A Folding Inhibitor of the HIV-1 Protease

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ABSTRACT **Because the human immunodeficiency virus type 1 protease (HIV-1-PR) is an essential enzyme in the viral life cycle, its inhibition can control AIDS. The folding of single-domain proteins, like each of the monomers forming the HIV-1-PR homodimer, is controlled by local elementary structures (LES, folding units stabilized by strongly interacting, highly conserved, as a rule hydrophobic, amino acids). These LES have evolved over myriad generations to recognize and strongly attract each other, so as to make the protein fold fast and be stable in its native conformation. Consequently, peptides displaying a sequence identical to those segments of the monomers associated with LES are expected to act as competitive inhibitors and thus destabilize the native structure of the enzyme. These inhibitors are unlikely to lead to escape mutants as they bind to the protease monomers through highly conserved amino acids, which play an essential role in the folding process. The properties of one of the most promising inhibitors of the folding of the HIV-1-PR monomers found among these peptides are demonstrated with the help of spectrophotometric assays and circular dichroism spectroscopy. Proteins 2006;62:928–933.**

Key words: HIV-1 protease; folding inhibitors; protein folding

INTRODUCTION

Human immunodeficiency virus type 1 protease (HIV-1-PR) is a homodimer, that is, a protein whose native conformation is built of two (identical) disjoint chains (see Fig. 1), each made out of 99 amino acids. Sedimentation equilibrium experiments have shown that at neutral pH, the protease folds according to a three-state mechanism ($2U \rightarrow 2N \rightarrow N_2$), populating consistently the monomeric native conformation N. The dimer dissociation constant is $K_D = 5.8\ \mu M$ at 4°C, while the folding temperature of the monomer (i.e., the temperature at which the free energy of the native monomeric state N is equal to that of the unfolded state U) is $T_f = 52.5$°C.[1,2] Recent NMR studies have also found folded monomers of several mutants.[3,4] At low pH, on the other hand, calorimetric experiments have shown[5] that there is a single transition at $T = 59$°C (pH 3.4, 25 μM protein, 100 mM NaCl) between the dimeric native state and a monomeric unfolded state.

The pH of the solution that surrounds the HIV-1-PR affects its catalytic capabilities. It has been shown[6] that the activity of the protease increases if the pH of the solution is lowered. Considering that the active site of the protease is at the interface between the two monomeric units, it is most likely that the affinity of the protease to the substrate is correlated with the structure of the dimer. The value of the dimerization constant K_D is very controversial.[1,2] Anyway it seems it ranges from the order of nanomoles to that of micromoles in going from acidic to neutral conditions. One can thus guess that the activity of the protease is higher at low pH because the dimeric state is more populated. Increasing the value of pH, acid residues acquire a negative charge. In particular, the pair of Asp25, which lies close on the interface, repels each other through the Coulomb force. The overall effect is to increase the dissociation constant (measured by sedimentation equilibrium experiments), which assumes the value $K_D = 5.8\ \mu M$ at pH 7 (and $T = 4$°C[1,2]), further increasing at higher temperatures. Consequently, one expects a detectable ratio of folded monomers in solution. Thus, the destabilization of the monomer will lead to enzyme inhibition.[7]

Drug resistance has severely limited the effectiveness of conventional (active site–centered) HIV-1-PR inhibitors in AIDS therapy.[8] Experimental evidence has shown that drug-resistant mutations can occur only at specific positions that are critical for drug binding but are tolerated as far as folding and thus viral activity is concerned. Likely resistance-evading drugs can thus be searched among molecules interacting strongly with those conserved residues that play an important role in the folding of the protease. Following this viewpoint, we have recently proposed a general strategy based on the inhibition of folding.[7]

Model studies of single-domain globular proteins[9–12] indicate that folding proceeds following a hierarchical

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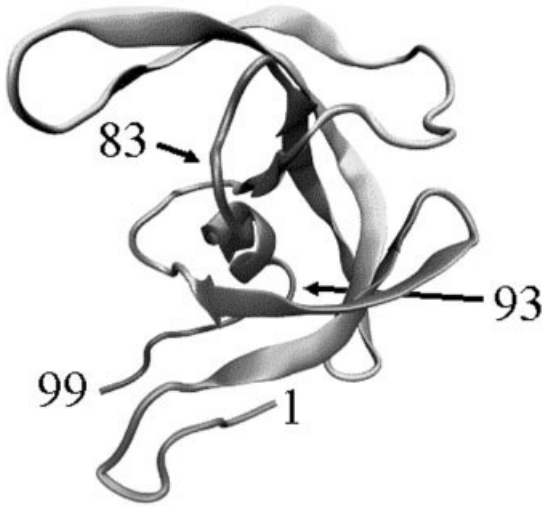

Fig. 1. Monomer of the HIV-1-PR. The inhibitor peptide I has a sequence idential to that of the segment whose ends (residues 83 and 93) are indicated here.

succession of events starting from the formation of local elementary structures (LES, stabilized by strongly interacting, highly conserved, as a rule hydrophobic, so-called "hot" amino acids). The docking of these LES, which is again controlled by these "hot" amino acids, leads to the formation of the (postcritical) folding nucleus.[12] Mutations of the "hot" amino acids give rise, in general, to protein denaturation.[13] Strong support for the soundness of this hierarchical scenario is found in a number of studies presenting circumstantial evidence.[14–22] Even proteins that apparently fold according to a nonhierarchical pathway, such as those following the "nucleation-condensation model"[23] [e.g., chymotripsin inhibitor 2 (CI2)], have been suggested[19] to fold thorough a well-defined sequence of events. In particular, a theoretical study (Sutto L, Tiana G, Broglia RA. Hierarchy of events in protein folding: beyond the Go model. To be published) of the 66-mer CI2 protein (a study similar to that carried out in connection with the HIV-1-PR[7]), has shown the hierarchical folding of this protein. The study has identified segments S_1 = 29–34, S_2 = 45–52, and likely S_3 = 55–64 of CI2, as those associated with LES, the corresponding peptides p-S_i (i = 1–3) being efficient inhibitors. In any case, it will be a matter of systematic future research to assess whether the present folding-inhibition approach can be applied also to proteins folding according to the nucleation-condensation paradigm.

In any case, the hierarchical scenario applies also to each of the monomers forming three-state folding homodimers, like the HIV-1-PR,[22] as has been shown in detail with the help of extensive Go-model simulations[7] (see also the detailed all-atom Go model simulations[24]).

The hierarchical model also suggests that it is possible to destabilize the native conformation of a protein with the help of peptides whose sequences are identical to those of the LES of the protein.[25] Such peptides (p-LES) interact with the protein (in particular with their complementary LES) with similar energy to that which stabilizes its folding nucleus,[a] thus competing with its formation. Given this fact, it is unlikely that the virus can develop drug resistance through mutations, because, to prevent interaction between the LES or between the p-LES and the LES, one has to mutate "hot" amino acids.

Based on these general criteria, molecular dynamics and Monte Carlo simulations, along with evidence taken from site-directed mutagenesis and sequence analysis, lead to the identification of three segments of the HIV-1-PR monomers that are likely connected with the LES of the protease. These segments are S_2 = 24–34, S_7 = 75–78, and S_8 = 83–93 (cf. Broglia et al.[7]). As discussed,[7] S_7 is too short to lead to a specific inhibitor, while S_2 contains the active site (residue 25–27), a fact that has not been taken into account in studying the folding of model proteins[10–12] or in simulations of folding inhibitors[25] (for details, see first column of the Results and Discussion section in Broglia et al.[7]). On the other hand, S_8 is well structured in its native conformation, as well as in simulations of the peptide p-S_8 alone in the solvent.[7] Consequently, segment S_8 = 83–93 of the protease is expected to be the most promising candidate as monomer inhibitor. In the following we show its efficiency as inhibitor of the enzyme.

MATERIALS AND METHODS

Recombinant **HIV-1-PR**, expressed in *Escherichia coli* (Bachem UK, Ltd., catalog H-9040)[26,27] contained five mutations to restrict autoproteolysis (Q7K, L33I, L36I) and to restrict cysteine thiol oxidation (C67A and C95A). The enzyme was stored (at −70°C) as solution with concentration 0.1 μg/μL in dilute HCl (pH = 1.6).

A **chromogenic substrate** for HIV-1-PR (HIV Protease Substrate III, Bachem UK, Ltd., catalog H-9035) with sequence H-His-Lys-Ala-Arg-Val-Leu-Phe(NO_2)-Phe-Glu-Ala-Nle-Ser-NH_2 was obtained as a 1-mg desiccate, diluted with 0.1 mL of dimethylsulfoxide (DMSO), and stored at −20°C. Protease-assisted cleavage between the

[a]This is because the folding nucleus, resulting from the docking of the LES, contains a large fraction of the stabilization energy of the protein, and is strongly decoupled from the rest of the residues of the protein. This result is the basis of the hierarchic folding mechanism. Consequently, the interaction energy of a p-LES peptide with the rest of the protein is, essentially, the sum of the interaction energy with the complementary LES. In other words, while the protein energetics is nonadditive in terms of individual amino acids, it becomes almost additive concerning the LES of the protein. In particular, as shown in Broglia et al.,[7] the all-atom molecular dynamics energy map expressed in terms of the folding nucleus, the flap region, and the outer region around the C-terminal can be expressed in terms of a codiagonal matrix (see Table 1 in Broglia et al.[7]), with small nondiagonal matrix elements between the FN and the rest of the protein. Furthermore, the submatrix associated with the FN divides essentially into two blocks, one associated with S_7 and another associated with S_2 and S_8. The corresponding off-diagonal (cooperative) matrix elements connecting the (S_2– S_8) and the S_7 structures are only a fraction of the S_2– S_8 stabilization energy. Furthermore, this energy is rather similar to that associated with peptides p-S_8 and p-S_2 alone in the solvent, in the conformation corresponding to the native state. Summing up, the interaction between S_2 and S_8 depends rather weakly on the rest of the protein (cf. for details, Broglia RA, Tiana G, Simona F, Sutto L, Provasi D. Stability, folding and escape mutants of HIV-1-PR: input for the design of nonconventional inhibitors. To be published).

TABLE I. Peptides Used in This Work

Peptide	HIVI-Pr sites	Sequence	Molecular weight
I	83–93	NIIGRNLLTQI	1295.54
K_1	61–70	QILIEICGHK	1194.46
K_2	9–19	PLVTIKIGGQL	1179.46
K_3	Not from HIV-1-PR	LSQETFDLWKLLPEN	1874.12

Peptide I is the proposed inhibitor; control peptides, either from the HIV-I-PR sequence (K_1 and K_2) and not (K_3) were also tested.

Leu and the Phe(NO_2) residues of substrate entails a blue-shift of the absorption maximum (277 nm to 272 nm) that can be adequately monitored observing the continuous decrease of absorbance at 300 nm.[28–30] A regression of the absorbance at 300 nm against substrate concentration allows us to check that the absorbance scales linearly up to a concentration of 800 μM, and to estimate the molar absorption coefficient of the whole substrate [$\epsilon_S = 3000 \pm 600$ (M cm)$^{-1}$]. To determine the molar absorption coefficient of the cleaved products, reactions with different initial substrate concentration were followed for at least 2 h. The absorbance at 300 nm after complete peptidolysis allows us to determine differential extinction coefficient $\Delta\epsilon = 500 \pm 90$ (M cm)$^{-1}$ between the whole substrate and the cleaved products. This compares well with a difference of extinction coefficient at 310 nm between the cleaved and the complete substrate of 1200 ± 100 (M cm)$^{-1}$, reported[28] for a similar substrate.

Inhibitor peptide (peptide **I**, cf. Table I) from the sequence of the HIV-1-PR wild-type [Protein Data Bank (PDB) code: 1BVG] was synthesized by Fmoc solid-phase peptide synthesis with acetyl and amide as terminal protection group and was estimated to be > 95% pure by analytical high-performance liquid chromatography (HPLC) after purification. After that, 1 mg of inhibitor peptide was dissolved in 100 μL of DMSO; 4 μL of this solution were then diluted with 16 μL of DMSO and 180 μL of the buffer used for assay. The obtained solution (150 μM of peptide **I**) was used for the experiments.

Control peptides were also synthesized by Fmoc solid-phase peptide synthesis. Two of them, called K_1 and K_2 (cf. Table I), are also from the primary sequence of the HIV-1-PR, but from regions well outside the local elementary structures identified in Broglia et al.[7] MD simulations indicate that any peptide of a length similar to that of the inhibitors (≈10 amino acids) displaying a sequence identical to a segment of the protein not corresponding to LES, does not have any particular effect on the folding of the protease.[7] In keeping with this result, the two chosen control peptides (K_1 and K_2) fulfill the general criteria not to correspond to a LES, nor to contain the active site amino acids (25–28). Summing up, we expect peptides K_1 and K_2 not to be able to destabilize appreciably the native state, since they cannot compete with the formation of the folding nucleus. A third peptide, K_3, used also as control peptide, is not related in any way to the protease and has been chosen for the sole purpose of testing whether the mere presence of a peptide (uncorrelated this time to the protease) can affect its activity. Note that peptide K_2 is rather hydrophobic and only > 70% purity could be achieved.

The **assay buffer** was prepared, following the method by Leuthardt and Roesel,[27] by adding 0.8 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol to a 20 mM phosphate buffer (pH 6).

Experimental Methods

Each measure was performed recording the absorbance at 300 nm in a standard UV-vis spectrophotometer (Jasco V-560). The sample had a total volume of 70 μL in Spectrosil Far UV Quartz (170–2700 nm) cuvettes (3.3-mm optical path). The sample in the cuvette was exposed to a constant temperature (25 ± 0.05°C) provided by continuous circulation of water from a water bath to the cell holder via a circulation pump.

For the determination of the kinetic parameters, we measured at least six different concentrations of substrate, spanning the range from 100 μM to 600 μM. After proper thermal stabilization of the substrate dissolved in the buffer, the absorbance at 300 nm was checked to be stable, then the reaction initiated by adding 4 μL of the enzyme solution.

For each sample we followed at least 1200 s and determined the initial rate v_i by a linear fit of the first 200 s. We repeated twice the determination of the kinetic parameters of the enzyme.

The assay was then repeated in presence of the inhibitor peptide. We followed the same procedure, incubating for 60 s the inhibitor peptide (3, 10, and 20 μM) with the protein before adding it to the substrate.

Circular Dichroism (CD) Spectrum

Ultraviolet CD spectra were recorded on a Jasco J-810 spectropolarimeter in nitrogen atmosphere at room temperature using 0.1 cm path-length quartz cell. Each spectrum was recorded between 260 nm and 200 nm. The data were collected at a rate of 10 nm/min with a wavelength step of 0.2 nm and a time constant of 2 s. The spectra were corrected with respect to the baseline and normalized to the amino acidic concentration. The protein and the peptide were dissolved in a 20 mM phosphate buffer with 0.8 M NaCl at the same concentration used for the activity assays. The CD spectra were analyzed in terms of contribution of secondary structure elements[31] using the K2D method based on comparison with CD spectra of proteins and peptides with known secondary structure.

RESULTS AND DISCUSSION

Enzyme kinetics and inhibition constants are analyzed in the framework of the Michaelis–Menten equation; that is, one assumes that the reaction can be described by the relation

$$E + S \leftrightarrows ES \rightarrow E + P, \tag{1}$$

where E, S, ES, and P stand for enzyme, substrate, enzyme–substrate complex, and product, respectively. The

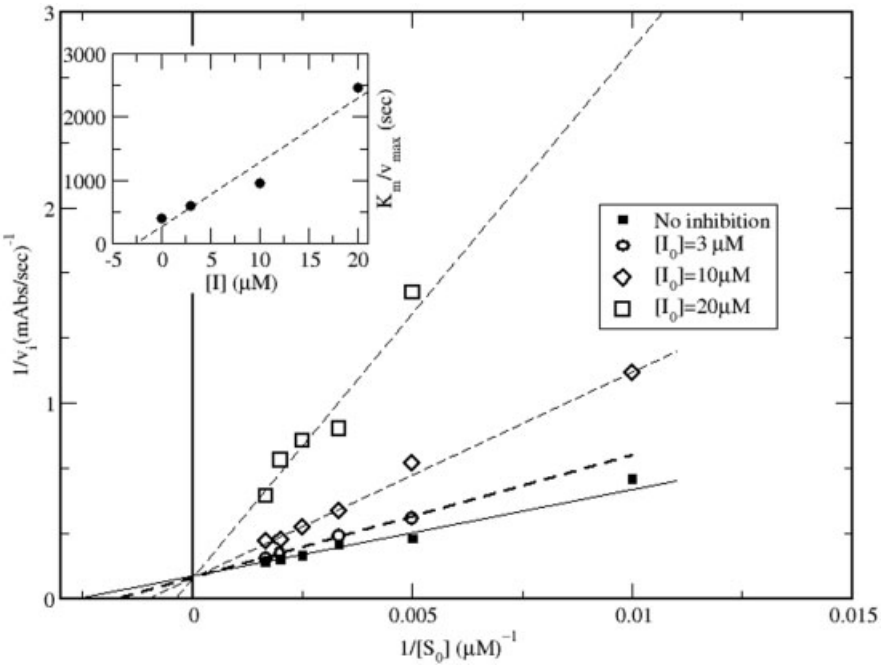

Fig. 2. Inhibitory activity of the peptide. The Lineweaver–Burk plot associated with the protease (■) and the protease complexed with inhibitor **I** at 3 μ*M* (○), 10 μ*M* (◇), and 20 μ*M* (□). The lines are the fits to the experimental points. The initial velocities, v_i, are expressed in terms of micromoles per second, while the substrate concentration $[S_0]$ is in micromoles. We report in the inset the values of K_m/v_{max} as a function of the inhibitor concentration [**I**]. The linear fit to the data gives a $K_i = 2.58 \pm 0.78$ μ*M*, with a correlation coefficient $r = 0.94$ and a p value for the F test of 0.029.

TABLE II. Kinetic Parameters for the Reactions Assessed

	No inhibitor	[I] = 3 μ*M*	[I] = 10 μ*M*	[I] = 20 μ*M*
K_m (μ*M*)	380 ± 80	680 ± 92	980 ± 290	2600 ± 2000
v_{max} (mAbs/min)	8.57 ± 0.88	10.37 ± 0.8	9.29 ± 1.9	9.71 ± 11.0
v_{max} (μmol/s)	0.94 ± 0.19	1.14 ± 0.09	1.02 ± 0.27	1.07 ± 1.2

The table contains the kinetic data for the HIV-1-PR hydrolization of the HIV-1-PR substrate III, without and with different concentrations [I] of inhibitor. The values of K_m for the inhibited reactions have to be regarded as apparent dissociation constants. The values for v_{max} are reported as measured (mAbs/min) and converted using the differential extinction coefficient $\Delta\varepsilon = 500 \pm 90$ $(M\ \mathrm{cm})^{-1}$. Values and errors are obtained using nonlinear fits.

rate in the production of the product [*P*] for short times, is then described by

$$v_i = v_{max}[S](K_m + [S])^{-1}, \quad (2)$$

where K_m represents the dissociation constant of the enzyme substrate complex, and $v_{max} = k_{cat}\ [E]_0$ is the maximum catalytic rate, attained for saturating substrate concentrations [*S*]. The quantity k_{cat} is the catalytic constant (i.e., the first-order rate constant for the chemical conversion of the *ES* complex into the *EP* complex). The value of K_m of the enzyme–substrate can be obtained by transforming the reaction rate and concentration data to a double-reciprocal plot (see Fig. 2).

A fit of the data provides estimates for K_m and v_{max} (380 ± 80 μ*M* and 8.57 ± 0.88 mAbs/min, respectively). We note that this value of K_m is considerably larger than the one reported in the literature for the same enzyme–substrate system. It should be stressed, however, that the experimental conditions in Pennington[30] are different as far as pH and ionic strength are concerned.

In the presence of an inhibitor, Eq. (2) still holds, where now the parameters v_{max} represent the apparent maximum catalytic rate for the inhibited reaction, and K_m should be interpreted as an apparent dissociation constant. To test the inhibitory properties of peptide **I**, we have measured these kinetic parameters K_m^{app} and v_{max} for three different concentrations of the peptide. The data recorded are plotted in Figure 2, along with the data obtained without inhibitors. The results of the fits are also reported in Table II.

It is observed that the values of v_{max} are almost constant and only the dissociation constants K_m increase with increasing concentration of peptide **I**. The observed kinetics is thus compatible with a competitive inhibition mechanism, where

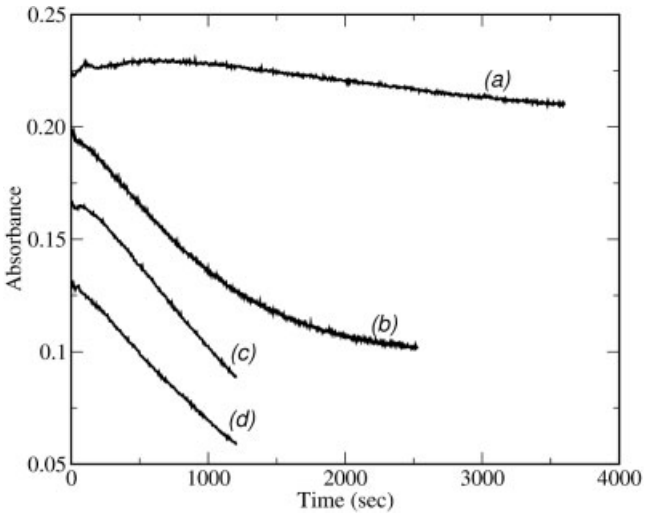

Fig. 3. Control peptides. The enzymatic kinetics of the inhibited protease alone (curve *b*), of the protease inhibited with peptide **I** (*a*), and of the protease together with control peptides K_1 (*c*) and K_2 (*d*), measured as change in absorbance of the chromogenic substrate as a function of time. All the curves have been measured at $[S_0] = 125\ \mu M$, and have been shifted along the *y* axis in order to be inspected easily. In all samples, the concentration of peptides was 3 μM.

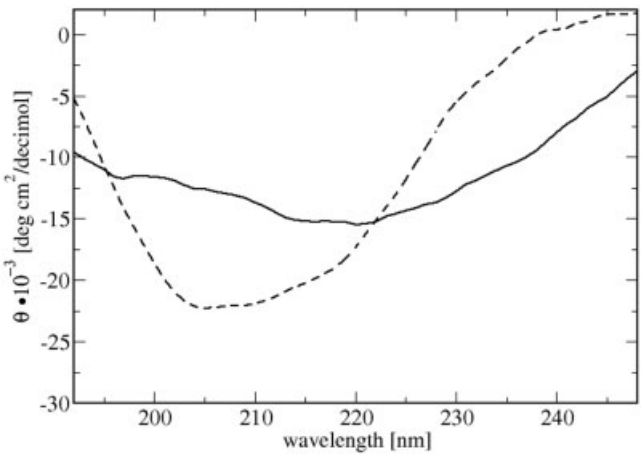

Fig. 4. Evidence of unfolding. The CD spectrum of the protease (dashed curve) and of the solution composed of the protease and peptide **I** (continuous curve) in the ratio 1:3, from which the spectrum of the peptide has been subtracted.

$$K_m^{\mathrm{app}} v_{\max}^{-1} = K_m\, v_{\max}^{-1}(1 + [I]\, K_I^{-1}). \qquad (3)$$

A competitive kind of inhibition is expected, since the binding of the p-LES denaturing the protein is mutually exclusive with respect to its proteolytic activity. Using this equation, we can estimate from a linear regression of the slopes of the Lineweaver–Burk plot, the value $K_i = 2.58 \pm 0.78\ \mu M$ (cf. inset to Fig. 2) that gives the dissociation constant for the enzyme–inhibitor complex. The results are reported in Table II. It should be stressed that, due to the saturation of the linear relationship between absorbance and concentration at around 800 μM, the most concentrated sample is only twice K_m, limiting the numerical accuracy of the estimated parameters. On the other hand, the data clearly point to a competitive inhibition mechanism, where the binding of peptide *I* to the protein, causing its unfolding, competes with its folded, active conformation.

We have made similar measurements using the **control peptides** K_1, K_2, and K_3 instead of peptide **I**, and found no appreciable variation in the kinetic parameters with respect to the uninhibited case (see Fig. 3). In presence of peptide K_1, K_2, or K_3 the reaction displayed initial rates $v_i = 1.98$ mAbs/min, $v_i = 2.06$ mAbs/min, and $v_i = 2.01$ mAbs/min, respectively, essentially identical to the value of v_i obtained for the uninhibited enzyme in identical conditions. Due to the limited amount of enzyme available, we have not fully characterized the kinetics of the reaction in the presence of the control peptides, but just checked that for a given value of [*S*] the reactions were not affected.

To provide evidence demonstrating that the inhibition mechanism of the peptide indeed prevents the proper folding of the enzyme, we measured a **circular dichroism** spectrum of the protein alone and after incubation with the peptide. The CD spectrum of the protease (cf. Fig. 4) under the same conditions used for the activity assay indicate a β-sheet content of 30%, consistent with the β character of the native conformation.[32] Figure 4 also displays the CD spectrum of the solution of the protease plus the **I** inhibitor (to which the spectrum of the peptide **I** alone has been subtracted, thus assuming that the structure of the free peptide does not change appreciably when it binds to the protein, as expected for a LES, as well as evinced from all-atom simulations of the peptide p-S_8 carried out with GROMACS, taking explicitly into account the solvent[7]) at the same concentrations and under the same conditions as those of the activity assay. It shows a loss of β structure (to a β-sheet content value of 14%), indicating that the protein is to a large extent in a nonfolded conformation. These numbers compare well with those predicted by model calculations.[7]

CONCLUSIONS

The peptide **I** (≡ NIIGRNLLTQI) displaying a sequence identical to that of one of the LES (83–93) of each of the two identical chains forming the HIV-1-PR homodimer is found to be a highly specific and efficient inhibitor ($K_i = 2.58 \pm 0.78\ \mu M$) of the folding of the 99mers, and thus of the whole enzyme. A remarkable property of this inhibitor is that it is unlikely that it would allow for escape mutants. In fact, the only mutations that will prevent **I** from acting are likely to involve protein denaturation.

Obvious disadvantages of the inhibitor are its length, its hydrophobicity and its peptidic character, as it is not clear how to prevent the degradation by enzymes. Consequently, there are a number of clear tasks lying ahead in the quest to develop the lead into a potential drug. One is to investigate whether the shortening of peptide **I**, by leaving out some residues either at the beginning or at the end (or both), leads to peptides that still inhibit folding with similar specificity and effectiveness as peptide **I** does and, at the same time, are more soluble. Another is to

develop molecules mimetic to the present inhibitor or eventually to shorter peptides derived from it. A third one is to check membrane permeability so as to secure internalization.

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