Kinetics of the dimerization of retroviral proteases: The “fireman’s grip” and dimerization

MAREK INGR,^{1,2} TAT’ÁNA UHLÍKOVÁ,¹ KVIDO STRÍŠOVSKÝ,^{1,2} EVA MAJEROVÁ,³
AND JAN KONVALINKA^{1,2}

¹Institute of Organic Chemistry and Biochemistry, Academy of Science of the Czech Republic, 166 10 Praha 6, Czech Republic

²Department of Biochemistry, School of Science, Charles University, 128 43 Praha 2, Czech Republic

³Biological Testing Branch, Science Applications International Corp. (SAIC), National Cancer Institute-Frederick Cancer Research Development Center (NCI-FCRDC), Frederick, Maryland 21784, USA

(RECEIVED May 7, 2003; FINAL REVISION July 8, 2003; ACCEPTED July 8, 2003)

Abstract

All retroviral proteases belong to the family of aspartic proteases. They are active as homodimers, each unit contributing one catalytic aspartate to the active site dyad. An important feature of all aspartic proteases is a conserved complex scaffold of hydrogen bonds supporting the active site, called the “fireman’s grip,” which involves the hydroxyl groups of two threonine (serine) residues in the active site Asp-Thr(Ser)-Gly triplets. It was shown previously that the fireman’s grip is indispensable for the dimer stability of HIV protease. The retroviral proteases harboring Ser in their active site triplet are less active and, under natural conditions, are expressed in higher enzyme/substrate ratio than those having Asp-Thr-Gly triplet. To analyze whether this observation can be attributed to the different influence of Thr or Ser on dimerization, we prepared two pairs of the wild-type and mutant proteases from HIV and myeloblastosis-associated virus harboring either Ser or Thr in their Asp-Thr(Ser)-Gly triplet. The equilibrium dimerization constants differed by an order of magnitude within the relevant pairs. The proteases with Thr in their active site triplets were found to be ~10 times more thermodynamically stable. The dimer association contributes to this difference more than does the dissociation. We propose that the fireman’s grip might be important in the initial phases of dimer formation to help properly orientate the two subunits of a retroviral protease. The methyl group of threonine might contribute significantly to fixing such an intermediate conformation.

Keywords: Retroviral protease; dimerization; HIV protease; fireman’s grip; kinetic assay; fluorescence; pressure

Retroviral proteases (PRs) belong to the family of retropepsins (Dunn 1998), which are dimeric aspartic proteases. The

two monomers are interconnected by their C and N termini, which form an antiparallel β -sheet that is primarily responsible for the dimer stability (Wlodawer et al. 1989). The proteases are expressed as parts of the viral polyproteins Gag or Gag-Pol, from which they cleave themselves before cleaving the rest of the polyproteins to yield the structural proteins and replication enzymes. The Gag-Pol polyprotein is a product of ribosomal frame-shifting or readthrough suppression, which takes place with 5% to 10% frequency. In contrast, in the case of alpharetroviruses (e.g., myeloblastosis-associated virus [MAV]), the protease is a part of the Gag polyprotein and is thus expressed in equimolar ratio with its Gag substrate. In the case of betaretroviruses (Mason-Pfizer monkey virus [MPMV]) the protease also is expressed by frame-shifting event. It was observed previously that the activities of the alpharetroviral proteases are con-

Reprint requests to: Jan Konvalinka, Institute of Organic Chemistry and Biochemistry, Flemingovo nám. 2, 166 10 Praha 6, Czech Republic; e-mail: jan.konvalinka@uochb.cas.cz; fax: 420-220-183-203.

Abbreviations: HIV-1-PR(T), wild-type HIV-1-PR; HIV-1-PR(S), HIV-1-PR harboring Thr26Ser mutation; MAV-PR(S), wild-type MAV-PR; MAV-PR(T), MAV-PR harboring Ser38Thr mutation; MPMV-PR(T), wild-type MPMV-PR, 12-kD form; DABCYL, 4-[[4’-(dimethylamino)phenyl]azo]-benzoic acid; EDANS, 5-[(2’-aminoethyl)-amino]naphthalene-sulfonic acid; MAV, myeloblastosis-associated virus; MPMV, Mason-Pfizer monkey virus; PAL, peptide amide linker, 5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)valeric acid; PheSta, phenylstatin; PR, protease; RSV, Rous sarcoma virus; TBTU, *O*-(benzotriazol-1-yl)-N,N,N’,N’-tetramethyluronium tetrafluoroborate; Fmoc, 9-fluorenylmethoxycarbonyl; TFA, trifluoroacetic acid; PMSF, phenylmethylsulfonyl fluoride.

Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.03171903>.

siderably lower than those of lentiviral proteases, which was interpreted to be a compensation for the difference in their relative levels of synthesis and thus concentration in the virion (Arad et al. 1995).

Because the PRs are active in the form of noncovalently bound homodimers, chemical compounds preventing dimerization became possible antiviral agents. Moreover, the dimerization seems to be the key event in the activation of the polyprotein processing in retroviruses (Kräusslich 1991). Hence, many analyses of dimerization equilibrium and rate constants have been performed, especially for HIV-1 PR. The experimental approaches can be divided into two groups: substrate-independent methods, which involve techniques such as ultracentrifugation (Holzman et al. 1991; Grant et al. 1992; Towler et al. 1998; Xie et al. 1999; Stříšovský et al. 2000), and substrate (inhibitor)-dependent methods, based on kinetic measurements, most often fluorometric ones (Cheng et al. 1990; Zhang et al. 1991; Jordan et al. 1992; Kuzmič 1993; Darke et al. 1994; Pargellis et al. 1994; Uhlíková et al. 1996).

The method of sedimentation equilibrium is attractive due to its independence of substrate. The result is therefore less influenced by stabilization of the dimeric form by substrate binding. On the other hand, the experiments require relatively high concentrations of pure protein. The K_d values for HIV-1 and HIV-2 proteases determined by this method are summarized in Table 1. The majority of these values are in the micromolar range. These results, however, seem to be inconsistent with the fact that most activity assays routinely use nanomolar concentrations of HIV-PRs for activity determinations.

Although the kinetic measurements are more sensitive, require a lower concentration of protein, and are easier to perform, their interpretation can be obscured by phenomena such as the stabilization of the dimer by a substrate or an inhibitor. This effect is difficult to separate from genuine dimer stability, and therefore, the result may depend on the ligand used. On the other hand, the advantage of kinetic studies is that both of the rate constants, k_1 and k_2 , describing the association and dissociation processes can be determined.

As shown in Table 1, results of both K_d and rate constants determined kinetically with fluorogenic substrate or inhibitor have been reported by several groups for both HIV-1 and HIV-2 proteases (Cheng et al. 1990; Zhang et al. 1991; Jordan et al. 1992; Kuzmič 1993; Darke et al. 1994; Pargellis et al. 1994; Uhlíková et al. 1996. For summary, see Darke 1994). The reported K_d values from kinetic studies are typically of the order of 10^{-7} to 10^{-9} M, that is, on average, three orders of magnitude lower than the values derived from sedimentation analysis. The largest difference between two HIV-1 K_d values is five orders of magnitude. This major disagreement may be partially explained by the incorrect assumption of very fast association and dissociation processes in the publication by Jordan et al. (1992), in which the lowest values were determined. Another reason for the observed differences may be the inconsistency of experimental conditions in different experiments, especially pH varying from 4.5 to 7.0. However, there still remain discrepancies in the reported K_d values that are difficult to explain.

There are two major structural features believed to be responsible for the dimer stability of PRs: the N and C

Table 1. Values of K_d from previous studies

Reference	Protease	pH	K_d	$\tau_{1/2}$ (sec)	Remark
Holzman et al. 1991	HIV-2	4.5	87 μ M		
		7.5	28 μ M		
Grant et al. 1992	HIV-1	5.0	<10 nM		
Towler et al. 1998	HIV-1	7.0	9 μ M		
	HERV-K10	7.0	52.5 μ M		
Xie et al. 1999	HIV-1	7.0	52.7 μ M		Autolysis products interfered, K_d taken as upper limit
Stříšovský et al. 2000	HIV-1	6.0	77 μ M		
Cheng et al. 1990	HIV-1	7.0	50 nM	198	
Zhang et al. 1991	HIV-1		3.6 nM		Very fast processes assumed
Jordan et al. 1992	HIV-1		<1 nM		
	HIV-2		<1 nM		
Darke et al. 1994	HIV-1	6.5	16 nM		Fluorogenic inhibitor used
		7.0	39 nM		
Pargellis et al. 1994	HIV-1	5.5	5.2 nM	533	0 M NaCl
			4.0 nM	745	1.5 M NaCl
Kuzmič et al. 1993	HIV-1	6.4	440 nM	23.9	
Uhlíková et al. 1996	HIV-1	6.4	112 nM		Integral kinetics
		4.5	19 nM		

The top part of the table shows the results of sedimentation studies; the bottom part the results of kinetic assays. $\tau_{1/2}$ is the half-time of dimer dissociation.

termini intertwined in an antiparallel β -sheet, and a hydrogen bond scaffold supporting the active site called the fireman's grip. It is formed by the Asp-Thr(Ser)-Gly triplet in which the oxygen of side chain of the active site Thr (Thr26 in the case of HIV-1 PR) forms a hydrogen bond with the main-chain amide of the active site Thr (Thr26') of the other molecule in the dimer. Similar interactions take place between the side chain of this Thr (Thr26) and the main-chain carbonyl of the preceding Leu (Leu24' in the case of HIV-1 PR) of the other molecule in the dimer. Both hydrogen bonds are dependent on the side-chain hydroxyls of the Thr residues. An X-ray crystallographic model of the three-dimensional structure of HIV-1 PR and a detailed view of the active site region depicting the described hydrogen bond network are shown in Figure 1. This complex structure maintains the productive conformation of the active site (Pearl and Blundell 1984; Pearl and Taylor 1987).

Previous studies showed that the Thr26Ser mutation of HIV-1-PR decreases the enzyme activity fivefold to 10-fold (Konvalinka et al. 1995; Rose et al. 1995). The opposite phenomenon was observed for the Ser38Thr mutation of MAV and Rous sarcoma virus (RSV) proteases (Arad et al. 1995; Ridky et al. 1996). A recent study (Střišovský et al. 2000) provides evidence that the fireman's grip contributes to dimerization, which could thus be responsible for these differences in activity. Střišovský et al. (2000) showed, by using sedimentation analysis and activity assays, that mutation of Thr26 of wild-type HIV-PR to Ala and, in lesser extent, also to Ser and Cys decreases the dimer stability considerably. This finding makes it important to address whether this is a general phenomenon, that is, whether the

presence of either Thr or Ser in the active site triplet Asp-Thr(Ser)-Gly indeed regulates the dimer stability of PRs. Therefore, we undertook a comparative analysis of the equilibrium and rate constants of dimerization of three prototype PRs, from HIV, MAV, and MPMV. We examined the hypothesis that the presence of either Thr or Ser in the active site triplet of PRs plays a regulatory role in the PR dimerization and therefore in its activation. We also asked whether Ser-Thr changes affect the K_d of the dimer by affecting the association or the dissociation rate constants. Answering this question may give clues to the folding pathway that leads to the active PR dimer and thus triggers polyprotein processing in retroviruses.

Results

Design, fluorometric, and kinetic characterization of fluorogenic substrates

New fluorogenic substrates were designed for the purpose of the present study. Our aim was to prepare substrates with a good fluorometric response and good kinetic properties. The design was based on the previously known peptide substrates with convenient values of K_m and k_{cat} (Konvalinka et al. 1991, 1992). Donor and acceptor of energy for nonradiative energy transfer were attached to the termini of the substrates, 5-[(2'-aminoethyl)-amino]naphthalenesulfonic acid (EDANS) as a donor and 4-[[4'-(dimethylamino)phenyl]azo]-benzoic acid (DABCYL) as an acceptor. Emission and excitation spectra were recorded for both the uncleaved and cleaved substrates (data not shown). The fluorescence of the cleaved form is 11 times higher than that of the noncleaved form. The wavelengths of maxima for excitation and emission, 347 and 500 nm, respectively, roughly correspond with those tabulated for EDANS. The substrates were also characterized kinetically. Determined values of Michaelis constant K_m and the turnover number k_{cat} are presented in Table 2. Both substrates are suitable for wild-type MAV-PR (MAV-PR[S]). For the other enzymes, however, only peptide FS1 can be used because the cleavage of FS2 is too slow or does not occur at all.

Equilibrium constant of the monomer-dimer process

The equilibrium between monomeric and dimeric forms of a PR is described by the chemical equation

The equilibrium concentration of dimer in solution is given by the equation

$$[D] = \frac{1}{8}(K_d + 4E - \sqrt{K_d(K_d + 8E)}), \quad (2)$$

Figure 1. (A) Structure of the dimer of HIV-1 protease. The individual subunits are depicted by different colors. Dimerization domain (D), flaps (F), and active site (A) are indicated. (B) Detail of the active site of HIV-1 protease with fireman's grip. Hydrogen bridges are denoted by dashed lines. Source is Protein Data Bank entry 3 PHV.

Table 2. Values of K_m and k_{cat} for the fluorogenic substrates FS1 (Glu[EDANS]ThrHisGlnValTyr↓PheValArgLysAlaLys [DABCYL]-NH₂) and FS2 (Glu[EDANS]ThrProGlnValTyr↓PheValArgLysAlaLys [DABCYL]-NH₂) and their chromogenic analogues CS1 (AlaThrHisGlnValTyr(p-nitro-Phe)ValArgLysAla) and CS2 (AlaThrProGlnValTyr(p-nitro-Phe)ValArgLysAla)

Enzyme	Substrate	K_m (μM)	k_{cat} (sec ⁻¹)
MAV-PR(S)	FS1	2.2 ± 0.6	0.7 ± 0.1
MAV-PR(S)	FS2	3.5 ± 0.5	8.6 ± 1.4
MAV-PR(S)	CS1	75 ^a	5 ^a
MAV-PR(S)	CS2	9 ^a	5 ^a
HIV-1-PR(T)	FS1	3.0 ± 0.4	13.3 ± 1.4
HIV-1-PR(T)	CS1	15 ^b	14 ^b
HIV-1-PR(S)	FS1	4.2 ± 1.2	4.8 ± 0.7
MPMV-PR(T)	FS1	0.9 ± 0.1	1.9 ± 0.2
MPMV-PR(T)	CS1	60 ^c	0.17 ^c

For experimental details see Materials and Methods. Several values were adopted from ^aKonvalinka et al. (1991); ^bKonvalinka et al. (1990); and ^cZábranský (1999).

where K_d is the equilibrium constant, $[D]$ the concentration of dimer, and E the overall (analytical) concentration of the enzyme considered as monomer. We introduce the constants α and β (Kuzmič 1993):

$$\alpha = \sqrt{K_d(K_d + 8E)} \quad (3a)$$

$$\beta = K_d + 4E. \quad (3b)$$

Equation 2 now has the form

$$[D] = \frac{1}{8}(\beta - \alpha). \quad (4)$$

Thus, the equilibrium constant has to be calculated from the ratio of the initial and equilibrium dimer concentration with the aid of equation 4. Let us denote Q_{eq} as the ratio $[D_{eq}]/[D_0]$, where $[D_{eq}]$ is the equilibrium dimer concentration and $[D_0]$ is the initial dimer concentration, that is, the equilibrium concentration of the stock solution multiplied by the dilution factor. Q_{eq} fulfills the following equation:

$$Q_{eq} = \frac{E_0}{E} \frac{K_d + 4E - \sqrt{K_d(K_d + 8E)}}{K_d + 4E_0 - \sqrt{K_d(K_d + 8E_0)}} \quad (5)$$

After some rearrangements, the quadratic equation for K_d is obtained, which has two roots, one of them physically meaningful:

$$K_d = \frac{-B \pm \sqrt{B^2 - 4AC}}{2A}, \quad (6)$$

$$A = Q_{eq}(Q_{eq}[E + E_0]^2 - EE_0[Q_{eq} + 1]^2), \quad (7a)$$

$$B = -4EE_0(E + E_0)Q_{eq}(Q_{eq} - 1)^2, \quad (7b)$$

$$C = 4E^2E_0^2(Q_{eq} - 1)^4. \quad (7c)$$

Here E is the cuvette concentration, and E_0 is the concentration of the stock solution.

Equation 6 was used for the practical determination of the dimerization equilibrium constant K_d . The parameter Q_{eq} was calculated from the initial ($t_{preincubation} = 0$) and limit ($t_{preincubation} \rightarrow \infty$) values of reaction rates. Final enzyme concentrations after the dilution were chosen to cover the significant range properly, that is, to reach different values of the degree of dimer dissociation from low to high. Substrate concentration was kept as low as possible, generally below the value of K_m , to prevent possible substrate stabilization of the dimer.

A typical dependence of the initial rate on the preincubation time is shown in Figure 2, together with the inferred equilibrium rate. The value of K_d was calculated for every series of measurements, and the final result was then obtained as an arithmetic average of these values. These data, together with number of determinations and the initial (stock) and final concentrations, are presented in Table 3. A schematic plot of these values for all the studied enzymes is given in Figure 3. The wild-type forms of HIV-PR and MPMV-PR prefer the dimer form more than the wild type of MAV-PR, with the difference being approximately an order of magnitude. The two enzymes that can be compared to their fireman's grip mutants indicate that the dimers of T-variants of PRs are about an order of magnitude more stable than those of the S-variants. Wild-type MPMV-PR (MPMV-PR[T]) is not compared with its mutant, but the value of K_d for this enzyme is close to that of the other T-variants.

Rate constant of dimer association and dissociation

The kinetics of the dimerization–monomerization process is described by the following differential equation:

Figure 2. Initial reaction rate as a function of a preincubation time (circles). Dashed line indicates the equilibrium rate.

Table 3. Values of equilibrium constants of dimerization (K_d) and kinetic parameters (k_2) of dimer dissociation and association

Enzyme	Number of experiments	Stock enzyme concentration (μM)	Assay enzyme concentration (nM)	K_d (nM)	K_2 (10^{-4} sec^{-1})	$\tau_{1/2}$ (min)	k_1 ($10^3 \text{ M}^{-1} \text{ sec}^{-1}$)
MAV-PR(S)	6	18.4	12–62	412 ± 122	7.47 ± 1.07	15.5 ± 2.2	1.81 ± 0.79
MAV-PR(T)	5	13–27	21–51	31.5 ± 3.3	7.81 ± 1.49	14.8 ± 2.8	24.8 ± 18.3
HIV-1-PR(T)	7	3–21	1–7	15.1 ± 4.3	6.78 ± 1.97	17.0 ± 5.0	44.9 ± 25.8
HIV-1-PR(S)	4	0.75–10	2–10	122 ± 28	12.1 ± 1.09	9.5 ± 0.8	10.0 ± 3.19
MPMV-PR(T)	4	2.78	4–19	13.5 ± 8.3	28.1 ± 10.8	4.1 ± 1.6	208.1 ± 207.9

Specific experimental conditions for each enzyme are indicated.

$$\frac{d[D]}{dt} = k_1[M]^2 - k_2[D] \quad (8)$$

Using $[M] = E - 2[D]$ ($[M]$ is the monomer concentration), this equation can be solved yielding the time dependence of dimer concentration, $[D]$

$$[D] = \frac{1}{8} \frac{(\beta - \alpha)\gamma - (\beta + \alpha)e^{-k_1\alpha t}}{\gamma - e^{-k_1\alpha t}}. \quad (9)$$

The symbol γ is defined as

$$\gamma = \frac{E(\beta_0 - \alpha_0) - E_0(\beta + \alpha)}{E(\beta_0 - \alpha_0) - E_0(\beta - \alpha)} \quad (10)$$

and α_0 and β_0 are defined analogously to α and β in equation 3, a and b, where E is substituted by E_0 .

To determine the kinetic constants k_1 and k_2 , we make use of the approximate, but generally accepted, relation among these constants and K_d :

$$K_d = \frac{k_2}{k_1} \quad (11)$$

By rearranging equation 9 with the aid of equation 11, we obtain the equation

$$W \equiv \frac{K_d}{\alpha} \ln \left(\frac{EQ(\beta_0 - \alpha_0) - E_0(\beta + \alpha)}{EQ(\beta_0 - \alpha_0) - E_0(\beta - \alpha)} \frac{1}{\gamma} \right) = k_2 t, \quad (12)$$

where Q , the ratio of current and initial dimer concentrations, is a generalization of the experimental quantity Q_{eq} from equation 5 and is determined as a ratio of the reaction rate at a certain time and at the initial time. The experimental quantity W depends linearly on time through the proportionality constant k_2 . Thus, this constant can be determined by linear regression. By using equation 11, we obtain the other rate constant k_1 .

The determination of the rate constant k_2 makes use of equation 12. In contrast to K_d , the values of initial reaction rate for all preincubation times are necessary. For every

experimental point, the quantity W is calculated and linear regression of its dependence on time is performed. The constant k_2 is obtained as a slope of this dependence. The calculated values of the rate constant k_2 are shown in Table 3. In addition, values of the rate constant of dissociation k_1 are presented there. The last quantity presented in this table is the halftime of dimer dissociation provided that the dissociation is an independent process and no association takes place. Thus, this quantity is calculated in the following way:

$$\tau_{1/2} = \frac{\log 2}{k_2}. \quad (13)$$

It is, in fact, only a different expression of k_2 with a more transparent meaning.

The final averaged values of kinetic parameters are presented in Table 3. It can be seen that MPMV-PR(T) is the most quickly dissociating enzyme, whereas the other two wild-type enzymes dissociate more slowly, MAV-PR(S) being slightly faster than HIV-PR(T). Comparison of wild-type enzymes and fireman's grip mutants of HIV-PR and MAV-PR shows that the dissociation rate constants are less affected by the mutations than the are K_d values. Thus, although HIV-PR(S) dissociates approximately twice as quickly as HIV-PR(T), there is no difference between the rate of dissociation of MAV-PR(S) and MAV-PR harboring Ser38Thr mutation (MAV-PR[T]). In contrast, the association rate constant k_1 , calculated from the values of K_d and k_2

Figure 3. Values of K_d for the five studied enzymes: logarithmic plot. Each pair of the wild-type enzyme and corresponding mutant is connected for clarity.

(see Table 3), is strongly affected by the mutations. In summary, despite the higher experimental error of k_1 determination, the results show that the Thr→Ser mutation in fireman's grip leads to the decrease of the rate of association rather than to the increase of the rate of dissociation.

Discussion

In this article, we present a study of thermodynamic and kinetic characteristics of dimerization of three PRs and their mutant forms. These quantities have not been previously reported for these enzymes, with the exception of the wild-type HIV-1-PR. Even for this protease, which is one of the most thoroughly studied enzymes, the values provided by different experimenters and different methods are in a considerable disagreement with each other. The problems with K_d determination arise especially from the very low value of this constant, because the protein concentrations in enzymatic assays cannot differ much from K_d . The reason is that the total enzyme concentration is divided equally between monomer and dimer when it is equal to K_d (cf. equation 2). Moreover, the concentration range in which neither of the two forms prevails is rather limited, approximately one order of magnitude on each side. For example, when the enzyme concentration $E = 10K_d$, 80% of the protein is present as dimer, whereas for $E = 0.1K_d$, the dimer forms only 15% of the total enzyme. For the determination of low values of K_d (10^{-7} to 10^{-9}), the experimental enzyme concentration has to be kept close to these values, which consequently complicates the detection of the response, and thus, it allows only fluorescent detection in kinetic studies. Methods such as NMR or light scattering, which require high protein concentrations, are almost excluded from these studies unless the protease has a dimerization defect leading to a much higher K_d (Ishima et al. 2001; Schatz et al. 2001; Louis et al. 2003).

The results presented in this article were obtained under optimal conditions of pH and ionic strength for each wild-type enzyme, and the substrate concentrations were kept below the K_d of the dimer. Two different substrates for MAV PR gave similar results. Other artifacts that might interfere with the interpretation also are unlikely. Autoprocessing, precipitation, or denaturation of the enzyme or its adsorption on the walls of preincubation tubes are excluded by those experiments performed with higher enzyme concentrations, because they run to a well-pronounced equilibrium.

In the experimental results presented, there are two principal sources of the experimental error, the error of enzyme titration and the error of determination of the ratio of equilibrium and initial rates. The error of K_d was estimated using the equation

$$\Delta K_d = \left| \frac{\partial K_d}{\partial E_0} \right| \Delta E_0 + \left| \frac{\partial K_d}{\partial Q} \right| \Delta Q, \quad (14)$$

where ΔK_d means the error of the equilibrium constant, ΔE_0 is the error of the initial concentration obtained from the nonlinear regression procedure, and ΔQ is the error estimate of the equilibrium and initial rates ratio. The error of enzyme titration depends on the availability of a tight-binding inhibitor of the corresponding enzyme. In the case of MAV and MPMV enzymes, for which only less tightly binding inhibitors are available, the error is higher. Furthermore, the fluorometric detection of enzyme activity, especially the signal-to-noise ratio, is supported either by high enzyme activity or lower dimer stability, because the experiment then can be carried out with more enzyme. Accordingly, the lowest experimental error was obtained for the enzymes with high activity and low K_d value (such as HIV-1-PR[T]) and the highest for MAV-PR(T) and MPMV-PR(T).

As regards the rate constant determination, we decided to determine experimentally the dissociation constant k_2 and then calculate k_1 by using the value of K_d . The reason is that dimer dissociation is the prevalent process immediately after the dilution of the enzyme solution, and k_2 is thus less affected by the experimental error. Moreover, dimer dissociation obeys first-order kinetics, which are concentration independent. This fact precludes the influence of enzyme-titration error on k_2 , when only short-time measurements are taken into account. The values of k_1 , on the other hand, are influenced by all the errors of the both previous determinations, which makes them less reliable for enzymes in which both the measurements are troublesome, as, for example, MPMV-PR(T).

Because of the complexity of the mathematical procedure used to determine k_2 and its weak dependence on the enzyme concentration, the experimental error of this constant was estimated as a standard deviation of the mean of a set of independent experimental results. The errors of the dependent quantities, $\tau_{1/2}$ and k_1 , were evaluated by using the following expressions:

$$\Delta \tau_{1/2} = \frac{\Delta k_2 \ln 2}{k_2^2}, \quad (15a)$$

$$\Delta k_1 = \frac{K_d \Delta k_2 + k_2 \Delta K_d}{K_d^2}. \quad (15b)$$

The determined values of K_d (see Fig. 3) differ considerably among the studied enzymes. Physically meaningful dependencies can, however, be observed. First, the K_d of wild-type HIV-1-PR (HIV-1-PR[T]) agrees well with the other kinetically determined values of this constant in similar conditions, especially as published by Pargellis et al. (1994) and

Uhlířková et al. (1996). However, the majority of the sedimentation experiments provide values that are higher by three to four orders of magnitude. We hypothesize that this disagreement is caused by the pressure dependence of the equilibrium constant, as found, for example, in the case of LexA repressor (Mohana-Borges et al. 2000). High pressure in the cuvette forces proteins to decrease their volume, leading to unfolding or dissociation of multimeric structures (Silva et al. 2002). The equilibrium constant K_d depends on pressure via the standard molar Gibbs reaction energy $\Delta_r G^0$, $K_d = \exp(-\Delta_r G^0/RT)$, where R is the molar gas constant ($R = 8.314 \text{ J mole}^{-1}\text{K}^{-1}$) and T is thermodynamic temperature. The partial derivative of $\Delta_r G^0$ with respect to the pressure is $\partial\Delta_r G^0/\partial p = \Delta_r V^0$, where $\Delta_r V^0$ is the molar reaction change of volume. Thus, if an approximation is made that this quantity is independent of pressure, the pressure dependence of $\Delta_r G^0$ is as follows: $\Delta_r G^0(p) = \Delta_r G^0(p_0) + \Delta_r V^0(p - p_0)$. Now it is possible to compare results of kinetic and sedimentation studies, and estimate the value of $\Delta_r V^0$ that might cause the observed difference. If an example of the kinetic result of the present study ($K_d = 15 \text{ nM}$) and the sedimentation result of Střišovský et al. (2000; $K_d = 77 \text{ } \mu\text{M}$) are taken, the former gives $\Delta_r G^0 = 45 \text{ kJ/mole}$; the latter, $\Delta_r G^0 = 24 \text{ kJ/mole}$. The pressure during ultracentrifugation is in the order of 5 MPa. Thus, the corresponding $\Delta_r V^0$ is $\sim -4.3 \times 10^{-3} \text{ m}^3/\text{mole}$, which is $-7.1 \times 10^{-27} \text{ m}^3$ per a reaction of one dimer molecule. According to the calculation method presented by Zamyatnin (1972), the volume of the dimer is about $2.7 \times 10^{-26} \text{ m}^3$. Similar volume estimates can be deduced also from the Protein Data Bank crystal structures. Hence, the calculated volume change after dissociation of a single PR dimer decreases by one fourth. Although this estimate of the volume change is higher than the values measured for other proteins (Foguel et al. 1998; Mohana-Borges et al. 2000; Suarez et al. 2001), the volume change might account for the experimentally observed high K_d values, when sedimentation analysis is used for K_d determination.

The dimer of HIV-1-PR(T) is by more than an order of magnitude more stable than that of MAV-PR(S), but is comparable with MPMV-PR(T) dimer. This observation means that in the concentration range between $\sim 10^{-6}$ and 10^{-9} M , the HIV-1 and MPMV PRs are considerably more active as a direct consequence of the process of dimerization. Dimerization could play an important role in regulation of activity. The concentration of the protease in the viral particle, estimated to be $\sim 0.1 \text{ mM}$ (Konvalinka et al. 1995), at first glance seems too high for such regulation. However, dimerization might be important for activity regulation in the cell before the formation of an immature viral particle, although the quantification of this process is difficult. It was reported that N-terminal extension of the protease may change both its activity and dimerization properties (Zybarth and Carter 1995; Schatz et al. 2001). The requirement for dimerization

prevents the cleavage of cellular proteins and the premature processing of viral polyproteins by viral PR. It was shown (Burstein et al. 1991; Kräusslich 1991) that introduction of a tethered PR dimer into a retroviral provirus lead to the loss of infectivity and particle formation, clearly due to the premature activation of the polyprotein processing.

Our experiments show that the dimers of the T-version of MAV and HIV PRs are more stable than are their S-analogs, with the difference in K_d being about an order of magnitude for both enzymes. The structural reason for this phenomenon is far from clear, but the observation itself is in correspondence with the previous virological expectations. Thus, the expression strategy of a retrovirus (PR in frame with its substrate or not) correlates well with the amino acid after the active-site aspartate (serine or threonine), as well as with the K_d value. Hence, the change in this amino acid might be a direct cause of the different dimer stability, which in turn helps to regulate the replication of the virus. Our results thus strongly support the hypothesis that Thr→Ser mutation in fireman's grip destabilizes the dimer of the protease and vice versa, and this mechanism is used in the nature for regulation of proteolytic activity of PRs.

The determined values of the kinetic constant k_2 (and also $\tau_{1/2}$) summarized in Table 3 show that the increased dimer stability of the T-version of PRs is caused by a higher rate of the dimer association rather than slower dissociation. Thus, the respective amino acid does not probably stabilize the dimer considerably but plays an important role in the mechanism of dimer association. It is conceivable that association and dissociation are not strictly reciprocal but proceed by different pathways. Provided that the dimer is kept together mainly by the interactions in the N- and C-terminal dimerization domain and the flap region (see Fig. 1A), it is not surprising that the influence of the fireman's grip mutation to the dissociation process is small. However, these two domains are probably insufficient to induce the dimer formation on their own. We hypothesize that the fireman's grip provides an aid to dimerization, mediating the initial contact of the two monomer molecules and adjusting them to the proper conformation and/or orientation. Several possible mechanisms for this phenomenon can be proposed. One of them can be inferred from a detail of this structure (Fig. 1B). This domain is capable of binding the two chains quite firmly by a network of hydrogen bridges and forms some predimeric intermediate. Recent structural analyses (Ishima et al. 2001; Louis et al. 2003) have demonstrated the existence of a folded monomer conformationally very similar to the subunits of a dimer. Thus, the conformation change of the monomer subunit is likely to be small and the initial intermediate may play a role in orientation of the particles to the proper positions. The fireman's grip domain is situated in an optimal position for this purpose (as opposed to, e.g., dimerization domain at the PR termini), because it is close to the mass center of the dimer. Hence, once

the subunits are connected in this region and even oriented properly, the dimerization process might be facilitated.

The remarkable difference between threonine and serine in the fireman's grip motif also can be anticipated from Figure 1B. Methyl groups of threonines may come into a tight contact during the dimer formation. An interaction of these groups may restrict the rotation around the joint connecting the two monomers and, therefore, stabilize the nascent dimer. The conformation of the threonine side chains need not change in a major way during this process, because even in the final dimer structure, they are still close to one of the favorable rotamers, as can be deduced from the rotamer library (<http://www.fccc.edu/research/labs/dunbrack/bbdep.html>; see also Dunbrack Jr. and Karplus 1993; Dunbrack Jr. and Cohen 1997). If threonine is substituted by serine, the methyl groups are missing. The nascent intermediate is lacking their interaction, and its geometry may therefore be less favorable for establishing the contacts with the other domains important for dimerization.

Materials and methods

Expression and purification of recombinant enzymes

DNA coding for MAV-PR was a kind gift of Jiří Hejnar (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic), and the DNA coding for the Ser38Thr mutant of MAV-PR was kindly provided by Moshe Kotler (Department of Molecular Genetics, Hebrew University, Hadassah Medical School, Jerusalem, Israel). The DNA coding regions of MAV-PR(S) and MAV-PR(T) were PCR-amplified and cloned into the expression vector pET 22b (Novagen) by using 5'-AGAAGCTTCTCGAGCTATAAATTTGTCAAGCG-3' as a downstream primer and 5'-TAGGTACCATATGGGATCCCCGGGACATTAT-3' as the upstream primers for MAV-PR, cloned with 65 codon extension at the 5'-end, and 5'-TAGGTACCATATGTTAGCGATGACAATGGAG-3' for the mutated MAV-PR(T) (without extension). Restriction endonucleases *XhoI* and *NdeI* were used, respectively. Coding areas of the constructs were checked by DNA sequencing. The expression and purification protocol follows the procedure of Pichová et al. (1992) with minor modifications. Bacterial cells BL21(DE3) were transformed by the expression plasmids and grown to the optical density of 1.0. The expression was induced by 1 mM IPTG (isopropyl- β -D-galactopyranoside); after 3 h of expression cells were harvested, washed by PBS buffer (10 mM PO_4^{3-} , 100 mM NaCl at pH 7.2), and resuspended in buffer A (50 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA at pH 8.0) with the addition of phenylmethylsulfonyl fluoride (PMSF, 10 $\mu\text{g/mL}$). Cells were disrupted by two cycles of freezing/thawing (-70°C), 30-min incubation with chicken egg lysozyme (0.5 mg/mL), and another 30-min incubation with 1% sodium deoxycholate (0.05 mL per 1 mL of the cell suspension) followed by three 1-min sonication cycles. Suspension was centrifuged for 10 min at 1000g at 4°C ; pellet was resuspended in 3 mL buffer B (50 mM Tris-HCl, 50 mM PO_4^{3-} , 30 mM NaCl, 2 mM EDTA, 0.1% [v/v] mercaptoethanol at pH 7.5) and then dissolved in buffer B containing 9 M urea. The solution was dialyzed against buffer C (20 mM Tris-HCl, 10 mM PO_4^{3-} , 20 mM NaCl, 1 mM EDTA, 0.05% [v/v] mercaptoethanol, 5% [v/v] glycerol at

pH 7.0) and centrifuged (20,000g, 4°C , 30 min). The refolding was repeated three times. Supernatants were collected and purified by batchwise anion-exchange chromatography on DEAE-Sephadex in buffer C, and the unbound material was further dialyzed into the buffer D (10 mM sodium acetate, 1 mM EDTA, 0.05% 2-mercaptoethanol at pH 5.0), loaded on the column of SP-Sepharose and eluted by NaCl gradient (0 to 0.4M) in buffer D.

HIV-1-PR(T) and HIV-1-PR harboring Thr26Ser mutation (HIV-1-PR(S)) were prepared as described in Stříšovský et al. (2000). MPMV-PR (the 12-kD form; Zábranský et al. 1998) was a kind gift of Iva Pichová (Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic).

Titration of enzyme by tight-binding inhibitor

One milliliter of assay buffer AB (0.1 M sodium acetate, x M NaCl, 1 mM EDTA, 10% [v/v] glycerol at pH 5.0; $x = 2.0$ for both wild-type and mutated MAV-PR[S] and [T] and 0.3 for the other enzymes) was mixed with a constant amount (typically 10 to 20 μL of 3 mM stock solution) of the peptide substrate AlaThrHisGlnValTyr (p-nitro-Phe) ValArgLysAla (5 mg/mL) and a variable amount (0 to 100 μL) of a tight binding competitive inhibitor (Majer et al. 1993). Enzyme-specific peptide inhibitors were used for this purpose: QF34, 1 μM (Konvalinka et al. 1997) for HIV-1-PR(T) and HIV-1-PR (S); ProProCysVal (PheSta) AlaMetThrMet, 13 μM for MAV-PR(S) and MAV-PR (T); and ProTyrVal (PheSta) AlaMetThr, 36 μM for MPMV-PR(T). The reaction was started by the addition of 10 μL of the enzyme and was monitored spectrophotometrically. Typically, the reaction was performed for several different values of inhibitor concentration, and the initial velocity was determined for each of them. The enzyme concentration was determined by nonlinear regression of the dependence of the initial velocity on the inhibitor concentration.

Fluorogenic substrates for kinetic studies

Two peptide substrates were designed by using an internally quenching pair of fluorescent groups EDANS (donor) and DABCYL (acceptor) as reported (Matayoshi et al. 1990). The sequences of the two substrates are as follows: FS1, (Glu[EDANS]ThrHisGlnValTyr↓PheValArgLysAlaLys[DABCYL]-NH₂); FS2, (Glu[EDANS]ThrProGlnValTyr↓PheValArgLysAlaLys [DABCYL]-NH₂; the arrow denotes the cleavage site). The fluorogenic substrates were synthesized on the solid phase by using standard *O*-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) mediated 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on polystyrene support with peptide amide linker (PAL), 5-(4-Fmoc-aminomethyl-3,5-dimethoxyphenoxy)valeric acid. Both the fluorophore (EDANS) and the quencher (DABCYL) were introduced onto the corresponding amino acids (Glu and Lys) prior to their attachment; hence, FmocGlu(EDANS)-OH and FmocLys(DABCYL)-OH were used for the coupling. The peptides were cleaved off the resin and simultaneously deprotected by 5% thioanisole, 3% ethanedithiol, 2% anisole, and 90% trifluoroacetic acid (TFA) and were finally purified by high-performance liquid chromatography (Vydac C18 column) using water/acetonitrile/0.1% TFA as mobile phase in gradient elution (Gulnik et al. 1997).

Kinetic characterization of fluorogenic substrates

A series of reactions was performed in which increasing amounts of the substrate (typically 3 to 15 μL of 2 mM stock solution) was

mixed with 3 mL of assay buffer AB (see above), and the reaction was started by addition of 3 μ L of the enzyme. The fluorescence increase was monitored for 300 sec, and the initial reaction rate was determined. The constants K_m and k_{cat} were calculated by fitting the data to the Michaelis-Menten equation by nonlinear regression.

Determination of dimerization parameters

A stock concentration of the enzyme was dialyzed into the assay buffer AB (see above) and titrated by tight-binding inhibitor. To determine the dimerization parameters K_d and k_2 , several series of individual reaction runs were performed, each of them for different enzyme concentration. Every experiment consisted of several reactions differing in the preincubation time. The reaction for the zero preincubation time: 3 mL of the assay buffer was mixed with known amount (1 or 2 μ L) of fluorogenic substrate (~2 mM), and the reaction was started by the addition of the corresponding amount of enzyme. The course of the reaction was monitored fluorometrically for 300 sec. The reaction mixtures for the other reactions were prepared mixing 3 mL of the assay buffer with the corresponding amount of the enzyme in plastic tubes. These samples were preincubated in 37°C, each one for a different time (2 to 75 min). After preincubation, the content of the tube was transferred into the cuvette, and substrate was added in the same amount as in the first reaction; the reaction course was monitored. Initial reaction rate was determined graphically as the negative value of the slope of this dependence for $t_{\text{reaction}} = 0$. After completing the whole series, the limit reaction rate for $t_{\text{preincubation}} \rightarrow \infty$ was estimated. The dimerization parameters were derived from the set of initial reaction velocities for all these series by a mathematical procedure described in Results.

Acknowledgments

We thank Iva Pichová, Helena Bauerová, and Aleš Zábranský for providing MPMV protease. We are grateful to Jiří Hejnar and Moshe Kotler for providing the DNA coding for the wild-type and mutant MAV protease. We also thank Pavel Majer for his kind assistance in the synthesis of fluorogenic substrates and Martin Lepšík for his help in preparation of Figure 1. This work was performed under the research project Z4 055 905 and supported by grants from the Ministry of Health Care of the Czech Republic (NI/6339-3) and from the Fifth Framework of the European Commission (QLRT-2000-02360).

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Arad, G., Chorev, M., Shtorch, A., Goldblum, A., and Kotler, M. 1995. Point mutation in avian sarcoma leukemia virus protease which increases its activity but impairs infectious virus production. *J. Gen. Virol.* **76**: 1917–1925.
- Burstein, H., Bizub, D., and Skalka, A.M. 1991. Assembly and processing of avian retroviral Gag polypeptides containing linked protease dimers. *J. Virol.* **65**: 6165–6172.
- Cheng, Y.S., Yin, F.H., Foundling, S., Blomstrom, D., and Kettner, C.A. 1990. Stability and activity of human immunodeficiency virus protease: Comparison of the natural dimer with a homologous, single-chain tethered dimer. *Proc. Natl. Acad. Sci.* **87**: 9660–9664.
- Darke, P.L. 1994. Stability of dimeric retroviral proteases. *Methods Enzymol.* **241**: 104–127.
- Darke, P.L., Jordan, S.P., Hall, D.L., Zugay, J.A., Shafer, J.A., and Kuo, L.C. 1994. Dissociation and association of the HIV-1 protease dimer subunits: Equilibria and rates. *Biochemistry* **33**: 98–105.
- Dunbrack Jr., R.L. and Cohen, F.E. 1997. Bayesian statistical analysis of protein side-chain rotamer preferences. *Protein Sci.* **6**: 1661–1681.
- Dunbrack Jr., R.L. and Karplus, M. 1993. Backbone-dependent rotamer library for proteins: Application to side-chain prediction. *J. Mol. Biol.* **230**: 543–574.
- Dunn, B.M. 1998. Human immunodeficiency virus 1 retropepsin. In *Handbook of proteolytic enzymes* (eds. A.J. Barrett et al.), pp. 919–928. Academic Press, London.
- Foguel, D., Silva, J.L., and de Prat-Gay, G. 1998. Characterization of a partially folded monomer of the DNA-binding domain of human papilloma virus E2 protein obtained at high pressure. *J. Biol. Chem.* **273**: 9050–9057.
- Grant, S.K., Deckman, I.C., Culp, J.S., Minnich, M.D., Brooks, I.S., Hensley, P., Debouck, C., and Meek, T.D. 1992. Use of protein unfolding studies to determine the conformational and dimeric stabilities of HIV-1 and SIV proteases. *Biochemistry* **31**: 9491–9501.
- Gulnik, S.V., Suvorov, L.I., Majer, P., Collins, J., Kane, B.P., Johnson, D.G., and Erickson, J.W. 1997. Design of sensitive fluorogenic substrates for human cathepsin D. *FEBS Lett.* **413**: 379–384.
- Holzman, T.F., Kohlbrenner, W.E., Weigl, D., Rittenhouse, J., Kempf, D., and Erickson, J. 1991. Inhibitor stabilization of human immunodeficiency virus type-2 proteinase dimer formation. *J. Biol. Chem.* **266**: 19217–19220.
- Ishima, R., Ghirlando, R., Tozser, J., Gronenborn, A.M., Torchia, D.A., and Louis, J.M. 2001. Folded monomer of HIV-1 protease. *J. Biol. Chem.* **276**: 49110–49116.
- Jordan, S.P., Zugay, J., Darke, P.L., and Kuo, L.C. 1992. Activity and dimerization of human immunodeficiency virus protease as a function of solvent composition and enzyme concentration. *J. Biol. Chem.* **267**: 20028–20032.
- Konvalinka, J., Strop, P., Velek, J., Cerna, V., Kostka, V., Phylip, L.H., Richards, A.D., Dunn, B.M., and Kay, J. 1990. Sub-site preferences of the aspartic proteinase from the human immunodeficiency virus, HIV-1. *FEBS Lett.* **268**: 35–38.
- Konvalinka, J., Bláha, I., Škrabana, R., Sedláček, J., Pichová, I., Kaprálek, F., Kostka, V., and Štřop, P. 1991. Subsite specificity of the proteinase from myeloblastosis associated virus. *FEBS Lett.* **282**: 73–76.
- Konvalinka, J., Hořejší, M., Andreánský, M., Novek, P., Pichová, I., Bláha, I., Fábry, M., Sedláček, J., Foundling, S., and Štřop, P. 1992. An engineered retroviral proteinase from myeloblastosis-associated virus acquires pH dependence and substrate specificity of the HIV-1 proteinase. *EMBO J.* **11**: 1141–1144.
- Konvalinka, J., Litterst, M.A., Welker, R., Kottler, H., Rippmann, F., Heuser, A.M., and Krausslich, H.G. 1995. An active-site mutation in the human immunodeficiency virus type 1 proteinase (PR) causes reduced PR activity and loss of PR-mediated cytotoxicity without apparent effect on virus maturation and infectivity. *J. Virol.* **69**: 7180–7186.
- Konvalinka, J., Litera, J., Weber, J., Vondrášek, J., Hradilek, M., Souček, M., Pichová, I., Majer, P., Štřop, P., Sedláček, J., et al. 1997. Configurations of diastereomeric hydroxyethylene isosteres strongly affect biological activities of a series of specific inhibitors of human-immunodeficiency-virus proteinase. *Eur. J. Biochem.* **250**: 559–566.
- Kräusslich, H.G. 1991. Human immunodeficiency virus proteinase dimer as component of the viral polypeptide prevents particle assembly and viral infectivity. *Proc. Natl. Acad. Sci.* **88**: 3213–3217.
- Kuzmič, P. 1993. Kinetic assay for HIV proteinase subunit dissociation. *Biochem. Biophys. Res. Commun.* **191**: 998–1003.
- Louis, J.M., Ishima, R., Nesheiwat, I., Pannell, L.K., Lynch, S.M., Torchia, D.A., and Gronenborn, A.M. 2003. Revisiting monomeric HIV-1 protease: Characterization and redesign for improved properties. *J. Biol. Chem.* **278**: 6085–6092.
- Majer, P., Urban, J., Gregorova, E., Konvalinka, J., Novek, P., Stehlikova, J., Andreánský, M., Sedláček, J., and Štřop, P. 1993. Specificity mapping of HIV-1 protease by reduced bond inhibitors. *Arch. Biochem. Biophys.* **304**: 1–8.
- Matayoshi, E.D., Wang, G.T., Krafft, G.A., and Erickson, J. 1990. Novel fluorogenic substrates for assaying retroviral proteases by resonance energy transfer. *Science* **247**: 954–958.
- Mohana-Borges, R., Pacheco, A.B., Sousa, F.J., Foguel, D., Almeida, D.F., and Silva, J.L. 2000. LexA repressor forms stable dimers in solution: The role of specific DNA in tightening protein–protein interactions. *J. Biol. Chem.* **275**: 4708–4712.
- Pargellis, C.A., Morelock, M.M., Graham, E.T., Kinkade, P., Pav, S., Lubbe, K., Lamarre, D., and Anderson, P.C. 1994. Determination of kinetic rate con-

- stants for the binding of inhibitors to HIV-1 protease and for the association and dissociation of active homodimer. *Biochemistry* **33**: 12527–12534.
- Pearl, L. and Blundell, T. 1984. The active site of aspartic proteinases. *FEBS Lett.* **174**: 96–101.
- Pearl, L.H. and Taylor, W.R. 1987. A structural model for the retroviral proteases. *Nature* **329**: 351–354.
- Pichová, I., Štrop, P., Sedláček, J., Kaprálek, F., Beneš, V., Trávníček, M., Pavlíčková, L., Souček, M., Kostka, V., and Foundling, S. 1992. Isolation, biochemical characterization and crystallization of the p15gag proteinase of myeloblastosis associated virus expressed in *E. coli*. *Int. J. Biochem.* **24**: 235–242.
- Ridky, T.W., Bizub-Bender, D., Cameron, C.E., Weber, I.T., Wlodawer, A., Copeland, T., Skalka, A.M., and Leis, J. 1996. Programming the Rous sarcoma virus protease to cleave new substrate sequences. *J. Biol. Chem.* **271**: 10538–10544.
- Rose, J.R., Babe, L.M., and Craik, C.S. 1995. Defining the level of human immunodeficiency virus type 1 (HIV-1) protease activity required for HIV-1 particle maturation and infectivity. *J. Virol.* **69**: 2751–2758.
- Schatz, G.W., Reinking, J., Zippin, J., Nicholson, L.K., and Vogt, V.M. 2001. Importance of the N terminus of Rous sarcoma virus protease for structure and enzymatic function. *J. Virol.* **75**: 4761–4770.
- Silva, J.L., Oliveira, A.C., Gomes, A.M., Lima, L.M., Mohana-Borges, R., Pacheco, A.B., and Foguel, D. 2002. Pressure induces folding intermediates that are crucial for protein-DNA recognition and virus assembly. *Biochim. Biophys. Acta* **1595**: 250–265.
- Stříšovský, K., Tessmer, U., Langner, J., Konvalinka, J., and Kräusslich, H.G. 2000. Systematic mutational analysis of the active-site threonine of HIV-1 protease: Re-thinking the “fireman’s grip” hypothesis. *Protein Sci.* **9**: 1631–1641.
- Suarez, M.C., Lehrer, S.S., and Silva, J.L. 2001. Local heterogeneity in the pressure denaturation of the coiled-coil tropomyosin because of subdomain folding units. *Biochemistry* **40**: 1300–1307.
- Towler, E.M., Gulnik, S.V., Bhat, T.N., Xie, D., Gustschina, E., Sumpter, T.R., Robertson, N., Jones, C., Sauter, M., Mueller-Lantzsch, N., et al. 1998. Functional characterization of the protease of human endogenous retrovirus, K10: Can it complement HIV-1 protease? *Biochemistry* **37**: 17137–17144.
- Uhlíková, T., Konvalinka, J., Pichová, I., Souček, M., Kräusslich, H.G., and Vondrášek, J. 1996. A modular approach to HIV-1 proteinase inhibitor design. *Biochem. Biophys. Res. Commun.* **222**: 38–43.
- Wlodawer, A., Miller, M., Jaskolski, M., Sathyanarayana, B.K., Baldwin, E., Weber, I.T., Selk, L.M., Clawson, L., Schneider, J., and Kent, S.B. 1989. Conserved folding in retroviral proteases: Crystal structure of a synthetic HIV-1 protease. *Science* **245**: 616–621.
- Xie, D., Gulnik, S., Gustchina, E., Yu, B., Shao, W., Qoronfleh, W., Nathan, A., and Erickson, J.W. 1999. Drug resistance mutations can effect dimer stability of HIV-1 protease at neutral pH. *Protein Sci.* **8**: 1702–1707.
- Zábranský, A. 1999. “Analysis of the protease from Mason-Pfizer monkey retrovirus.” PhD thesis, Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic.
- Zábranský, A., Andreánský, M., Hrušková-Heidingsfeldová, O., Havlíček, V., Hunter, E., Ruml, T., and Pichová, I. 1998. Three active forms of aspartic proteinase from Mason-Pfizer monkey virus. *Virology* **245**: 250–256.
- Zamyatnin, A.A. 1972. Protein volume in solution. *Prog. Biophys. Mol. Biol.* **24**: 107–123.
- Zhang, Z.Y., Poorman, R.A., Maggiora, L.L., Heinrikson, R.L., and Kezdy, F.J. 1991. Dissociative inhibition of dimeric enzymes: Kinetic characterization of the inhibition of HIV-1 protease by its COOH-terminal tetrapeptide. *J. Biol. Chem.* **266**: 15591–15594.
- Zybarth, G. and Carter, C. 1995. Domains upstream of the protease (PR) in human immunodeficiency virus type 1 Gag-Pol influence PR autoprocessing. *J. Virol.* **69**: 3878–3884.