Effectors of HIV-1 Protease Peptidolytic Activity

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ABSTRACT: High concentrations of salts dramatically affect the interaction of small ligands with HIV-1 protease. For instance, the K_m and k_{cat} values for Abz−Thr−Ile−Nle−*p*-nitro-Phe−Gln−Arg-NH_2 (S) increased 120-fold and 3-fold, respectively, as the NaCl concentration in the assay decreased from 4.0 to 0.5 M. The K_d value for the competitive inhibitor amprenavir increased 12-fold over this concentration range of NaCl. The bimolecular rate constant for association of enzyme with amprenavir was independent of NaCl concentration, whereas the dissociation rate constant decreased with increasing NaCl concentration. Polyanionic polymers such as heparin or poly A substituted for NaCl. For example, the value of k_{cat}/K_m for S was 0.18 μM^{-1} s^{-1} when the enzyme (<10 nM) was assayed in the standard buffer supplemented with 5 mM NaCl. If 0.01% poly A were included, the value of k_{cat}/K_m increased to 8.6 μM^{-1} s^{-1}. A DNA oligomer (23-mer) with an hexachlorofluoresceinyl moiety linked to the 5′ end was studied as a model polyanionic polymer. The enzyme bound HF23 (K_d < 1 nM) with concomitant quenching of the hexachlorofluoresceinyl fluorescence. The stoichiometry for binding was 3 mol of enzyme per mol of oligomer. The hydrolytic activity of the enzyme with this oligomer was similar to that observed with poly A or high salt concentration when the molar ratio of oligomer to enzyme was greater than one. The results presented herein demonstrate that polyanionic polymers substitute for salts as effectors of HIV protease.

Inhibitors of HIV-1[1] protease have been successfully developed as chemotherapeutic agents for intervention in the treatment of AIDS. Currently, six protease inhibitors are drugs for the treatment of AIDS (*1*). During the course of the development of these drugs, the protease has been studied extensively by structural (*2*, *3*), genetic (*4*, *5*), and biochemical (*6*−*11*) techniques. The enzyme is an aspartyl protease in which the homodimeric active site is composed of aspartyl groups from each monomer (*12*, *13*). Meek and co-workers (*6*−*11*) have probed the chemical mechanism of HIV-1 protease for several representative peptidyl substrate analogues with initial velocity studies, isotope exchange studies, and solvent isotope effects. They suggested the rate-limiting step was an enzyme isomerization or product release step (*10*). Other workers have suggested that the chemical step is rate limiting for selected substrate analogues (*14*). One common feature of all biochemical studies has been the requirement for relatively high NaCl concentration (100−1500 mM) for high catalytic activity. Because the effect of salt was primarily on the K_m value, it was attributed to a nonselective "salting-out" of the hydrophobic substrate onto the enzyme (*6*, *15*). Support for this interpretation was the observation that the salting-out effect follows the Hofmeister series (*15*). Subsequently, Szeltner and Polgar have proposed that part of the salt effect on the catalytic activity of HIV protease is the result of increased conformational stability of the enzyme through preferential hydration of the enzyme. They proposed that this effect could result in higher substrate affinity at a rigid active site because of entropic effects (*16*). Both of these mechanisms attribute the salt effect to changes in solvent structure. Alternatively, the salt effect could be the result of direct binding of the salt to a site on the enzyme that allosterically enhances the value of k_{cat}/K_m. This mechanism suggests that it may be possible to find small molecules that enhance the value of k_{cat}/K_m of the enzyme at concentrations that are significantly less than those needed to demonstrate an effect with NaCl. We report herein that polyanionic polymers at low concentrations were potent activators of HIV protease. By directly interacting with the protein, these effectors increased the value of k_{cat}/K_m of the enzyme to a level that was comparable to that observed with high concentrations of NaCl (>2.0 M). These results suggested that the effect of salts on the value of k_{cat}/K_m of the enzyme were in part the result of direct interactions with the enzyme.

MATERIALS AND METHODS

Materials. Heparin, poly A, dextran sulfate (MW = 10 000), polygalacturonate, poly-L-aspartate (MW = 8400), tripolyphosphate, tetrapolyphosphate, phosphate glasses 5, 18, 28, 48, carboxymethylcellulose, poly-L-glutamate (MW = 13 300), polyethylenimine, poly-L-lysine (MW = 14 400), poly-L-arginine (MW = 13 000), poly-D-lysine (MW = 10 700), high viscosity methyl cellulose, poly-D-alanine, and G-10 resin were from the Sigma Chemical Co. Different

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[1] Abbreviations: HIV-1, human immunodeficiency virus, type 1; AIDS, acquired immune deficiency syndrome; HF23, 5′-hexachlorofluorescein-TT TTT TAC AAC GTC GTG ACT CTT-3′; poly A, polyadenylic acid (5′); S, 2-aminobenzoyl-Thr−Ile−Nle−Phe(*p*-NO_2)−Gln−Arg-NH_2; MES, 2-(morpholino)ethanesulfonic acid; TRIS, tris-(hydroxymethyl)aminomethane; I, amprenavir; T, saquinavir; D, catalytically active dimeric form of HIV-1 protease; P, products of enzymatic hydrolysis of S.

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grades and batches of MES buffer were purchased from Sigma Chemical Co, Calbiochem, and GIBCO Laboratories. Sigma ULTRA MES was used for most experiments. The 10-mer of L-glutamate was purchased as an HPLC-purified product from SynPEP. HF23 (5′-hexachlorofluorescein-TT TTT TAC AAC GTC GTG ACT CTT-3′) was purchased as a gel purified product from Oligos Etc. [^{14}C]-amprenavir was synthesized at GlaxoSmithKline. Amprenavir and saquinavir were from GlaxoSmithKline compound stores. Solutions of these compounds were prepared in dimethyl sulfoxide. The concentration of amprenavir was determined spectrophotometrically in 0.1 N NaOH with a $\epsilon_{265} = 16.7$ mM^{-1} cm^{-1}. Solutions of saquinavir were calibrated by titration of enzymatic activity that had been normalized with amprenavir. Other chemicals were purchased from commercial sources as ACS reagent grade. 2-aminobenzoyl-Thr−Ile−Nle−Phe-(*p*-NO_2)−Gln−Arg-NH_2 (S), 2-aminobenzoyl-Thr−Ile−Nle, and Phe(*p*-NO_2)−Gln−Arg-NH_2 were purchased from SynPep or Enzyme Systems Products. Dimethyl sulfoxide solutions of the peptides were prepared gravimetrically.

General Methods. The standard buffer was 0.02 M MES TRIS at pH 5.5. The standard temperature was 25 °C. Steady-state fluorescence data were collected with a Kontron SFM 25 spectrofluorometer. Product was monitored with $\lambda_{ex} =$ 340 nm and $\lambda_{em} =$ 420 nm; hexachlorofluorescein was monitored with $\lambda_{ex} =$ 530 nm and $\lambda_{em} =$ 550 nm. Fluorescence time-courses presented were representative of at least three experiments. Rapid reactions were monitored with an Applied Photophysics SX.17MV spectrophotometer (Leatherhead, UK). Hexachlorofluorescein was monitored spectrofluorometrically by this instrument with a slit width of 2 mm, an excitation path length of 2 mm, $\lambda_{ex} =$ 500 nm, and $\lambda_{em} >$ 535 nm. Equations described below were fitted to the data by nonlinear least squares using SigmaPlot from Jandel Scientific (Corte Madera, CA). The fits of the parameters to the data are reported as the fitted value with the standard error of the least significant figure in parentheses.

Enzyme Expression and Purification. The construct used for expression of HIV-1 protease in *Escherichia coli* was similar to that reported by Wan et al. (*17*) except that a hexahistidinyl tag was substituted for the glutathione-*S*-transferase fusion partner. The hexahistidinyl tag was followed by the heptapeptide GTVSFNF containing an HIV-1 protease processing site and by the 99 amino acids of mature protease. Amprenavir significantly enhanced the recovery of the recombinant fusion protein expressed in *E. coli*. However, most of the protease was insoluble. The insoluble material was recovered from the lysed cells by centrifugation. The pellet was dissolved in 7 M urea. The protease was purified by metal chelating chromatography and size exclusion chromatography in buffers containing 7 M urea. The denatured protein was folded and the hexahistidinyl tag was cleaved by incubating at 4 °C for 3 h in a solution made by rapidly diluting the protein (14-fold) into 50 mM MES, pH 5.5, 2 mM 2-mercaptoethanol, 5% ethylene glycol, 10% glycerol. The catalytically active dimeric enzyme was concentrated and purified by metal chelating chromatography. These preparations of enzyme were judged to be greater than 95% homogeneous from polyacrylamide gel electrophoresis using a 4 to 12% gradient gel (Invitrogen). Further details of the purification procedure will be published elsewhere.

Scheme 1: Kinetic Constants for Substrate Hydrolysis

$$D + S \rightleftharpoons DS \quad : k_1 \; k_{-1}$$

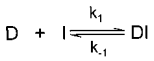
$$DS \longrightarrow D + P : k_{cat}$$

The enzyme (28 μM) was stored as 100 μL samples at −80 °C in 50 mM HEPES at pH 7.0, 10% glycerol, 5% ethylene glycol, 175 mM imidazole, and 1 mM β-mercaptoethanol (enzyme buffer). The enzyme sample was discarded after four freeze−thaw cycles. The concentration of enzyme was determined by titration of enzymatic activity with amprenavir whose concentration was determined spectrophotometrically. Enzyme concentrations are expressed in terms of amprenavir binding sites.

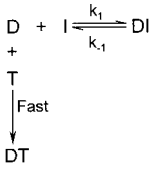
Enzymatic Activity. The enzyme was assayed in the standard assay buffer (1 mL of 0.5 M NaCl, 0.02 M MES-TRIS at pH 5.5 with 2.5 μM S) by monitoring product formation spectrofluorometrically ($\lambda_{ex} =$ 340 nm, $\lambda_{em} =$ 420 nm) at 25 °C (*8*). A working solution of enzyme (1.4 or 0.14 μM enzyme dimer) was prepared by dilution of the purified enzyme into the standard buffer (0.02 M MES TRIS, pH 5.5) at 5 °C. The hydrolysis reaction was initiated by dilution of the working enzyme solution into the assay buffer. The rate of fluorescence increase per second was converted to micromolar substrate hydrolyzed per second using the change in fluorescence for complete hydrolysis of substrate. Purified enzyme was not transferred into the buffer being studied prior to use. Consequently, the reaction contained variable amounts of the enzyme buffer. The percentage of enzyme buffer in the reaction was equal to 100 times the concentration of enzyme in the reaction divided by 28 μM.

Steady-State Parameters for Hydrolysis of S by HIV Protease (D). Values for the steady-state kinetic parameters were determined by analysis of the complete time-course for S hydrolysis in which product was monitored by the associated fluorescence increase ($\lambda_{ex} =$ 340 nm, $\lambda_{em} =$ 420 nm). The indicated buffer (1 mL) with substrate (0.5 to 40 μM) was equilibrated to 25 °C in a quartz cuvette in the chamber of the SFM 25 spectrofluorometer. The hydrolytic reaction was initiated by addition of enzyme. The time-course data were analyzed with Dynafit according to Scheme 1 (*18*, *19*). In this scheme, D is the catalytically active dimeric form of the enzyme, S is the fluorogenic substrate, and P is the two products of the reaction. The products of the reaction were ineffective competitive inhibitors with K_m/K_i values greater than 200. Consequently, product inhibition was not included in Scheme 1. Scheme 1 is a simplified form of the mechanism used by Kuzmic in which the effects of mechanical stirring and enzyme monomerization on catalytic activity have been accounted for (*20*). Our data was collected in the absence of rigorous stirring and over a time period of less than 150 s. Under these conditions, inactivation by stirring was minimal, and dissociation of dimeric enzyme was not significant. This was demonstrated to be the case by addition of a second sample of substrate to the spent reaction. In all cases, the activity of the enzyme at the end of the reaction was similar to that at the beginning of the reaction. The initial concentration of substrate was typically ∼4-fold greater than the K_m value for the reaction. For this analysis, the bimolecular rate constant for association of enzyme with substrate (k_1) was assumed to have a fixed value of 100 μM^{-1} s^{-1} (*20*). The K_m value for the reaction was calculated as the

Scheme 2: One-Step Mechanism for Binding of Amprenavir (I) to D



Scheme 3: Competition of T (Sequinavir) for I (Amprenavir) in DI to Determine the Value of k_{-1}



ratio of the sum of the values of k_{cat} and k_{-1} to the value of k_1. Errors for the K_m value were calculated by error propagation from the respective errors for the individual rate constants (*21*). The substrate was dissolved in dimethyl sulfoxide. Dimethyl sulfoxide was a competitive inhibitor of the reaction with a K_i value of 143 (7) mM. To minimize inhibition, the concentration of dimethyl sulfoxide added with the substrate was below 30 mM.

Kinetic Constants for Binding of Amprenavir to the Enzyme. The binding of amprenavir to HIV protease is associated with a small fluorescence increase ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 340$ nm) that was used to monitor the formation of D·amprenavir. The inhibitor binding process was analyzed by the one-step mechanism of Scheme 2 (*22*−*23*).

With the concentration of amprenavir greater than the concentration of enzyme, a pseudo first-order rate constant described the observed fluorescence changes. The dependence of the pseudo first-order rate constant on amprenavir concentration was given by eq 1.

$$k_{obs} = k_{-1} + k_1[I] \quad (1)$$

The reaction was initiated with the stopped-flow spectrofluorometer by mixing an equal volume of enzyme in the indicated buffer with an equal volume of amprenavir in the same buffer.

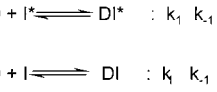
The dissociation of D·amprenavir was measured by the spectrofluorometric change associated with the displacement of amprenavir by excess saquinavir (Scheme 3), where I was amprenavir and T was saquinavir.

Addition of a large excess of saquinavir ([T] = 21 μM in Scheme 3) to a solution of D·amprenavir, which was prepared from 140 nM D and 190 nM amprenavir, resulted in a time-dependent fluorescence decrease that was described by a first-order process (k_{-1}). The fluorescence drift of the enzyme−inhibitor complexes was accounted for by including a linear term in the exponential fitting function (vt in eq 2).

$$F(t) = F_{int} - \Delta F_{amp}(1 - e^{-k_{-1}t}) + vt \quad (2)$$

The dissociation of D·amprenavir was measured by an alternative method. In this method, D·amprenavir was generated with radiolabeled amprenavir (I*). The time-course for displacement of radiolabeled amprenavir from this complex by cold amprenavir (I) was then monitored. Initially, 0.5 μM I* was preincubated with 0.6 μM D for 5 min at 25 °C to form DI* in the selected buffer. The displacement reaction was initiated by addition of 6.8 μM I. At selected times, DI and DI* were separated from I and I* by rapid chromatography ($t \sim 30$ s) through 0.5 mL of G-10 resin equilibrated in the indicated buffer. Under these conditions, over 99% of the unbound amprenavir was absorbed onto the G-10 resin, and over 80% of the protein was recovered. The amprenavir could only be removed from the resin with organic solvents. These data were analyzed according to Scheme 4 with the Dynafit program.

Scheme 4: Competition of I for Radiolabeled I (I*) in EI* to Determine the Value of $k-1$



Titration Data. The enzyme was titrated with poly A and HF23. In the former case, residual activity in the supernatant was determined after centrifugation, whereas in the latter case the quenching of the fluorescence of the hexachlorofluoresceinyl moiety ($\lambda_{ex} = 530$ nm, $\lambda_{em} = 550$ nm) was monitored. These data were analyzed as an equilibrium titration of a fixed concentration of an effector (poly A or HF23) with a ligand (enzyme). The receptor is assumed for simplicity to have *n* independent sites. The binding curve is described by eq 3 (*24*).

$$RL(L_t) = RL_\infty\left(\frac{1}{2nR_t}\right)\{(nR_t + K + [L_t]) - ((nR_t + K + [L_t])^2 - 4nR_t[L_t])^{0.5}\} \quad (3)$$

$RL(L_t)$ was the signal change for a total ligand concentration L_t, and RL_∞ was the signal change at infinite concentration of ligand, *n* was the number of equivalent binding sites on R, and L_t and R_t were total concentrations of L and R, respectively.

RESULTS

Effect of NaCl Concentration on the Values of the Steady-State Kinetic Parameters for S Hydrolysis. The steady-state data were described by the mechanism of Scheme 1 at all concentrations of NaCl tested at pH 5.5. The K_m value for S increased over 100-fold as the NaCl concentration was decreased from 4.0 to 0.5 M, whereas the value of k_{cat} increased less than 3-fold over this range of NaCl concentration (Table 1). The value of k_{cat}/K_m decreased from a value of 50 μM^{-1} s^{-1} for 4.0 M NaCl to a value of 0.176 μM^{-1} s^{-1} for 0.005 M NaCl.

Effect of NaCl Concentration on the Values of the Kinetic Parameters for Amprenavir Binding to the Enzyme. Amprenavir inhibits HIV protease with high potency (66 pM (*23*)). The kinetics of association of this inhibitor can be monitored by the associated small increase in protein/amprenavir fluorescence ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 340$ nm). An example of the time course for the fluorescence change observed upon mixing 0.4 μM amprenavir with 0.2 μM protease in 0.4 M NaCl is presented in the inset of Figure 1. The pseudo first-order rate constant describing this reaction was linearly dependent on amprenavir concentration (Figure 1). These results were described by Scheme 2 and eq 1 with $k_1 = 60$ (3) μM^{-1} s^{-1}. Because the value for k_{-1} was too small to estimate from these data, two alternative methods were used to estimate this value. In the first method, the dissociation of D·amprenavir was monitored by the fluores-

Table 1: Kinetic Parameters for Substrate Hydrolysis by HIV Protease at Selected Salt Concentrations[a]

[NaCl], M	k_{cat}, s^{-1}	K_m, μM	k_{cat}/K_m, $\mu M^{-1} s^{-1}$
0.005	nd[b]	>20	0.176 (1)
0.5[c]	nd[b]	>10	1.00 (1)
0.5[d]	15.1 (2)	14.9 (4)	0.99
0.5[e]	16.2 (2)	16.9 (4)	0.96
1.0	12.2 (2)	4.9 (1)	2.5
2.0	9.0 (1)	1.16 (3)	8
4.0	6.5 (1)	0.13(1)	50

[a] k_{cat} and K_m values or k_{cat}/K_m values were determined from the progress curves for substrate hydrolysis in the standard buffer supplemented with the indicated concentrations of NaCl. For analysis of the data, the value of k_1 was fixed at 100 $\mu M^{-1} s^{-1}$. The value for K_m was calculated from the ratio of the value of $k_{-1} + k_{cat}$ to that of k_1. The enzyme concentrations were 280 nM D for 0.005 M NaCl, 140 nM D for 0.5 M NaCl, 42 nM D for 1.0, and 2.0 M NaCl and 28 nM D for 4.0 M NaCl. [b] Not determined. [c] Substrate concentration (5 μM) was much less than the K_m value. [d] Substrate concentration (40 μM) was much greater than the K_m value. [e] The standard buffer was supplemented with 0.2% PEG-8000.

cence change associated with the formation of D·saquinavir (Figure 1B). Thus, excess saquinavir (21 μM) was added to a solution of D·amprenavir (140 nM) in 1.0 M NaCl. The dissociation of D·amprenavir was monitored by the fluorescence decrease (λ_{ex} = 280 nm, λ_{em} = 340 nm). These data were described by Scheme 3. Equation 2, which also accounted for the drift in fluorescence at the end of the reaction, was fitted to these data with k_{-1} = 0.00110 (1) s^{-1}. In the second method, the value of k_{-1} was estimated from the time-course for equilibration of D·radiolabeled amprenavir in 1.0 M NaCl with excess unlabeled amprenavir. These data were analyzed by Scheme 4 with Dynafit to give k_{-1} = 0.0021 (3) s^{-1}. The values for k_1 and k_{-1} determined in the presence of different NaCl concentrations are summarized in Table 2. The values of the dissociation constants of D·amprenavir were calculated from the ratio of k_{-1} to k_1. The K_i value for amprenavir (70 pM) in 0.5 M NaCl was similar to the K_d value determined from the ratio of k_{-1} to k_1 (35 pM) which confirmed that the same process was being measured by the two methods (Table 2). The concentration of NaCl had a large effect on the K_d value for dissociation of D·amprenavir. Only the dissociation rate constant (k_{-1}) was affected significantly by NaCl concentration (Table 2).

Polyanionic Polymers Substitute for NaCl as Effectors for the Enzyme. The catalytic activity of HIV protease has been proposed to increase with increasing NaCl concentrations because of the "salting-out" of the substrate on the enzyme. To investigate this effect further, we sought to find effectors that could substitute for NaCl at lower concentrations. Initial studies found that the value of k_{cat}/K_m ([D] <10 nM) in the absence of NaCl was very dependent on the source of MES buffer. Of the sources of MES buffers tested, the enzyme had the least activity in Sigma Ultra MES buffer. These results suggested buffers from different sources were contaminated with either an inhibitor of or an activator of the enzyme. The former was established to be the case by the observation that enzymatic activity was stimulated in Sigma Ultra MES buffer by polyanionic polymers such as heparin. High concentrations of NaCl in the buffer eliminated these effects.

Selected polyanionic, polycationic, and neutral polymers were tested as effectors of protease activity (Table 3).

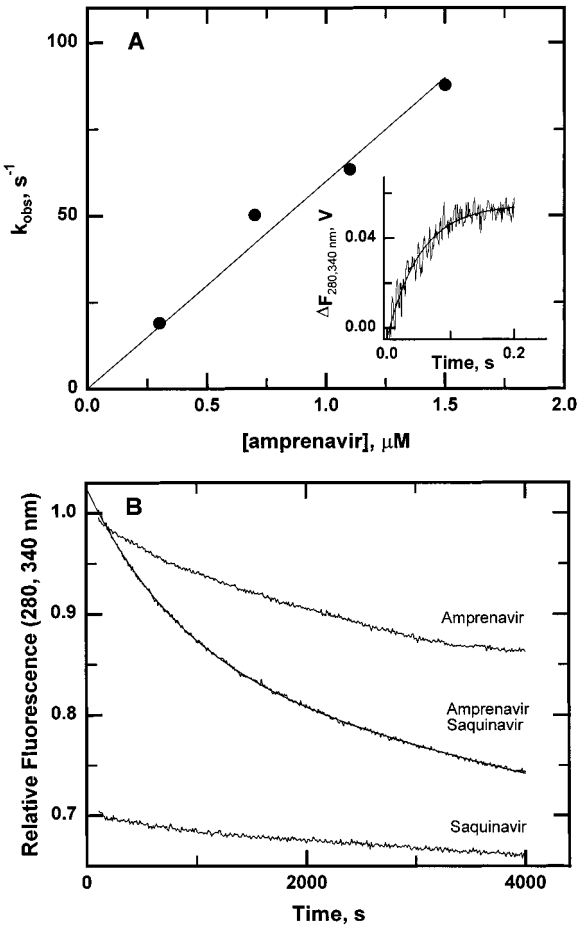


FIGURE 1: Fluorescence changes associated with the binding of inhibitors to D. (A) Binding of amprenavir to D. The time course for the binding of amprenavir was monitored by the fluorescence changes associated with complex formation (λ_{ex} = 280 nm, λ_e > 335 nm). The inset presents a typical example of the time course of the fluorescence changes upon reaction of 0.4 μM amprenavir with 0.2 μM D in 0.4 M NaCl and 0.1 M MES buffer at pH 5.5. The smooth line is the theoretical curve for a process described by a first-order rate constant of 19 (1) s^{-1}. The main figure shows the dependence of the values of this pseudo first-order rate constant on amprenavir concentration. The solid line corresponds to a bimolecular rate constant of 60. (3) $\mu M^{-1} s^{-1}$. (B) Dissociation of D·amprenavir. The reaction was monitored by the fluorescence change associated with the conversion of D·amprenavir to D·saquinavir (λ_{ex} = 280 nm, λ_{em} = 340 nm). D·amprenavir was formed from 190 nM amprenavir and 140 nM D in 0.02 M MES-TRIS buffer with 1.0 M NaCl at pH 5.5. The reaction was initiated by the addition of 21 μM saquinavir (middle tracing). Control tracings for addition of saquinavir to D alone (lower tracing) and for addition of amprenavir to D alone (upper tracing) are presented. Superimposed on the experimental tracing is the theoretical curve described by eq 2 with F_{int} = 0.835 (1), ΔF_{amp} = 0.191 (1), k = 1.10 (1) × 10^{-3} s^{-1}, and v = −2.38 (4) × 10^{-5} relative fluorescence units/s.

Polyanionic polymers were effective activators of the enzyme, polycationic polymers were less effective, and neutral polymers were ineffective activators. The activation of the enzyme by polyanionic polymers was relatively nonselective with heparin, poly A, and poly-L-aspartate behaving similarly. The concentration of polymer required for activation was much less than that of NaCl. For example, 0.025% NaCl had no effect on the catalytic activity, whereas 0.025% heparin activated the enzyme maximally. 0.5 M NaCl (~2.5%) was required for this level of activity.

Table 2: Kinetic Constants for Binding of Amprenavir to HIV Protease at Selected Concentrations of NaCl[a]

[NaCl], M	k_{-1}, s^{-1}	k_1, $\mu M^{-1}s^{-1}$	K_d, pM[b]
0.005	0.0060 (1) 0.0032 (2)[c]	65 (3)	92
0.4[d]	nd[e]	60 (3)	nd[e]
0.5	0.00252 (7) 0.0020 (2)[c]	71 (3)	35
0.5[f]	nd[e]	nd[e]	70 (2)[g]
1.0	0.00110 (1) 0.0021 (3)[c]	84 (2)	12
2.0	0.00055 (2) 0.00037 (4)[c]	82 (4)	7
4.0	nd[e] 0.00024 (4)[c]	79 (5)	3[h]

[a] Parameter values were calculated from spectrofluorometric data unless otherwise noted. [b] Calculated as the ratio of k_{-1} to k_1 using constants determined from spectrofluorometric data. [c] Calculated from the time-course for displacement of radiolabeled amprenavir from the enzyme−amprenavir complex (0.5 μM) by cold amprenavir (6.8 μM). [d] The standard buffer was supplemented with 0.1 M MES. [e] Not determined. [f] The standard buffer was supplemented with 0.2% PEG-8000. [g] K_i for inhibition of S hydrolysis in the presence of 70 pM enzyme. [h] Calculated as the ratio of k_{-1} to k_1 using k_{-1} from the radiolabeled data and k_1 from the spectrofluorometric data.

Table 3: Effectors of HIV Protease Peptidolytic Activity[a]

polymer	V_0/E_t, s^{-1}
NaCl (0.025%)	0.06
NaCl (1 M)	5.1
none	0.06
Anionic Polymers	
heparin (0.01%)	3.1
poly A (0.025%)	2.9
HF23 (0.008%)	2.1
dextran sulfate (0.025%)	1.6
polygalacturonate (0.025%)	2.3
poly-L-aspartate (0.01%)	3.8
tripolyphosphate (0.01%)	0.15
tetrapolyphosphate (0.01%)	0.07
$(P_i)_5$ (0.01%)	0.48
$(P_i)_{18}$ (0.01%)	2.7
$(P_i)_{28}$ (0.01%)	2.8
$(P_i)_{48}$ (0.01%)	3.3
carboxymethyl cellulose (0.01%)	3.0
poly-L-glutamate (0.01%)	0.06
Cationic Polymers	
polyethylenimine (0.01%)	0.41
poly-L-lysine (0.01%)	0.57
poly-L-arginine (0.01%)	0.57
poly-D-lysine (0.01%)	0.57
Neutral Polymers	
methyl cellulose (0.01%)	0.06
poly(ethylene glycol) 8000 (0.01%)	0.06
poly-DL-alanine (0.01%)	0.06

[a] Assayed conditions were 2.8 nM D and 2.5 μM S in the standard buffer supplemented with 5 mM NaCl unless otherwise noted.

The steady-state kinetic parameters for S hydrolysis by the enzyme with selected effectors are summarized in Table 4. On the basis of the value of k_{cat}/K_m of the enzyme in the presence of these effectors, 4.0 M NaCl was superior. However, the activity of the enzyme in poly A (0.01%) approached that in 4.0 M NaCl (23%). Consequently, a more detailed study of the activation of the enzyme by poly A was undertaken.

Requirements for Activation of the Enzyme by Poly A. The hydrolytic activity of the enzyme in 0.02 M MES-TRIS at pH 5.5 was very low. Supplementing this buffer with 0.01% poly A resulted in a dramatic increase in hydrolytic activity (Figure 2A). Even though the hydrolytic activity of the enzyme was stimulated by poly A alone, maximal stimulation required a low concentration of NaCl (Figure 2B). Concentrations of NaCl (80 mM) that resulted in significant stimulation of the enzyme in the absence of poly A inhibited the poly A effect (Figure 2A). The concentration of NaCl that yielded maximal stimulation of the catalytic activity in the presence of poly A was approximately 5 mM (Figure 2B). At this concentration of NaCl, the hydrolytic activity of 2.8 nM enzyme was hyperbolically dependent on the concentration of poly A (Figure 2B) with 4.9 (6) $\times 10^{-5}$% poly A yielding 50% of the maximal stimulation. Similar results were observed when the reaction was initiated by dilution of enzyme from 0.14 or 1.4 μM stock solution. Because the fraction of enzyme present as the dimer is potentially different in these stock solutions, the effects of poly A on the value of k_{cat}/K_m were not the result of stabilization of the catalytically active dimeric form of the enzyme.

Table 4: Kinetic Constants for Substrate Hydrolysis by HIV Protease with Selected Effectors[a]

effector	k_{cat}, s^{-1}	K_m, μM	k_{cat}/K_m, μM^{-1} s^{-1}
0.005 M NaCl	nd[b]	>20	0.176 (1)
4.0 M NaCl	6.5 (1)	0.13 (1)	50
0.01% heparin	3.3 (2)	0.9 (1)	3.6
0.01% PEI	nd[b]	>20	0.213 (9)
0.01% poly A	3.36 (7)	0.39 (3)	8.6
0.01% HF23	4.93 (9)	1.7 (1)	2.9

[a] Determined from the progress curve for substrate hydrolysis as described in Materials and Methods. [b] Not determined.

Aggregation of the Enzyme with Poly A. The finding that poly A enhanced the hydrolytic activity of the enzyme suggested that the enzyme and/or substrate was interacting with poly A. Because poly A did not affect the fluorescence of the substrate, these components of the assay were probably not interacting. However, low speed centrifugation (15000*g* for 20 min) of a mixture of 1.4 μM enzyme and 0.01% poly A resulted in significant loss of the enzyme from the supernatant. If the enzyme sample was treated with 0.01% poly A and 50 mM NaCl, the activity of the treated sample before centrifugation was similar to that of the untreated sample. Centrifugation removed most of the enzyme from the treated sample (>90%). If the NaCl concentration was increased to 250 mM in the poly A treated sample, most of the enzyme remained in the supernatant after centrifugation (Figure 3). With NaCl concentrations lower than 50 mM, centrifugation removed a smaller fraction of the poly A-treated enzyme. The dependence of this phenomenon on NaCl concentration between 0 and 50 mM was similar to the dependence of enzymatic activity on NaCl concentration (Figure 2B and Figure 3). The minimal concentration of poly A required for effective removal of 1.4 μM enzyme by low speed centrifugation from the standard buffer supplemented with 50 mM NaCl was 0.0006% poly A (Figure 3, inset). Equation 3 was fitted to these data to give $K_d = 20$ (2) nM and $n = 2800$ μM (*1*) sites per % poly A. This corresponded to a relative molecular weight of 3400 (10 adenylic acid monomers) for the size of poly A binding to the enzyme.

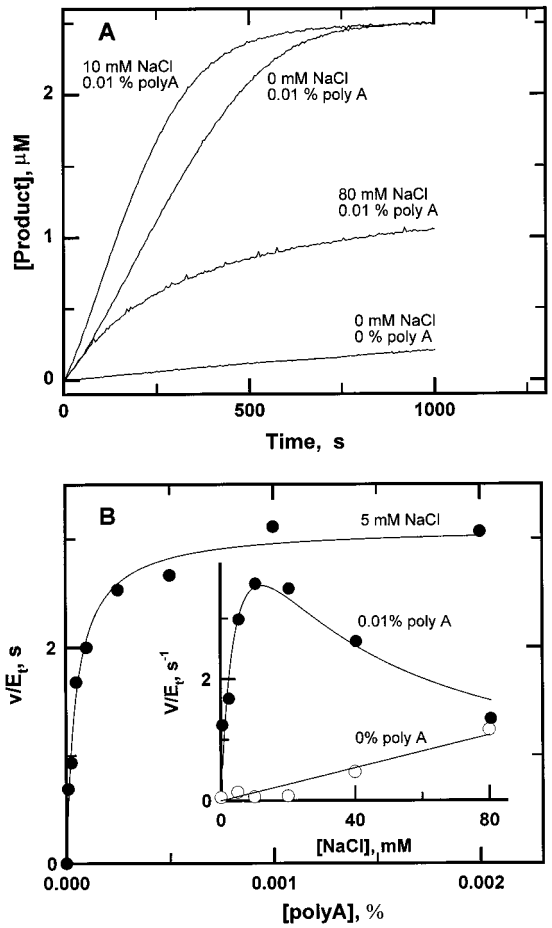


FIGURE 2: Enhancement of the rate of enzymatic hydrolysis of S by poly A. (A) The effect of NaCl and poly A on the rate of enzymatic hydrolysis of S. In the absence of NaCl and poly A, 2.8 nM D did not hydrolyze 2.5 μM S significantly in the standard buffer. In the presence of 0.01% poly A, the enzyme catalyzed substrate hydrolysis at a significant rate after an initial lag. Supplementing the buffer with 10 mM NaCl enhanced the rate of substrate hydrolysis significantly and eliminated the lag. Supplementing the buffer with 80 mM NaCl depressed the initial rate of substrate hydrolysis significantly. (B) Dependencies of the initial rates of substrate hydrolysis on the concentrations of NaCl and poly A. The rate of hydrolysis of 2.5 μM S by 2.8 nM E in the standard buffer supplemented with 5 mM NaCl was saturated at high concentrations of poly A. A simple binding isotherm was fitted to these data to give a maximal value for v/E_t of 3.1 (1) s^{-1} and a concentration of poly A of 4.9 (6) $\times 10^{-5}$% for 50% of the maximal effect (solid line). In the presence of 0.01% poly A, the rate of S (2.5 μM) hydrolysis was biphasically dependent on NaCl concentration. The rate was maximal at approximately 10 mM NaCl (inset, closed circles). In the absence of poly A, the initial rate of substrate hydrolysis increased with increasing linearly with NaCl concentrations (inset, open circles).

The Enzyme-poly A Aggregate Had Reduced Catalytic Activity. Experiments described above demonstrated that the enzyme and poly A formed a large aggregate that was readily removed from solution by low speed centrifugation. Because the centrifugation studies were performed at enzyme concentrations much greater than those employed for the determination of catalytic activity, it was possible that two independent phenomenon were being monitored, i.e., the aggregate formed at high enzyme concentration was not the species with enhanced catalytic activity. To address this possibility, 2.8 μM enzyme was preincubated with 10 mM NaCl in the presence or absence of 0.01% poly A. The

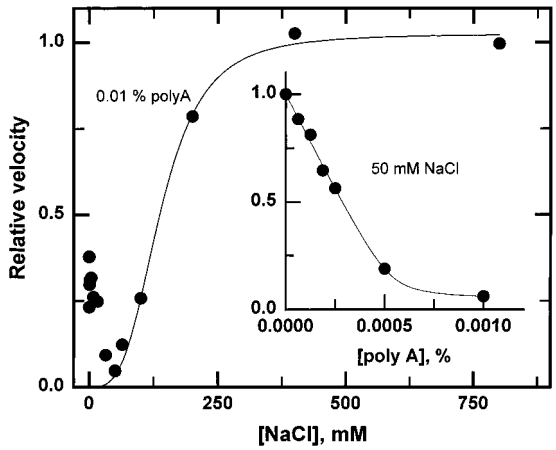


FIGURE 3: Aggregation of D with poly A. D (1.4 μM) was incubated with 0.01% poly A and the indicated concentrations of NaCl in the standard buffer for 5 min. This mixture was centrifuged at 15000*g* for 20 min. The supernatant was assayed for enzymatic activity in the presence of 1.0 M NaCl and 2.5 μM S. The activity in the supernatant relative to that of enzyme prior to treatment or centrifugation was determined as a function of NaCl concentration. The solid line through the data for NaCl concentration greater than 50 mM was generated using the logistic equation. Similar data were collected for varying concentrations of poly A at 50 mM NaCl (inset). Equation 3 was fitted to these data with $K_d = 20$ (2) nM and $n = 2800$ μM (1) sites per % poly A. (solid line).

enzymatic activity in these mixtures was determined after a 500-fold dilution into an assay that contained either 0.01% poly A, 10 mM NaCl, and 2.5 μM S or 1.0 M NaCl and 2.5 μM S. In the latter assay, the preincubation mixtures had the same catalytic activity (v/E_t was 2.96 and 2.71 s^{-1}, respectively), whereas in the former assay only the preincubation mixture that did not contain poly A retained high catalytic activity (v/E_t was 1.46 and 0.12 s^{-1}, respectively). These results demonstrated that the aggregate did not have enhanced catalytic activity.

HF23 as an Effector. Because the chain lengths of our preparation of poly A were heterogeneous, a quantitative study with this polymer was not pursued. Instead, a DNA oligomer of defined length (23-mer) with a fluorescence probe (hexachlorofluorescein) linked to the 5′ end (HF23) was chosen as a model for studying the interaction of D with a polyanionic polymer. Perturbations in the fluorescence of the hexachlorofluoresceinyl moiety were used to monitor the binding of the enzyme to the polymer. The enzyme efficiently quenched the fluorescence of HF23 (Figure 4A). Because the fluorescence quenching was abrogated in the presence of excess poly A (Figure 4A), which would compete for a polyanionic site but presumably not a hexachlorofluoresceinyl binding site, the quenching was attributed to binding of the enzyme to the DNA and not to the hexachlorofluoresceinyl moiety. Titration of the fluorescence with enzyme indicated that multiple enzyme dimers were required for complete quenching of the fluorescence. The affinity of the enzyme for HF23 was high. Because formation of the complex between 18 nM HF23 and 14 nM D was too rapid to be monitored ($\lambda_{ex} = 500$ nm, $\lambda_{em} > 535$ nm) by the stopped flow spectrofluorometer, the association rate constant was estimated to be greater than 1 nM^{-1} s^{-1}. The dissociation rate constant was estimated from competition experiments with poly A to be ~ 0.02 s^{-1}. Thus, the dissociation constant

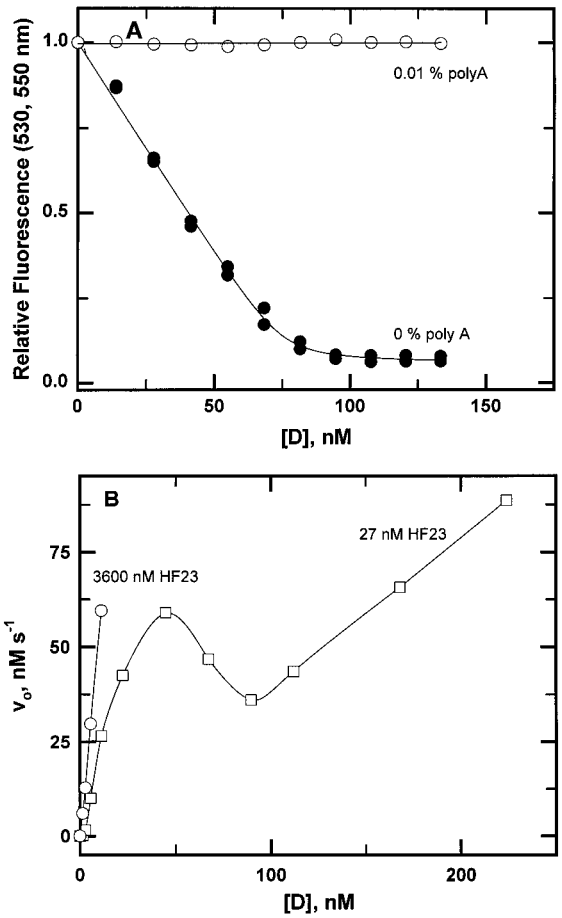


FIGURE 4: Titration of HF23 with enzyme. (A) Binding of HF23 to the enzyme. The fluorescence of HF23 (λ_{ex} = 530 nm, λ_{em} > 550 nm) was monitored. The standard buffer was supplemented with 5 mM NaCl. HF23 (27 nM) was titrated with D in the presence (open circles) or absence (closed circles) of 0.01% poly A. Equation 3 was fitted to the data collected in the absence of poly A to yield a stoichiometry value of 2.79 (5) enzyme molecules per HF23. (B) Activity of the enzyme in the presence of HF23. Enzymatic activity was determined as a function of enzyme concentration in the presence of 3600 nM HF23 (circles) or 27 nM HF23 (squares). The standard buffer was supplemented with 5 mM NaCl and 2.5 μM S. The reaction was initiated by addition of enzyme from a 1.4 μM solution.

for formation of a 1:1 complex between HF23 and D was < 0.02 nM. The stoichiometry was 2.79 (5) enzyme dimers per HF23 molecule (eq 3).

The catalytic activity of D was modulated upon binding to HF23 (Figure 4B). In the presence of excess HF23 the hydrolytic activity was linearly dependent on the concentration of enzyme. In the presence of limiting HF23, this activity had a complicated dependence on enzyme concentration that was correlated with the fluorescence titration data. At ratios of D to HF23 less than one, the hydrolytic activity increased linearly with increasing enzyme concentration. For ratios of D to HF23 between one and three, the hydrolytic activity decreased with increasing enzyme concentration. For ratios of D to HF23 greater than three, the hydrolytic activity increased with increasing enzyme concentration (Figure 4B). The increase in hydrolytic activity with enzyme concentration after titration of HF23 (~3 mol enzyme/mol HF23) was similar to that measured in the absence of HF23 (see Figure 5 for the enzyme concentration dependence of substrate hydrolysis in the absence of effector).

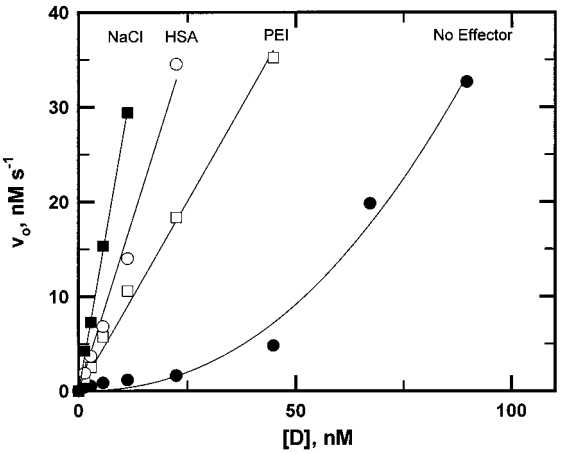


FIGURE 5: Enzyme concentration dependence of the initial velocity of substrate hydrolysis in the presence of selected effectors. The initial velocity of hydrolysis of 2.5 μM S was determined in the presence of 0.4 M NaCl (closed squares), 9 μM human serum albumin (open circles), 0.025% polyethylenimine (open squares), and no added effector (closed circles). The standard buffer was supplemented with 5 mM NaCl. The reaction was initiated by dilution of enzyme from a 1.4 μM working solution.

The activity of the enzyme with 9 μM HF23 as the effector was decreased 50% by addition of 100 mM NaCl. However, the residual activity in the presence of HF23 and NaCl was still 2-fold greater than the activity in the presence of NaCl alone. In contrast to these results, the catalytic activity of enzyme stimulated by poly A was diminished by 80 mM NaCl to that expected for 80 mM NaCl alone (Figure 2B). These results demonstrated that stimulation of HIV protease activity by polyanionic polymeric effectors could occur in the presence of relatively high concentrations of NaCl.

Inhibition of HIV Protease Activity by Amprenavir with 0.01% Poly A as an Effector. Because the enzyme and poly A formed an aggregate at high concentrations of enzyme, it was not meaningful to determine the dissociation constant of the enzyme for amprenavir by the methods used for Table 2. Consequently, the K_i of the enzyme for amprenavir was determined at low enzyme concentration with 0.01% poly A as the effector. The IC_{50} value with 1 μM S was 800 (3) pM. Assuming a competitive model for the binding of substrate and amprenavir, the K_d of the enzyme for amprenavir under these conditions was calculated to be 200 pM, which was similar to the value of 70 pM determined with 0.5 M NaCl as the effector (Table 2).

Dependence of the Initial Velocity on Enzyme Concentration in the Presence of Selected Effectors. The dependence of the initial velocity of hydrolysis of 2.5 μM S on enzyme concentration was determined in the presence of NaCl, polyethylenimine, and human serum albumin, or in the absence of an effector. The initial velocity was linearly dependent on enzyme concentration in the presence of added effector, whereas the initial velocity was nonlinearly dependent on enzyme concentration in the absence of added effector (Figure 5). The nonlinearity of the activity of the enzyme in the absence of effector was not the result of nonselective absorption of the enzyme onto the quartz assay cuvette.

DISCUSSION

We have confirmed the observation of others (*6*, *15*) that the activity of HIV protease is greatly enhanced by NaCl.

NaCl affected both the values of k_{cat} and K_m but primarily affected the value of K_m. As the concentration of NaCl in the assay was increased form 0.5 to 4.0 M, the k_{cat} value decreased from 15 to 6 s^{-1} and the K_m value decreased from 15 to 0.13 μM. The effect of NaCl on the K_m value has been attributed to a "salting out " of the hydrophobic substrate onto the enzyme (*6*, *15*). This suggestion is supported by the finding that the effect follows the Hofmeister Series (*15*). Because the effect of salt on the k_{cat} value was small, the large effect of salt on the K_m value must be the result of changes in the bimolecular association rate constant (k_1) and/or the dissociation rate constant (k_{-1}). Steady-state methods yielded reliable estimates for the values of k_{cat} and K_m but did not yield estimates for k_1 or k_{-1}. Nonetheless, steady-state kinetics provides an estimate for the minimal value for k_1. For the simple mechanism of Scheme 1, k_{cat}/K_m is equal to $k_1(k_{cat}/(k_{cat} + k_{-1})$. Consequently, the value of k_{cat}/K_m is the minimal value ($k_{-1} = 0$) of k_1. The minimal value of k_1 with 4.0 M NaCl in the assay was 50 μM^{-1} s^{-1}. This value was similar to that value for the bimolecular rate constant association of amprenavir with enzyme determined with the same conditions ($\sim$60 μM^{-1} s^{-1}, Table 2). The value of this constant was independent of salt concentration, whereas the value of the dissociation rate constant decreased as the salt concentration was increased. If the binding of amprenavir to the enzyme is a model for the binding of substrate to the enzyme, these results suggested that the salt dependence of the binding of substrate to the enzyme could be explained by a large effect of salt on k_{-1} with little effect on k_1.

In contrast to the "salting out" mechanism, Szeltner and Polgar (*16*) have suggested an alternative mechanism for the activation of HIV protease by NaCl that is based on the observation that increasing concentrations of NaCl increase the conformational stability of the enzyme. In this mechanism, the increased conformational stability of the enzyme in NaCl results in greater affinity of the enzyme for substrate resulting from entropic effects. Presumably increased salt concentration results in an inchoate binding site that more closely resembles the binding site of E·S. This interpretation predicts a large effect of salt on the association rate constant. We found that most of the effect of NaCl concentration was on the dissociation rate constant for E·S. This suggested that in the presence of high salt the incipient binding site of the enzyme for substrate was very similar to that in the absence of NaCl. The increased stability of E·S in the presence of salt resulted from a decreased rate of dissociation of E·S. This result is not readily reconciled with the conformational stability hypothesis of Szeltner and Polgar (*16*).

Polyanionic polymers increased the value of k_{cat}/K_m nearly as effectively as high salt. For example, the K_m value and the k_{cat} values of the enzyme for S in the presence of 0.01% poly A were comparable to those values determined for 4.0 M NaCl (Table 4). Unfortunately, a study with polyanionic polymers analogous to that with salt on the association and dissociation rate constants was not feasible because the enzyme formed aggregates with polyanionic polymers at the enzyme concentrations required for these studies. Nonetheless, the finding that low concentrations of polyanionic polymers activate the enzyme to a level comparable to that observed with high salt suggested that the activation process could result from a common mechanism. The relative effectiveness of these two classes of reagents as activators of the enzyme was estimated as follows. The enzymatic efficiency of HIV protease with 2.0 M NaCl was similar to that with 0.001% poly A. Upon the basis of NaCl concentration and the concentration of AMP monomers in poly A, the AMP monomer was approximately 10^5-fold more efficient effector than NaCl. This indicated that a strongly cooperative effect was involved with binding polyanionic polymers in which many negative charges are held in juxtaposition versus binding of chloride. Furthermore, the finding that the enzymatic activity was stimulated by a variety of effectors that were active at low concentrations suggested that the effect of NaCl was not only the result of salting-out of the substrate/inhibitor on the enzyme.

Finally, RNA is an effector for cleavage of the p15 structural protein by HIV protease (*25*). The enhanced activity was attributed to formation of a complex between RNA and p15 that is processed more efficiently by the enzyme (*25*) and not to formation of a complex between RNA and the enzyme. Nonetheless, polyanionic polymers may function as activators of HIV protease in the cellular milieu that contains relatively high salt concentrations. Even though the effect of 0.01% poly A on catalytic activity was eliminated by high salt concentrations by presumably disruption of E·poly A, the effect of high HF23 concentrations (3.6 μM) was not completely abrogated by 100 mM NaCl. Thus, the concentrations of nucleic acids may be sufficiently high in the cellular milieu to contribute to the activation of HIV-protease.

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