increase more than the rate of transport, then mass transport might pose a greater problem at higher temperatures. Inhibitors characterized by high association rates required protein immobilization levels of only about 800 RU and flow rates of 90 µl/min for resolution of the rate constants at higher temperatures (paper IV). In practice, the limits of individual rate constants that could be determined for the current studies were $k_{on} \sim 5 \times 10^6$ M$^{-1}$s$^{-1}$ and $k_{off} \sim 1$ s$^{-1}$ for resistance studies performed on the Biacore 3000 instrument (paper III) and $k_{on} \sim 2 \times 10^7$ M$^{-1}$s$^{-1}$ for thermodynamic studies using the Biacore S51 instrument (paper IV).

Correlating interaction characteristics to cell culture data
Finding characteristics of enzymes, inhibitors and enzyme-inhibitor interactions that correspond to \textit{in vivo} inhibition of the virus should expedite the discovery of drug leads. In addition, comparison of the viral inhibition in cell culture to the characteristics of interactions or interaction components provides a means of assessing the physiological relevance of the model system. Therefore, data from the current investigation (papers II, III and IV), and the associated drug discovery project [22, 53], were compared to data from cell culture studies (paper V). Inhibition of viral replication in cell culture was determined by measuring the cytopathogenicity of the HIV-1 virus at different concentrations of inhibitor [92]. The concentration of inhibitor corresponding to 50% inhibition of viral replication (ED$_{50}$ value) was determined. In addition, the minimal theoretical concentration of free enzyme in the viral particles was calculated for each inhibitor using the interaction kinetic constants and assuming $8 \times 10^{-7}$ M enzyme and inhibitor.
Resistance studies of HIV-1 protease and inhibitors

Resistance to anti-viral drugs can arise when mutations occur in the genes encoding viral proteins. The development of resistance towards HIV-1 protease inhibitors is associated with mutations in the gene encoding the protease and in residues corresponding to protease cleavage sites [12, 16]. The present study focuses on the effects of substitutions in the protease on the kinetic characteristics of interactions. The inhibition of wild type and resistant variants of HIV-1 protease (single, double and multiple combinations of G48V, V82A, I84V and L90M) by a panel of structurally similar inhibitors was studied. Interaction kinetics were further characterized using a SPR biosensor assay to determine affinity, association and dissociation rate constants.

Enzymatic activity and inhibition studies

The results of inhibition studies provide a measure of the effectiveness of an inhibitor, and conventionally involve determining the inhibition constant, $K_i$. Many inhibition studies of the HIV-1 protease include structurally diverse inhibitors or relatively small sets of similar inhibitors [e.g., Ref. 45, 40]. Because the comparison of data from different assays is difficult, it is not easy to draw general conclusions relating inhibitor structure to resistance. In addition, we were interested in examining the efficacy of inhibitors towards enzymes containing multiple substitutions. Therefore, inhibition studies were performed to determine the efficacy of a series of structurally similar inhibitors against the wild type and resistant variants of HIV protease (paper II).

Due to the requirement of substrate in the inhibition assays, it was necessary to account for the catalytic effects of mutations. In addition, changes in both the catalytic efficiencies of the enzymes and the effectiveness of the inhibitors occur with resistance. Therefore, a comparison between wild-type and enzyme variants was made by analyzing both the $K_i$ values for different inhibitors, and the catalytic efficiencies of the enzyme variants as compared to wild-type protease (paper II, tables 2 & 3). Resistant variants have a lower catalytic efficiency (1-30 %) as compared to wild type (100 %) enzyme, due to lower $k_{cat}$ values for all variants and higher $K_M$ values for all except G48V. The discrepancy in catalytic efficiency between the resistant and wild type enzymes indicates that the wild type may have a catalytic overcapacity in vivo.

Inhibition of wild type, V82A, I84V and G48V/L90M enzymes by a series of cyclic sulfamide and cyclic urea compounds was investigated. In general, $K_i$ values were higher for the resistant variants as compared to wild
type, indicating less effective inhibition. The enzymes showed similar structure-inhibition profiles for both types of cyclic compounds. For example, the effects of enlarging the S2/S2’ group of the inhibitor was paralleled in the cyclic urea and the cyclic sulfamide series. The I84V variant was the least sensitive to inhibition by the cyclic compounds as compared to wild type, V82A and G48V/L90M variants. Nevertheless, no structural features of the compounds that contributed to the inhibition profiles could be discerned.

Extension of the analysis to include a greater number of enzyme variants did not clarify the structure-resistance relationship for this set of inhibitors. The effect of variations at multiple residues could not be predicted from the single residue variants. However, increasing the number of substitutions clearly decreased the effectiveness of the inhibitors.

A complementary analysis of this data set was performed in paper V. The linear inhibitors included as control compounds (paper II) clearly differed from the cyclic inhibitors (Figure 6). This may either indicate inherent differences between the groups of compounds or simply be an experimental effect, since the linear inhibitors were chosen based on a known high affinity with wild type enzyme. The analysis of interactions with wild type, G48V, V82A and I84V/L90M indicated that, among the cyclic compounds, the grouping of compounds was based on non-structural characteristics. An analysis of the interactions of the inhibitors for which measurements were performed with all of the enzyme variants clearly separated A024 and A030 from the others. This was possibly due to the lower $K_i$ values of these inhibitors for the L90M enzyme variant as compared to wild type protease, a characteristic unique to these inhibitors in this data set.

![Figure 6](image)

**Figure 6** Cluster analysis of inhibition of wild type, G48V, V82A and I84V/L90M variants of HIV-1 protease by a panel of cyclic and linear inhibitors
It was concluded from this study with structurally similar inhibitors that there is no simple structure-activity relationship associated with resistance. The characteristics of interactions for each inhibitor/enzyme combination must be determined separately, and the design of inhibitors against resistant variants of the protease must be directed at the specific variant of interest. It was also concluded that the $K_i$ values were not sufficient for describing general structural features of inhibitors that may be associated with resistance to a particular enzyme variant. In addition, a model system for the design of inhibitors against resistant variants of the HIV-1 protease requires a more complex analysis of the interactions. Consequently, it was proposed that alternative analysis methods providing additional kinetic information should be tried.

**Binding studies: resolving affinity into association and dissociation rate constants**

Biosensor-based SPR methods allowing resolution of affinity into the interaction rate constants have been useful for kinetic characterization of protein-ligand interactions [36, 52, 79]. Therefore, a biosensor-based method for wild type HIV protease [52] was applied to the characterization of resistant variants of the HIV protease. Initially, it was necessary to confirm that the method was suitable for comparison of the enzyme variants. The analysis was therefore limited to clinical inhibitors, which allowed comparisons with both data obtained from the inhibition studies described above and with data obtained with the HIV protease (Q7K) [52]. Because the clinical inhibitors were structurally diverse, an analysis from the perspective of resistance and interaction kinetics, but not structural aspects of the inhibitors, was performed. This study successfully demonstrated that analysis of variants that differed considerably in activity (paper II), kinetic characteristics (including rate constants) and stability [49, 89] were possible. Only small modifications of the previously used biosensor method, accounting for binding capacity (related to specific activity) and differences in affinity (inhibitor concentration used), were required. In addition, the results from the inhibition and the biosensor-based methods were consistent. For example, both studies indicated that interactions with saquinavir and amprenavir were least effected by the V82A mutation and that the G48V variant was less sensitive to inhibition by saquinavir as compared to wild type.

Resolution of affinity into individual rate constants provided additional information about the interactions that was relevant for understanding resistance. Inhibitors exhibited distinctive characteristics that were maintained with different enzyme variants. Amprenavir and ritonavir were
characterized by fast association rates, whereas saquinavir and nelfinavir had the slowest association and dissociation rates (Figure 7). Increased dissociation rates were associated with resistant variants for all inhibitors. This is consistent with data obtained for saquinavir-resistant enzyme variants using stopped-flow spectroscopy [57]. In addition, inhibitors having high association rates with the wild type protease (amprenavir, ritonavir and indinavir) also had decreased association rates with enzymes containing multiple substitutions. Due to successful use of the SPR based biosensor method for analysis of resistant variants of HIV protease, future studies with a larger set of inhibitors are expected to provide important information about the relationship between kinetics and resistance.

**Strategies for inhibiting resistant targets**

A number of approaches can be examined for improving the effectiveness of inhibitors towards resistant targets. Specificity has been proposed as an important consideration for the discovery of effective inhibitors of resistant HIV protease. The development of resistance against inhibitors that interact specifically with highly conserved residues may be less likely than the development of resistance to inhibitors that interact specifically with residues that are prone to variation [84]. From this perspective, lopinavir, which interacts with conserved residue Asp 29 [78], should be a promising inhibitor. Unfortunately, enzyme variants found during treatment with other protease inhibitors have also been reported to have reduced susceptibility to lopinavir [37].

Another consideration is increasing the flexibility of inhibitors. The active site of the protease exhibits some flexibility [94] and contains a number of residues that are prone to variation (i.e., V82, I84). Therefore, inhibitors that are capable of adjusting to a changing active site should be able to inhibit a greater number of variants. It has been proposed that decreasing the rigidity of inhibitors may in turn decrease susceptibility to resistance [28, 46].

Finally, optimization of the interaction kinetics may improve inhibitors [36, 53]. The resistance studies performed in paper III indicate that inhibitors capable of maintaining slow dissociation rates with a wide range of protein variants may be less prone to resistance. This theory will be exciting to test and to apply to the discovery of new protease inhibitors.

In addition, analysis of resistant enzymes using the biosensor method also raised questions about the nature of the interactions and forces contributing to association and dissociation rates. For example, would the design of effective inhibitors of the resistant proteases be assisted by a better understanding of the forces driving these interactions? Could such information contribute to the understanding of molecular interactions in
general? For this reason, an analysis of the clinically relevant inhibitors using the SPR based biosensor method was extended to characterization of the energetics of binding.

Figure 7 Interaction kinetic plots for interactions between resistant HIV-1 protease and inhibitors.
The inhibitors are: saquinavir (S), nelfinavir (N) and ritonavir (R) in the upper plot, and indinavir (I) and amprenavir (A) in the lower plot. The enzyme variants are: wild type (circle), L90M (square), G48V (diamond), V82A (triangle), I84V/L90M (open diamond) and G48V/V82A/I84V/L90M (open circle). Arrows indicate increased resistance.
Thermodynamic analysis of interactions

The energetic properties of binding can be described in terms of free energy (G), enthalpy (H) and entropy (S). For binding to occur spontaneously, the change in free energy, $\Delta G$, of the interaction must be negative (Figure 8). The enthalpy and entropy are measures of the heat and the disorder of the system, respectively. Changes in enthalpy ($\Delta H$) and entropy ($\Delta S$) are related to changes in free energy at constant temperature and pressure according to equation T1.

$$\Delta G = \Delta H - T\Delta S$$  \hspace{1cm} T1

Similarly, free energy changes of association and dissociation can be resolved into enthalpy ($\Delta H_{on}$ and $\Delta H_{off}$) and entropy ($\Delta S_{on}$ and $\Delta S_{off}$) (eq T2).

$$\Delta G_{on} = \Delta H_{on} - T\Delta S_{on}$$

$$\Delta G_{off} = \Delta H_{off} - T\Delta S_{off}$$  \hspace{1cm} T2

Figure 8 Binding Energetics

The free energy change of binding ($\Delta G$) is proportional to $K_{on}$ according to equation T3 and the free energy changes of association and dissociation ($\Delta G_{on}$ and $\Delta G_{off}$) are inversely proportional to $k_{on}$ and $k_{off}$ according to equation T4.
Translating kinetics into energetics

Just as affinity ($K_D$) can be resolved into association and dissociation rate constants ($k_{on}$ and $k_{off}$), the free energy change of the interactions ($\Delta G$) can be resolved into the free energy change of association and dissociation ($\Delta G_{on}$ and $\Delta G_{off}$) as $\Delta G = \Delta G_{off} - \Delta G_{on}$ (Figure 8). In addition, the free energy changes ($\Delta G$, $\Delta G_{on}$ and $\Delta G_{off}$) are directly related to the affinity (eq T3) and the association and dissociation rate constants (eq T4).

$$K_D = \exp \left( \frac{\Delta G}{RT} \right)$$ \hspace{1cm} \text{T3}

$$k_{on} \left( \frac{h}{T \cdot k_b} \right) = \exp \left( \frac{\Delta G_{on}}{-RT} \right) \quad \& \quad k_{off} \left( \frac{h}{T \cdot k_b} \right) = \exp \left( \frac{\Delta G_{off}}{-RT} \right)$$ \hspace{1cm} \text{T4}

The enthalpy and entropy of interactions can be determined by analyzing the affinity at different temperatures. In the simplified case where $\Delta H$ and $\Delta S$ are independent of temperature, a plot of the natural log of the affinity against the inverse of the absolute temperature ($\ln K_D$ vs. $1/T$, van’t Hoff analysis) yields a straight line and equation T5 can be fitted to the data points.

$$\ln K_D = \frac{\Delta H}{RT} - \frac{\Delta S}{R}$$ \hspace{1cm} \text{T5}

However, $\Delta H$ and $\Delta S$ are often temperature dependent and analysis requires an equation accounting for higher complexity and a change in the heat capacity, $\Delta C_p$ (eq T6a-c) \[85\].

$$\Delta H_{T_2} = \Delta H_{T_1} + \Delta C_p(T_2 - T_1)$$ \hspace{1cm} \text{T6a}

$$\Delta S_{T_2} = \Delta S_{T_1} + \Delta C_p \ln(T_2/T_1)$$ \hspace{1cm} \text{T6b}

$$\ln K_{D_{T_2}} = \left[ \frac{(\Delta H_{T_1} - \Delta C_p T_1)(1/T_2 - 1/T_1) - \Delta C_p \ln(T_2/T_1)}{R} \right] + \ln K_{D_{T_1}}$$ \hspace{1cm} \text{T6c}
In addition, the enthalpy of interactions of ligands with proteins in conformational equilibrium (potentially temperature dependent) may be determined using the more complex analysis including \( \Delta C_p \) [72]. Conformational changes of HIV protease, due to movement of the flap region of the enzyme [73, 82, 91], further motivate the use of a more complex analysis.

Equation T6c uses the difference between two temperatures to determine \( \Delta C_p, \Delta H \) and \( \Delta S \), and is therefore sensitive to errors in the measured data. A more sound approach is to rearrange equations T6a-b into \( \Delta H = \Delta C_p T + \alpha \) and \( \Delta S = \Delta C_p \ln T + \beta \), where \( \alpha \) and \( \beta \) are constants, and to use these expressions for \( \Delta H \) and \( \Delta S \) in equation T5. The resulting equation (eq T7) can be fitted to the data points using non-linear regression analysis (personal communication, Karl Andersson).

\[
\ln K_D = \frac{1}{R} \left[ \frac{1}{T} \left( \Delta C_p T + \alpha \right) - \left( \Delta C_p \ln T + \beta \right) \right]
\]  

T7

The enthalpy, entropy and heat capacity changes of association and dissociation can be determined in a similar fashion using equations T8 and T9, where the subscript ‘r’ indicates the association (on) or dissociation (off).

\[
\ln \left( \frac{k_r * h}{k_b * T} \right) = -\Delta H_r / RT + \Delta S_r / R
\]  

T8

\[
\ln \left( \frac{k_r * h}{k_b * T} \right)_{T_2} = \frac{\left[ \Delta H_{r,T_1} - \Delta C_p, T_1 \right] (1/T_1 - 1/T_2) + \Delta C_p, \ln(T_2/T_1)}{R} + \ln \left( \frac{k_r * h}{k_b * T} \right)_{T_1}
\]  

T9

Direct comparison of two temperatures may be avoided by using equation T10, analogous to equation T7 above.

\[
\ln k_r = -\left( \frac{k_b * T}{h} \right) \left[ \frac{1}{T} \left( \Delta C_p, T + \alpha \right) - \left( \Delta C_p, \ln T + \beta \right) \right]
\]  

T10
We proposed that resolving free energy into enthalpy and entropy and dissecting the contributions of these factors to the association and dissociation of inhibitor would help to clarify the details of the interactions between HIV protease and inhibitors. The influence of temperature on the interactions between HIV-1 protease and inhibitors was therefore analyzed using biosensor technology (paper IV). Interaction kinetic analyses yielding affinity, association and dissociation rate constants were performed for seven clinically relevant inhibitors at temperatures between 5 and 35 °C. Kinetic data at 25 °C were consistent with those previously obtained for amprenavir, indinavir, nelfinavir, ritonavir and saquinavir (paper III). Lopinavir and atazanavir both had affinity, association and dissociation rate constants in the same range as the other clinical inhibitors. These inhibitors were characterized by slow dissociation rates and, for lopinavir, fast association rates (Figure 9). Analyses of the energetic characteristics of HIV-protease-inhibitor interactions were performed as described in paper IV. The free energy, enthalpy and entropy of interactions (ΔG, ΔH and ΔS) were found to be similar for the clinical inhibitors studied and some aspects of the energetic characteristics were consistent with observations from studies performed using isothermal calorimetry (ITC) [64, 80]. The observed differences are not easily explained, but may be due to differences in the assay conditions, stabilization techniques or enzyme variant used. In addition, the difficulty of comparing estimates of enthalpy determined from van’t Hoff and calorimetry analyses, even when assay conditions are identical, is well established [e.g., Ref. 8, 27, 62]. Characterization of the energetics of association and dissociation involved resolving the free energies of association and dissociation, ΔG_{on} and ΔG_{off}, which were similar for all inhibitors, into enthalpy and entropy terms (ΔH_{on}, ΔH_{off}, TΔS_{on} and TΔS_{off}).

Interactions were more complex than anticipated
Determination of the affinity, association and dissociation rate constants added an extra dimension to the characterization of HIV-protease-inhibitor interactions. Higher complexity was revealed for the interactions with amprenavir, ritonavir and indinavir. This complexity of the kinetics and thermodynamics of interactions influenced the determination of enthalpy and entropy contributions to affinity, association and dissociation. In addition, the assumption that enthalpy and entropy were temperature dependent was further supported, as the more complex analyses including heat capacity change provided a better description of the data (Figure 10).
Interactions with lopinavir (diamonds), ritonavir (squares) and nelfinavir (triangles) illustrate different influences of temperature on the interaction kinetics and interaction kinetics for lopinavir and atazanavir (circles) are presented for the first time here. Interactions with lopinavir were least influenced by temperature over the range studied. Interactions with nelfinavir were more influenced and showed greater variation in interaction rate constants. Interactions with ritonavir were more complex and non-linear trends were observed over the temperature range. Interaction kinetics for saquinavir, indinavir and amprenavir are given in paper IV. The analysis temperatures are labeled 5, 15, 25 or 35 °C.

Saquinavir, nelfinavir and lopinavir exhibited less complexity than amprenavir, ritonavir and indinavir throughout the analyses. Since the inhibitors differ structurally, the observed differences could not be assigned to structural characteristics of the two groups of inhibitors. The central region of ritonavir and lopinavir, corresponding to the S1 and S1’, are identical (Figure 4), indicating that more distal sites are responsible for the different characteristics between the two groups. We propose that analysis of compounds that are structurally related could give insight into the differences between these two groups of inhibitors. In addition, the non-linearity observed with amprenavir, indinavir and ritonavir might indicate temperature-dependent structural changes of the protease during inhibitor binding of these three inhibitors. Therefore, analysis of the interactions with enzyme variants associated with altered flexibility as compared to the wild type would be also interesting.
Figure 10 Temperature dependence of the interaction between ritonavir and HIV-1 protease
Equations T5 (linear) and T6c (non-linear) fit to the data points

Distinctive interaction energetics
Resolution of the energetics of the association and the dissociation of inhibitors revealed distinguishing characteristics for the interaction of lopinavir with HIV-protease. The enthalpic contribution for both association and dissociation of lopinavir was less than that of the other inhibitors (Figure 11). The entropic contribution to association of lopinavir was positive, whereas it was negative for the other inhibitors.

One could speculate that the differences in the entropy of interactions might be due to the amount of solvent released upon inhibitor binding. Differences in molecular weight between lopinavir and ritonavir may contribute to the amount of solvent released. The occurrence of van der Waals interactions between an isopropyl group of ritonavir and Val 82 [78] in the S3 binding subsite of the protease might also contribute to these differences. As a comparison, the molecular weight of amprenavir is similar to that of lopinavir, and amprenavir is not reported to have van der Waals interactions with Val 82 in the S3 subsite of the protease. However, the energetics of association and dissociation of amprenavir resemble those for ritonavir, not lopinavir, indicating that the speculations made here are too simplistic.
Figure 11 Free energy, enthalpy and entropy contributions to affinity, association and dissociation
The free energy change, $\Delta G$, black bars; enthalpy change, $\Delta H$, grey bars; and entropy change, $\Delta S$, white bars. Inhibitors are amprenavir (A), indinavir (I), lopinavir (L), nelfinavir (N), ritonavir (R) and saquinavir (S).

An additional difference between lopinavir and the other inhibitors is the formation of hydrogen bonds between a cyclic urea in the S2 position of lopinavir and Asp 29 of the protease [78]. However, as hydrogen bond formation is generally isoenthalpic, hydrogen bonds are more likely to contribute to specificity of the interactions than to enthalpic differences between bound and non-bound ligand [18].

Clearly, the interactions are complex and generalizations about the unique interaction characteristics for lopinavir cannot be made at this point. Nevertheless, resolution of the free energy of association and dissociation into enthalpy and entropy terms distinguishes lopinavir from the other clinical inhibitors and indicates that resolution of the energetics of association and dissociation may be useful for characterization of inhibitors. Further studies with a greater number of inhibitors and enzyme variants will improve our understanding of the energetic features of the interactions and help in the discovery of new protease inhibitors.
Application of model systems for drug discovery

Development of a model system capable of describing or predicting the effectiveness of an inhibitor against viral replication \textit{in vivo} would simplify and expedite the process of drug discovery and help keep pace with an ever changing virus, like HIV. A goal of the present study was to determine which characteristics of interactions between ligand and target are important for understanding viral inhibition \textit{in vivo}. This required careful selection and validation of the target, as well as of the assay. Furthermore, the identity of the target may be enigmatic, since the target may change as resistance arises or be present as a heterogeneous mix \textit{in vivo}. Although a thorough description of all aspects to be considered is outside the scope of this work, it is still important to put the current study into a larger perspective.

The “right” target

A key component of a relevant model system is the target protein. The importance of selecting and defining the target can be illustrated by the HCV protease model system. Although studies have previously been performed with a truncated version of the HCV NS3 protease containing only the protease domain, the full-length protein as studied in paper I is more physiologically relevant \cite{5, 30}. Crystal structures have revealed that the binding pocket of the active site of the HCV protease is formed in the interface between the protease and helicase domains of the NS3 protein \cite{90}. Subsequent studies with the full-length protein have resulted in the discovery of smaller compounds that are more effective inhibitors of the protease than are those found using only the protease domain \cite{31}.

A challenge in the development of anti-AIDS drugs with long-term efficacy is the ability of the viral components, including the protease, to mutate rapidly under evolutionary pressure during drug treatment. Because of this genetic flexibility, the HIV protease is not one target, but rather many closely related targets. Although inhibitors of HIV protease have contributed to the tremendous success of highly active antiviral therapy (HAART) since 1995, drug resistance has emerged as the major cause for failure of these therapies \cite{81}. Therefore, discovery of inhibitors of specific resistant variants, or a pool of closely related variants, is required for the next generation of effective anti-AIDS drugs.

The “right” assay and conditions

When developing model systems for the analysis of molecular interactions it is essential to choose conditions that are relevant for the system and
molecules being studied. The conditions used during molecular interaction analyses are often dictated either by the type of method performed or the specific molecules involved. For example, the conditions yielding optimal enzymatic activity may not be suitable for growing cells and determining ED$_{50}$ values. Cell culture experiments must be performed in physiologically relevant media (pH 7.4 and an ionic strength of about 0.14 M) whereas kinetic assays are often performed under conditions yielding the highest enzyme activity, pH 5.0 and 1 M NaCl for HIV-1 protease (Table 3). Furthermore, cell culture studies must be performed at physiological temperature, but many other assays require lower temperatures due to instability of assay components (usually protein) or temperature control capabilities of instruments. The ionic strength, pH, temperature and buffer environment will influence the kinetic parameters. If the influence of these conditions differs among inhibitors, it may be difficult to correlate ED$_{50}$ values with interaction data obtained under different assay conditions.

A benefit of the biosensor-based method used in the present studies is increased flexibility in choosing assay conditions. The assay is not limited to conditions that are optimal for enzyme activity measurements, as is the case with the inhibition assay. Although both are equilibrium dissociation constants for the enzyme-inhibitor complex, the $K_i$ values obtained in the inhibition assay differed from the $K_D$ values obtained from binding studies (paper II and III). Some of the differences between the inhibition and binding studies were due to improved definition of high affinity interactions with the biosensor method [53]. Additional differences between the two systems are thought to depend upon differences in the assay conditions. Because the conditions used for the biosensor assay are closer to those used in cell culture studies, the interaction data obtained is proposed to be more physiologically relevant.

Recent analysis of a set of HIV-1 protease inhibitors indicated that $K_D$ values from the biosensor assay correlated better to ED$_{50}$ values than did $K_i$ values (paper V). An even better correlation was found when the kinetic parameters were used to estimate the free enzyme concentration in the viral particle. This validated our use of the biosensor system as an improved description of viral inhibition and further illustrated the importance of defining the system being studied.
Conclusions and future prospects

The information obtained in the current study of viral proteases can be applied to a more general understanding of molecular interactions. More importantly, the improved description of protein-inhibitor interactions presented here can be applied to the development of effective protease inhibitors. This is expected to contribute to the discovery of improved antiviral drugs, and ultimately to the improvement of worldwide health.

On a less grandiose scale, it is important to illuminate the specific contributions that the present investigations make to the science of protein-ligand interactions:

- To begin with, the influence of the choice of target for the study of protein-ligand interactions was demonstrated. Full-length NS3 protein of HCV provided a more relevant target and a better model for the development of HCV protease inhibitors. It was also established that resistant variants of the HIV protease must be analyzed case wise for each inhibitor-protease variant combination, since the effect of structural changes in inhibitors and the protease on the resistance profile are not predictable.

- Although K<sub>i</sub> values are the standard measure of an inhibitors’ efficacy, the studies presented here show that they do not provide the best depiction of viral inhibition. The affinity obtained from the biosensor based-binding studies correlated better with viral inhibition in cell culture, ED<sub>50</sub> values, and was therefore a more physiologically relevant model system. Approximating the free enzyme concentration in the virus particle further improved the correlation with ED<sub>50</sub> values.

- The resolution of affinity into association and dissociation rate constants provided a more detailed picture of the interactions between resistant protease and inhibitor. Increased dissociation rates were identified as the primary kinetic contribution to the development of resistance. Therefore, inhibitors that maintain slow dissociation rates to a number of variants may prove to be less susceptible to resistance.
A model system was developed for analysis of the energetics (free energy, enthalpy and entropy) of interactions using the biosensor-based technology. This analysis provided an added dimension to our characterization of protease-inhibitor interactions, and complex interactions were revealed. Through the resolution of enthalpy and entropy contributions to association and dissociation, an inhibitor with unique interaction energetics was identified. This demonstrates the potential of this method to disclose differences in interactions with similar kinetic characteristics, and indicates that it will be useful in the drug discovery project.

What next?
Many interesting possibilities exist for expansion of the studies presented here. Fortunately, time, resources and financial considerations are only practical limitations, and do not hinder the development of ideas. The most obvious proposals for future studies are direct continuations of the current investigations:
1. Analysis of the interaction characteristics of resistant variants of HIV-1 protease with a larger set of closely related inhibitors
2. Extend the thermodynamic study (paper IV) to a larger set of inhibitors to elucidate the complex nature of the observed interactions
3. Perform a thermodynamic analysis with resistant variants of HIV-1 protease
4. Investigate the correlation of resistance and energetic characteristics of interactions with ED₅₀ data

The model systems developed here for studying resistance and energetics can be applied to the discovery of new inhibitors. In addition, inhibitors can be designed and tested, based on the current findings.

The present investigations have also provided information that may be applied to the characterization of other protein-ligand interactions. Analyses of other proteases are in progress within the related drug discovery project (D Backman, M Geitmann, T Gossas, M Lindgren and A Poliakov, personal communications), but thus far, resistance and thermodynamic studies of other proteases have not been performed. Investigation of molecular interactions that may decrease the efficacy of protease inhibitors in vivo, such as inhibitor binding to human serum albumin or other host proteins, would also be interesting. Finally, insight into the general characteristics of molecular interactions may be gained by applying the results and perspectives from the current studies to analysis of a diverse range of proteins and ligands.
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