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Elucidation of HIV-1 protease resistance by characterization of interaction kinetics between inhibitors and enzyme variants

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Abstract

The kinetics of the interaction between drug-resistant variants of HIV-1 protease (G48V, V82A, L90M, I84V/L90M, and G48V/V82A/I84V/L90M) and clinically used inhibitors (amprenavir, indinavir, nelfinavir, ritonavir, and saquinavir) were determined using biosensor technology. The enzyme variants were immobilized on a biosensor chip and the association and dissociation rate constants (k_{on} and k_{off}) and affinities (K_D) for interactions with inhibitors were determined. A unique interaction kinetic profile was observed for each variant/inhibitor combination. Substitution of single amino acids in the protease primarily resulted in reduced affinity through increased k_{off} for the inhibitors. For inhibitors characterized by fast association rates to wild-type protease (ritonavir, amprenavir, and indinavir), additional substitutions resulted in a further reduction of affinity by a combination of decreased k_{on} and increased k_{off}. For inhibitors characterized by slow dissociation rates to wild-type enzyme (saquinavir and nelfinavir), the decrease of affinity conferred by additional mutations was attributed to increased k_{off} values. Development of resistance thus appears to be associated with a change of the distinctive kinetic parameter contributing to high affinity. Further inhibitor design should focus on improving the "weak point" of the lead compound, that being either k_{on} or k_{off}.

Keywords: HIV protease; Drug resistance; Protease inhibitors; Biosensors; Interaction kinetics

1. Introduction

Resistance is a serious obstacle for the continued success of antiviral pharmaceuticals in the treatment of AIDS (Tomasselli and Heinrikson, 2000; Richman, 2001). The protease is strikingly tolerant to mutations, with substitutions observed in almost 50% of the residues and over 20 residues associated with resistance to clinically available inhibitors (Shafer et al., 1999; Parikh et al., 2001). Cross-resistance, which occurs when resistance to one inhibitor results in resistance to an additional inhibitor, is also a limitation in the long-term treatment of AIDS patients. Although many studies revealing characteristic mutations that confer resistance to protease inhibitors have been performed (for reviews see Boden and Markowitz, 1998; Tomasselli and Heinrikson, 2000; Miller, 2001), the mechanism for reduced efficacy of inhibitors resulting in resistance and cross-resistance is not known. Consequently, design of new antiviral drugs that are effective against the numerous variants of HIV protease requires a better understanding of the development of resistance.

In order to investigate the correlation between inhibitor structure and resistance, an extensive study of the inhibition of resistant variants of HIV-1 protease by a series of inhibitors has previously been performed (Ahlsén et al., 2002). The variants were designed on the basis of mutations known to be involved in resistance to clinically used inhibitors, and with respect to the location of the corresponding amino acid in the protein (Fig. 1). Accordingly, single and multiple combinations of G48V, V82A, I84V, and L90M were introduced into wild-type HIV-1 protease. Substitution of Gly48 by Val has been found after exposure of the virus to saquinavir, rendering the enzyme less susceptible to inhibition (Jacobsen

Abbreviations: AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; RU, resonance units; R_{max}, maximum binding level; k_{on}, association rate constant; k_{off}, dissociation rate constant; k_t,, mass transport coefficient

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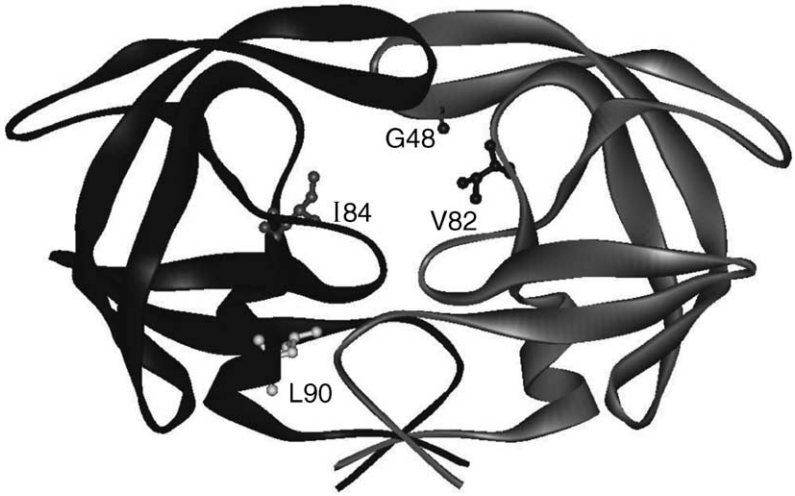


Fig. 1. HIV-1 protease with the wild-type residues G48, V82, I84, and L90 shown in one subunit, all residues and substitutions occur in both subunits.

et al., 1995). Residue Gly48 is located in the "flaps" of the protease and contributes to the formation of the S2/S2′ and S3/S3′ binding sites (Wlodawer and Erickson, 1993). Substitution of residue Val82 by an assortment of amino acids (Ala/Thr/Phe/Ser) and substitution of Ile84 by Val has been reported in association with treatment with ritonavir and indinavir (Markowitz et al., 1995; Condra et al., 1996; Molla et al., 1996; Shafer et al., 1999). The I84V substitution has also been reported after treatment with amprenavir (Maguire et al., 2002). These residues are both located in the active site and interact directly with the P1/P1′ groups of the substrate or inhibitor (Wlodawer and Erickson, 1993). Development of resistance to all studied inhibitors has been correlated with the mutation of residue Leu90, located at the dimer interface, to methionine (Jacobsen et al., 1995; Condra et al., 1996; Shafer et al., 2001). Furthermore, recent studies have shown that V82A, I84V, and L90M substitutions, among others, are frequently found in cross-resistant strains (Hertogs et al., 2000) and that cross-resistance between indinavir, ritonavir, nelfinavir, and saquinavir is higher than to amprenavir (Race et al., 1999). The systematic study of structural changes in the enzyme and inhibitors did not reveal a clear relationship between structure and inhibition (Ahlsén et al., 2002). It was concluded that the inhibition constants (K_i values) did not provide adequate information for understanding resistance of HIV protease to inhibitors, and that a new approach should be attempted.

We have previously used surface plasmon resonance biosensor technology for screening and kinetic characterization of HIV-1 protease inhibitors (Markgren et al., 1998, 2000, 2001, 2002). These studies have revealed association and dissociation rates as important features of drug–target interactions that may be used for design of inhibitors (Markgren et al., 2002). It was proposed that a similar analysis, with particular focus on the structural features of resistant enzyme variants rather than on the characteristics of different inhibitors, could give insight into the development of resistance. Therefore, the same set of resistant enzyme variants used previously (Ahlsén et al., 2002) was adopted in the present study and the rate constants for the interactions with five clinically used protease inhibitors were determined by biosensor technology.

2. Material and methods

2.1. Enzymes and inhibitors

The gene encoding wild-type HIV-1 protease from strain HXB and mutant genes for G48V, V82A, I84V, L90M, G48V/L90M, I84V/L90M, and G48V/V82A/I84V/L90M variants were constructed, expressed, and purified essentially as described previously (Nillroth et al., 1997; Danielson et al., 1998). Amprenavir was obtained from Vertex Pharmaceuticals Inc. (Cambridge, MA, USA), indinavir from Merck Sharp & Dome Ltd. (Herts, UK), nelfinavir from Agouron Pharmaceuticals Inc. (La Jolla, CA, USA), ritonavir from Abbott Laboratories Ltd. (Kent, UK), and saquinavir from Roche Registration Ltd. (Herts, UK).

2.2. Interaction studies

The interactions between HIV-1 protease and inhibitors were studied using a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden), essentially as described previously (Markgren et al., 2001). Injection times of 180–360 s were used, the analysis temperature was 20 or 25 °C, and at least three blank injections were included for each measurement series. To assess the quality of each run, various report point values (baseline level, binding level for various times during the sample injection, etc.) were plotted versus cycle number. Cycles that clearly deviated from the other cycles were omitted. The enzyme was immobilized at a level of 2000–4500 resonance units (RU).

A series of different inhibitor concentrations was measured for each enzyme/inhibitor combination. Experiments were performed at least four times. Generally, 0–160 nM inhibitor was used, but in some cases up to 400 nM was required in order to reach a maximum binding level (R_{max}). Triplicates of either 10 or 100 nM inhibitor were included as a control for both the dilution series and the sensor surface.

2.3. Data analysis

Each concentration series was analyzed using simultaneous non-linear regression analysis (global fitting) with BIAevaluation 3.1.0 Software (Biacore AB, Uppsala, Sweden). The analysis was based on a mathematical model assuming 1:1 binding and included a mass transport coefficient (Glaser, 1993; Karlsson and Fält, 1997), as was previously found to be appropriate for wild-type enzyme (Markgren et al., 2001). Additional equations were employed when it was suspected that a mass transport coefficient was superfluous (simple Langmuir), or when binding was completely mass transport-limited (Karlsson, 1999). In all cases, several analyses were carried out on the same set of sensorgrams and the kinetic parameters from the fits with the lowest χ^2 values were selected, after which a visual comparison of the simulated and experimental sensorgrams was made to verify that the parameters were reasonable. The degree of the influence of mass transport on the calculated association rate was determined by calculating the ratio of k_{on} multiplied by the maximum binding level the (R_{max}) and the mass transport coefficient (k_t), as described by Karlsson (1999). The values of the kinetic parameters were judged to be unreliable when this ratio was above five.

3. Results

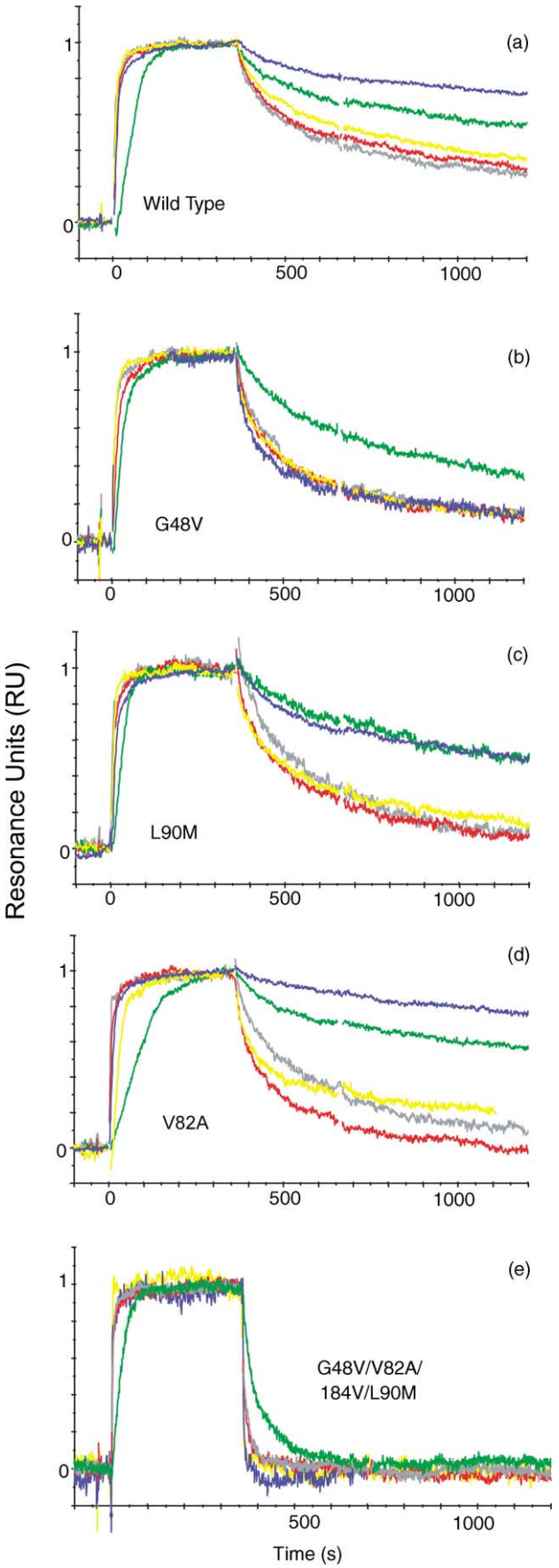
3.1. Sensor surfaces and sensorgrams

The interaction kinetic parameters for the wild-type enzyme (Table 1) were essentially the same as to those previously obtained for the Q7K variant with clinically used inhibitors (Markgren et al., 2001). It was, therefore, possible to minimize the number of amino acid substitutions in

Table 1
Kinetic constants for interactions between HIV protease and inhibitors

Enzyme	Inhibitor	n	K_D (nM)	k_{on} ($M^{-1}\,s^{-1}$)	k_{off} (s^{-1})	k_{off}/k_{on} (nM)
Wild-type	Amprenavir	4	0.96 ± 0.20	*>5.00 × 10^6*	*>4.79 × 10^−3*	*0.96*
	Indinavir	4	1.51 ± 0.36	2.37 × 10^6 ± 2.61 × 10^5	3.44 × 10^−3 ± 7.87 × 10^−4	1.45
	Nelfinavir	3	1.87 ± 0.59	3.79 × 10^5 ± 1.28 × 10^5	5.65 × 10^−4 ± 8.72 × 10^−5	1.49
	Ritonavir	4	0.90 ± 0.27	*>5.00 × 10^6*	*>4.51 × 10^−3*	*0.90*
	Saquinavir	5	0.44 ± 0.09	1.05 × 10^6 ± 1.74 × 10^5	4.07 × 10^−4 ± 4.43 × 10^−5	0.39
L90M	Amprenavir	4	2.62 ± 0.34	*>5.00 × 10^6*	*>1.31 × 10^−2*	*2.62*
	Indinavir	4	4.31 ± 0.33	*>5.00 × 10^6*	*>2.15 × 10^−2*	*4.31*
	Nelfinavir	4	3.73 ± 0.56	7.94 × 10^5 ± 1.16 × 10^5	3.02 × 10^−3 ± 6.31 × 10^−4	3.80
	Ritonavir	4	4.09 ± 0.88	*>5.00 × 10^6*	*>2.05 × 10^−2*	*4.09*
	Saquinavir	4	1.29 ± 0.21	1.69 × 10^6 ± 6.86 × 10^5	2.22 × 10^−3 ± 9.56 × 10^−4	1.31
G48V	Amprenavir	4	2.22 ± 0.69	3.31 × 10^6 ± 7.49 × 10^5	6.27 × 10^−3 ± 1.22 × 10^−3	1.89
	Indinavir	4	7.70 ± 1.66	*2.30 × 10^6 ± 5.71 × 10^5*	*2.04 × 10^−2 ± 6.99 × 10^−3*	*8.89*
	Nelfinavir	4	5.60 ± 1.52	5.28 × 10^5 ± 1.90 × 10^5	2.11 × 10^−3 ± 3.97 × 10^−4	3.99
	Ritonavir	3	4.17 ± 1.47	1.93 × 10^6 ± 1.87 × 10^5	7.99 × 10^−3 ± 2.65 × 10^−3	4.53
	Saquinavir	4	5.12 ± 1.51	2.10 × 10^6 ± 6.63 × 10^5	8.24 × 10^−3 ± 2.55 × 10^−3	3.92
V82A	Amprenavir	4	3.31 ± 1.03	*2.60 × 10^6 ± 4.97 × 10^5*	*6.03 × 10^−3 ± 1.04 × 10^−3*	*2.32*
	Indinavir	4	8.61 ± 2.67	*3.51 × 10^6 ± 1.91 × 10^5*	*1.48 × 10^−2 ± 1.10 × 10^−3*	*4.20*
	Nelfinavir	4	4.08 ± 0.80	5.57 × 10^5 ± 1.99 × 10^5	1.84 × 10^−3 ± 3.51 × 10^−4	3.29
	Ritonavir	4	3.03 ± 0.60	*>5.00 × 10^6*	*>1.52 × 10^−2*	*3.03*
	Saquinavir	4	0.60 ± 0.16	7.12 × 10^5 ± 3.33 × 10^5	2.76 × 10^−4 ± 5.52 × 10^−5	0.39
I84V/L90M	Amprenavir	4	12.5 ± 2.4	4.80 × 10^6 ± 2.00 × 10^6	6.56 × 10^−2 ± 3.69 × 10^−2	13.67
	Indinavir	5	65.7 ± 29.2	2.67 × 10^6 ± 1.97 × 10^6	5.90 × 10^−2 ± 3.03 × 10^−2	22.12
	Nelfinavir	4	23.6 ± 14.5	1.53 × 10^6 ± 2.96 × 10^5	3.61 × 10^−2 ± 2.25 × 10^−2	23.60
	Ritonavir	4	69.2 ± 29.6	1.20 × 10^6 ± 3.58 × 10^5	5.43 × 10^−2 ± 1.57 × 10^−2	45.42
	Saquinavir	4	17.0 ± 7.1	2.69 × 10^6 ± 1.99 × 10^6	2.05 × 10^−2 ± 9.41 × 10^−3	7.64
G48V/V82A/I84V/L90M	Amprenavir	5	84.4 ± 10.2	1.01 × 10^6 ± 1.33 × 10^5	8.24 × 10^−2 ± 1.04 × 10^−2	81.42
	Indinavir	4	127 ± 6.0	7.22 × 10^5 ± 5.40 × 10^4	9.21 × 10^−2 ± 7.77 × 10^−3	127.65
	Nelfinavir	3	105 ± 11.0	5.60 × 10^5 ± 2.92 × 10^5	5.20 × 10^−2 ± 2.11 × 10^−2	92.74
	Ritonavir	2	>400	n.d.	n.d.	n.d.
	Saquinavir	3	535 ± 150	*3.28 × 10^5 ± 1.14 × 10^5*	*1.49 × 10^−1 ± 3.44 × 10^−2*	*453.96*

The values are presented with standard errors based on 'n' replicates. Rate constants of mass transport-limited interactions are presented in italics. The k_{off} values for interactions with k_{on} values >5.00 × 10^6 $M^{-1}\,s^{-1}$ were calculated from the K_D value using $k_{off} = K_D \times k_{on}$. n.d.—not determined.



the enzyme by using the wild-type enzyme for construction of these variants rather than the Q7K variant.

The immobilization procedure used here resulted in stable surfaces with all the enzyme variants, indicating that different interaction kinetic constants were not a result of dissimilar surface stabilities. It was necessary to optimize the quantity of immobilized protein for each enzyme variant to obtain comparable binding levels for saturated surfaces. Enzyme was immobilized at a level of 2000–3000 RU, based on levels previously determined to be optimal for the Q7K variant. This resulted in satisfactory binding responses (5–20 RU) for all variants analyzed, with the exception of I84V/L90M and the quadruple mutant, which required 4500 RU immobilized enzyme. Saturation of the enzyme surface was achieved with up to 160 nM of inhibitor, except with the I84V/L90M and the quadruple mutants for which up to 400 nM inhibitor was required. It was not possible to obtain adequate binding responses for either the I84V or the G48V/L90M mutants despite attempts to increase both the inhibitor concentration and the amount of immobilized enzyme. These were, therefore, omitted from the current study. Surfaces with inhibitor bound to the immobilized enzyme were successfully regenerated using ethylene glycol.

The differences in kinetic characteristics of interactions between the inhibitors and the enzyme variants could be compared by simply overlaying normalized sensorgrams obtained with the same concentration of each inhibitor (Fig. 2). A comparative analysis of sensorgrams was most easily interpreted in the dissociation phase; essentially only a function of the dissociation rate, whereas the signal in the association phase of sensorgrams is a function of both association and dissociation rates. The analysis of overlaid sensorgrams revealed that the dissociation rate for saquinavir increased as a result of substituting Gly48 by Val, or Leu90 by Met (Fig. 2a–c). Similarly, substitution of Val82 by Ala resulted in increased dissociation rates for indinavir and ritonavir (Fig. 2d), whereas it had little effect on the interaction with amprenavir, nelfinavir, or saquinavir. Additional substitutions resulted in greater increases of the dissociation rates for all inhibitors, with nelfinavir being least sensitive to any of these changes. Simultaneous substitution of G48V, V82A, I84V, and L90M resulted in an enzyme with no significant affinity for any of the inhibitors (Fig. 2e). The observation that nelfinavir had the slowest association rate of all inhibitors for all mutants, although non-conclusive in Fig. 2, was later substantiated by determination of the kinetic constants.

Fig. 2. Comparison of the interaction kinetics for clinical inhibitors and variants of HIV-1 protease, sensorgrams are normalized to give relative responses between zero and one. Sensorgrams are shown for the wild-type and single mutants with 80 nM inhibitor, and for quadruple mutant with 100 nM inhibitor. Saquinavir—blue, nelfinavir—green, ritonavir—yellow, indinavir—red, and amprenavir—gray. (a) Wild-type, (b) G48V, (c) L90M, (d) V82A, and (e) G48V/V82A/I84V/L90M.

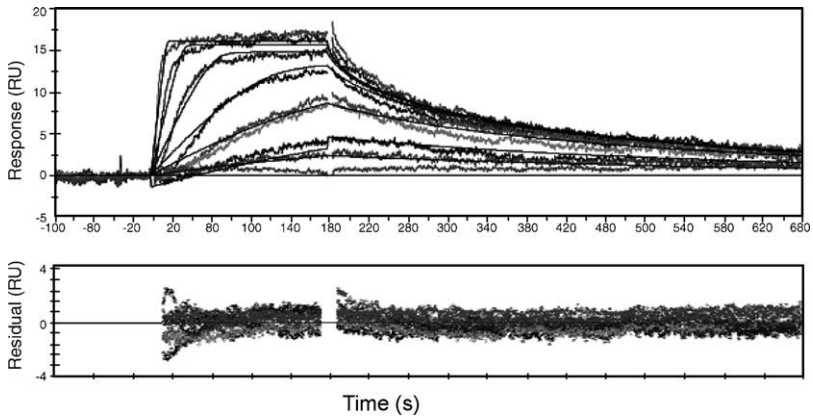


Fig. 3. Sensorgrams for the interaction between indinavir (0–160 nM) and L90M HIV-1 protease. The predicted sensorgrams for the global fit and the corresponding residuals are shown.

3.2. Interaction kinetic constants

Quantitative determination of kinetic constants was required for a more comprehensive analysis of the interactions between the inhibitors and the enzyme variants. By using an equation describing a 1:1 interaction and accounting for the rate of mass transport, it was possible to determine k_{on}, k_{off}, and K_D values (Table 1). The K_D values for the independent averages from replicate experiments (first column of Table 1) are presented, as well as the ratio of the rate constants (k_{off}/k_{on}) (last column of Table 1). A representative set of sensorgrams and the corresponding fitted curves and residuals is presented in Fig. 3.

Affinities (K_D values) for these interactions could be determined for all inhibitors and enzyme variants and were found to range from approximately 0.4 to over 500 nM. Some of the rate constants were difficult to determine accurately because the association rates were higher than the rate of mass transport, a phenomena hereafter referred to as "mass transport-limited". It was estimated that the maximum determinable value for k_{on} was $5 \times 10^6\,M^{-1}\,s^{-1}$ and all values exceeding this limit were, therefore, reported as $>5 \times 10^6\,M^{-1}\,s^{-1}$. The corresponding minimum values of k_{off} for these interactions were calculated from K_D values. Even when it was possible to determine both k_{on} and k_{off} directly, the values were in some cases influenced by mass transport. Such values were identified by determining the $k_{on} \times R_{max}$ to k_t ratios, and are indicated in italics in Table 1.

Single amino acid substitutions reduced the affinity of the interactions with the studied inhibitors two- to fivefold, while additional substitutions reduced the affinity 40- to 100-fold (Table 1). However, the affinity for saquinavir did not follow this trend; the V82A substitution had little effect on the affinity while the G48V substitution reduced affinity 10-fold. The quadruple mutant had a 1000-fold lower affinity for saquinavir as compared to wild-type, the lowest affinity determined in this study. In addition, the affinity of both amprenavir and nelfinavir only decreased 10-fold in response to the combined substitution of I84V and L90M.

The relationship between the interaction rate constants for the different mutants and inhibitors was rather complicated. The values of k_{on} and k_{off} were, therefore, plotted in an interaction kinetic plot (Fig. 4) to aid in visualization of the results. The affinity is also represented in the plot, as indicated by the diagonal lines. By analyzing this interaction map, it was found that the different inhibitors had typical interaction characteristics that were maintained even when the enzyme was modified. Nelfinavir and saquinavir, located in the lower left section of the plot, had the slowest association and dissociation rates. In contrast, amprenavir and ritonavir had the fastest association rates. The graph reveals that for all enzyme variants, loss of affinity for saquinavir and nelfinavir was due to increased dissociation rates. This was also the case with indinavir, with the exception of the interaction with the quadruple mutant, for which decreased association rate also contributed to loss of affinity. The loss of affinity for ritonavir and amprenavir to enzyme with single and multiple substitutions appeared to be due to a combined effect of association and dissociation rates.

Substitution of single residues generally led to three- to sixfold increases of k_{off}, except interactions between saquinavir and V82A, for which k_{off} was unchanged, or G48V, for which k_{off} was increased 20-fold. Multiple substitutions contributed to further increase of k_{off}. Combined substitution of both I84V and L90M resulted in association rates (1.2×10^6–$4.8 \times 10^6\,M^{-1}\,s^{-1}$) and dissociation rates (2.0×10^{-2}–$6.6 \times 10^{-2}\,s^{-1}$) that were similar for all inhibitors, a phenomenon that was also observed when all four residues were substituted.

4. Discussion

4.1. Resistance

The kinetics of the interaction between drug-resistant variants of HIV-1 protease and clinically relevant inhibitors were characterized using biosensor technology. In

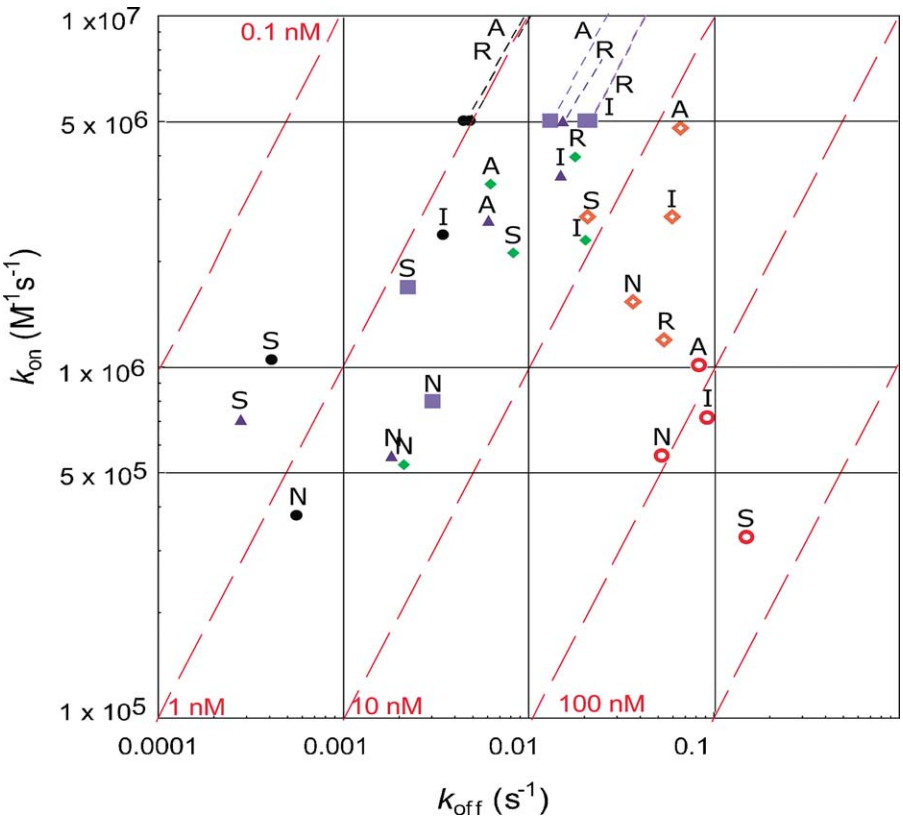


Fig. 4. Interaction kinetic plot for interactions between HIV-1 protease and inhibitors. The ratios of k_{off}/k_{on} (K_D) are represented as diagonal lines in the plot, and correspond to the last column in Table 1. The inhibitors are labeled by the first letter in the name: saquinavir—S, nelfinavir—N, ritonavir—R, indinavir—I, and amprenavir—A. The enzyme variants are: wild-type—black circle, L90M—purple square, G48V—green diamond, V82A—blue triangle, I84V/L90M—open orange diamond, and G48V/V82A/I84V/L90M—open red circle. Data points located on the upper frame of the figure have correct K_D values, shown as dashed lines, and values for k_{on} set at $>5 \times 10^6\ M^{-1}\ s^{-1}$.

general, resistance for a certain inhibitor correlates with decreased affinity, conventionally estimated as K_i values in inhibition assays. Affinities determined by biosensor methodology (K_D) were also reduced upon introduction of resistance-associated substitutions in the protease gene. Since affinity is the ratio between k_{off} and k_{on}, reduction of affinity can be due to an increase in dissociation rate (k_{off}), a decrease in association rate (k_{on}), or a combination of both. The aim of the present study was to attain increased understanding of resistance by breaking down the affinity into k_{off} and k_{on}, and to evaluate how these correlate with resistance. Our work thus focused on the effects of amino acid substitutions in HIV-1 protease on these kinetic parameters.

The interaction kinetic characteristics were visualized by mapping the k_{off} and k_{on} values for all inhibitors and enzyme variants (Fig. 4). This revealed distinguishing features of the interaction kinetics for the inhibitors with wild-type HIV-1 protease that were essentially maintained with the studied enzyme variants. Saquinavir and nelfinavir were distinguished by low association and dissociation rates, and decrease of affinity, due to substitution of residues in the protease, was attributed to increased dissociation rates. Amprenavir, ritonavir, and indinavir maintained characteristically fast association rates for interactions with the protease even when single substitutions were introduced. Additional substitutions resulted in further decrease of affinity by influencing both k_{off} and k_{on}. Amprenavir appeared to be most resilient to changes in the enzyme.

To better understand the relationship between changes in the kinetic constants and resistance, it was helpful to consider the structural context of substituted residues in the protease. Substitution of Leu90 to Met, a residue situated in the dimer interface, has been associated with disturbance of the dimer interface and reduced stability of the dimer (Mahalingam et al., 1999, 2001; Xie et al., 1999). In this study, the substitution of Leu90 to Met appeared to have a non-specific effect on the binding of inhibitors, all losing affinity to the same extent, apparently as a result of increased dissociation rates. This may explain why it is implied in either resistance or cross-resistance to all inhibitors.

Gly48 is located in the flap region of the protease, which is important for shaping the binding pocket of the active site and stabilizing the enzyme–substrate complex. Substitution of this residue by valine introduces a bulky side chain into the P3/P3′ binding pocket (Markland et al., 2000) and results in resistance towards saquinavir, but not the other inhibitors.

Although the kinetics for all inhibitors were changed as a result of the G48V substitution, the effect of the interaction with saquinavir was striking due to a 20-fold increase in the dissociation rate. The observation that increased dissociation rate accounted for the loss of affinity of saquinavir to G48V- or L90M-substituted enzyme is consistent with that for stopped-flow data reported by Maschera et al. (1996).

Two of the substituted residues (82 and 84) were localized in the active site, and contribute directly to binding of inhibitors through hydrophobic interactions in the P1/P1′ sites. The substitutions studied here result in reducing the size of the residue while maintaining the hydrophobic nature of the residues. The loss of affinity to inhibitors by these variants could, therefore, be attributed to reduced hydrophobic and van der Waals interactions. Our data with V82A show that the reduced affinity was an effect of different changes in dissociation and association rates for the various inhibitors. The inhibitors known to be influenced by this mutation (indinavir and ritonavir) lost affinity primarily due to increased dissociation rates. For inhibitors not associated with resistance to the V82A mutant, the kinetic constants were essentially the same as with wild-type enzyme (saquinavir) or had reduced affinity due to lowered association rate (amprenavir).

Combining the substitutions resulted in a synergistic effect on the kinetics of the enzyme/inhibitor interactions. Interestingly, the interaction kinetics for the targets became more similar for all interactions with each additional substitution. This can be visualized in Fig. 4 as a clustering in the interaction kinetics for the interactions with I84V/L90M mutant and, even more distinctly, for the interactions with quadruple mutant. It is hypothesized that the loss of distinguishing features of the interactions is related to the appearance of cross-resistance.

In general, resistance was correlated with an increase in the dissociation rate. Resistance resulting from multiple substitutions could arise as a result of decreases in association rate as well as increases in dissociation rate; however, our data suggest that there was no correlation with decreases in association rate above $5 \times 10^6\,M^{-1}\,s^{-1}$ and resistance. For example, decreased affinity of amprenavir to enzyme with substitutions at V82A or G84V was primarily due to decreased k_{on}, but was not associated with resistance.

In conclusion, resolution of the affinity into individual rate constants provided a greater understanding of the interaction between HIV protease and inhibitors than previous studies, where the inhibition constant alone was determined (Ahlsén et al., 2002). The key experiments presented here also offered insights into the mechanism of drug resistance for HIV protease, creating a platform for further studies. Analysis of interactions between drug-resistant HIV protease and a larger data set of structurally related inhibitors, similar to those performed with wild-type HIV-1 protease (Markgren et al., 2002), would enable a more comprehensive understanding of the interactions. In addition, these results may provide potentially helpful information for the design of new antiviral drugs. It is proposed that design and optimization of new protease inhibitors be based on dissociation and association rates. For interactions with fast association rates (or fast dissociation rates), decreasing the dissociation rate would provide a more effective means of increasing affinity than increasing association rates. For interactions with slow dissociation rates, affinity of inhibitors could be improved by increasing the association rates.

4.2. Methodology

An experimental strategy previously found to be successful for a stabilized wild-type HIV-1 protease (Markgren et al., 2001) was used as a foundation for this study of enzyme variants. Determination of kinetic constants using a surface plasmon-based biosensor requires stable, sensitive sensor surfaces, correction for unspecific signals, and application of suitable interaction models for data analysis (Karlsson and Fält, 1997; Morton and Myszka, 1998; Myszka, 1999; Rich and Myszka, 2000). Stable sensor surfaces were obtained with all the enzyme variants used in the present study and different immobilization levels were required to obtain comparable binding levels for each enzyme variant. The need to optimize immobilization levels may be due to differences in the relative amount of active protease in the enzyme preparation, much alike the necessity of increasing the enzyme concentration of certain variants in the inhibition study (Ahlsén et al., 2002). Although increasing the amount of immobilized protein enhances the signal, the amount of immobilized enzyme should be kept as low as possible in order to minimize mass transport problems (Morton and Myszka, 1998; Myszka, 1999), which occur when interactions are significantly faster than the rate of transport. Apparent differences in the value of the association rate at which mass transport becomes limiting for each enzyme variant may, therefore, be explained by differences in the availability of active enzyme on the sensor surface. By using an interaction model accounting for the influence of mass transport, association and dissociation rates could be determined for most combinations of enzyme variants and inhibitors.

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References

Ahlsén, G., Hultén, J., Shuman, C.F., Poliakov, A., Lindgren, M.T., Alterman, M., Samuelsson, B., Hallberg, A., Danielson, U.H., 2002. Resistance profiles of cyclic and linear inhibitors of HIV-1 protease. Antivir. Chem. Chemother. 13, 27–37.

Boden, D., Markowitz, M., 1998. Resistance to human immunodeficiency virus type 1 protease inhibitors. Antimicrob. Agents Chemother. 42, 2775–2783.

Condra, J.H., Holder, D.J., Schleif, W.A., Blahy, O.M., Danovich, R.M., Gabryelski, L.J., Graham, D.J., Laird, D., Quintero, J.C., Rhodes, A., Robbins, H.L., Roth, E., Shivaprakash, M., Yang, T., Chodakewitz, J.A., Deutsch, P.J., Leavitt, R.Y., Massari, F.E., Mellors, J.W., Squires, K.E., Steigbigel, R.T., Teppler, H., Emini, E.A., 1996. Genetic correlates of in vivo viral resistance to indinavir, a human immunodeficiency virus type 1 protease inhibitor. J. Virol. 70, 8270–8276.

Danielson, H., Lindgren, M.T., Markgren, P.-O., Nillroth, U., 1998. Investigation of an allosteric site of HIV-1 proteinase involved in inhibition by Cu^{2+}. Adv. Exp. Med. Biol. 436, 99–103.

Glaser, R.W., 1993. Antigen-antibody binding and mass transport by convection and diffusion to a surface: a two-dimensional computer model of binding and dissociation kinetics. Anal. Biochem. 213, 152–161.

Hertogs, K., Bloor, S., Kemp, S.D., Van den Eynde, C., Alcorn, T.M., Pauwels, R., Van Houtte, M., Staszewski, S., Miller, V., Larder, B.A., 2000. Phenotypic and genotypic analysis of clinical HIV-1 isolates reveals extensive protease inhibitor cross-resistance: a survey of over 6000 samples. AIDS 14, 1203–1210.

Jacobsen, H., Yasargil, K., Winslow, D.L., Craig, J.C., Kröhn, A., Duncan, I.B., Mous, J., 1995. Characterization of human immunodeficiency virus type 1 protease mutants with decreased sensitivity to proteinase inhibitor Ro 31-8959. Virology 206, 527–534.

Karlsson, R., 1999. Affinity analysis of non-steady-state data obtained under mass transport limited conditions using BIAcore technology. J. Mol. Recognit. 12, 285–292.

Karlsson, R., Fält, A., 1997. Experimental design for kinetic analysis of protein–protein interactions with surface plasmon resonance biosensors. J. Immunol. Methods 200, 121–133.

Maguire, M., Shortino, D., Klein, A., Harris, W., Manohitharajah, V., Tisdale, M., Elston, R., Yeo, J., Randall, S., Xu, F., Parker, H., May, J., Snowden, W., 2002. Emergence of resistance to protease inhibitor amprenavir in human immunodeficiency virus type 1-infected patients: selection of four alternative viral protease genotypes and influence of viral susceptibility to coadministered reverse transcriptase nucleoside inhibitors. Antimicrob. Agents Chemother. 46, 731–738.

Mahalingam, B., Louis, J.M., Reed, C.C., Adomat, J.M., Krouse, J., Wang, Y.F., Harrison, R.W., Weber, I.T., 1999. Structural and kinetic analysis of drug resistant mutants of HIV-1 protease. Eur. J. Biochem. 263, 238–245.

Mahalingam, B., Louis, J.M., Hung, J., Harrison, R.W., Weber, I.T., 2001. Structural implications of drug-resistant mutants of HIV-1 protease: high-resolution crystal structures of the mutant protease/substrate analogue complexes. Proteins 43, 455–464.

Markgren, P.-O., Hämäläinen, M., Danielson, U.H., 1998. Screening of compounds interacting with HIV-1 proteinase using optical biosensor technology. Anal. Biochem. 265, 340–350.

Markgren, P.-O., Hämäläinen, M., Danielson, U.H., 2000. Kinetic analysis of the interaction between HIV-1 protease and inhibitors using optical biosensor technology. Anal. Biochem. 279, 71–78.

Markgren, P.-O., Lindgren, M.T., Gertow, K., Karlsson, R., Hämäläinen, M., Danielson, U.H., 2001. Determination of interaction kinetic constants for HIV-1 protease inhibitors using optical biosensor technology. Anal. Biochem. 291, 207–218.

Markgren, P.-O., Schaal, W., Hämäläinen, M., Karlen, A., Hallberg, A., Samuelsson, B., Danielson, U.H., 2002. Relationships between structure and interaction kinetics for HIV-1 protease inhibitors. J. Med. Chem. 45, 5430–5439.

Markland, W., Rao, B.G., Parsons, J.D., Black, J., Zuchowski, L., Tisdale, M., Tung, R., 2000. Structural and kinetic analyses of the protease from an amprenavir-resistant human immunodeficiency virus type 1 mutant rendered resistant to saquinavir and resensitized to amprenavir. J. Virol. 74, 7636–7641.

Markowitz, M., Mo, H., Kempf, D.J., Norbeck, D.W., Bhat, T.N., Erickson, J.W., Ho, D.D., 1995. Selection and analysis of human immunodeficiency virus type 1 variants with increased resistance to ABT-538, a novel protease inhibitor. J. Virol. 69, 701–706.

Maschera, B., Darby, G., Palu, G., Wright, L.L., Tisdale, M., Myers, R., Blair, E.D., Furfine, E.S., 1996. Human immunodeficiency virus. Mutations in the viral protease that confer resistance to saquinavir increase the dissociation rate constant of the protease–saquinavir complex. J. Biol. Chem. 271, 33231–33235.

Miller, V., 2001. International perspectives on antiretroviral resistance. Resistance to protease inhibitors. J. Acquir. Immune Defic. Syndr. 26 (Suppl. 1), S34–S50.

Molla, A., Korneyeva, M., Gao, Q., Vasavanonda, S., Schipper, P.J., Mo, H.M., Markowitz, M., Chernyavskiy, T., Niu, P., Lyons, N., Hsu, A., Granneman, G.R., Ho, D.D., Boucher, C.A., Leonard, J.M., Norbeck, D.W., Kempf, D.J., 1996. Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. Nat. Med. 2, 760–766.

Morton, T.A., Myszka, D.G., 1998. Kinetic analysis of macromolecular interactions using surface plasmon resonance biosensors. Methods Enzymol. 295, 268–294.

Myszka, D.G., 1999. Improving biosensor analysis. J. Mol. Recognit. 12, 279–284.

Nillroth, U., Vrang, L., Markgren, P.-O., Hultén, J., Hallberg, A., Danielson, U.H., 1997. Human immunodeficiency virus type 1 proteinase resistance to symmetric cyclic urea inhibitor analogs. Antimicrob. Agents Chemother. 41, 2383–2388.

Parikh, U., Calef, C., Larder, B., Schinazi, R., Mellors, J.W., 2001. Mutations in retroviral genes associated with drug resistance. In: HIV Sequence Compendium 2001. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM, p. 191.

Race, E., Dam, E., Obry, V., Paulous, S., Clavel, F., 1999. Analysis of HIV cross-resistance to protease inhibitors using a rapid single-cycle recombinant virus assay for patients failing on combination therapies. AIDS 13, 2061–2068.

Rich, R.L., Myszka, D.G., 2000. Advances in surface plasmon resonance biosensor analysis. Curr. Opin. Biotechnol. 11, 54–61.

Richman, D.D., 2001. HIV chemotherapy. Nature 410, 995–1001.

Shafer, R.W., Hsu, P., Patick, A.K., Craig, C., Brendel, V., 1999. Identification of biased amino acid substitution patterns in human immunodeficiency virus type 1 isolates from patients treated with protease inhibitors. J. Virol. 73, 6197–6202.

Shafer, R.W., Dupnik, K., Winters, M.A., Eshleman, S.H., 2001. HIV-1 reverse transcriptase and protease sequencing for drug resistance studies. In: HIV Sequence Compendium 2001. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM, p. 83.

Tomasselli, A.G., Heinrikson, R.L., 2000. Targeting the HIV-protease in AIDS therapy: a current clinical perspective. Biochim. Biophys. Acta 1477, 189–214.

Wlodawer, A., Erickson, J.W., 1993. Structure-based inhibitors of HIV-1 protease. Annu. Rev. Biochem. 62, 543–585.

Xie, D., Gulnik, S., Gustchina, E., Yu, B., Shao, W., Qoronfleh, W., Nathan, A., Erickson, J.W., 1999. Drug resistance mutations can effect dimer stability of HIV-1 protease at neutral pH. Protein Sci. 8, 1702–1707.