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Effect of Salt on the Kinetic Parameters of Retroviral and Mammalian Aspartic Acid Proteases

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A continuous spectrophotometric method has been used to measure the rates of hydrolysis of three synthetic peptide substrates by the retroviral proteases of human immunodeficiency virus type 1 (HIV-1) and of avian myeloblastosis virus (AMV) as well as the mammalian aspartic acid protease pepsin. The kinetic parameter k_{cat}/K_m for these reactions is markedly increased by increasing the sodium chloride concentration. In contrast to earlier reports, no evidence for a bell-shaped dependence of the rate on sodium chloride concentration was observed up to the highest salt concentration (5.0 M) used. Detailed kinetic analysis of the hydrolysis of the synthetic peptide Thr–Phe–Gln–Ala–Phe(NO_2)–Pro–Leu–Arg–Glu–Ala catalyzed by AMV protease and by pepsin at concentrations of sodium chloride between 2 and 5 M showed that k_{cat} remained constant, whereas K_m for both enzymes and K_i for the inhibition by pepstatin A of the AMV protease-catalyzed hydrolysis decreased by ca. 65-fold under these conditions. The observation of similar sodium chloride effects on k_{cat}/K_m for the homodimeric retroviral proteases and the monomeric enzyme pepsin is not consistent with a salt effect on the monomer–dimer equilibrium of the retroviral proteases. Furthermore, the lack of a salt effect on k_{cat} also suggests that increasing the concentration of sodium chloride does not significantly alter the secondary structure nor the extent of dimerization of the retroviral proteases. The sodium chloride effect on K_m and K_i is discussed in terms of a mechanism in which hydrophobic interactions between the inhibitor or substrate and the enzyme are enhanced by a salting out effect on the substrate and/or the active site region of the enzyme.

INTRODUCTION

Retroviral proteases are dimeric proteins that consist of two identical subunits 99–130 amino acids in length (*1–3*). These enzymes, which belong to the aspartic acid protease family, are catalytically active only in the dimeric form with each polypeptide subunit contributing one of the two aspartyl residues that are essential for enzymatic activity. The biological function of retroviral proteases is the processing of the polyprotein products of the *gag* and *gag–pol* genes to smaller proteins by cleaving specific peptide sequences (*1–3*). Thus, these enzymes play

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a key role in the life cycle of retroviruses including the human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS). Specific inhibitors for HIV-1 protease have been shown to inhibit the replication and maturation of the virus (*2, 4*). Synthetic peptides that correspond to cleavage sites in the Gag and Gag–Pol polyproteins are substrates for these enzymes (*5–7*), as are analogous peptides in which an aromatic amino acid at the scissile peptide bond is replaced with the chromogenic amino acid *p*-nitrophenylalanine (Phe(NO_2)) (*8–12*).

Enzymatic activity of retroviral proteases has been shown to be sensitive to the concentration of sodium chloride (*7, 9, 12–17*) as well as several other ions (*7, 13, 16*). The rates of hydrolysis of synthetic substrates catalyzed by the proteases of avian myeloblastosis virus (AMV) and HIV-1 were reported to exhibit a bell-shaped dependence on sodium chloride concentration with a maximal rate between 2 and 3 M NaCl (*7, 13*). Detailed kinetics studies of the reactions catalyzed by HIV-1 protease at concentrations of sodium chloride between 0.2 and 2.5 M showed that K_m decreased with increasing ionic strength whereas k_{cat} remained unchanged (*9, 13, 16, 17*). In contrast, AMV protease-catalyzed hydrolysis was reported to display an increase in k_{cat} and a decrease in K_m over a similar range of sodium chloride concentrations (*12*). The value of K_i for inhibition of HIV-1 protease by pepstatin A, a known inhibitor of aspartic acid proteases (*18*), also decreases with increasing sodium chloride concentration (*13*).

Unlike retroviral proteases, mammalian aspartic acid proteases are catalytically active, single-chain proteins. The best known member of this group is the mammalian gastric enzyme pepsin (*18*). Although the kinetics and inhibition of several mammalian aspartic acid proteases have been extensively studied, a salt effect on pepsin-catalyzed hydrolysis has been described only recently and without detailed kinetic analysis (*7*). A plot of the rate of the pepsin-catalyzed hydrolysis of a synthetic peptide substrate as a function of salt concentration was reported to be a bell-shaped curve similar to those observed for the retroviral proteases.

We have determined the effect of sodium chloride on the kinetic parameters for hydrolysis reactions catalyzed by the proteases of HIV-1 and AMV and by pepsin under comparable conditions. Our studies utilized a continuous optical assay method. In contrast to the bell-shaped dependence on sodium chloride previously reported (*7, 13*), we have observed that k_{cat}/K_m for reactions catalyzed by these three enzymes increases monotonically with increasing salt concentration between 0 and 5 M NaCl. Increasing ionic strength decreases K_m and has little or no effect on k_{cat} for both AMV protease and pepsin, again in contrast to an earlier report that suggested a salt effect on k_{cat} as well as K_m for the AMV enzyme, but in agreement with published data for the protease of HIV-1. As described in more detail later, we ascribe the bell-shaped curves and apparent discrepancies in k_{cat} to the artifacts resulting from incomplete solubility of the substrates and the use of data in a concentration range inappropriate for the separation of kinetic parameters. The present study also provides good evidence that the salt effect is a general phenomenon for aspartyl proteases and is not restricted to the retroviral enzymes.

MATERIALS AND METHODS

Materials. Peptides I (Ac–Lys–Ala–Ser–Gln–Asn–Phe(NO_2)–Pro–Val–Val–NH_2), II (Thr–Phe–Gln–Ala–Phe(NO_2)–Pro–Leu–Arg–Glu–Ala), and III (Phe–Gly–His–Phe(NO_2)–Phe–Ala–PheOCH_3) were custom synthesized and purified by the American Peptide Co., Inc. (Santa Clara, CA). The three peptides were homogeneous by HPLC and contained ca. 80% peptide by weight. HIV-1 protease was obtained as described (*19*). Lyophilized AMV protease was purchased from Molecular Genetic Resources (Tampa, FL). Chromatographically purified porcine pepsin and pepstatin A were purchased from Sigma Chemical Co., (Saint Louis, MO).

Enzyme stock solutions. Stock solutions of AMV protease and pepsin were prepared by dissolving 1–2 mg of the lyophilized enzymes in 1 ml of water and 100 mM HCl, respectively. HIV-1 protease was obtained as a stock solution of 85–300 μg/ml protein in 50 mM 2-morpholinoethanesulfonic acid (Mes) buffer, pH 6.5, containing 5% glycerol, 1 mM EDTA, and 1 mM DTT as described (*19*).

Kinetics. Reactions were carried out in 100 μl microspectrophotometric cells with a 10-mm light path (Hellma Cells, Inc., Jamaica, NY) as described (*8*). Kinetics of reactions catalyzed by AMV and HIV-1 proteases were measured in 50 mM MES buffer, pH 6.0, containing 5% glycerol and 1 mM EDTA. Pepsin kinetics were measured in 50 mM citrate buffer, pH 4.0. In a typical experiment, 120 μl of buffer containing the desired amount of sodium chloride and substrate was incubated at 37°C for 10 min. Reaction was initiated by addition of the desired amount of enzyme (5–10 μl). Whenever possible, substrate concentrations above and below K_m were used to enable the separation of the kinetic parameters K_m and V_{max}. Concentrations of substrate solutions were determined spectrophotometrically using $\varepsilon_{280} = 12{,}000$ for the *p*-nitrophenyl chromophore (*8*). Kinetic parameters V_{max} and K_m were evaluated by fitting initial rate data to the Michaelis–Menten equation using the curve fitting program MLAB (*20*) or Enzfitter (published by Biosoft, Cambridge, UK).

Inhibition of AMV protease-catalyzed hydrolysis of peptide II by pepstatin was investigated at substrate concentrations much less than K_m (35 μM), where the reaction follows pseudo-first-order kinetics with an observed rate constant, $k_{obs} = k_{cat}E_0/K_m$ in the absence of inhibitor or $k_{obs} = k_{cat}E_0/K_m(1 + [I]/K_i)$ in the presence of inhibitor. Enzyme and inhibitor were incubated at 37°C for 10 min in 110 μl of buffer prior to initiation of the reaction by addition of 15 μl of the substrate solution in water (292 μM). Plots of $1/k_{obs}$ vs pepstatin concentration were linear. The inhibition constant K_i was obtained by fitting the equation $k_{obs} = k_{cat}E_0/K_m(1 + [I]/K_i)$ to the observed pseudo-first-order rate constants at pepstatin concentrations above and below K_i using the program MLAB (*20*).

Product analysis. Products of the pepsin-catalyzed hydrolysis of peptides I and II were analyzed by chromatography on a Vydac C_{18} 5-μm reverse phase column (0.46 × 15 cm) eluted with a linear gradient from 0 to 40% of acetonitrile in water, both containing 0.1% trifluoroacetic acid, over 25 min at a flow rate of 1.5 ml/min. The major products (retention times 8.9 and 15.2 min), which had been previously characterized (*8*), were those resulting from cleavage of the peptide bond between

Ac-Lys-Ala-Ser-Gln-Asn-Phe(NO_2)---Pro-Val-Val-NH_2

Peptide I

Thr-Phe-Gln-Ala-Phe(NO_2)-Pro---Leu-Arg-Glu-Ala

Peptide II

Phe-Gly-His-Phe(NO_2)---Phe-Ala-PheOCH_3

Peptide III

FIG. 1. Amino acid sequences of peptide substrates. Dashed lines represent the scissile peptide bonds.

Phe(NO_2) and Pro of peptides I and II. Interestingly, a minor product (retention time 13.5 min, ~3% at zero salt) was isolated from the pepsin-catalyzed hydrolysis of peptide II. This product increased to ~15% at 4 M sodium chloride. Its uv spectrum (λ_{max} 272 nm) and FAB mass spectrum (m/z 777) indicated that it had resulted from cleavage of the Ala–Phe(NO_2) peptide bond. The ratio of this peptide to the major Phe(NO_2) containing product did not change during the course of a given reaction, indicating that it was a primary reaction product that did not undergo further enzyme-catalyzed hydrolysis.

RESULTS

In order to compare the salt effects on different aspartyl proteases, the retroviral proteases of AMV and HIV-1 and the mammalian enzyme pepsin were investigated under similar conditions. Since these enzymes exhibit different substrate specificities, three different chromogenic substrates (Fig. 1) were used, namely, peptide I for HIV-1 protease, peptide II for AMV protease, and peptide III, a substrate for pepsin reported by Fruton and co-workers (*21*). Since peptides I and II are also substrates for pepsin, cross-comparison of these substrates with pepsin and the appropriate retroviral proteases was possible.

The solubility of the HIV-1 protease substrate, peptide I, is very limited, especially at high salt concentrations. With this peptide, a concentration of 35–40 μM can be achieved at concentrations of sodium chloride between 0.1 and 4.44 M at 37°C. Thus, only pseudo-first-order kinetics (apparent rate constant $k_{cat}E_0/K_m$) could be observed for the HIV-1 protease-catalyzed hydrolysis of peptide I (*8*). Similarly, at concentrations of peptide II below K_m, good first-order kinetics were observed for the AMV protease-catalyzed hydrolysis of this specific substrate. Hydrolysis of peptide I by pepsin (at the peptide bond between Phe(NO_2) and Pro) exhibits a salt effect that is similar to that observed for the retroviral protease-

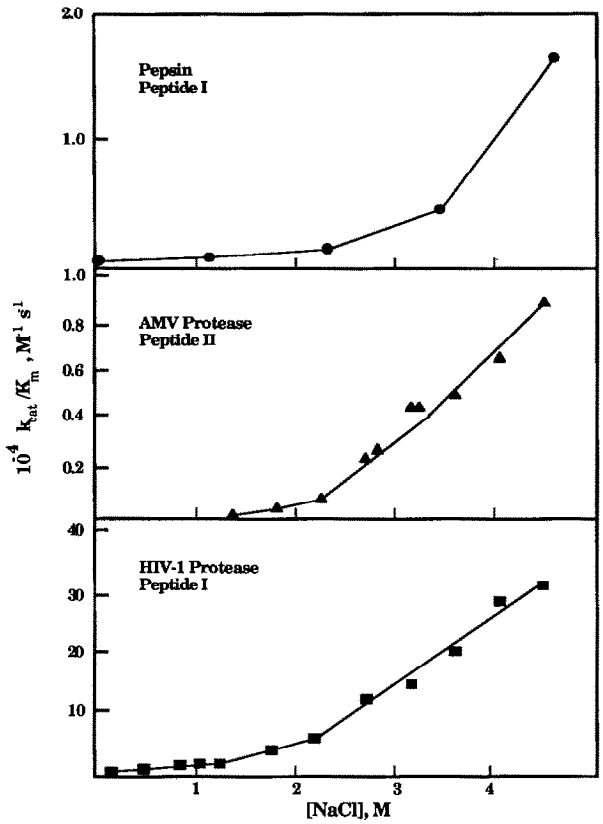

FIG. 2. Effect of sodium chloride concentration on k_{cat}/K_m, measured under pseudo-first-order conditions; the observed rate constant, $k_{obs} = k_{cat}E_0/K_m$, for the hydrolyses of peptide I catalyzed by pepsin (●) and HIV-1 protease (■) and of peptide II catalyzed by AMV protease (▲).

catalyzed reactions. Figure 2 shows plots of k_{cat}/K_m, determined under pseudo-first-order kinetic conditions, vs sodium chloride concentration for the hydrolysis reactions of peptide I catalyzed by HIV-1 protease and pepsin, and of peptide II catalyzed by AMV protease.

Although K_m for peptide II is $\geqslant$5 mM at concentrations of sodium chloride <2 M and only pseudo-first-order kinetics could be observed with the AMV protease under these conditions, saturation kinetics were observed at higher sodium chloride concentrations between 2 and 5 M. The kinetic parameters K_m and k_{cat} for the AMV protease-catalyzed hydrolysis of peptide II at various concentrations of sodium chloride are summarized in Table 1. Also listed in Table 1 are the values of K_i for inhibition by pepstatin A of the AMV protease-catalyzed hydrolysis of peptide II at various concentrations of sodium chloride. As in the case of peptide II and the AMV protease, saturation kinetics were observed and kinetic parameters were separated for the hydrolysis of this substrate catalyzed by pepsin at concentrations of sodium chloride between 2 and 5 M. These results are shown in Table 2.

TABLE 1

Effect of Sodium Chloride Concentration on the Kinetic Parameters for Hydrolysis of Peptide II Catalyzed by AMV Protease[a]

[NaCl] (M)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	K_i^b (μM)
1.0				30.3 ± 4.0
1.5	1.02 ± 0.27	3900 ± 1700	262	
2.0	1.04 ± 0.10	1950 ± 380	533	11.7 ± 1.2
2.7	1.54 ± 0.11	597 ± 70	2,580	
2.8				4.35 ± 0.65
3.1	1.74 ± 0.11	446 ± 52	3,900	
3.6	1.28 ± 0.04	174 ± 16	7,360	1.65 ± 0.45
4.0	1.30 ± 0.05	150 ± 17	8,670	
4.4	1.18 ± 0.03	105 ± 7	11,200	
4.6				0.585 ± 0.066
4.9	0.94 ± 0.02	60 ± 4	15,700	

[a] 50 mM MES buffer, pH 6.00, 5% glycerol, 1 mM EDTA at 37°C; the indicated uncertainties are standard deviations in k_{cat} and K_m.

[b] Inhibition by pepstatin A of the hydrolysis reaction catalyzed by AMV protease.

TABLE 2

Effect of Sodium Chloride Concentration on the Kinetic Parameters of Pepsin-Catalyzed Hydrolysis[a]

[NaCl] (M)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/k_m (M^{-1} s^{-1})
	Pepsin-catalyzed hydrolysis of peptide III		
0.0			287,000[b]
0.75			345,000[b]
1.50			584,000[b]
	Pepsin-catalyzed hydrolysis of peptide II		
0.0			1,490[b]
1.0			2,080[b]
2.0	1.66 ± 0.11	276 ± 45	6,020
3.0	1.45 ± 0.08	130 ± 20	11,200
4.0	1.86 ± 0.02	71 ± 17	26,100
5.0	1.60 ± 0.13	26 ± 6	61,500

[a] 50 mM citrate buffer, pH 4.0, at 37°C; the indicated uncertainties are standard deviations in k_{cat} and K_m.

[b] Values were derived from pseudo-first-order kinetic data and the average of three to five measurements; the measurements were reproducible within 2–6%.

Although the chromogenic pepsin substrate, peptide III, is fairly soluble in 50 mM buffer at pH 4 (>10 mM), its solubility becomes poor in the presence of sodium chloride (e.g., 20 μM in 2 M salt). Thus, this substrate could only be investigated over a very limited range (0–1.5 M) of sodium chloride concentration. Under these conditions, enzyme saturation could not be achieved, and only pseudo-first-order kinetics could be observed. The kinetic parameter k_{cat}/K_m exhibited an approximately twofold increase between 0 and 1.5 M sodium chloride (cf. Table 2). The magnitude of this effect was similar to that found for the retroviral proteases in the same range of sodium chloride concentrations.

DISCUSSION

The results presented in Fig. 2 show that increasing concentrations of sodium chloride markedly increase k_{cat}/K_m for hydrolysis of synthetic peptide substrates by the retroviral proteases of AMV and HIV-1 and the mammalian aspartic acid protease pepsin. This kinetic parameter for hydrolysis of peptides I and II catalyzed by HIV-1 and AMV protease was increased ca. 160-fold and 180-fold upon increasing the sodium chloride concentration from 0 to 4.4 M and 1.0 to 4.9 M, respectively. A similar, although somewhat smaller (40- to 60-fold) effect on k_{cat}/K_m is observed for the pepsin-catalyzed hydrolysis of peptides I and II between 0 and 5 M sodium chloride. Although pepsin-catalyzed hydrolysis of peptide III could be studied only between 0 and 1.5 M NaCl due to the poor solubility of this substrate at higher concentrations of sodium chloride, the observed 2-fold acceleration of k_{cat}/K_m over this range of salt concentration is consistent with data in the same range obtained with peptides I and II, as well as with the results for HIV-1 protease and peptide II (Fig. 2). Thus, existence of a sodium chloride effect on k_{cat}/K_m for aspartic acid proteases is independent of the source of enzyme and the peptide substrate used.

The monotonic increases in the kinetic parameter k_{cat}/K_m with increasing sodium chloride concentration for pepsin as well as AMV and HIV-1 proteases (Fig. 2) cannot be reconciled with the previously reported bell-shaped curves for the salt effect on the rate of peptide hydrolysis catalyzed by these three enzymes (*7, 13, 15*). Although the earlier experiments employed different (nonchromogenic) substrates, it is unlikely that the shape of the curves describing the salt effect would be very similar for the three substrates investigated in the present work and yet entirely different for those described previously. A more likely explanation involves the solubility of the substrates. In previous studies, rates were determined by point assays conducted at a constant substrate concentration in a volume of 25 to 50 μl. In such an assay system, it is difficult to determine whether the substrate is completely soluble at each salt concentration. The solubilities of peptide substrates are expected to decrease as the salt concentration increases, as is the case with peptides I–III. Thus, at high salt concentrations it is possible that the actual concentration of substrate available to the enzyme is determined by its solubility rather than the amount of substrate added. This solubility decreases with increasing ionic strength. Hence, a bell-shaped curve is observed when the experiment is

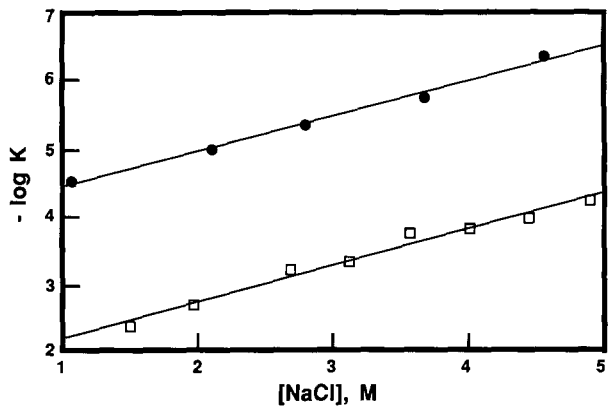

FIG. 3. Comparison of the effect of sodium chloride concentration on K_m (□) for the AMV protease-catalyzed hydrolysis of peptide II and K_i (●) for the inhibition of this reaction by pepstatin A.

carried out at a nominally constant concentration of added substrate. The use of chromogenic substrates avoids this possible source of error, since their solubilities can easily be evaluated spectrophotometrically at each salt concentration studied.

Although the individual kinetic parameters, K_m and k_{cat}, could not be separated for all the substrates because of solubility limitations and/or high K_m values, it was possible to evaluate these parameters for the hydrolyses of peptide II catalyzed by the AMV protease and by pepsin (Tables 1 and 2). As has been observed for the hydrolysis of other peptide substrates catalyzed by the HIV-1 protease (*9, 13, 16, 17*), K_m decreases with increasing sodium chloride concentration, whereas k_{cat} remains unchanged. In contrast, Strop *et al.* (*12*) reported an increase in k_{cat} as well as a decrease in K_m for the AMV protease-catalyzed hydrolysis of Ala–Thr–His–Gln–Val–Tyr–Phe(NO_2)–Val–Arg–Lys–Ala, upon increasing the sodium chloride concentration from 0 and 2.5 M. Examination of the results and experimental procedure of that sutdy (*12*) revealed that the highest substrate concentration used was well below the reported K_m at sodium chloride concentrations of 0 and 1 M. Hence, it is not possible to resolve the kinetic parameters at these salt concentrations, and thus the reported values are unreliable. On the other hand, the reported K_m values at sodium chloride concentrations of 2 and 2.5 M were within the range of the substrate concentrations used. At both these salt concentrations, where the individual kinetic parameters could be reliably evaluated, k_{cat} values were identical.

Increasing concentrations of sodium chloride exert an identical effect on K_m for the AMV protease-catalyzed hydrolysis of peptide II and K_i for the inhibition of this reaction by pepstatin A (Table 1). Both K_i and K_m decrease ~20-fold upon increasing the sodium chloride concentration from 2 to ~4.5 M. A plot of $-\log K_i$ and $-\log K_m$ vs sodium chloride concentration produced two parallel lines (Fig. 3). The identical effect of sodium chloride on K_m and K_i indicates that the salt effect on pepstatin binding is a good model for the salt effect on whatever equilibrium process(es) may be involved in the formation of the kinetically competent en-

zyme–substrate complex. This conclusion is further confirmed by the lack of any sodium chloride effect on the kinetic parameter k_{cat} for hydrolysis of peptide II catalyzed by the AMV protease and by pepsin. Thus, an effect of salt on the monomer–dimer equilibrium of the retroviral proteases or on the conformation of the monomeric enzyme pepsin is unlikely. The most attractive mechanism for the salt effect involves either forcing the hydrophobic substrate from the aqueous phase into the less polar active site of the enzyme or the removal of water from the active site upon forming the enzyme–substrate or enzyme–inhibitor complex. Thus, the steady state concentration of this complex is increased. A similar interpretation of the salt effect was previously suggested based on the observed preponderance of hydrophobic residues in substrates and inhibitors of HIV-1 protease, and in the enzyme's active site (*17*).

Studies of the kinetic isotope effect for HIV-1 protease at pH values near neutrality indicate that the rate-determining catalytic step involves a change in a covalent chemical bond (*17, 22*). However, multiple preequilibrium events, such as conformational changes of the initially formed enzyme–substrate complex and possible hydration of the substrate to form an enzyme-bound tetrahedral intermediate, may precede the formation of the complex involved in this rate determining step (*17, 22*). A common feature of aspartic acid proteases, as demonstrated by X-ray structural studies, is a flap region which undergoes large movements upon binding inhibitors such as pepstatin. In the presence of an inhibitor, the active site is closed by the flaps to form a hydrophobic tube in which the inhibitor makes extensive hydrophobic contacts with the enzyme and has very little contact with the bulk of the solvent (*2, 23–27*). A water molecule between the two γ-carboxyl side chains of two aspartic acid residues at the active site in the absence of inhibitor (*23–25*) is replaced by the hydroxyl group of a statine residue upon binding of pepstatin. The binding of pepstatin and several related analogues to aspartic acid proteases occurs via a two-step mechanism (*18*). The initial faster step is the formation of a loose enzyme–inhibitor complex which subsequently changes to a tight complex in a slower process. The extrusion of water from the active site of aspartic acid proteases was proposed to be the origin of the slow-binding process for this class of inhibitors. A tight-binding Michaelis complex in which the carbonyl oxygen of the scissile peptide bond is directly hydrogen bonded to one of the active site aspartic acid carboxyl groups, or one in which tetrahedral intermediate formation with water has already occurred, should be on the catalytic pathway, and formation of such a complex should be analogous to the binding of pepstatin.

Hydrophobic interactions between small organic molecules in water are known to be enhanced in the presence of salts such as lithium and sodium chloride (*28*). Such an enhancement is due to the "salting out effect" of the less polar solute to minimize its contact with water. The observation of Wondrak *et al.* (*16*) that the effect of ionic strength on the activity of the HIV-1 protease was dependent on the salting out ability of the ions used is consistent with such a mechanism for the salt effect. The observed magnitude of the effect suggests that hydrophobic interactions between the aspartic acid proteases and their substrates play a primary role in substrate recognition and binding by these enzymes.

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