Conformational Stability and Catalytic Activity of HIV-1 Protease Are Both Enhanced at High Salt Concentration*

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The activity of human immunodeficiency virus protease is markedly increased at elevated salt concentration. The structural basis of this effect has been explored by several independent methods by using both the wild-type enzyme and its triple mutant (Q7K/L33I/L63I) (Mildner, A. M., Rothrock, D. J., Leone, J. W., Ban-now, C. A., Lull, J. M., Reardon, I. M., Sarchic, J. L., Howe, W. J., Tomich, C.-S. C., Smith, C. W., Heinrikson, R. L., and Tomasselli, A. G. (1994) Biochemistry 33, 9405-9413), designed to better resist autolysis. Monitoring the intrinsic fluorescence of the two enzymes during urea-mediated denaturation has shown that at high NaCl concentration, both the conformational stability (ΔG‡) and the transition midpoint (D50) between the folded and unfolded states increase, indicating that the salt stabilizes the enzyme structure. These equilibrium data are supported by kinetic studies on the urea-mediated unfolding. Toward this end, we used both the fluorescence change, red shifting in the maximum of the emission spectrum, and far and near-UV CD. The salt effects observed in urea-mediated unfolding reactions prevail upon heat denaturation. All these findings support the existence of a two-state equilibrium between the folded and unfolded proteins. The pH dependence of fluorescence intensity indicated that the conformation of human immunodeficiency virus type 1 protease should change in the catalytically competent pH region. It is concluded that preferential unfolding stabilizes the protease structure in the presence of salt, providing entropic contribution to enhance the catalytic activity.

The protease encoded by HIV-1 is involved in the specific processing of large viral polyproteins into individual structural proteins and enzymes. This prompted extensive investigations on HIV-1 protease as a potential therapeutic target of AIDS. The enzyme is a member of the aspartic protease family and exists as homodimer of identical polypeptide chains, each consisting of 99 amino acid residues (cf. Refs. 1 and 2). High salt concentration was found to enhance the catalytic activity, primarily by lowering the Michaelis constant (Km), and it was suggested that the salting-out effect of NaCl decreased Km and increased kcat/Km, the specificity rate constant (3, 4). We have pointed out that Km values may be markedly dependent on pH, while the rate enhancement by salt is practically independent of Km and pH (5). Therefore, other factors, for example the conformational stability of the protein, may also be important. Indeed, raising the NaCl concentration frequently stabilizes the protein structure by preferential hydration (6). However, the effects of ionic strength on the conformational stability of HIV-1 protease have not yet been studied. To reveal whether or not the enhanced catalytic activity is associated with a more stable structure, we have examined the stability of the enzyme against urea, pH, and heat denaturation at different salt concentrations. A mutant enzyme designed to resist autolysis (7) has been used in most experiments.

EXPERIMENTAL PROCEDURES

HIV-1 Protease—The enzyme was expressed in Escherichia coli and purified as described (5). The construction containing three mutations (Q7K/L33I/L63I) in the HIV-1 protease was kindly supplied by Dr. Tomasselli (7), and the enzyme was purified from inclusion bodies with the same method as used for the wild-type protease. The protein concentration was determined at 280 nm (5). The activity of enzymes was measured spectrophotometrically with the chromogenic substrate Lys-Ala-Arg-Val-Leu-Phe(NO2)-Gln-Ala-Nle as described (5). The specificity rate constant (kcat/Km) was 290 ± 20 μ mol min⁻¹ s⁻¹ with both the native and mutant enzymes. In some cases, the reactions were monitored spectrophotometrically with 2-aminobenzoyl-Thr-Ile-Nle-Phe(NO2)-Gln-Arg using an excitation wavelength of 337 nm (bandwidth of 1.5 nm) and an emission wavelength of 420 nm (bandwidth of 10 nm). The substrates were prepared by solid-phase synthesis (Applied Biosystems Inc. Model 431A) and purified by high pressure liquid chromatography.

Intrinsic Fluorescence Measurements—Unfolding of HIV protease in urea or at extreme pH values was monitored at 25 °C by a Jasco FP 777 spectrofluorometer equipped with a thermostated cell holder. The excitation wavelength was 280 nm (bandwidth of 5 nm), and the emission wavelength was 347 nm (bandwidth of 10 nm). Fluorescence intensities (Iobs) were measured in a four-component buffer of 25 mM formic acid, 25 mM acetic acid, 25 mM Mes, and 75 mM Tris containing 1 mM EDTA and 5% (v/v) glycerol (standard buffer). The pH of the buffer mixture was adjusted with 1.0 N HCl or 1.0 N NaOH. This system gave virtually constant ionic strength throughout the pH range employed. Small differences were corrected by the addition of NaCl to give identical conductivity values. For measuring emission spectra, the following conditions were used: 280-nm excitation wavelength, 1.5-nm excitation bandwidth, 320-370-nm emission wavelength, 3-nm emission bandwidth, scanning speed of 100 nm/min, response time of 0.5 s, and photomultiplier gain "medium." Urea-dependent Denaturation—Unfolding of HIV protease at increasing urea concentrations was determined as described (8). The equilibrium unfolding measurements, assuming a two-state reaction (Equation 1),

\[
f_{u} = f_{i} \frac{(1 - f_{u})}{(1 - f_{i})}
\]

were carried out in 1.0 ml of ~140 μmol protein solutions after incubation for 60–180 min at 25 °C. No change in fluorescence was observed after that time. For each value of the measured fluorescence intensity (Iu), the fraction of unfolded protein (fu) was calculated from Equation 2,

\[
f_u = f_i - I_{u}/I_i - I_u
\]
where $I_n$ and $I_i$ are the fluorescence intensities, respectively, of the fully unfolded protein obtained at high concentrations of urea and the fully folded protein acquired in the absence of the denaturant. The values of $I_n$ and $I_i$ were obtained at the base lines of the transition curves, at which $I_{m0}$ became practically invariant at changing urea concentrations. The fluorescence values were corrected by substraction of the fluorescence of the corresponding buffer or urea solution in the absence of protein.

For equilibrium unfolding, the change of $f_u$ with increasing urea concentration follows Equation 3 (9, 10).

$$ f_u = 1 - \exp(m[D] - \Delta G^0/RT)/(1 + \exp(m[D] - \Delta G^0/RT)) $$  
(Eq. 3)

where $R$ is the gas constant (8.314 J/degree/mol), $T$ is the absolute temperature, and $m$ is a measure of the dependence of $\Delta G$ on the denaturant concentration. $\Delta G^0$ stands for the conformational stability of the protein at zero concentration of denaturant ([D] = 0) and indicates how much more stable the native conformation is compared with the unfolded protein. The urea concentration at the midpoint of the unfolding curve can be calculated from Equation 4.

$$ [D]_{1/2} = \Delta G^0/m $$  
(Eq. 4)

Denaturation Rate—The unfolding of HIV-1 protease was monitored spectrofluorometrically using 280-nm excitation (bandwidth of 1.5 nm) and 347-nm emission (bandwidth of 5 nm) wavelengths. The reaction was initiated by the addition of 100 µl of enzyme (0.16 mg/ml) to 900 µl of 7 M urea in standard buffer containing 0.1 or 1.0 M NaCl. The first-order rate constant for unfolding was calculated by nonlinear regression using the GraFit computer program (10). To establish the reversibility of denaturation, a 50-µl reaction mixture was added to 950 µl of assay mixture to determine the regained activity at pH 5.5 using the fluorogenic substrate 2-aminobenzoyl-Thr-Ile-Nle-Phet(NO)₂-Gin-Arg.

Circular Dicroism—CD spectral measurements in the near- and far-UV regions were made on a Jasco J-720 spectropolarimeter using 0.1- and 1.0-cm path length cuvettes for far- and near-UV, respectively. Protein concentration was typically 5–10 µM. Because of the high absorbance of urea, the measurements in the far-UV region could not be carried out below 210 nm. Spectral scans were run at a rate of 10 nm/min. Unfolding kinetics were recorded at 270 and 230.5 nm in the near- and far-UV regions, respectively.

Thermal Inactivation—The mutant protease (2.13 µM) was incubated in standard buffer at 53 °C. Aliquots were taken at appropriate times, and the remaining activity was determined by measuring the initial rate with the chromogenic substrate (347 nm) at 20 °C in 50 mM acetate buffer, pH 4.0, containing 1 mM EDTA; 1 mM dithioerythritol; 0.5 M NaCl, and 5% glycerol. The activity decreased according to a first-order reaction. The rate constants were calculated by nonlinear regression data analysis using the GraFit computer program (10).

Calculation of Isoelectric Points—Evaluation of the ionization of amino acid residues at different pH values and determination of the isoelectric points of HIV-1 proteases were carried out by using the ISOELECTRIC program (25).

RESULTS

Unfolding of HIV-1 Protease in Urea—For measuring the differences in conformational stabilities of a protein under different conditions, denaturation curves are especially useful. Since the fluorescence intensities of the native and denatured proteins are different (cf. Ref. 8), the unfolding of HIV-1 protease in urea or at extreme pH values can be monitored spectrofluorometrically. Many globular proteins have been found to follow a two-state mechanism (Equation 1), in which only the folded and unfolded states are present at significant concentrations in the transition region. We have confirmed the results of a previous study (11) that the denaturation of HIV-1 protease is reversible, i.e. the folded and unfolded enzyme conformations are at equilibrium, indicating that Equation 3 is applicable to this system (8, 12).

For studying the effects of salt concentration on the stability of HIV-1 protease, we have used both the wild-type enzyme and its mutated form (Q7K/L33I/L63I), which is much less sensitive to autoxidation without significant alteration in the specific activity (7). The unfolding of HIV-1 protease and its mutated form was measured at increasing urea concentration, and the unfolded fraction, $f_u$ (Equation 2), was plotted against the concentration of denaturant (Equation 3). Representative experiments are shown in Fig. 1, and the parameters calculated from the denaturation curves are given in Table I. The relative stabilities of the enzymes are illuminated by the transition midpoints of denaturation ($D_{1/2}$) and by $\Delta G^0$ determined at 0.1 and 1.0 M NaCl as well as at pH 5.0 and 7.0. The data show that 1) both enzymes are stabilized by the higher ionic strength; 2) the effects of salt concentration on both proteases are more significant at pH 7.0 than at pH 5.0; 3) both enzymes are more stable at pH 5.0 than at pH 7.0; and 4) the mutant protease is slightly more stable than the wild-type enzyme.

The unfolding of HIV protease has also been examined by measuring the rate of the fluorescence change in 6.3 M urea, assuming that the more stable structure denatures more slowly (13). The reactions measured at various pH values gave perfect first-order rate constants with both the wild-type and mutant enzymes. It is seen in Fig. 2 that the unfolding rate constant is lower at higher ionic strength, i.e., the enzymes are stabilized in the presence of salt. Their greatest conformational stability is found around pH 5. In the vicinity of the minimum of the rate constants, the effects of ionic strength are less pronounced than at the extreme pH values. Furthermore, the difference in the stability of the wild-type and mutant enzymes is smaller in the presence of 1.0 M NaCl than at the lower ionic strength. These kinetic results are consistent with the preceding equilibrium data (Fig. 1 and Table I).
The spectral change during denaturation in 6.65 M urea has also been determined. Fig. 3 shows that upon unfolding of the protein, the fluorescence intensity decreases throughout the emission wavelength range and that the intensity maximum of the spectrum shifts toward longer wavelengths. The rate constants of unfolding measured at different wavelengths (330, 340, 347, and 360 nm) are identical within experimental error (2.93 ± 0.12 s⁻¹), in accordance with the two-state mechanism (Equation 1). The rate of the shift in wavelength maximum upon denaturation (Fig. 4) provided the same first-order rate constant (2.90 ± 0.30 s⁻¹) as obtained from the intensity changes.

Measuring Unfolding by Circular Dichroism—Fluorescence data monitor the changes in local environments of tryptophan residues, which primarily reflect perturbations in the tertiary structure. Similar information may be obtained from near-UV CD measurements (250–310 nm), which reveal the environmental changes of aromatic residues. Far-UV CD (190–250 nm), on the other hand, monitors predominantly alterations in the secondary structure. The spectrum of the denatured enzyme cannot be determined in the far-UV region below 210 nm because of the high absorbance of urea. The unfolding rate constants for the mutant enzyme were determined both in the near- and far-UV regions at 270 and 230.5 nm, respectively, at pH 5.00 in the presence of 0.1 M NaCl and 5.25 M urea. The results have shown that the rate constants obtained with the two CD and the fluorescence methods are similar (1.4 ± 0.12 s⁻¹).

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### Table 1

| NaCl | Native | Q7K/L331/L631 | ΔΔG° | ΔD1/2 |
|------|--------|--------------|------|-------|
|      | m      | m            |      |       |
| pH 7.0 |       |              |      |       |
| 0.1 M | ΔG°    | ΔD1/2        |      |       |
| 1.0 M | ΔG°    | ΔD1/2        |      |       |

### Fig. 2

pH dependence of the unfolding rate constant of HIV-1 protease in 6.3 M urea. The concentrations of native (○, ●) and mutant (▲, △) enzymes were 0.26 and 0.24 μM, respectively. ○ and ▲, reactions in 0.1 M NaCl; ● and △, reactions in 1.0 M NaCl.

### Fig. 3

Changes in the fluorescence spectrum of the mutant HIV-1 protease upon urea denaturation. The spectra were measured in 6.65 M urea at pH 5.0 in the presence of 0.1 M NaCl at 30, 100, 180, 290, 360, 540, 1200, and 1800 s. The enzyme concentration was 10.6 μM, and the scanning speed was 100 nm/min.

### Fig. 4

Change with time of the maximum wavelength of the intrinsic fluorescence of the mutant HIV-1 protease upon urea denaturation. The values are taken from the curves in Fig. 3.

**Stability and Activity of HIV-1 Protease**

The parameters are calculated from Equation 3.

| pH 7.0 | 4.10 ± 0.13 | 10.40 ± 0.71 | 1.38 ± 0.42 |
| pH 5.0 | 4.02 ± 0.26 | 14.35 ± 1.38 | 0.42 ± 0.25 |

a Difference between the parameters for the mutant and native enzymes. b Difference between the ΔG° or ΔD1/2 values obtained at 1.0 and 0.1 M NaCl.
s\(^{-1}\)) when the protease concentrations do not differ too much (5–7 \(\mu\)M). The fluorescence change monitored at lower enzyme concentration (0.57 \(\mu\)M) provided a higher unfolding rate constant (2.35 \(\times\) 10\(^{-3}\) s\(^{-1}\)), indicating that the HIV-1 protease is more stable when used at higher concentrations. This is consistent with the observation that upon urea denaturation, the transition midpoint of HIV-1 protease shifts toward higher urea concentration when applied at higher enzyme concentration (11).

Thermal Inactivation—The change in the activity of the mutant HIV-1 protease has been measured during incubation at 53°C. The decrease in initial rate followed first-order kinetics, as shown in Fig. 5. The rate constants determined under various conditions are compiled in Table II. It is shown that the rate of inactivation is considerably higher at pH 3.0 and 7.0 than at pH 5.0, which is in accord with the urea-dependent unfolding. In contrast, the addition of salt stabilizes the enzyme conformation at pH 3.0, but slightly reduces it at pH 5.0, while the enzyme is precipitated at pH 7.0.

pH Dependence of the Intrinsic Fluorescence—The fluorescence intensity of the mutant HIV-1 protease (Q7K/L33I/L63I) is only slightly affected by salt concentration (Fig. 1 and Table I). This is apparent from both the pH dependence of protein structure on salt concentration has not yet been measured.

Unfolding studies using urea denaturation have clearly indicated that the stability of both the wild-type and mutant HIV-1 proteases is significantly enhanced at higher ionic strength (Fig. 1 and Table I). This is apparent from both the \(\Delta G^\circ\) and \(D_m\) values. The \(\Delta G^\circ\) values obtained in this study are \(-4\) times less than that found previously for the wild-type HIV-1 protease (11). The latter value of 14.2 kcal/mol (the conversion factor between joules and calories is 4.18) calculated with a more complicated equation, which also involved protein concentration, was determined at pH 6.0 in the presence of 0.2 m NaCl and 2% glycerol, not too different from the conditions employed here. The value of 14.2 kcal/mol is rather high since the conformational stability of a globular protein is generally between 5 and 15 kcal/mol (21–63 kJ/mol), with one of the most stable proteins, bovine pancreatic trypsin inhibitor, having 14.3 kcal/mol energy (18). Our results indicate that the HIV-1 protease belongs to a class of much less stable proteins. The transition midpoint of urea denaturation (\(D_m = 2.6\) m) of the previous study (11), however, is in accordance with our findings.

The preferable autolysis sites of the wild-type HIV-1 protease have been changed in the mutant (Q7K/L33I/L63I), so that the specificity for cleaving the sensitive peptide bonds has been lost (7, 19). It is, therefore, interesting that the mutant enzyme is stabilized not only against proteolysis, but also against urea denaturation. Since the leucine-isoleucine exchange is a fairly conservative modification, the enhanced conformational stabil-
ity may primarily be attributed to Lys-7. In fact, creation of specific cation- or anion-binding sites on the surface of a protein through genetic engineering may increase the conformational stability by strengthening the water shell around the protein (18).

It cannot be ruled out that some autolysis occurs during unfolding, and this promotes denaturation, in particular with the wild-type enzyme. However, autolysis may not be significant because the unfolded enzyme can be renatured to at least 70% in the case of the wild-type enzyme (11), which we have confirmed, and we have obtained even higher activity with the mutant enzyme (data not shown). Furthermore, the recovery of activity is somewhat better in the presence of 1.0 M NaCl relative to 0.1 M NaCl. Since the enzyme is more active at high salt concentration, greater autolysis and lower recovery of activity would be expected in 1.0 M NaCl if autolysis were a crucial factor. Hence, the stabilization by NaCl appears to overbalance the effect of autolysis.

The generally used equilibrium method requires relatively long incubation of the enzyme at different denaturant concentrations. In the transition region, where both folded and unfolded proteases are present, the danger of proteolysis is imminent. Therefore, we have developed a simple kinetic method that markedly diminishes the residence of the protein in the transition region. Specifically, the enzyme is incubated at high urea concentration, while the rate of unfolding is monitored fluorometrically. Under this condition, the reaction rapidly proceeds through the transition region, thereby considerably reducing the time allowed for autolysis. This simple method has shown that the high salt concentration lowers the unfolding rate constants and enabled us to readily encompass a wider pH range (Fig. 2), where a minimum rate constant representing the maximum stability of the protease was revealed near pH 5.0. In all cases, the rate constants were perfectly first-order, consistent with a two-state denaturation mechanism (Equation 1).

Further evidence for the two-state mechanism has been obtained by analyzing the change of the emission spectrum during urea denaturation. The two tryptophan residues of HIV-1 protease are not uniformly exposed to water; Trp-6 is more buried than Trp-42, as can be judged from the three-dimensional structure of the enzyme (20). If the unfolding reactions of two tryptophan residues are not simultaneous, the kinetics measured at various emission wavelengths may be different (21). In contrast, the rate of unfolding is independent of the wavelength (Fig. 3), and the rate constants are identical within experimental error.

The intrinsic fluorescence of HIV-1 protease is approximately constant between pH 4.5 and 7.5 (Fig. 6). The pH-rate profiles of the enzyme reactions conform to bell-shaped curves, with \( pK_a \) values of ~3 and 4.8–6.2 with different substrates (5, 22, 23). These \( pK_a \) values are attributed to the ionization of the catalytically competent aspartic acids. It is clear, however, from Fig. 6 that at pH ~3, there is a considerable conformational change, which may arise from the ionization of one of the catalytic aspartic acids or some other acidic group involved in the stabilization of the protein structure, for example by ion pair formation (11).

From the pH dependence of fluorescence intensity, it appears that the tryptophans are buried to the greatest extent between pH 4.5 and 7.5 (Fig. 6), where the enzyme is most compact. The compact form of a protein is expected to be more stable than a loose form. However, the urea-mediated denaturation (Figs. 1 and 2) indicates that the HIV-1 protease is most stable at pH 5 and is significantly less stable at pH 7. This apparent contradiction may be resolved by considering that the fluorescence intensity at a given pH reflects the static form of the enzyme, while the urea-mediated denaturation is a dynamic probe, which offers information about the unfolding reaction.

The stabilization of proteins by NaCl can be attributed to preferential hydration of the surface of the molecule (6). A substantial portion of the protective water shell is apparently released at high temperature, and this may, in part, explain why the salt effects are less important at 53 °C.

This study has shown that the enzyme is most stable around pH 5 and less stable under more acidic and more alkaline conditions. Furthermore, the less stable the enzyme is, the more effectively it is stabilized. Proteins are generally most stable near their isoelectric points, where the net charge of the protein is low (18, 24). In contrast, HIV-1 protease is most stable at pH 5, which is far from pl 8.66 and 9.35, the calculated isoelectric points of the wild-type and mutant enzymes, respectively. In the case of HIV-1 protease, the sum of the charges rather than the equality of the positive and negative species controls the stability. Specifically, the total number of charges is practically unchanged between pH 5 and the isoelectric point. Thus, the number of charges of the mutant enzyme is 13.87, 21.03, 21.35, and 21.70 at pH 3.00, 5.00, 7.00, and 9.35, respectively. The reduced stability at pH 3.0 is explicable in terms of diminished hydration. It may also be inferred from the calculated data that the stabilization by a water shell could be similar at pH 5.00 and 9.35. However, the stability is substantially decreased with increasing pH above 5, and this may indicate that some ion pairs of conformational importance decompose, such as Asp-29–Arg-8, Asp-29–Arg-8, and/or the C- and N-terminal electrostatic interaction between Pro-1 and Phe-99 and between Pro-1 and Phe-99.

The most intriguing question concerns the effects of salt on the catalytic activity. One factor of the promotion of catalysis may be the salting in of substrate to the enzyme surface, which is supported by the lower \( K_m \) at higher ionic strength (3, 4). We propose that an additional factor to be considered is the conformational stability of the enzyme. In the light of the present data, it is clear that the enhanced ionic strength increases not only the catalytic activity, as has been shown previously (5, 14–16), but also the stability of the enzyme structure. The contribution of the increase in stability to catalysis may be surprising because of the implication in the catalysis of the flexible flaps that cover the substrate in the binding cleft. This requires flexibility rather than stability. On the other hand, better binding occurs at a rigid active site because of entropic reasons. In the case of HIV-1 protease, the latter factor appears to overrule the former one. Some flexibility in the active site of HIV-1 protease is indispensable in order that the enzyme be able to accommodate to the unlike sites of its natural polyprotein substrates. A proper balance between flexibility and rigidity should hold when a sufficiently wide specificity and catalytic efficiency are simultaneously required.

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