

Biosensor Studies of Ligand Interactions with Structurally Flexible Enzymes

Applications for Antiviral Drug Development

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Abstract

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The use of a surface plasmon biosensor fills a missing link in kinetic studies of enzymes, since it measures directly the interaction between biomolecules and allows determination of parameters that are determined only indirectly in activity assays. The present thesis deals with kinetic and dynamic aspects of ligand binding to two viral enzymes: the human cytomegalovirus (HCMV) protease and the human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT). The improved description of interactions presented herein will contribute to the discovery and development of antiviral drugs.

The biosensor method provided new insights into the interaction between serine proteases and a peptide substrate, as well as substrate-induced conformational changes of the enzymes. The direct binding assay served as a tool for characterising the binding mechanism of HCMV protease inhibitors.

Kinetic details of the interaction between HIV-1 RT and non-nucleoside reverse transcriptase inhibitors (NNRTIs) were unravelled. The recorded sensorgrams revealed several forms of complexity. A general binding model for the analysis was derived from the data, describing a two-state mechanism for the enzyme and a high- and a low-affinity interaction with the inhibitor. Interaction kinetic constants were determined for the clinically used NNRTIs and several investigational inhibitors.

The established method was applied to investigate the mechanism of resistance against NNRTIs. Amino acid substitutions in the NNRTI-binding site resulted in both decreased association rates and increased dissociation rates for the inhibitors. The K103N and the L100I substitution also interfered with the formation of the binding site, thereby facilitating inhibitor binding and unbinding.

Finally, thermodynamic analysis revealed that, despite the hydrophobic character of the interaction, NNRTI binding was mainly enthalpy-driven at equilibrium. Large entropy contributions in the association and dissociation indicated that binding is associated with a dynamic effect in the enzyme.

Keywords: SPR biosensor, HIV-1 reverse transcriptase, HCMV protease, interaction kinetics, drug discovery, non-nucleoside inhibitor, resistance

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List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I Geitmann, M. and Danielson, U. H. (2004) Studies of substrate-induced conformational changes in human cytomegalovirus protease using optical biosensor technology. *Anal. Biochem.*, 332, 203-214.
- II Geitmann, M., Unge, T. and Danielson, U. H. Biosensor-based kinetic characterization of the interaction between HIV-1 reverse transcriptase and non-nucleoside inhibitors. *Submitted for publication.*
- III Geitmann, M., Unge, T. and Danielson, U. H. Interaction kinetic characterization of HIV-1 reverse transcriptase non-nucleoside inhibitor resistance. *Submitted for publication.*
- IV Geitmann, M. and Danielson, U. H. Thermodynamic analysis of non-nucleoside inhibitor binding to HIV-1 reverse transcriptase. *Submitted for publication.*

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
DABCYL	4-(4'-Dimethylaminophenylazo)benzoyl
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
<i>E</i>	Enzyme
<i>E. coli</i>	<i>Escherichia coli</i>
ED_{50}	Effective dose required for 50 % of the max. effect
EDANS	(2' Aminoethyl)amino-naphtalene-1-sufonic acid
$EI, EI^*, E_RI, E_RI^*, E_RI'$	Enzyme inhibitor complex
EP_2	Acylenzyme
E_R	Binding-competent form of the enzyme
ES	Enzyme substrate complex, Michaelis complex
E_T	Non-binding-competent form of the enzyme
h	Planck's constant 6.626×10^{-34} Js
HAART	Highly active anti-retroviral therapy
HCMV	Human cytomegalovirus
HIV-1	Human immunodeficiency virus type 1
<i>I</i>	Inhibitor
IC_{50}	Inhibitor conc. that results in 50 % enzyme inhibition
k_b	Boltzmann constant, 1.381×10^{-23} JK ⁻¹
k_{cat}	Turnover number, rate of catalysis
K_D	Equilibrium dissociation constant
K_i	Inhibition constant
K_m	Michaelis-Menten constant
k_{off}, k_{-1}, k_{-2}	Dissociation rate constant
k_{on}, k_1, k_2	Association rate constants
k_p, k_{-p}	Pre-equilibrium rate constant
K_p	Pre-equilibrium constant
K_s	Dissociation constant of the Michaelis complex
NMR	Nuclear magnetic resonance
NNIBP	Non-nucleoside inhibitor-binding pocket
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
P_1, P_2	Product
PETT	Phenylethylthioazolythiourea

R	Gas constant, $8.3145 \text{ JK}^{-1}\text{mol}^{-1}$
RNA	Ribonucleic acid
RNase H	Ribonuclease H
RT	Reverse transcriptase
RU	Resonance unit
S	Substrate
SPR	Surface plasmon resonance
T	Absolute temperature
wt	Wild type
ΔC_p	Heat capacity change
ΔG	Free energy change
ΔS	Entropy change
ΔH	Enthalpy change

Amino acids are referred to by their three- or one-letter abbreviations:

Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartate	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamate	Glu	E	Serine	Ser	S
Glutamine	Gln	Q	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

Introduction

The discovery of enzyme inhibitors and their further development into drugs is aided by a detailed understanding of the interaction between the drug target and the inhibitor. The appearance of new technologies is enhancing our ability to identify pathogenic targets associated with human diseases, and a significant portion of these targets to be discovered is likely to be enzymes. Study of the catalytic mechanisms and methods for inhibition of these proteins will therefore remain a basis of pharmaceutical science for the future. The present work focuses on kinetic and dynamic aspects of the interaction between viral enzymes and ligand molecules and will contribute to the understanding of the complex world of biomolecular interactions.

Protein Conformations and Dynamics

During recent decades a large amount of structural information for proteins has been accumulated by NMR and crystallography. However, scientists commonly agree that static structures alone are not sufficient to describe the character of these macromolecules. Knowledge of the dynamic nature of proteins is essential to understanding of their wide range of functions and behaviors, such as catalysis, recognition, binding, signalling, biosynthesis and transport. Even in their native states, proteins exhibit a surprising degree of flexibility, ranging from local structural fluctuations, which occur as a consequence of thermal motion, to large-scale conformational changes resulting from collective motions (Frauenfelder *et al.*, 1991). Dynamic processes in macromolecules cover a large time scale regime, including very fast fluctuations of individual atoms on the picosecond time scale, loop and domain motions on the nanosecond time scale, conformational rearrangements on the millisecond time scale and breathing modes on a timescale slower than seconds (Yon *et al.*, 1998; Kern *et al.*, 2003). Multiple conformational changes are a general feature of proteins, in particular of enzymes, receptors, and proteins associated with transport and coordinated motions. These native dynamics are often vital to the function of proteins, and must therefore be characterised in order to clarify protein machinery at the molecular level (Berendsen *et al.*, 2000; Karplus, 2000; Villa *et al.*, 2001; Hammes, 2002; Gutteridge *et al.*, 2005).

Classic techniques to study conformational changes in proteins involve fluorimetry, stopped flow spectroscopy, circular dichroism and NMR. These methods usually monitor average conformations of proteins. Recent advances in the fields of x-ray crystallography (Parak 2003, Schotte 2004), NMR (Barbar, 1999; Ishima *et al.*, 2000; Volkman *et al.*, 2001), mass spectrometry (Apuy *et al.*, 2001; Kaltashov *et al.*, 2002; Konermann *et al.*, 2003), single molecule spectroscopy (Orrit, 2003; Barkai *et al.*, 2004; Kulzer *et al.*, 2004;) and computer-based molecular dynamic simulation (Norberg *et al.*, 2003; Lindorff-Larsen *et al.*, 2005) have addressed dynamic properties of proteins and made it possible to identify single conformational species of proteins.

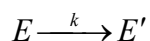
In parallel to this development, the concept of conformational ensembles (Tsai *et al.*, 1999; Kumar *et al.*, 2000; Gunasekaran *et al.*, 2004) has gained popularity against the static model of proteins that has been manifested by the power of crystal structure elucidation. This model, which is hardly new (Weber, 1972), assumes that macromolecules in solution exist in multiple, equilibrating conformations that can be described with standard statistical distributions. The process of ligand binding shifts this equilibrium from the statistical distribution of the free conformations towards the stabilised bound conformations. This concept has been adopted mainly for allosteric regulated proteins, but could apply essentially to all non-structural proteins (Kern *et al.*, 2003; Gunasekaran *et al.*, 2004).

Two types of flexibility in proteins will be addressed in the present work: The first is induced by the changes in the local environment when a ligand binds to a protein, which results in conformational changes. The second type of flexibility of proteins is the existence of different conformational states, which are selected upon binding of a ligand. For the elucidation of these complex biomolecular interactions a biosensor approach was chosen based on surface plasmon resonance technique.

Basic interaction kinetics

Rate constants

A rate constant is a quantitative characteristic of a chemical or physical process. In the simplest case, the first-order rate constant k describes the fraction of the molecules in the originating compartment that is travelling to another compartment per unit time, for example a conformational change of an enzyme from state E to state E' .



This irreversible conversion from E to E' can be described by

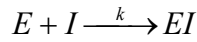
$$-\frac{d[E]}{dt} = k \cdot [E] = \frac{d[E']}{dt} \quad (1)$$

or after integration

$$[E](t) = [E]_0 \cdot e^{-k \cdot t} \text{ and } [E'](t) = [E]_0 \cdot (1 - e^{-k \cdot t}) \quad (2)$$

where $[E]$ and $[E']$ are the concentrations of molecules in state E and E' , respectively, at time t , and $[E]_0$ the initial amount in state E .

In the case of two interacting partners, E and I , forming a complex, EI , according to



k is a second-order rate constant where

$$-\frac{d[E]}{dt} = k \cdot [E] \cdot [I] = \frac{d[E']}{dt} \quad (3)$$

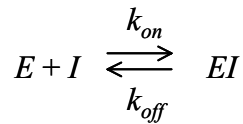
If the concentration of one of the reactants, $[I]$, is constant or $[I] \gg [E]$ the solution simplifies to

$$[E](t) = [E]_0 \cdot e^{-[I] \cdot k \cdot t} \text{ and } [EI](t) = [E]_0 \cdot (1 - e^{-[I] \cdot k \cdot t}) \quad (4)$$

where $[I] \cdot k$ is a pseudo-first-order rate constant.

Reversible interactions

Many reactions are reversible, and the reverse reaction must be accounted for in the rate equation. In the case of a reversible interaction between an inhibitor I and an enzyme E , the interaction is described by an association rate constant k_{on} and a dissociation rate constant k_{off} .



K_D is the dissociation constant of the equilibrium and defined by

$$K_D = \frac{k_{off}}{k_{on}} = \frac{[E] \cdot [I]}{[EI]} \quad (5)$$

The corresponding differential equation for the change of the free enzyme E and the complex EI

$$-\frac{d[E]}{dt} = k_{on} \cdot [E] \cdot [I] - k_{off} \cdot [EI] = \frac{d[EI]}{dt} \quad (6)$$

can be integrated to give the relationship between time and the amount of E or EI , respectively:

$$[E](t) = [E]_0 \cdot \left(1 - \frac{[I] \cdot k_{on}}{[I] \cdot k_{on} + k_{off}} \cdot \left(1 - e^{-([I] \cdot k_{on} + k_{off}) t} \right) \right) \quad (7)$$

$$[EI](t) = [E]_0 \cdot \frac{[I] \cdot k_{on}}{[I] \cdot k_{on} + k_{off}} \cdot \left(1 - e^{-([I] \cdot k_{on} + k_{off}) t} \right) \quad (8)$$

Basic thermodynamics

The temperature dependence of rate constants for chemical reactions was originally described by van't Hoff (1884) and Arrhenius (1889). Adding the concept of Gibbs free energy of reactions, Boltzmann's theory of the distribution of energy among molecules and quantum-mechanical principles, a general relationship between a rate constant k and the absolute temperature T can be formulated:

$$\ln k = \ln \left(\frac{k_b \cdot T}{h} \right) + \frac{\Delta S}{R} - \frac{\Delta H}{R \cdot T} \quad (9)$$

In this equation, k_b is the Boltzmann constant, h is Planck's constant and R is the gas constant. Furthermore, ΔS is the entropy and ΔH is the enthalpy part of the Gibbs energy ΔG of the reaction according to

$$\Delta G = \Delta H - T \cdot \Delta S \quad (10)$$

These equations can be combined with transition state theory, based on the work of Eyring (1935). The transition state in a reversible reaction system is the state of highest energy that has to be passed by the forward and the reverse reaction, as depicted in an energy diagram in Figure 1.

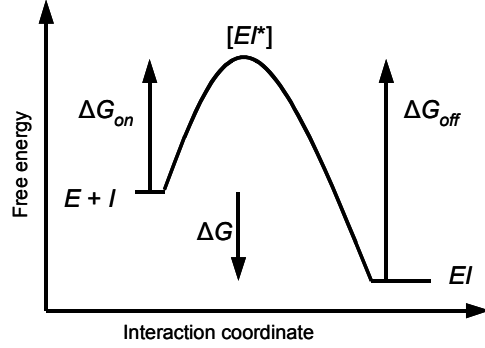


Figure 1. Reaction profile according to transition-state theory. The example shows the binding of an inhibitor I to an enzyme E to give an enzyme-inhibitor complex EI . The reaction proceeds through a transition state $[EI^*]$.

The free energy change of the reaction (ΔG) is then the difference between the free energy changes to reach the transition state from the site of the free reactants (ΔG_{on}) and from the site of the products (ΔG_{off}):

$$\Delta G = \Delta G_{on} - \Delta G_{off} \quad (11)$$

Combining Equations 5 with 9 for the association and dissociation rate constants of the example in Figure 1, the relationship between the dissociation constant K_D and the temperature can be derived as

$$\ln K_D = \frac{\Delta H}{R \cdot T} - \frac{\Delta S}{R} \quad (12)$$

where $\Delta H = \Delta H_{on} - \Delta H_{off}$ and $\Delta S = \Delta S_{on} - \Delta S_{off}$.

Drug-receptor interaction theories

In order to understand and to classify the interaction between enzymes (receptors) and drug molecules (or in general: ligands), a number of hypotheses have been proposed describing the relationship between binding and action (Kenakin, 1997, 2004; Silverman, 2004). There is no single theory that adequately explains all aspects of ligand-receptor interactions, nor are the different concepts exclusive of each other. The following brief overview reflects more a historical development in this field.

The *occupancy theory* (Gaddum, 1926; Clark, 1926; Ariëns, 1954; Stephenson, 1956) describes drug action in terms of the affinity between a drug

and a drug target and the efficacy of the drug to initiate a certain biological effect. According to this theory, the intensity of the pharmacological response is proportional to the number of receptors occupied by the drug times the efficacy of the drug. Alternatively, the response was suggested to be proportional to the number of encounters of the drug with its receptor per unit time and not to the number of occupied receptors. This theory is known as the *rate theory* (Paton, 1961).

From these basic theories, the *operational model* (Black *et al.*, 1983) has been developed, which proposes that production of the response is modelled by two or more successive saturable hyperbolic functions. The first involves ligand binding to the receptor and the second is the operational binding of the drug-receptor complex to a possible series of hyperbolic functions leading to the response.

The *induced fit theory* (Koshland, 1958, 1961) was initially formulated for the interaction between enzymes and substrates, but it applies to drug-receptor interactions as well. In this model, the binding of a ligand to a receptor causes a conformational change that orients the essential binding sites, which elicits a certain effect in the receptor (Figure 2). In case of enzyme-substrate interactions the catalytic groups are aligned in their correct orientation first upon binding. This theory is an alternative to the *lock and key mechanism* (Fischer, 1894), which assumes enzyme-ligand complementarity.

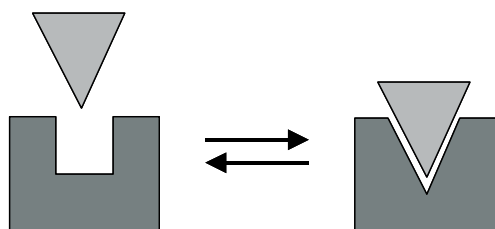


Figure 2. Induced fit binding of a ligand to a receptor.

The *activation-aggregation theory* (Monod *et al.*, 1965) suggests that the receptor exists in a dynamic equilibrium between two different conformational states, *R* and *T*, of which *R* is active and *T* is inactive form. Binding of a ligand to one of the conformations shifts the equilibrium to the stabilised form of the receptor, either to the inactive (in case of an antagonistic ligand) or to the active form (agonistic ligand).

The concept of *allostery* (Greek, *allos* = other, *stereos* = solid or space) commonly involves receptors that have multiple binding sites which cooperate with each other, usually by conformational changes (Kern *et al.*, 2003). The binding of an allosteric effector can either cause positive cooperativity

(activator) or negative cooperativity (inhibitor) to the activity of another binding site. Allosteric inhibitors or activators are usually structurally different from substrates (heterotrophic) and bind at a separate site away from the active site of the enzyme. Even though only a few enzymes are officially classified as allosteric enzymes, the concept of allostery can be regarded as an intrinsic property of almost all proteins as a result of ligand-induced redistribution of dynamic conformational ensembles (Gunasekaran *et al.*, 2004).

Enzyme inhibitors

In general, enzyme inhibitors are substances that decrease the rate of an enzyme-catalysed reaction when present in the reaction mixture (Cornish-Bowden, 1995). Common classifications of enzyme inhibitors are based either on their structure, their kinetic behaviour or their mechanistic properties. Regarding the structure, one can, for example, distinguish between substrate-based and product-based inhibitors, transition state analogues or inhibitors that have a completely different structure than the substrate. Inhibitors can either bind *reversibly* or *irreversibly* to an enzyme. The latter case may involve the formation of a covalent bond and the substance is then rather called *inactivator*. The name *mechanism-based enzyme inactivator* includes that the inactivator depends on the mechanism of the targeted enzyme. Reversible inhibitors that behave in a way that is difficult to distinguish from irreversible inhibition are called *tight-binding inhibitors*. Kinetically, three main types of reversible inhibition can be distinguished: *competitive inhibition*, *uncompetitive* and *mixed inhibition*, according to their effect on the kinetic parameters of the catalytic reaction. Also, an inhibitor can cause *complete inhibition* (*linear inhibition*) or only *partial inhibition* of the enzyme activity upon binding.

AIDS

The human immunodeficiency virus (HIV)

AIDS (Acquired Immunodeficiency Syndrome) is a worldwide pandemic caused by an infection with HIV. At the time of writing the virus is estimated to be carried by about 40 million people and more than 20 million people have died of AIDS since the first diagnosis in 1981 (WHO 2004).

HIV belongs to the *Lentivirus* (“slow” virus) genus of the family *Retroviridae*, characterised by a genome consisting of two identical plus sense RNA molecules. Two species of the virus have been recognized, referred to as HIV-1 and HIV-2. They differ in their genome and in their infectivity,

with HIV-1 being well over three times as infective as HIV-2 (Gilbert *et al.*, 2003). While HIV-1 is found throughout the world, HIV-2 infections are mainly restricted to Western Africa and India.

Transmitted by body fluids, HIV infects cells of the human immune system that display a certain receptor type (CD4 lymphocytes). After entry into the host cell the HIV genome is reverse transcribed by the virus-encoded reverse transcriptase and integrated into the host genome (Luciw, 1996; Nielsen *et al.*, 2005). Primary infection with HIV causes an acute viremia, followed by a phase of clinical latency which can last for several years. The continuously high production of virus particles depletes T-helper cells and thereby progressively destroys the body's immune system. The patient becomes prone to a range of diseases that the body can normally control, e.g. pneumonia, tuberculosis, meningitis and cytomegalovirus infection (Cavert *et al.*, 1997; Carmichael *et al.*, 1997). These opportunistic infections are usually the cause of death of AIDS patients.

The human cytomegalovirus (HCMV)

Also known as *Human herpes virus 5* (HHV 5), HCMV is a double stranded DNA virus of the *Herpesviridae* family (Gold *et al.*, 1982) and is one of the eight herpesviruses that infect people. It has a worldwide distribution and infects between 70 and 100 % of the population (De Jong *et al.*, 1998; Khoshnevis *et al.*, 2002). Transmission occurs from person to person via body fluids. The virus usually stays dormant in the body and imposes no harm to most healthy persons, but can cause morbidity and mortality in immunocompromised individuals and congenitally infected newborns. Pneumonia, gastrointestinal disease, retinitis and encephalitis are common manifestations of disease. HCMV infection is a particular concern among individuals infected with HIV.

Virus-encoded enzymes as drug targets

Enzymes play an essential role in the metabolism of all living organisms as they accelerate the rates of specific chemical reactions. A virus, whose replication is dependent on living cells, commonly utilises enzymes of the host cell but also its own enzymes. The genome of a virus therefore codes not only for proteins needed to package its genetic material but also for enzymes needed by the virus to reproduce during the infective cycle. Since these virus-encoded enzymes are specific for the virus and the infected cell and may be essential for virus replication, they are attractive targets for infectious disease therapies (De Clercq, 2001; Copeland *et al.*, 2002). This approach is likely to yield more specific, less toxic compounds, with a narrow spectrum of antiviral activity, but also with a higher likelihood of resistance develop-

ment (De Clercq, 2002; Provencher *et al.*, 2004). In contrast to the evolution in other fields, the development of antiviral chemotherapy has been slow and it was not until intense pressure was placed on research and industry to deal with HIV that a wide range of new antiviral drugs have become available during the past two decades (Herdewijn *et al.*, 2002). There are currently almost 40 antiviral compounds officially approved for clinical use, most of which inhibit enzymatic reactions (De Clercq, 2004a). At least half of them are used for the treatment of HIV, targeting the HIV protease, the HIV reverse transcriptase or the viral entry process. Four antiviral drugs target the polymerase of the HCMV and one blocks translation.

Despite increasing concern about toxicities and resistance, the use of these drugs has resulted in a dramatic decrease in the number of AIDS-related deaths in patients fortunate enough to have access to the medications. However, this possibility is beyond the reach of the majority of people living with HIV infection throughout the world, an ethical issue that should be of great concern and encourage everyone to find solutions to overcome this problem.

Two viral enzymes have been in the focus of the present work, the HCMV protease and the HIV-1 reverse transcriptase. This thesis deals with the binding mechanism of ligand molecules to these enzymes, and the new knowledge accumulated herein might contribute to the rational design and development of new drugs against the associated diseases.

The human cytomegalovirus protease

Like all viruses of the herpes family, the HCMV encodes a protease essential for viral capsid formation and viral replication (Preston *et al.*, 1983; Liu *et al.*, 1991; Gao *et al.*, 1994; Matusick-Kumar *et al.*, 1995; Welch *et al.*, 1993; Gibson *et al.*, 1997). This protease is synthesised as a 708 amino acid precursor protein that undergoes autoproteolytic processing during viral assembly (Welch *et al.*, 1991). The precursor consists of the 28 kDa catalytic domain of the protease at its N-terminal end, also referred to as assemblin, joined by an 8 kDa linker domain to a 38 kDa assembly protein precursor which is necessary for the process of virus assembly and also transcribed separately (Welch *et al.*, 1991). The mature protease domain is released from the precursor by sequential cleavages at two sites. The protease is subsequently cleaved at the internal I site, which inactivates it (Baum *et al.*, 1993).

The HCMV protease is a serine protease and cleaves specifically between an alanine and a serine (Waxman *et al.*, 2000). Determination of the structure showed that its fold and active site is different from other non-herpes serine proteases (Tong *et al.*, 1996; Qui *et al.*, 1996; Shieh *et al.*, 1996; Chen *et al.*, 1996). Unlike classical serine proteases, such as chymotrypsin, which have two distinct β -barrel domains, the structure of the HCMV protease has a single domain (Figure 3). The active site consists of a catalytic triad with a

serine (Ser-132) and two histidines (His-63 and His-157), but His-63 is not absolutely required for catalysis (Shieh *et al.*, 1996). The catalytic machinery for activating the serine nucleophile is oriented similarly to that of other serine proteases, which is remarkable, considering the absence of significant homology in amino acid sequence and three-dimensional structure. Non-herpes serine proteases commonly have an aspartate residue as the third amino acid instead of the His, and usually have a much higher catalytic efficiency than the herpes proteases (Liang *et al.*, 1998; Margosiak *et al.*, 1996).

To gain full catalytic activity, the HCMV protease requires formation of a homodimer (Darke *et al.*, 1996; Margosiak *et al.*, 1996; Holwerda, 1999), most likely due to a stabilisation of the oxyanion hole in the active site (Batra *et al.*, 2001; De Oliveira *et al.*, 2003). The symmetry positions the two separate catalytic sites on opposite faces of the dimer. Also different from other serine proteases, the HCMV protease has an induced fit mechanism and undergoes large conformational changes upon complexation with a peptide substrate or a peptidomimetic inhibitor (Bonneau *et al.*, 1997; LaPlante *et al.*, 1999; Tong *et al.*, 1998; De Oliveira *et al.*, 2003).

Inspired by the effectiveness of inhibitors against the HIV protease (Patick *et al.*, 1998), the HCMV protease was also suggested to be a potential target for antiviral drug development (Waxman *et al.*, 2000; Holwerda, 1997; Martinez *et al.*, 2001), and extensive efforts have been put into biochemical, enzymatic and structural studies of this enzyme. However, inhibitors based on the peptide substrates have shown only modest inhibitory effect against the HCMV protease, possibly due to the shallowness of the substrate-binding pocket and the induced-fit mechanism (LaFemina *et al.*, 1996; Bonneau *et al.*, 1997; Ogilvie *et al.*, 1997). More efficient approaches involved mechanism-based inhibitors, chemically reactive with the catalytic serine Ser-132 (Borthwick *et al.*, 1998, 2003; Déziel *et al.*, 1998; Yoakim *et al.*, 1998; Ogilvie *et al.*, 1999; Jarvest *et al.*, 1999; Smith *et al.*, 1999) or two surface cysteines, Cys-161 (Pinto *et al.*, 1999) and Cys-202 (Ertl *et al.*, 1999). Also, inhibitors with redox properties (Baum *et al.*, 1996a, 1996b; Di Grandi *et al.*, 2003) or as yet unknown mechanism (Dhanak *et al.*, 1998, 2000; Guo *et al.*, 2000; Qian-Cutrone *et al.*, 1998; Matsumoto *et al.*, 2001; Gopalsamy *et al.*, 2004) were reported. Despite these many approaches to inhibit the HCMV protease, there are presently no drugs available for clinical use.

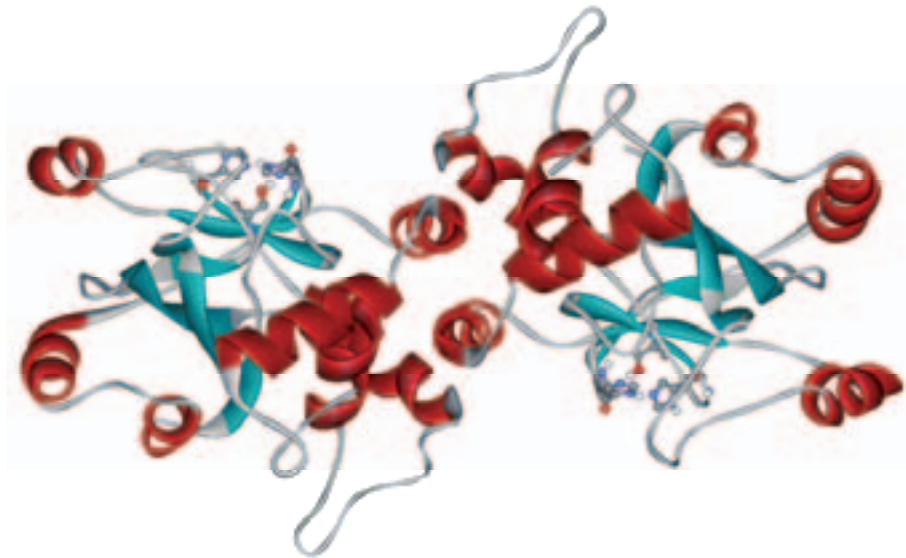


Figure 3. Three-dimensional structure of the HCMV protease dimer (PDB entry 1CMV). The single domain monomer consists of a seven-stranded β -barrel core (blue) surrounded by a chain of α -helices (red). Active site residues are depicted in ball and stick.

The reaction mechanisms of serine proteases

Serine proteases hydrolyse peptides and synthetic ester substrates by an acylenzyme mechanism (Fersht, 1999). Substrate (S) and enzyme (E) first form a non-covalent enzyme-substrate complex (ES), so called Michaelis-complex (Figure 4). Hydrolysis then occurs through a covalent tetrahedral intermediate resulting from the attack of the hydroxyl of the active site serine on the carbonyl carbon of the substrate. The resulting oxyanion is stabilised through formation of strong hydrogen bonds with the amide groups of the enzyme. The intermediate then collapses to give the acylenzyme (EP_2), releasing the amide or alcohol product (P_1). The acylenzyme then hydrolyses via a second tetrahedral intermediate to release the acid (P_2).

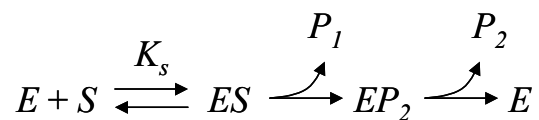


Figure 4. Reaction scheme of serine proteases.

The HIV-1 reverse transcriptase (HIV-1 RT)

As an essential event in the life cycle of HIV-1, the RT catalyses the conversion of genomic viral RNA into double-stranded DNA, which is subsequently integrated into a host cell genome by an integrase enzyme (Goff, 1990). This complex process is accomplished by the polymerase function of the RT, which synthesises DNA on both RNA and DNA templates, and by the ribonuclease H (RNase H) part, which performs a hydrolytic cleavage of the RNA component of intermediate RNA/DNA reverse transcription hybrids.

The HIV-1 RT is a heterodimer, composed of a 66 kDa subunit and its truncated N-coterminal 51 kDa form, designated as p66 and p51, respectively. The crystal structure of HIV-1 RT resembles a human right hand, and it reveals that both subunits each contain four common subdomains, termed the fingers, palm, thumb and connection (Kohlstaedt *et al.*, 1992) (Figure 5).

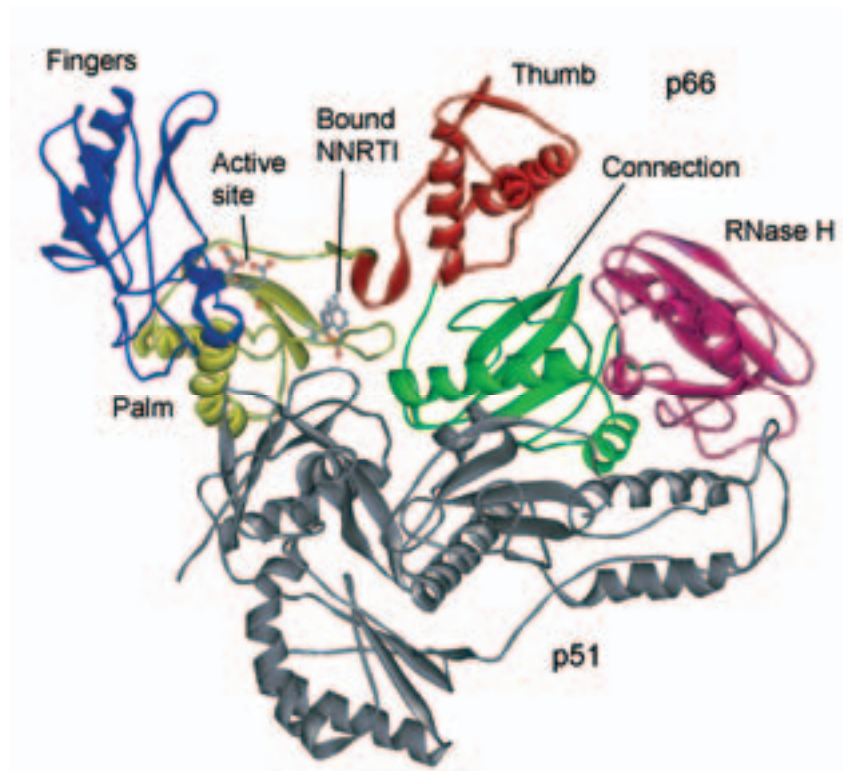


Figure 5. Structure of the HIV-1 reverse transcriptase in complex with the non-nucleoside inhibitor efavirenz (coordinates are from PDB entry 1IKW). The subdomains are indicated in the p66 subunit.

The p66 subunit has an extra C-terminal domain spanning the last 120 residues, which provides the RNase H activity. The overall folding of the

subdomains is similar in p66 and p51, but their spatial arrangements differ markedly. The p66 subunit has a large nucleic acid-binding cleft formed by the fingers-, palm- and thumb subdomain (Jacobo-Molina *et al.*, 1993). The polymerase active site of the enzyme resides within the p66 palm domain and contains the catalytic aspartic acid residues, Asp-110, Asp-185, and Asp-186 (Figure 5).

Reverse transcription is initiated from a cell-derived tRNA^{Lys3} primer molecule annealed to the viral RNA template (Mak *et al.*, 1997). After incorporation of a nucleotide into the RT-primer/template complex, an isomerisation is induced leading to a tight ternary complex, which is the rate-limiting step of nucleotide incorporation (Kati *et al.*, 1992; Rittinger *et al.*, 1995). Thumb movements then translocate the growing strand for subsequent nucleotide incorporations. The RNase H activity removes the original RNA template from the DNA/RNA hybrid, allowing the synthesis of the complementary DNA strand. Due to the lack of a proofreading function the process of reverse transcription is error prone, causing the high degree of genetic variability of HIV and a fast emergence of drug-resistant variants (Menéndez-Arias, 2002a, 2002b).

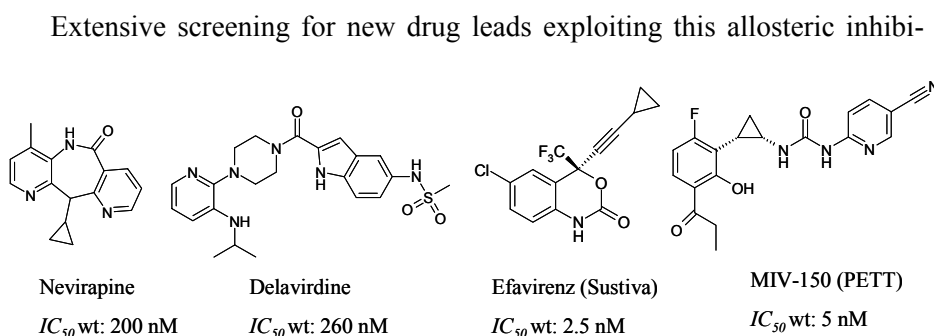
Since the polymerases of other viruses had previously been used for development of antiviral drugs, the potential of the reverse transcriptase as a target for anti-AIDS drugs was realised at an early stage (Herdewijn *et al.*, 2002). The enzyme has a broader substrate specificity than cellular DNA polymerases, which explains why the first drugs to be approved for the treatment of HIV infection were nucleoside analogues, so called NRTIs, causing chain termination upon being incorporated (Painter *et al.*, 2004). Today, the HIV-1 reverse transcriptase is still the target for most of the anti-HIV drugs currently used in chemotherapy (De Clercq, 2004a).

NNRTIs

The first non-nucleoside reverse transcriptase inhibitors (NNRTIs) were discovered by random screenings of compound libraries (Pauwels *et al.*, 1990; Merluzzi *et al.*, 1990). These relatively small inhibitor molecules were highly specific for the HIV-1 RT, and since they were found to act non-competitively with respect to substrates it was speculated that they bound to an allosteric site. This was confirmed by resolving the structure of HIV-1 RT in complex with NNRTIs using x-ray crystallography (Kohlstaedt *et al.*, 1992; Esnouf *et al.*, 1995, 1997; Chan *et al.*, 2001; Ding *et al.*, 1995; Hopkins *et al.*, 1996; Hsiou *et al.*, 1996, 1998, 2001; Das *et al.*, 1996, 2004; Ren *et al.*, 1995a, 1995b, 1998, 1999, 2000a, 2000b, 2000c, 2001, 2004; Lindberg *et al.*, 2002). NNRTIs were found to bind to a cavity – called the non-nucleoside inhibitor-binding pocket (NNIBP) - located between the β 6- β 10- β 9 and β 12- β 13- β 14 sheets of the p66 subunit and close to the active site (Figure 5). The NNIBP is unique to the HIV-1 RT and is not found in

other RTs or DNA polymerases. Neither is the cavity visible in the structure of the unliganded HIV-1 RT (Esnouf *et al.*, 1995; Rodgers *et al.*, 1995; Hsiou *et al.*, 1996). It is predominantly composed of hydrophobic residues (residues 98-108, 179-190, 229, 318) in the p66 subunit, but a small portion of the NNIBP is also from the p51 subunit (Thr-139). Besides favorable hydrophobic interactions with several residues, most NNRTIs form a hydrogen bond to Lys-101, which might be water-mediated. Comparison with structures of unliganded HIV-1 RT and in complex with template/primer (Ding *et al.*, 1998; Jacobo-Molina *et al.*, 1993) revealed that NNRTIs binding is accompanied by large rearrangements, mainly in the position of the thumb domain. Assuming a closed conformation in the unliganded enzyme, the thumb is locked in an open position in the NNRTI complex, which is extended beyond the upright configuration seen in the HIV-1 RT-DNA structure (Rogers *et al.*, 1995; Hsiou *et al.*, 1996). The flexibility of the thumb domain is of importance for the recognition of nucleic acid substrates and the translocation of the growing DNA strand during polymerisation. Moreover, the β 12- β 14 sheet of the p66 palm subdomain is deformed, which affects the precise positioning of the primer strand relative to the polymerase active site. Inhibitor binding also alters the position of the catalytic aspartates relative to the rest of the active site (Kohlstaedt *et al.*, 1992; Esnouf *et al.*, 1995). Pre-steady-state kinetic experiments have shown that non-nucleoside inhibitors block the chemical reaction and interfere with the binding of nucleotide, or the nucleotide-induced conformational change (Spence *et al.*, 1995). Molecular dynamics simulations support the hypothesis that NNRTIs inhibit the HIV-RT by enlarging the DNA-binding cleft and either restricting the mobility or altering the direction of motions of the p66 thumb domain (Bahar *et al.*, 1999; Shen *et al.*, 2003; Temiz *et al.*, 2002; Sluis-Cremer *et al.*, 2004). These different inhibition mechanisms are not exclusive of each other and may differ among different classes of NNRTIs.

Figure 6. Chemical structure of NNRTIs.



tion mechanism has identified more than 30 structurally different classes of compounds (De Clercq, 1999, 2004b, 2005; Balzarini, 2004; Pauwels, 2004; Campiani *et al.*, 2002; Buckheit, 2001; De Corte, 2005). Among these are

the phenylethylthioazolythiourea (PETT) analogues (Figure 6), which are potent inhibitors of the HIV-1 RT with favorable resistance profiles (Åhgren *et al.*, 1995; Högberg *et al.*, 1999). Nevertheless, only three NNRTIs, nevirapine, delavirdine and efavirenz (Figure 6) have so far been formally licensed for clinical use in the treatment of HIV-1 infections (De Clercq, 2004a), and are commonly used in combination with HIV protease inhibitors and NRTIs in highly active anti-retroviral therapy (HAART) (Moyle, 2001; De Clercq, 1999, 2004b). Inhibition constants of the most potent NNRTIs are in the low nanomolar range, which is surprising, given the relative weakness of the individual binding forces, in particular the absence of strong ionic interactions (Tronchet *et al.*, 2003).

NNRTI Resistance

Although NNRTIs are initially quite effective in reducing the viral load in HIV-1 infected individuals, treatment with these drugs rapidly selects for mutations because of a pre-existing population of mutant viruses within an individual (Conway *et al.*, 2001). Substitution of a single amino acid in the NNIBP can result in high-level resistance, causing an inevitable rebounding of the viral burden despite continued therapy (Johnson *et al.*, 2004; Cheung *et al.*, 2004; Schinazi *et al.* 2000). Structurally different NNRTIs elicit different spectra of resistance mutations because of differences in the size, shape and the specific amino acids they contact (Figure 7).

	L	K	V	V	Y	Y	G	
	100	103	106	108	181	188	190	
Nevirapine	I	N	A/M	I	C/I	C/L/H	A	
		K	V		Y	Y		P
		103	106		181	188		236
Delavirdine		N	M		C	L		L
	L	K	V	V	Y	Y	G	P
	100	103	106	108	181	188	190	225
Efavirenz	I	N	M	I	C/I	L	S/A	H

Figure 7. Amino acid substitutions in the HIV-1 RT associated with resistance against the three NNRTIs in clinical use.

However, there is considerable overlap in the mutations selected by different NNRTIs and, as a consequence, considerable cross-resistance (Deeks, 2001). The most common mutations found in patients receiving NNRTI-containing chemotherapy are K103N and Y181C, causing a two-fold to five hundred-fold loss of activity of the current inhibitors (De Clercq, 1999, 2004b). Some NNRTI resistance mutations may also compromise viral replication (Domaoal *et al.*, 2004), either by interfering with nucleoside binding (Kleim *et al.*, 1994) or by impairing the RNase H activity (Archer *et al.*, 2000).

Present investigations

Aims

The scientific objective of the present work was to characterise the interaction between viral enzymes and ligand molecules, with focus on applications to drug discovery and development. In particular, this included:

- Establishing a biosensor assay for ligand interaction studies with human cytomegalovirus protease and exploring the process of substrate binding to serine proteases.
- Resolving the binding mechanism of HIV-1 reverse transcriptase and non-nucleoside inhibitors by investigating kinetic, dynamic and thermodynamic aspects of the interaction using biosensor technology.
- Elucidating the mechanism of HIV-1 reverse transcriptase resistance towards non-nucleoside inhibitors.

Methodology

Recombinant viral enzymes

HCMV protease (Paper I)

E. coli (strain BL21, DE3) was used for expression of the HCMV protease gene, strain AD169, inserted into the expression vector pET11a. The gene included a mutation, A143V, to prevent autocleavage and loss of activity. During the expression, the enzyme formed inclusion bodies which were solubilised in 7 M urea. The enzyme was purified by a two-step chromatographic procedure utilising anion exchange in urea and DTT and size exclusion in 2 M NaSCN and DTT. Activation of the enzyme was accomplished by dialysis against urea followed by removal of urea and addition of 50 % glycerol. The enzyme activity was assayed with an internally cleaved

fluorogenic peptide substrate (Holskin *et al.*, 1995), which was also used for interaction studies:

DABCYL-Arg-Gly-Val-Val-Ala-↓-Ser-Ser-Arg-Leu-Ala-EDANS

HIV-1 reverse transcriptase (Paper II, III, IV)

The p66 subunit of the HIV-1 RT (BH10 isolate) was also expressed in *E. coli* using expression vector pET11a. Some of the described enzyme variants had an additional E478Q substitution in order to extinguish the RNase H activity. Soluble p66/p66 homodimers from the cell lysate were cleaved by chymotrypsin to give p66/p51 heterodimers. These were then purified by a three-step chromatographic procedure involving anion exchange, heparin affinity chromatography, and size exclusion.

Biosensor-based interaction analysis

The SPR biosensor

For the investigation of enzyme-ligand interactions a biosensor assay was developed based on the optical interface phenomenon surface plasmon resonance. Essentially, the SPR biosensor detects changes of the refractive index on the surface of a sensor chip caused by changes in mass (Homola, 2003). The sensor chip consists of a glass plate covered with a thin gold film to which a hydrophilic dextran matrix is covalently attached (Figure 8).

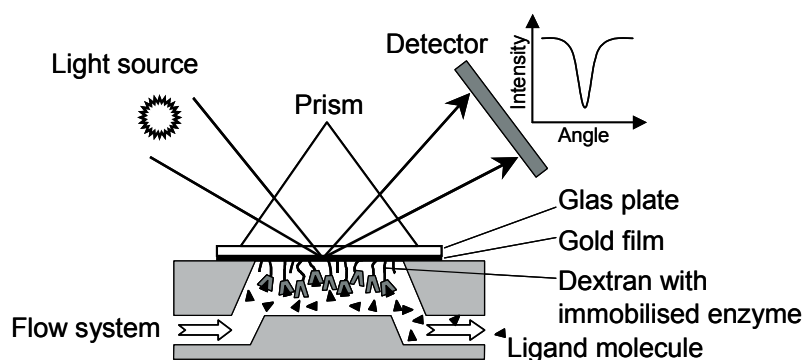


Figure 8. Schematic of an SPR biosensor.

The chip is connected to a microfluidic system that supplies the dextran surface with a constant flow of buffer and can address any reagents in solution to the chip surface via automated injections. The optical detection system operates from the opposite side of the chip and monitors the angle under which a light beam is absorbed from the gold/dextran interface instead of being reflected. This extinction of the light occurs due to the excitation of

collective oscillations of electrons parallel to the interface, a so-called plasma wave. Since the angle depends on electromagnetic parameters in the vicinity of both sides of the interface, changes in the refractive index at the dextran surface can be detected by this principle.

For interaction studies one of the binding partners is immobilised on the dextran surface. In the present experiments the HCMV protease and the HIV-1 reverse transcriptase, respectively, were covalently attached to a carboxylated dextran matrix by amine coupling. The other binding partner, here substrate or inhibitor, is injected through the flow system over the chip and accumulates closer to the surface upon interaction with the immobilised enzyme. For the majority of ligand molecules this accumulation leads to an increase in the refractive index at the sensor surface, and the SPR detector registers the progress of the interaction as a continuous change in the signal, which is reported in real time as sensorgrams. The amplitude of the signal is measured in resonance units (RU), where 1000 RU corresponds to approximately 1 ng/mm² protein. The kinetic constants of the interaction are calculated from the curvature of the sensorgrams during sample injection (association phase) and after sample injection (dissociation phase) (Figure 9). Since also conformational changes in the immobilised enzyme can cause changes in the SPR signal (Sota *et al.*, 1998; Mannen *et al.*, 2001; May *et al.*, 2002) this method can also be used to study dynamic aspects of biomolecular interactions.

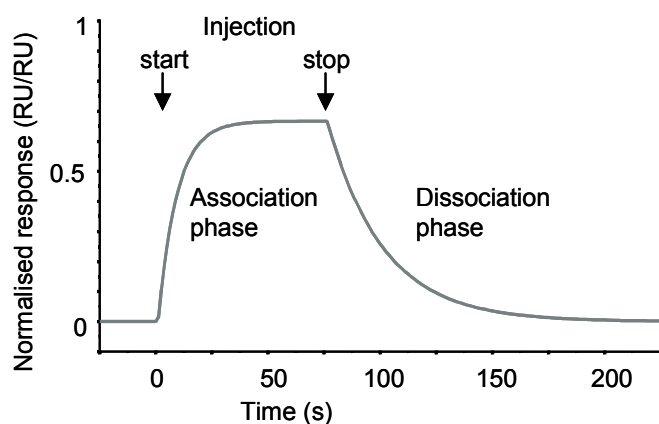


Figure 9. A typical sensorgram.

Experimental principles

A Biacore 2000 instrument (Biacore AB, Uppsala, Sweden) was used for the SPR biosensor measurements described in this thesis. The interaction experiments were performed on CM 5 sensor chips (Biacore) using a Hepes buffer, pH 7.4, with a supplement of 3 % DMSO as a running buffer in the

system. In order to ensure reliable and reproducible results, optimisation of the experimental parameters (flow rate, immobilisation level, system washing, regeneration, sample dispersion), control experiments (non-specific binding, immobilisation chemistry, solubility of ligands), quality controls (blank injections, replicates) and data processing (reference subtraction, blank subtraction) were conducted essentially as described previously (Myszka, 1999; Markgren, 2000; Shuman, 2003). The data was analysed using the BIAevaluation program (Biacore), which allows calculation of kinetic parameters by global fitting of data sets, either to the association phase (Paper I) or simultaneously to association and dissociation phases (Paper II-IV).

Key experiments

In order to resolve the kinetics of interactions with higher complexity than a simple 1:1 binding between enzyme and ligand, several parameters in the experimental arrangement were varied.

Ligand concentration (Paper I, II, III, IV)

Pseudo-first order rate constants in the interaction are dependent on the ligand concentration. The ligand was therefore typically injected in series of different concentrations. Simultaneous analysis of the obtained sensorgrams gave a more sound estimation of the kinetic parameters. The effect of varied ligand concentration was also important for the discrimination between different interaction models.

Contact time (Paper I, II)

The progress of the ligand association was interrupted at different times followed by detection of the dissociation phase. This approach was used to identify parallel binding processes and to investigate the validity of various interaction models for the data analysis.

Chemical environment (Paper I, II, III)

The flow system of the biosensor method provides the possibility of changing the chemical environment on the chip surface by defined injections or by changing the running buffer in the system. In Paper I this was used to study conformational changes in the immobilised enzyme by pH changes. In control experiments for Paper II the effect of the DMSO concentration in the running buffer was tested. Also, the impact of several reagents on the dissociation of NNRTIs was evaluated in order to find suitable regeneration conditions (Paper II and III).

Temperature (Paper IV)

Rate constants are dependent on the temperature (see Equation 9). Contributions of enthalpy and entropy to the free energy change of biomolecular in-

teractions can be resolved by a van't Hoff analysis. The biosensor can be equilibrated at different temperatures, which was exploited to determine thermodynamic parameters of the interaction between the HIV-1 RT and an NNRTI.

Crosslinking (Paper I, II)

The immobilised enzyme was modified by introduction of intermolecular and intramolecular chemical cross-links, which allowed conclusions about the role of enzyme flexibility in the studied interactions.

Activity (Paper I)

The catalytic properties of the immobilised enzyme could be studied by incubation of reaction components on the chip surface. This involved manual handling of injections and utilisation of the whole chip surface. The amount of immobilised enzyme on the chip could be quantified by SPR, which allowed a detailed kinetic analysis of the reaction.

Results and discussion

Ligand interactions with HCMV protease

Substrate binding of serine proteases (Paper I)

Details of the interaction between HCMV protease and a peptide substrate were studied using a biosensor, and the results were compared with the kinetic parameters determined by an activity assay. Incubation of the substrate directly on the chip surface revealed that the immobilised HCMV protease was proteolytically active, and the quantification of bound enzyme allowed determination of the steady state parameters K_m and k_{cat} of the reaction. Surprisingly, the K_m was found to be lower for the immobilised HCMV protease than for the free enzyme in solution (Table 1). However, it was larger than predicted by direct interaction analysis, which provided an estimate of the dissociation constant of the Michaelis complex, K_s , by fitting Equation 8 to the initial phase of the interaction sensorgrams.

Table 1. Kinetic constants for immobilised serine proteases.

	K_m solution (μ M)	K_m immobilised (μ M)	K_s (μ M)
HCMV protease	159 ± 10	83 ± 38	52 ± 18
α -Chymotrypsin	22.2 ± 0.7	31 ± 7.8	12.4 ± 3.6

Apparently, K_m , which is based on the enzyme activity and determined using Michaelis-Menten kinetics, does not reflect the apparent dissociation constant defined by

$$\frac{[E] \cdot [S]}{\sum [ES]} = K_m$$

where $[S]$ is the substrate concentration, $[E]$ is the amount of free enzyme and $\sum [ES]$ is the sum of all the bound enzyme species (Fersht, 1999). Similar results were obtained with another representative of the serine protease family, α -chymotrypsin, which was active towards the same peptide substrate and was used as a reference protease in the experiments. This discrepancy can be explained either by a substrate-induced activation of the enzyme, resulting in excessive estimates for K_m in the activity assay, or by the formation of non-productive enzyme-substrate complexes with a lower K_s than the correct Michaelis complex. It is also possible that structural rearrangements in the enzyme amplified the SPR binding signal giving wrong estimates of K_s . This was investigated further as described below.

Substrate-induced conformational changes (Paper I)

The recorded sensorgrams of the interaction between HCMV protease and the peptide substrate were clearly biphasic and composed of a fast changing signal and a slow changing signal (Figure 10A).

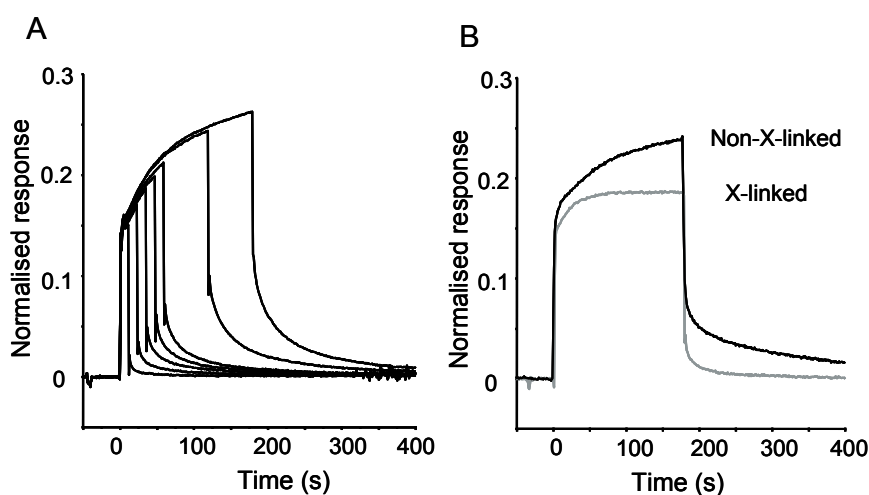


Figure 10. Sensorgrams of the interaction between immobilised HCMV protease and a peptide substrate. A) Different contact times. B) Comparison between cross-linked (grey) enzyme and non-cross-linked enzyme (black).

Due to the slow signal the experimental sensorgrams did not match simulated sensorgrams based on the estimated rate constants of the reaction. In order to explore the contribution of conformational changes to the observed signal the immobilised enzyme was cross-linked. This effectively reduced the enzyme's flexibility, as could be shown by eliciting structural alterations in the enzyme through pH changes. Cross-linking also reduced the amplitude of the slow SPR signal upon interaction with the substrate, but had no effect on the fast signal (Figure 10B). It was concluded that substrate binding induces a slow structural reorganisation of the HCMV protease. However, the conformational change does not noticeably alter the substrate-binding properties of the enzyme, as judged from the progress of sensorgrams with different contact times (Figure 10A). These findings supported earlier reports on the flexible structure of the HCMV protease, based on structural and computational studies (Tong *et al.*, 1998; De Oliveira *et al.*, 2003). Studies with peptidomimetic inhibitors have also suggested an induced-fit mechanism for substrate catalysis (Bonneau *et al.*, 1997; LaPlante *et al.*, 1999). Structural flexibility was indeed found to be crucial for the enzyme, as shown by the inactivation of the cross-linked HCMV protease despite little effect on the capacity to bind the substrate.

A tool for characterising the binding mechanism of inhibitors

The developed biosensor assay for the HCMV protease allowed interaction studies with inhibitor molecules, which can be applied for compound screening, kinetic analysis of binders and characterisation of binding mechanisms.

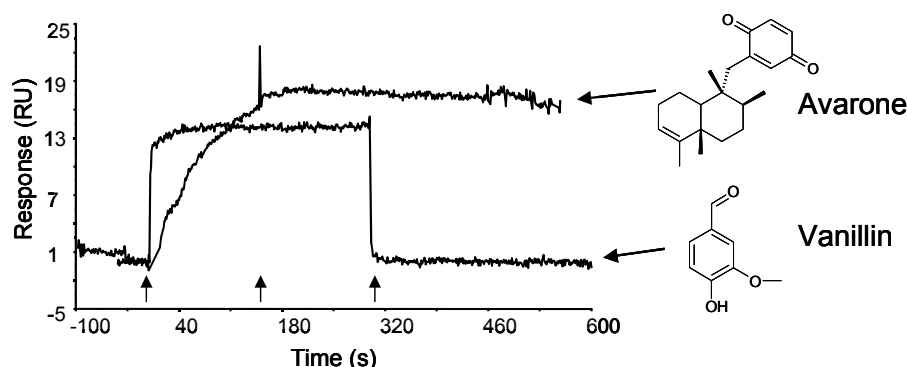


Figure 11. Sensorgrams of the interaction between HCMV protease and inhibitors (4.7 $\mu\text{g/ml}$ avarone; 1.88 mM vanillin).

Previous attempts to design potent inhibitors based on peptide substrates have not been successful, which was blamed partially on the enzymes's induced-fit mechanism (LaPlante *et al.*, 1999). Alternative inhibition mechanisms have therefore been in the focus of drug discovery approaches (Flynn

et al., 1997; Martinez *et al.*, 2001). Within the framework of this thesis two compounds were obtained from natural sources, vanillin and avarone (Figure 11), which inhibited the HCMV protease in the micromolar and sub-micromolar range, respectively (unpublished data). Interaction analysis with the biosensor (Figure 11) revealed that vanillin is a reversible binder, but does not elicit conformational changes as observed with the peptide substrate. Avarone was found to bind irreversibly to the HCMV protease with a 1:1 stoichiometry, most likely by arylation of one of the cysteine residues (Belisario *et al.*, 1994), as reported for related compounds (Ertl *et al.*, 1999). Binding of this inactivator to the HCMV protease did not affect substrate binding.

The binding mechanism of NNRTIs

Finding the right model (Paper II)

Since the SPR biosensor only produces an overall response signal, which is composed of all events that contribute to changes in the refractive index, the interpretation of the resulting sensorgrams is often challenging. The observation that the recorded binding signals for the interaction between immobilised HIV-1 RT and non-nucleoside inhibitors deviated from a simple 1:1 binding prompted the search for an interaction model that enabled a qualitative and quantitative description of the binding process.

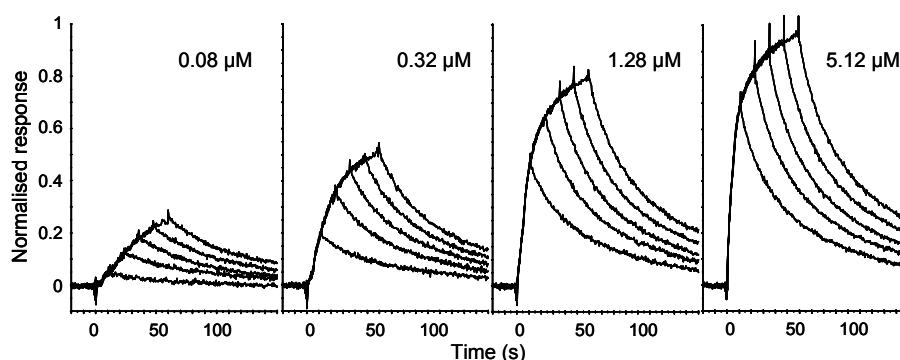


Figure 12. Overlay of sensorgrams of the interaction between HIV-1 reverse transcriptase (mutant K103N) and inhibitor (MIV-150) at various concentrations and contact times.

An initial study (Paper II) comprised four structurally different compounds (nevirapine, delavirdine, efavirenz and MIV-150) (Figure 6) and an NNRTI-resistant variant of the HIV-1 RT (K103N). This substitution resulted in faster dissociation rates for the inhibitors than for the wild type

enzyme, and therefore provided an effective regeneration of the biosensor surface for serial binding experiments. The evaluation of several interaction models was based on global analysis of recorded sensorgrams with various inhibitor concentrations and contact times (Figure 12). The tested inhibitors interacted with the enzyme with varying degrees of complexity. Whereas nevirapine binding, characterised by fast dissociation, could be described sufficiently by a simple 1:1 binding model (Figure 13A), proper fitting to the sensorgrams with delavirdine required two exponentials, assuming a model of non-competitive parallel interactions (Figure 13B). For efavirenz and MIV-150 an additional step had to be included in the model according to Figure 13C, suggesting a pre-equilibrium between two forms of the enzyme, a binding-competent (E_R) and a non-binding-competent form (E_T). Since the rate of conversion between the two forms is rate-limiting for the binding of inhibitor, the rates of the pre-equilibrium can be obtained from the analysis of the interaction data.

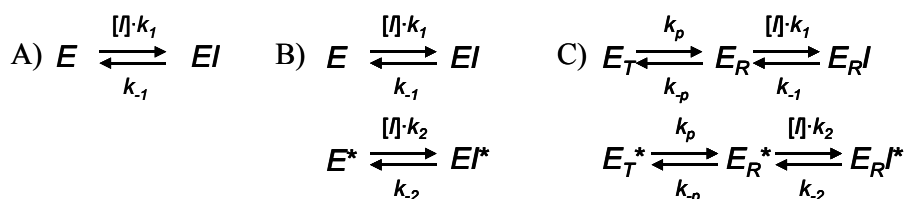


Figure 13. Interaction schemes used for the analysis of NNRTI binding. A) 1:1 interaction B) Non-competitive parallel interactions C) Non-competitive parallel interactions with pre-equilibrium.

An extended study with structurally diverse inhibitors (Paper III) supported the use of the binding model depicted in Figure 13C, being the simplest adequate model that could describe all interactions. As a result, the analysis provided rate constants for the pre-equilibrium (k_p , k_{-p}), as well as association and dissociation rate constants for a high-affinity interaction (k_1 , k_{-1}) and a low-affinity interaction (k_2 , k_{-2}). A simplified model, describing a single affinity binding with a pre-equilibrium, was sometimes applied in order to obtain stable fittings (Paper III).

Implications from the interaction model

Heterogeneity of the interaction

The recorded sensorgrams indicated the formation of multiple and kinetically distinct complexes between HIV-1 RT and most of the tested inhibitors (Paper II, III). This was supported by resolving the thermodynamic parameters of the observed high- and low-affinity interactions (Paper IV). A simple explanation is that the enzyme is structurally heterogeneous, possibly as a

consequence of immobilisation via random amino acids on the enzyme surface. Although the corresponding model, describing the formation of parallel and uncompetitive complexes, applied best to the binding data, it cannot be excluded that the observed signal reflects a sequential formation of distinct complexes in the binding process. As observed in sensorgrams with long contact times (Paper II), the binding response was interfered with by another process, a slow increase of the signal, which was not considered in the analysis and might have influenced the discrimination between different interaction models. The slow signal was interpreted to originate from structural rearrangements in the enzyme induced by inhibitor binding, since the total binding response exceeded the predicted 1:1 stoichiometry.

Different conformational states

The use of the two-state mechanism (Figure 13C) is reasonable, considering the fact that the NNIBP does not exist as such in the unliganded HIV-1 RT (Esnouf *et al.*, 1995; Rodgers *et al.*, 1995; Hsiou *et al.*, 1996), and the enzyme thus has to undergo a structural alteration before the inhibitor can bind. Therefore, only a pre-existing open conformation can rationalise the binding of NNRTIs to the HIV-1 RT, which suggests the existence of a dynamic equilibrium between different conformational states of the free enzyme, E_R and E_T . Inhibitor binding then shifts the equilibrium towards the liganded, and consequently inactive, form of the enzyme. Measurements of tryptophan fluorescence quenching upon inhibitor binding using stopped-flow spectroscopy supported the conformational shift, but the observed changes were faster than predicted by the biosensor (Paper II).

A bottleneck for the binding and unbinding of inhibitor

The pre-formation of the E_R state imposes an energy barrier for both the binding and the unbinding of the inhibitor, which can be visualised in an energy profile for the binding process (Figure 14). One can describe the mechanism of NNRTI binding even more pictorially with a cork being pressed through the bottleneck into an empty bottle. The more narrow the bottleneck the harder it is to press the cork in, and the less unlikely it is to get it out again. In fact, the bound inhibitor is buried deep in the NNIBP with little access to the solvent (see Figure 16B). It is indeed difficult to remove the inhibitor from the enzyme and most regeneration approaches, including several organic solvents, failed (Paper II). Typically, the determined values for the pre-equilibrium rate constants k_p and k_{-p} ranged around 0.2 s^{-1} and 5 s^{-1} , respectively. The energy barrier was predominantly determined by the inhibitor, and the tested inhibitors showed different values for the determined pre-equilibrium rate constants. For example, delavirdine and TMC 125 revealed large differences in the extent of limitation of the binding by the pre-equilibrium. Despite the similar size and solubility of these compounds (Jorgensen, 2004), E_R was energetically unfavourable for TMC 125

(with K103N mutant: $k_{-p}/k_p = 24.7 \pm 12.5$), but apparently not for delavirdine (with K103N mutant: $k_{-p}/k_p = 0.0011 \pm 0.0004$). The pre-equilibrium was also temperature-dependent, showing decreased k_{-p}/k_p at lower temperatures (Paper IV). Moreover, certain amino acid substitutions associated with resistance were found to influence the pre-equilibrium (Paper III).

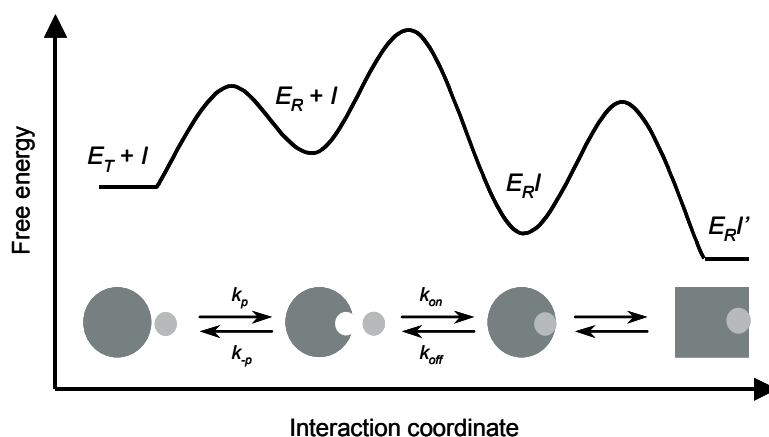


Figure 14. Energy profile of HIV-1 reverse transcriptase and NNRTI interaction. The inhibitor (I) binds only to one of the two free forms of the enzyme, E_R and E_T , representing an open and a closed conformation of the NNIBP. The formed complex $E_R I$ is assumed to undergo a structural rearrangement to give $E_R I'$.

The proposed interaction model is surely a simplification of the binding process, but it provides an explanation for why these inhibitor molecules bind so tightly despite the absence of strong electrostatic interactions. To further explore the nature of NNRTI binding and of the large impact these small molecules have on the HIV-1 RT, the forces that determine the interaction were investigated in the following.

Thermodynamic analysis of NNRTI binding

Determination of thermodynamic parameters (Paper IV)

A full characterisation of the interaction between HIV-1 RT and inhibitors requires determination of the thermodynamic parameters that describe the different forces involved in the complex formation. Experimentally, this was achieved by determination of the kinetic parameters at different temperatures, ranging from 5 to 35 °C, and using the PETT inhibitor MIV-150 (Figure 6) as a prototype inhibitor and two NNRTI-resistant variants of the HIV-1 RT, K103N and Y181C (Figure 16).

By combining Equations 9, 10 and 12 the obtained kinetic parameters could be converted into expressions of energy, providing the free energy changes for the association (ΔG_{on}), dissociation (ΔG_{off}) and at equilibrium

(ΔG). The free energy changes could be dissected into contributions of enthalpy (ΔH_{on} , ΔH_{off} , ΔH) and entropy (ΔS_{on} , ΔS_{off} , ΔS) by linear regression analysis using Equations 9 and 12. Alternatively, a non-linear regression analysis including a heat capacity change (ΔC_p) was performed, which accounted for non-linear tendencies in the van't Hoff plots. All parameters were determined separately for the high- and the low-affinity interaction of the heterogeneous binding.

The binding of MIV-150 is enthalpy-driven (Paper IV)

Generally, the binding of NNRTIs is dominated by hydrophobic interactions with various amino acid residues within the NNIBP. Like most NNRTIs, MIV-150 makes only a single hydrogen bond with Lys-101 (Ren *et al.*, 2000a). It was therefore surprising that for the binding of this compound to the HIV-1 RT the contribution of the enthalpy to the free energy change at equilibrium was found to be larger than that of the entropy (Figure 15A). However, both association and dissociation were governed by entropy terms that were larger than the gain or loss of enthalpy (Figure 15B, C).

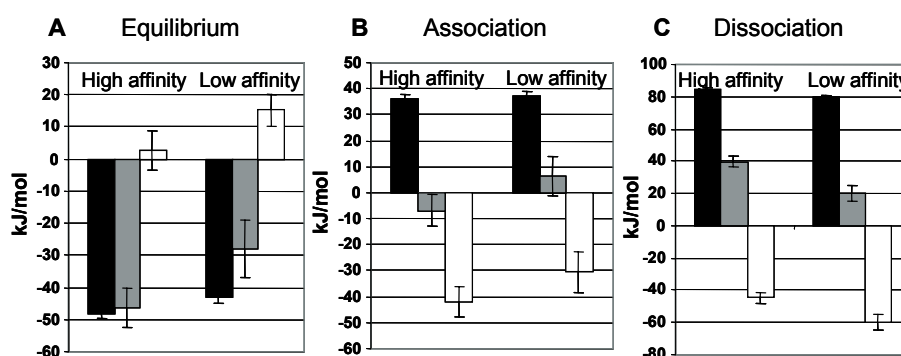


Figure 15. The contribution of changes in enthalpy (ΔH , grey bar), and entropy ($T\Delta S$, white bar) to the change in free energy (ΔG , black bar) for the high-affinity interaction (left) and low-affinity interaction (right) between MIV-150 and HIV-1 RT K103N at 25 °C, (A) equilibrium; (B) association; (C) dissociation.

It was concluded that the entropic solvent effect, which was expected to be rather positive for the association, was compensated by a dynamic effect of the enzyme. When binding to the HIV-1 RT, the inhibitor is suggested to constrain the enzyme in a state of decreased conformational flexibility. This is consistent with the reported rigidification of the otherwise flexible enzyme structure upon NNRTI binding, concerning mainly the thumb domain, which is sometimes referred to as “molecular arthritis theory” (Kohlstaedt *et al.* 1992; Bahar *et al.*, 1999; Temiz *et al.*, 2002; Shen *et al.*, 2003; Sluis-Cremer *et al.*, 2004). As a consequence, the entropy contributions of association and

dissociation partially cancelled out at equilibrium, and the net driving force was determined by the enthalpy change.

Apparent non-linearity in the van't Hoff plots revealed that, for the low-affinity interaction, the binding is entropy-driven at low temperatures. It can be speculated that the compensating dynamic effect of the enzyme, which accounts for the negative entropy upon inhibitor association, becomes less at low temperature, and therefore the inhibitor binding is rather governed by the increasing solvent effect of hydrophobic interactions.

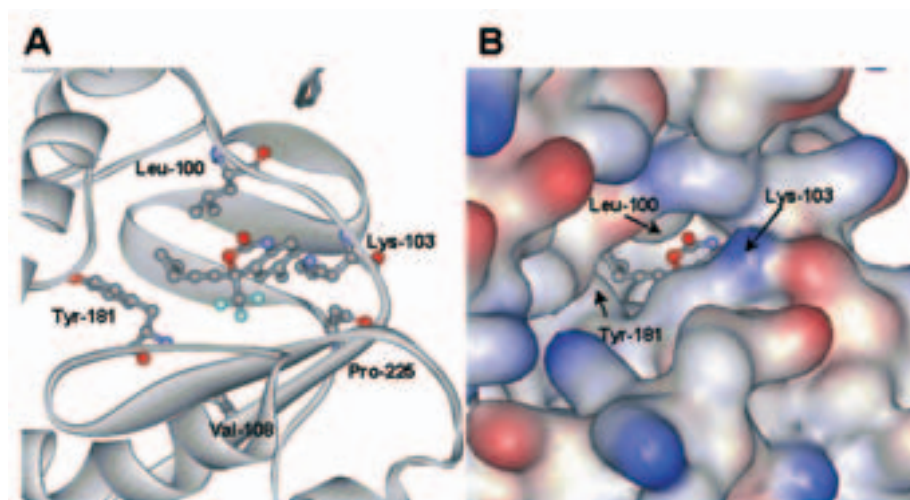


Figure 16. A) Position of the studied amino acid residues in the HIV-1 RT in complex with efavirenz. B) The protein surface is colored according to its electrostatic potential, red and blue for negatively and positively charged areas, respectively.

Resistance studies of HIV-1 RT and NNRTIs

A main contribution of the work presented in this thesis addresses the phenomenon of resistance against NNRTIs. Since the role in inhibitor binding of some amino acids prone to mutation is not obvious from crystal structures of HIV-1 RT, there has been a lot of speculation about the underlying mechanism, especially for the K103N substitution (Ren *et al.*, 1995a; Hsiou *et al.*, 2001; Lindberg *et al.*, 2002; Udier-Blagović *et al.*, 2004). The aim of the study described in Paper III was to find out which kinetic parameters of the interaction are affected by certain amino acid substitutions in the NNIBP. Another related question concerned the allosteric nature of the inhibitors: is the observed resistance an effect of decreased affinity or efficacy of the inhibitor? The present analysis was based on the interaction characteristics of a combination of 12 structurally diverse inhibitors (nevirapine, delavirdine, efavirenz, HBY 097, DPC 083, S-1153, TMC 125 and 5 PETT compounds) (Figure 6 and 19) with 7 enzyme variants, including the wild type and the

clinically relevant single mutants K103N, Y181C, L100I, and double mutants K103N/V108I, K103N/P225H, K103N/L100I (Figure 16).

Resistance substitutions interfere with different steps in the binding process (Paper III, IV)

The studied resistance mutations affected both the association and dissociation rates of the interaction between HIV-1 RT and NNRTIs, as shown for the inhibitors MIV-150 and efavirenz in Figure 17. For most inhibitors the effect on the dissociation was larger than on the association.

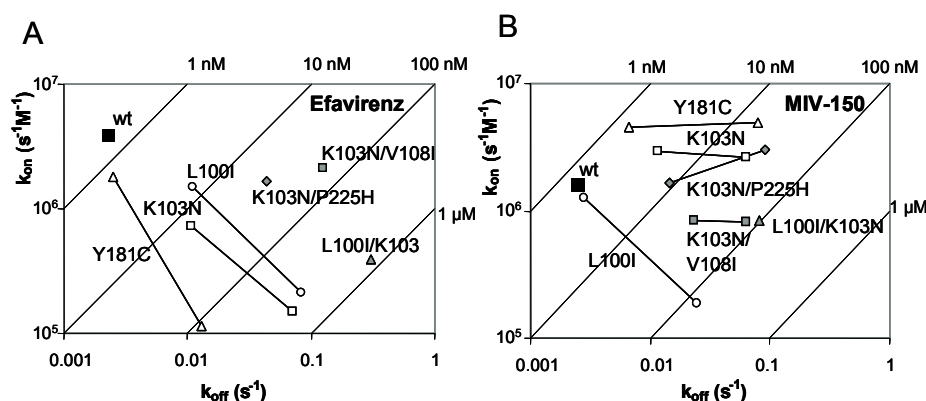


Figure 17. Interaction rate plot for the effect of mutations on the association and dissociation rates of efavirenz (A) and MIV-150 (B).

Upon comparing the obtained pre-equilibrium rate constants, the analysis revealed significant differences in the NNRTI resistance mechanism for different mutations. Although there were exceptions, it could be demonstrated that the substitutions Y181C, V108I and P225H affected primarily the association and dissociation rate constants of the interaction, whereas the K103N and the L100I substitution also influenced the pre-equilibrium between the two forms of the free enzyme, as schematised in Figure 18. It has been reported that an asparagine in position 103 forms a hydrogen bond with the side chain of Tyr-188, which does not exist in the wild type (Ren *et al.*, 1995a). It was thus hypothesised that the K103N substitution stabilises the closed conformation of the NNIBP, explaining its broad resistance against most classes of NNRTIs (Maga *et al.*, 1997; Hsiou *et al.*, 2001). In contrast, the present investigation revealed that this substitution, as well as the L100I, generally facilitates entry of an inhibitor by lowering the energy barrier imposed by the pre-formation of the binding competent enzyme E_R conformer. Positioned at the outer rim of the proposed NNIBP entrance (Figure 16B), Leu-100 and Lys-103 are suggested to play critical roles in an early step of the inhibitor binding process by steric interference (Shen *et al.*, 2003), and electrostatic interaction (Kroeger Smith *et al.*, 1995), respectively, thereby

forming a threshold at the NNIBP entrance. As a consequence of the facilitated inhibitor entry upon substitution of Lys-103 and Leu-100, also the dissociation increased, since the inhibitor has to take the same way back when unbinding from the enzyme.

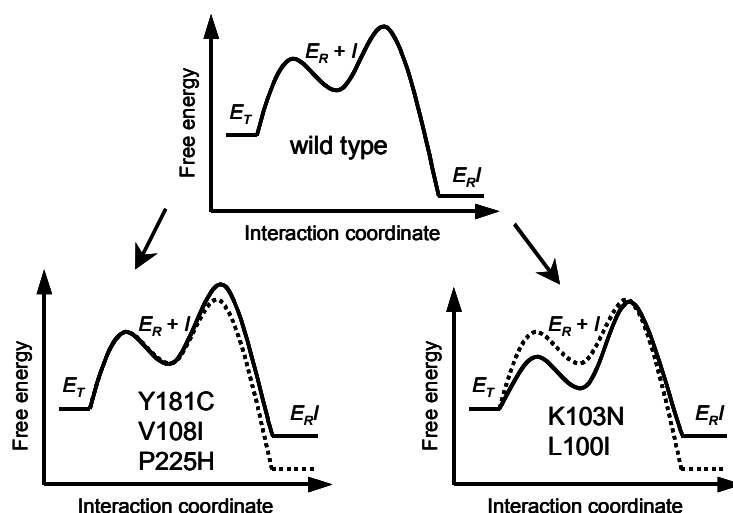


Figure 18. Effect of amino acid substitutions on the interaction between HIV-1 RT and NNRTIs.

The proposed difference in the resistance mechanism between K103N and Y181C was supported by a comparison of thermodynamic parameters in Paper IV. The higher affinity of MIV-150 for the Y181C mutant than for the K103N was demonstrated to originate primarily from a larger entropy contribution in the free energy change of the dissociation (Paper IV). Hence, the K103N variant assumes a less constrained conformation than the Y181C when the inhibitor is passing the bottleneck at the binding site entrance.

Decreasing the energy of the E_R state does not change the equilibrium between E_T and the $E_R I$ complex, and thus does not create resistance by itself. It can therefore be speculated that inhibition correlates rather to the dissociation rate of the compound than to an overall dissociation constant. Although a rational explanation has not been found for this, it would explain the large impact of these mutations on the activity of a broad range of inhibitors, despite the absence of excessive interaction in the determined structures.

Resistance profiles based on direct binding (Paper II-IV)

The present analysis generated resistance profiles for the three clinical inhibitors as well as several investigational compounds, some of which are currently in clinical trials and are promising candidates for becoming drugs in the near future (Tables 1, 2a and 2b in Paper III). The obtained resistance

profiles are unique in the sense that they are based on direct binding instead of the inhibitor's ability to impair the enzyme's activity, and thus reflect the affinity of the inhibitors rather than their efficacy. It has been reported that NNRTI binding does not completely extinguish the HIV-1 RT activity (Spence *et al.*, 1995), indicating that the relationship between binding and action might be complex for these allosteric inhibitors. A complete description of the interaction involves the rate constants for the pre-equilibrium between the non-binding and the binding competent form of the enzyme (k_p , k_{-p}), as well as association and dissociation rate constants (k_{on} , k_{off}). Provided that the efficacy is directly related to the affinity, the inhibition, determined as K_i , IC_{50} or ED_{50} , is suggested to correlate with an overall dissociation constant of the interaction according to

$$K_i, IC_{50}, ED_{50} \propto K_p \cdot K_D = \frac{k_{-p} \cdot k_{off}}{k_p \cdot k_{on}}$$

However, the observed heterogeneity of the interaction is expected to complicate this relationship. In general, resistance to a particular inhibitor, determined by enzyme activity assays or viral replication assays, correlated with decreased affinity data based on direct binding (Paper III). However, the reported values for inhibition vary a lot in the literature and apparently depend strongly on the assay conditions. A detailed analysis of the relationship between binding and inhibition therefore requires a more extensive data set and remains to be investigated.

Judged solely by their interaction characteristics, several of the tested investigational compounds showed improved properties when compared to the inhibitors in clinical use. Clearly, nevirapine (Figure 6) was the weakest binder to all enzyme variants, showing both low association rates and fast dissociation rates. DPC 083 (Cocuzza *et al.*, 2001) (Figure 19) showed generally lower dissociation rates than its analogue efavirenz. The PETT series (Högberg *et al.*, 1999) was characterised by high association rates, commonly $>10^6 \text{ s}^{-1}\text{M}^{-1}$, whereupon the thiourea PETT compound MSC-194 showed the slowest dissociation from all enzyme variants. HBY 097 (Hsiou *et al.*, 1998), S-1153 (Fujiwara *et al.*, 1998) and TMC 125 (Andries *et al.*, 2004) were resilient in particular toward the K103N mutant. TMC 125 binding was distinguished by low k_p values, and also slow dissociation rates with all studied resistance variants. Its resilience toward a broad spectrum of mutations associated with NNRTI resistance has been attributed to its structural flexibility and ability to adopt different conformations in the NNIBP (Das *et al.*, 2004). Based on the binding mechanism proposed herein, the present data suggest that this compound requires a high energy E_R state of the enzyme in order to bind and unbind. This might represent a rational concept for the design of new inhibitors with improved resistance profiles.

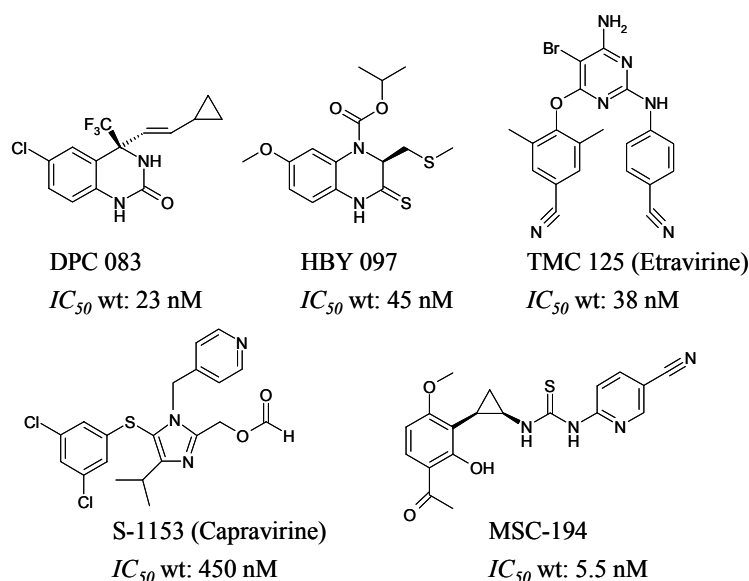


Figure 19. Chemical structure of NNRTIs

Conclusions and perspectives

What comes first, the binding or the conformational change?

In addition to resolving association and dissociation rate constants for biomolecular interactions, the SPR biosensor approach provides insights into dynamic events of the studied molecules. It could be demonstrated that the observed changes in the measured SPR signal were caused by substrate binding to the immobilised HCMV protease and by a subsequent structural reorganisation in the enzyme (Paper I). Moreover, it was shown that the binding of NNRTIs was preceded by a rate-limiting conformational change in the HIV-1 RT to form the binding-competent structure of the enzyme (Paper II). Indications of an NNRTI-induced structural alteration following the formation of the enzyme-inhibitor complex were also found (Paper II). The discussed relationship between receptor structure and ligand binding touches upon some fundamentals in the science of molecular interactions. Some drug-interaction concepts, e.g., the induced-fit theory and the activation-aggregation theory (see Introduction), can be reduced to the question: what comes first, the binding or the conformational change? In other words, does the inhibitor induce a conformational change after it has bound to the enzyme, or does it select a pre-existing conformer out of an ensemble of different conformers, thereby shifting the equilibrium away from the state of the

free enzyme? There is little experimental data that discriminates between induced-fit and population-shift models because the conformational changes are usually fast and the populations are often strongly skewed (Volkman *et al.*, 2001). Within the timescale that can be studied with the SPR biosensor, both cases were found to occur in the presented results. It was evident that structural flexibility was a prerequisite (two-state mechanism of the HIV-1 RT) and a consequence (substrate-induced conformational changes in the HCMV protease) of ligand binding. In particular, the present work supports the concept of enzymes in dynamic equilibria of various conformations, which are shifted from their native state upon interaction with other molecules. In conclusion, the biosensor provides a powerful tool for unravelling the relationship between structure and binding in biomolecular interactions.

Direct interaction studies – filling a missing link in enzyme kinetics and analysis of allosteric inhibitors

At the starting point of the present work structures were available for the studied enzymes in complex with ligands, showing, albeit in impressive detail, only time slices of the interaction between the involved binding partners. Kinetic parameters of the interaction with ligands had also been determined by activity assays and under steady state conditions. However, this experimental approach gives only indirect estimates of the parameters describing the formation of the enzyme-ligand complex; namely, K_m for the HCMV protease and the peptide substrate, and K_i , IC_{50} , ED_{50} values for the HIV-1 RT and NNRTIs. It is therefore not surprising that a direct binding assay, using both steady state and pre-steady state conditions, provided new insights into these biomolecular interactions and suggests a reassessment of previous assumptions. For example, as was demonstrated for the studied serine proteases, the concept of K_m as an apparent dissociation constant might be a simplification.

The biosensor has proven to be useful, in particular for binding studies of allosteric inhibitors against the HIV-1 RT. Since these compounds inhibit the enzyme only indirectly, their affinity may not correlate with their degree of inhibition. The potential of an inhibitor with high affinity but low efficacy might thus not be recognised in an inhibition assays.

A major advantage of the biosensor is that the measurement of direct binding does not depend on the activity of the involved enzyme. Therefore, the problem of physiologically irrelevant assay conditions, which are optimised to achieve maximal enzyme activity, and the lack of standardised conditions for better comparison between different results can easily be overcome.

Interestingly, the affinity between HIV-1 RT and NNRTIs is not only dependent on the assay conditions, but was also reported to vary for the different forms of the enzyme-substrate complex (Althaus *et al.*, 1994; Maga *et*

al., 2000). Vice versa, the presence of an inhibitor enhanced the affinity for the substrate (Divita *et al.*, 1993; Rittinger *et al.*, 1995). Using a biosensor approach with substrate present in the complex formation with the inhibitor, the validity of these results could be scrutinised simply in future studies. Furthermore, since NNRTIs are largely protein-bound *in vivo*, the addition of plasma protein to the assay buffer might enhance the ability to predict pharmacological properties from the interaction kinetics.

The improved description of enzyme-ligand interactions presented here and the establishment of the biosensor assay is expected to contribute to the development of antiviral drugs with improved kinetic properties.

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Summary in Swedish

De flesta av dagens antivirala läkemedel hämmar aktiviteten av enzymer som produceras av virus. Men nya och effektiva läkemedel behövs för att möta uppkomsten av resistenta varianter av dessa virus och nya virala sjukdomar. Ökad kunskap om mekanismerna bakom växelverkan mellan enzym och läkemedelssubstanser och framtagning av nya analysmetoder skapar förutsättningar för denna utveckling.

I detta arbete undersöks hur molekyler binds till två virala enzymer, proteaset från humant cytomegalovirus (HCMV), ett herpes virus, och det omvända transkriptaset (RT) från HIV typ 1, viruset som orsakar AIDS. Dessa enzymer har gemensamt att deras flexibla struktur är viktig i samband med bindningen av ligander. För interaktionsstudierna har en biosensor använts som är baserad på ytplasmonresonans teknik, där enzymet kopplas till en chipyta och ligandmolekylen sköljs över den genom ett mikroflödessystem. Med hjälp av en optisk detektor kan bindningen mellan de studerade molekylerna mätas direkt, och hastighetskonstanterna för växelverkan kan bestämmas genom analysen av bindningssignalerna.

I den första studien undersöks bindningen av ett peptid substrat till HCMV proteaset. Parametrarna för denna växelverkan som bestämdes med hjälp av biosensorn avviker från dem som kan mätas indirekt baserat på enzymets aktivitet, vilket antyder att interaktionen är mer komplex än tidigare antagits. Genom tvärbinding av enzymet kunde det visas att bindning av substrat orsakar konformationsändringar i enzymet. Denna biosensor-metod kan användas för bindingsstudier med inhibitorer, vilket exemplifierades med två enzymhämmare som har tagits fram ur naturprodukter.

I övriga delarbeten presenteras bindningstudier mellan HIV-1 RT och en viss klass av hämmare, så kallade icke-nukleosid hämmare. Dessa substanser binder sig till en hydrofob bindingsficka, som inte syns i kristallstrukturer av det obundna enzymet, och de hämmar enzymet genom att påverka dess struktur och flexibilitet.

Kinetiska detaljer kring denna komplexa interaktion kunde belysas och kvantificeras genom anpassning av en matematisk modell till de experimentella data, som presenteras i studie II. De fyra studerade hämmarna visar varierande grad av komplexitet i deras interaktion med HIV-1 RT. En generell modell för bestämningen av hastighetskonstanter har tagits fram. Den beskriver enzymet i jämvikt mellan två olika

konformationer, av vilken den ena kan binda hämmaren (öppen bindingsficka), men inte den andra (stängd bindingsficka). Dessutom kan hämmaren binda på två kinetiskt olika sätt, antingen med låg affinitet eller med hög affinitet.

Interaktionen mellan en serie av 12 olika hämmare och 7 olika varianter av HIV-1 RT presenteras i artikel III. Målet med denna studie var att undersöka vilka kinetiska parametrar som påverkas av ersättning av vissa aminosyror som är relaterade till utvecklingen av resistens mot icke-nukleosid hämmare. Utvärderingen av biosensorexperimenten visar att mutationerna i de flesta fall både sänker inbindingshastigheten och ökar dissociationshastigheten. De framtagna resistensprofilerna skiljer sig dock mellan de studerade hämmarna. Två av mutanterna (L100I och K103N) påverkade dessutom jämvikten mellan de två enzym konformationerna, så att hämmaren kan binda sig fortare men också släppas snabbare.

I den sista studien (IV) analyseras de krafter som ligger bakom bindningen mellan enzymet och hämmaren. Detta uppnåddes genom att undersöka växelverkans temperaturberoende. Resultaten visar att det bildade komplexet hålls ihop av krafter som kommer från direkta interaktioner mellan enzym och hämmare. Den indirekta hydrofoba dragningskraften mellan enzym och hämmare och den strukturella förvrängningen av enzymet när hämmaren binder sig jämnar delvis ut sig.

Sammanfattningsvis ger denna avhandling nya insikter om sambandet mellan enzymernas struktur och bindningen av ligander. Biosensor tekniken har visat sig vara av stor nytta för karakteriseringen av bindningsegenskaperna för olika typer av ligander. De presenterade resultaten kan användas för att ta fram nya läkemedel med förbättrade egenskaper.

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