Kosmotropic Salt Activation and Substrate Specificity of Poliovirus Protease 3C†

Iuri E. Gouvea,‡ Wagner A. S. Judice,‡ Maria H. S. Cezari,‡ Maria A. Juliano,‡ Tünde Juhász,§ Zoltán Szeltner,§ László Polgár,§ and Luiz Juliano*,‡

Department of Biophysics, Escola Paulista de Medicina, Universidade Federal de São Paulo - Rua Três de Maio, 100 - São Paulo, 04044-020, Brazil, and Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, H-1518 Budapest 112, P.O. Box 7, Hungary

Received April 24, 2006; Revised Manuscript Received July 30, 2006

ABSTRACT: Picornaviruses produce a large polyprotein, which is cleaved by virally encoded cysteine peptidases, picornain-2A and -3C. Picornain-3C has characteristics of both the serine peptidase chymotrypsin and the cysteine peptidase papain in that the 3D structure resembles chymotrypsin, but its nucleophile is a cysteine SH rather than a serine OH group. We investigated the specificity of poliovirus picornain-3C (PV3C) protease and the influence of kosmotropic salts on catalytic activity, using FRET peptides related to a cleavable segment of the virus polyprotein. The peptidase activity of PV3C was found to be 100-fold higher in the presence of 1.5 M sodium citrate. This activation was anion-dependent, following the Hofmeister series citrate^{3-} > SO_4^{2-} > HPO_4^{2-} > acetate$^-$ > HCO_3^- > Cl^-. The activation appeared to be independent of substrate sequence and arose primarily from an increase in k_{cat}. A shift to higher pH was also observed for the pK_1 of the enzyme pH-activity profile. Experiments with the fluorescent probe ANS (1-anilino-8-naphthalene sulfonate) showed that the protease bound the dye in the presence of 1 M sodium citrate but not in its absence or in the presence of 1 M NaCl. Structural changes in PV3C protease were detected using circular dichroism and the thermodynamic data indicated a more organized active site in the presence of sodium citrate. PV3C protease was also activated in D_2O, which was added to the activation by citrate. These effects seem to be related to nonspecific interactions between the solvent and the protein. Our data show that the catalytic efficiency of PV3C protease is modulated by the composition of the environment and that this modulation may play a role in the optimal processing of polyprotein for the virus assembly that occurs inside specific vesicles formed in poliovirus-infected cells.

Picornaviruses (name derived from "pico"(small) and "RNA") form a family of small closely related RNA$^+$ nonenveloped viruses responsible for several human and animal diseases, such as poliomyelitis (human polio virus, HPV), the common cold (human rhinovirus, HRV), hepatitis A (human hepatitis A, HAV), a rare form of myocardium inflammation (encephalomiocarditis virus, EMCV), and the highly contagious livestock foot and mouth disease virus (FMDV). The mRNA-like genomes of picornaviruses are translated as large polyproteins, which are processed by specific viral proteases, namely 2A, 3C and leader proteases, to produce structural and nonstructural proteins of the virus. The 3C protease or its precursor 3CD protease is responsible for carrying out the majority of proteolytic processing events, and all picornavirus geneomes encode this enzyme. The 2A protease (2A^{pro}) is encoded in enterovirus and HRV, and the leader protease (L^{pro}) is found in FMDV. The large number of studies regarding the details of picornavirus replication and proteolytic maturation has been thoroughly reviewed (*1*−*5*). The 3D structures of picornains have been solved and reviewed by Seipelt et al. (*6*) and, because of their obvious relationship to serine peptidases, grouped in the family C3 and clan PA. Picornains differ from the most extensively studied cysteine peptidases of the papain family that contain an Asn residue as the third member of the catalytic triad, whereas the PV3C protease contains Glu71. In this respect, picornains resemble the enzymes of the chymotrypsin family, which also have a carboxyl group in the catalytic triad. Moreover, in the catalytic triad of PV3C protease that employs His40, Glu71 and Cys 147, the imidazole group of His40 operates as a general base, as found with serine peptidases (*7*, *8*). In contrast to the expected decrease in the value of the specificity constant (k_{cat}/K_m) in D_2O, an inverse deuterium isotope effect was observed for the substrate hydrolysis by PV3C protease, and the increase in enzyme activity was associated with the stabilization of the protein structure (*7*). Moreover, the reported catalytic efficiency of PV3C protease on synthetic substrates is quite low compared to the activities of classic cysteine or serine proteases (*9*−*11*). The activity of PV3C protease seems to be dependent on the environmental composition because the 3C protease of HRV-14 (*12*) is activated by salts. In contrast, the leader protease (Lpro) of FMDV, which is highly sensitive to salts, is inactivated at 150 mM NaCl (*13*). However, the activity of the HRV 2A protease, also a cysteine protease with chymotrypsin-like structure, is insensitive to NaCl concentra-

† This work was supported by the Brazilian research agencies Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

* To whom correspondence should be addressed. Tel: +55 11 5576 4455. Fax: + 55 11 5575 9617. E-mail: juliano.biof@epm.br.

‡ Universidade Federal de São Paulo.

§ Hungarian Academy of Sciences.

10.1021/bi060793n CCC: $33.50
Published on Web 09/12/2006

tions below 5 M (*14*). The modulation of the activities of these viral proteases by the environmental composition is particularly relevant, taking into account that in poliovirus-infected cells the increase of the concentration of ions reduces overall protein synthesis (*15*) and induces the rearrangement of intracellular membranes into characteristic vesicles. These membrane vesicles form a complex that contains virus proteins and provides the structural basis for rapid and efficient RNA replication. In addition, the vesicles cause a severe structural reorganization of the cell, leading ultimately to cytopathology and cell death (*16*−*20*). All these observations prompted us to investigate the effects of salts, detergents, and glycosaminoglycans as possible regulators of the peptidase activity of PV3C protease using fluorescence-resonance-energy-transfer (FRET) peptides as substrates derived from the nonstructural 2C/3A cleavage site of poliovirus polyprotein. We also performed a thermodynamic analysis of PV3C protease activity as well investigations using circular dichroism and fluorescence spectroscopy in the presence of salt.

MATERIAL AND METHODS

Enzymes. Recombinant poliovirus picornain 3C (M27G) were expressed in *E. coli* BL21 (DE3) pLysS as previously described (*7*). The bacterial cells were harvested by centrifugation, suspended in 100 mL of buffer A (50 mM Tris-HCl at pH 8.0, 25 mM NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 5% glycerol) and sonicated. The cell extract was centrifuged for 30 min at 18000*g* and applied to a 6 mL Resource-Q column (Amersham Biosciences) equilibrated with buffer A. In contrast to most *E. coli* proteins, poliovirus picornain 3C protease failed to bind to the resin at this pH. The void volume containing the enzyme was concentrated by ultrafiltration on an Amicon PM10 membrane and applied to a Superdex-75 column (50 × 1.6 cm), previously equilibrated with buffer A in the presence of 100 mM NaCl. The enzyme was eluted with a flow rate of 0.5 mL/min. From 1000 mL of broth, 12.3 mg of PV3C-(M27G) protease was obtained. The enzyme concentration was determined as described previously (*7*).

Peptides. The FRET peptides based on the poliovirus 2C/3A protease cleavage site (Abz-EALFQGPLQ-EDDnp) were synthesized by solid-phase synthesis (*21*). An automated benchtop simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system, Shimadzu, Japan) was used to synthesize peptides using the Fmoc-procedure. All peptides obtained were purified by semipreparative HPLC on an ecosil C-18 column. The molecular mass and purity of synthesized peptides were checked by amino acid analysis and MALDI-TOF mass spectrometry using a Tof-Spec-E from Micromass, Manchester, U.K. Stock solutions of peptides were prepared in DMSO, and the concentrations were measured spectrophotometrically using the molar extinction coefficient of 17.300 M^{-1} cm^{-1} at 365 nm.

Enzyme assays. The hydrolysis of FRET peptides was assayed in a Hitachi F-2500 spectrofluorimeter, at 25 °C. The enzyme was preactivated in the presence of 1 mM DTE for 2 min before the addition of substrates. Fluorescence changes were monitored continuously at $\lambda_{ex} = 320$ nm and $\lambda_{em} = 420$ nm. The enzyme concentration was chosen at a level intended to hydrolyze less than 5% of the amount of added substrate. The slope was converted into micromoles of substrate hydrolyzed per minute on the basis of a calibration curve obtained from the complete hydrolysis of each peptide. The inner-filter effect was corrected using an empirical equation as previously described (*22*). The kinetic parameters K_m and k_{cat} with respective standard errors were calculated by the Michaelis−Menten equation using Grafit software (Erithacus Software, Horley, Surrey, U.K.).

Determination of the Substrate Cleavage Sites. The scissile bonds of hydrolyzed peptides were identified by isolation of the fragments using analytical HPLC (as described above), from amino acid sequencing (protein sequencer PPSQ-23, Shimadzu, Tokyo, Japan) and by MALDI-TOF mass spectrometry (Tof-Spec-E, Micromass).

Salt Influence on Catalytic Activity. The influence of NaCl, NaOAc, Na_2SO_4, NaH_2PO_4, and Na citrate on the catalytic activity of PV3C protease was investigated over a salt range of 0.5−1.5 M. The measurement of initial velocity of hydrolysis were made using 20 μM Abz-EALFQGPLQ-EDDnp as substrate at 25 °C in 50 mM Tris-HCl at pH 8, containing 1 mM EDTA with (v) or without (v_0) salt to obtain the ratio of v/v_0 reflecting the activation by salt.

pH and Temperature Dependence of Specificity Constant. The pH dependence of rate constants was measured at 25 °C in a four-component buffer comprised of 25 mM acetic acid, 25 mM Mes, 75 mM Tris, and 25 mM glycine containing 1 mM EDTA and 1 mM DTE (standard buffer) and adjusted to the required pH by the addition of 1 M NaOH or 1 M HCl. The reactions were performed under pseudo first-order conditions of hydrolysis of Abz-EALFQGPLQ-EDDnp. The data were fitted with the GraFit software to the appropriate equation as described earlier (*7*). The same procedure was employed to verify the effect of 1 M Na citrate on the pH dependence of PV3C protease activity.

Temperature dependence of the specificity constant (k_{cat}/K_m) was determined as described previously (*7*). The hydrolysis of Abz-EALFQGPLQ-EDDnp by PV3C protease was monitored under pseudo-first-order conditions with or without 1 M Na citrate in Tris-HCl buffer at pH 8.0 containing 1 mM EDTA and 1 mM DTE. Activation parameters were calculated from a linear plot of $\ln[(k_{cat}/K_m)/T]$ versus $1/T$ (eq 1)

$$\ln[(k_{cat}/K_m)/T] = \ln(R/N_A h) + \Delta S^*/R - \Delta H^*/RT \quad (1)$$

where R is the gas constant (8.314 J mol^{-1} K^{-1}), T is the absolute temperature, N_A is the Avogadro number, h is the Plank constant, ΔH^* ($\Delta H^* = -(\text{slope}) \times 8.314$ J mol^{-1}) is the enthalpy of activation, and ΔS^* ($\Delta S^* = (\text{intercept} - 23.76) \times 8.314$ J mol^{-1} K^{-1}) is the entropy of activation. The free energy of activation ΔG^*, was calculated from eq 2.

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (2)$$

ANS Binding. The extent of exposure of the hydrophobic surfaces of the enzyme was measured by its ability to increase the fluorescence of 1-anilino-8-naphthalene-sulfonic acid (ANS) (*23*). A stock solution of ANS was prepared in methanol, and the concentration of the dye was determined using a molar extinction coefficient of 5000 M^{-1} cm^{-1} at 350 nm. The excitation wavelength was set at 380 nm, and

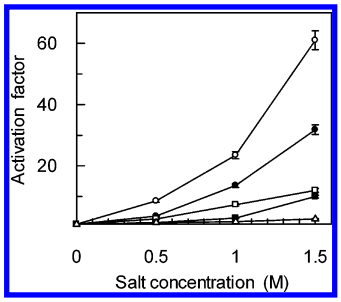

FIGURE 1: Effect of salts on PV3C protease activity. Activation factors (v/v_0) were determined by measuring the initial velocity of hydrolysis of 20 μM Abz-EALFQGPLQ-EDDnp by PV3C protease in the presence or absence of Na citrate (○), NaH_2PO_4 (●), Na_2SO_4 (□), NaOAc (■), and NaCl (△). The assay conditions are described in Materials and Methods.

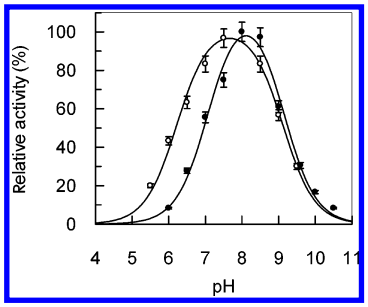

FIGURE 2: pH−k_{cat}/K_m profile for the reaction of PV3C protease with the substrate Abz-EALFQGPLQ-EDDnp in the absence (○) and presence (●) of 1 M Na citrate. The hydrolysis conditions are described in Materials and Methods.

the emission spectra were taken in the range of 400−600 nm. The molar ratio of protein to ANS was 1:50.

Size Exclusion Chromatography. A Superdex-200 column (Amersham Biosciences) was equilibrated with 50 mM Tris-HCl at pH 8.0 containing 100 mM NaCl or 50 mM Tris-HCl at pH 8.0 containing 0.5 Na citrate. Protein samples were incubated in each elution buffer for 10 min and subsequently applied to the appropriately equilibrated column. The samples were eluted at a rate of 0.5 mL/min with the same buffer used for equilibration. The elution was monitored at 280 nm. The molecular mass markers utilized were: blue dextran, thyroglobulin, catalase, albumin, carbonic anhydrase, and citochrome C (Amersham Biosciences).

Circular Dichroism. CD spectra were recorded with a Jasco J-810 spectropolarimeter equipped with a Peltier system of cell temperature control. The absorbance spectra of PV3C protease (1.1 mM) were collected in near-UV range (240−350 nm) using a 1 cm path length cell in the absence or presence of 1 M Na citrate in Tris-HCl buffer at pH 8.0. The system was routinely calibrated with an aqueous solution of recrystallized d_{10}-camphorsulfonic acid. Ellipticity is reported as molar ellipticity, [θ] (deg cm^2 dmol^{-1}). The spectrometer conditions were typically 100 mdeg sensibility; 0.5 nm resolution; 8 s response time; 50 nm min^{-1} scan rate, and 8 accumulations at 25 °C. The control baseline was obtained with solvent and all of the buffer components without the proteins. The absorbance spectra of PV3C protease in the absence of salt was also obtained in the far-UV range (190−250 nm).

Isotopic Effect. General base catalysis was tested in heavy water (99.9%). The deuterium oxide content of the reaction mixture was at least 97.5%. The pD of the deuterium oxide solution can be obtained from pH meter readings according to the relationship pD = pH(meter reading) + 0.4 (*24*).

RESULTS

Effects of Anions, pH, and Glycosaminoglycans on PV3C Protease Activity. The effects of the anions of the Hofmeister series, with Na^+ as the common cation, were determined at various concentrations on the hydrolytic activity of PV3C protease (Figure 1). Citrate was the most effective activator of PV3C protease on the hydrolysis of Abz-EALFQGPLQ-EDDnp, whereas HPO_4^{-2}, SO_4^{-2}, OAc^-, and Cl^- also activated the enzyme but were less efficient.

The effect of Na citrate on the pH dependence of PV3C protease activity was determined over the pH range of 5.0−10.5 (Figure 2). Optimal hydrolysis occurred around pH 7.7 in absence of salt and at 8.1 in the presence of 1 M Na citrate with bell-shaped curves, similar to those previously reported (*7*). Na citrate shifted the acid limb of the pH−activity curve by 0.86 pK_a units toward higher pH (pK_1 = 6.22 in absence of salt and 7.08 in the presence of 1 M Na citrate), whereas the pK_2 value of the basic limb did not significantly change (pK_2 = 9.20 in absence of salt and 9.30 in the presence of 1 M Na citrate).

Heparin sulfate and heparin, up to 20 μM concentration, did not exert any significant effect on the hydrolytic activity of PV3C protease either in the presence or in the absence of 1 M Na citrate. These results suggest that the activation by salts is not dependent on a particular positively charged surface in the protein where heparin or heparin sulfate could bind and activate the peptidase.

Effect of Na Citrate on PV3C Conformation. Kosmotropic (structure maker) salts as citrate and sulfate can produce nonpolar interactions in proteins, which may result in the association and, in some cases, the activation of monomers. This was observed with the human cytomegalovirus (hCMV) and the herpes simplex virus (HSV) proteases that form homodimers and are strongly activated in the presence of salt (*25*−*28*). To examine whether PV3C protease activation by citrate was due to dimerization, the enzyme was gel filtered on a Superdex-200 column in the absence and presence of 0.5 M Na citrate. As shown in Figure 3, PV3C protease was eluted at a molecular mass of 22.4 kD in the absence of Na citrate and at 20.0 kDa in its presence (0.5 M). This result does not support dimer formation and instead suggests that PV3C protease may adopt a more compact structure in the presence of salt. Alternatively, the increase in the retention time in the presence of Na citrate could be the result of a stronger interaction between the protease and the gel matrix.

Conformational change in PV3C protease, due to salt effects, was also investigated by monitoring the fluorescence of ANS when interacting with protease. As shown in Figure 4, the fluorescence emission spectra of the ANS−PV3C protease complex is significantly higher in the presence of Na citrate than in the presence of 1 M NaCl or in absence of these salts. These results suggest that Na citrate but not NaCl induces the formation of hydrophobic ANS binding sites, which increased the fluorescence of the ligand.

We further investigated the occurrence of conformational changes in PV3C protease, measuring the circular dichroism (CD) spectra of the PV3C protease protein (Figure 5). In the far-UV region (190−250 nm), only the spectra in the

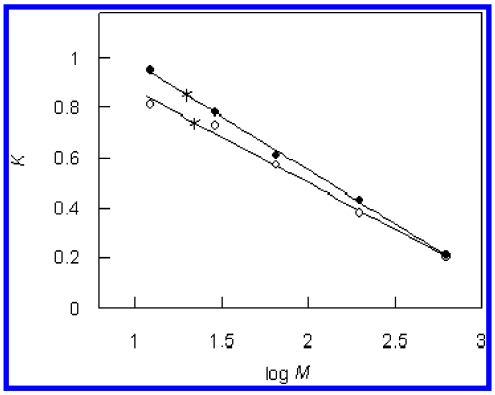

FIGURE 3: Estimation of the apparent molecular mass of PV3C protease. A Superdex-200 column was calibrated with molecular mass markers in 50 mM Tris-HCl at pH 8.0 containing 100 mM NaCl (○) and 50 mM Tris-HCl at pH 8.0 containing 0.5 M Na citrate (●) as described in Materials and Methods. K is defined as $(V_e - V_0)/V_s$, where V_e is the volume required to elute the molecule of interest, V_0 is the void volume of the column, and V_s is the volume of the stationary phase (*29*). PV3C protease (×) was eluted at 18.3 mL (apparent molecular mass of 22.4 kDa) in the absence of Na citrate and at 19.4 mL (apparent molecular mass of 20.0 kDa) in the presence of Na citrate.

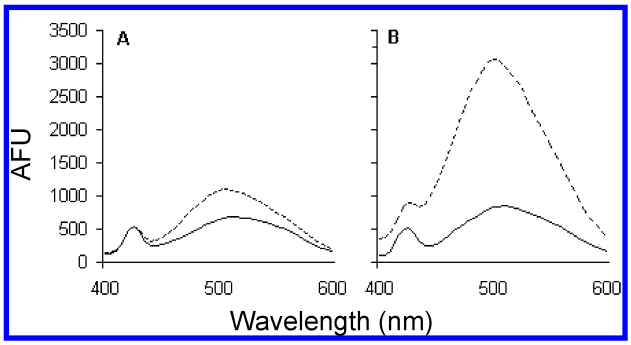

FIGURE 4: Comparative ANS fluorescence emission spectra. (A) ANS fluorescence emission spectra in absence (---) and presence of PV3C protease (—). (B) ANS−PV3C protease fluorescence emission spectra in the presence of 1 M NaCl (—) and 1 M Na citrate (---).

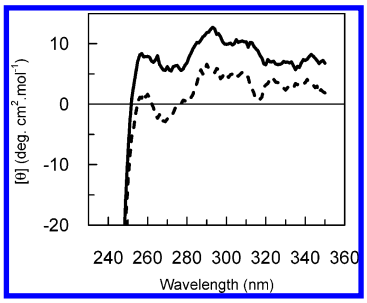

FIGURE 5: CD spectrometra of PV3C protease. Near-UV range CD spectrum in the absence (—) and presence (---) of 1 M Na citrate. Assay conditions are described in Materials and Methods.

absence of salt were obtained because of the high absorption of Na citrate. The CD spectra were deconvoluted (*30*) using CDNN software (*31*), indicating a high percentage of β-structure (53%) in the PV3C protease (data not show). The β-structure content obtained from CD is consistent with that also calculated from the crystal structure (*32*) using the DSSP algorithm available from Sequence Details of PDB (54.1% β structure). For other picornains, similar amounts of β-structure were found (*12*, *33*). In the near-UV range of 240−350 nm, which is characteristic of the tertiary protein structure, the CD spectrum of PV3C protease obtained in the absence of salt is different from that in the presence of 1 M Na citrate, suggesting that salt alters the environment of aromatic amino acids within the protease structure (Figure 5).

Influence of Na Citrate on PV3C Specificity. Table 1 shows the kinetic parameters of hydrolysis of the FRET peptide Abz-EALFQGPLQ-EDDnp (peptide I) and its six variants obtained by Ala scanning (peptides II to VIII). The hydrolysis of the susceptible peptides (I to IV and VI to VIII) was activated by Na citrate. The cleavage of the hydrolyzed peptides occurred only at the Q−G bond, either in the presence or in the absence of salt. We further examined the susceptibility to PV3C protease of peptides that resulted from the substitution of Gln at the P_1 position by amino acids other than Ala. All of them (peptides IX to XIII) were resistant to hydrolysis, except for peptide IX with Glu at the P_1 position, which was hydrolyzed but only after a long period of incubation. The S_1 subsite of PV3C protease presented a restricted specificity for Gln, and the S_1' and S_2' sites have a significant preference for Gly and Pro, respectively because their substitution by Ala (peptides VI and VII) resulted in substrates that were hydrolyzed with k_{cat}/K_m values 5 to 10 times lower than those of peptide I. In contrast, peptides III and VIII, in which Ala was located at positions P_3 and P_3', respectively, in place of Leu, were better hydrolyzed than peptide I. The activation of PV3C protease by Na citrate occurred essentially by increasing the k_{cat} value without significant alterations in K_m values. The enzyme activation by Na citrate was in the range 50−100, as shown in Table 1. The preferences of the PV3C protease subsites for the amino acids at each position of the substrates are in accordance with the proposed docking model obtained from the 3D structure of the enzyme (*32*) as well as to reported specificity studies using the mutagenesis of polyprotein at positions close to the cleavage site Gln−Gly (*34*, *35*). The hydrolysis of peptide IX at the Glu−Gly peptide bond was very slow but suggests that the hydrophobic character of the S_1 subsite and the hydrogen bond between His^{161} and the carboximide of Gln (*32*) is a very important requirement for the catalytic activity of PV-3C protease.

Solvent Isotopic Effects. Kinetic deuterium isotope effects have been widely studied to reveal a general base-catalyzed process, which is expected to be slower in deuterium oxide than in water by a factor of 2−3, as observed in serine protease catalysis (*36*). In PV3C protease, a mechanism similar to that in the papain family (thiolate-imidazolium ion par) was ruled out, and an inverse kinetic solvent isotopic effect was reported (*7*). Here, we investigated the Na citrate effect on the hydrolysis of Abz-EALFQGPLQ-EDDnp by PV3C protease in D_2O. Figure 6 shows that the enzyme is activated by Na citrate in D_2O to a larger extent than that in H_2O. The kinetic parameters for this reaction in D_2O were obtained both in the absence of Na citrate ($k_{cat} = 0.06\ s^{-1}$, $K_m = 17.9\ \mu M$, $k_{cat}/K_m = 3.4\ mM^{-1}\ s^{-1}$) and in the presence of 1 M Na citrate ($k_{cat} = 2.02\ s^{-1}$, $K_m = 20.3\ \mu M$, $k_{cat}/K_m = 99.5\ mM^{-1}\ s^{-1}$). The k_{cat} and k_{cat}/K_m values for peptide I in H_2O, both in the presence and in absence of Na citrate, are significantly lower than those in D_2O (Table 1). Therefore, the inverse kinetic solvent isotopic effect remains even in the presence of Na citrate. This result suggests that the enzyme activation mechanisms by D_2O and Na citrate are additive and that the demonstration of the possible general

Table 1: Kinetic Parameters for Hydrolysis by PV3C of FRET Peptides Derived from Abz-EALFQGPLQ-EDDnp in the Absence and Presence of 1 M Sodium Citrate[a]

		absence of salt			1 M sodium citrate			
no.	peptide	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}.s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}.s^{-1}$)	activation factor[b] (fold)
I	Abz-EALFQ↓GPLQ-EDDnp	0.03	14.0	2.2	0.87	16.0	54.0	24
	Scan of Ala							
II	Abz-**A**ALFQ↓GPLQ-EDDnp	0.01	18.2	0.5	0.96	20.0	48.0	104
III	Abz-E**A**FQ↓GPLQ-EDDnp	0.08	34.9	2.2	3.34	26.4	126.6	57
IV	Abz-EAL**A**Q↓GPLQ-EDDnp	0.01	41.3	0.3	0.66	43.5	15.1	48
V	Abz-EALF**A**GPLQ-EDDnp	resistant to hydrolysis						
VI	Abz-EALFQ↓**A**PLQ-EDDnp	<0.1 mM^{-1} s^{-1}			0.34	39.3	8.7	
VII	Abz-EALFQ↓G**A**LQ-EDDnp	<0.1 mM^{-1} s^{-1}			0.28	53.3	4.5	
VIII	Abz-EALFQ↓GP**A**Q-EDDnp	0.03	18.5	1.8	2.72	21.3	127.8	70
	Modifications at P_1 position							
IX	Abz-EALF**E**↓GPLQ-EDDnp	<0.1 mM^{-1} s^{-1}			<0.1 mM^{-1} s^{-1}			
X	Abz-EALF**R**GPLQ-EDDnp	resistant to hydrolysis						
XI	Abz-EALF**H**GPLQ-EDDnp							
XII	Abz-EALF**N**GPLQ-EDDnp							
XIII	Abz-EALF**Y**GPLQ-EDDnp							

[a] Conditions for hydrolysis: assays were performed at 25°C, in 50 mM Tris-HCl at pH 8,0. PV3C protease was preactivated with 1 mM DTE. The concentrations of the substrates employed in each determination were above and below the K_m values. [b] Activation factor is the ratio of k_{cat}/K_m values in the presence to absence of salt. The errors for determination of the kinetic parameters were lower than 5% and 10% in at least three determinations in the presence and absence of salt, respectively. The hydrolyzed bonds (↓) were determined by HPLC/mass spectroscopy.

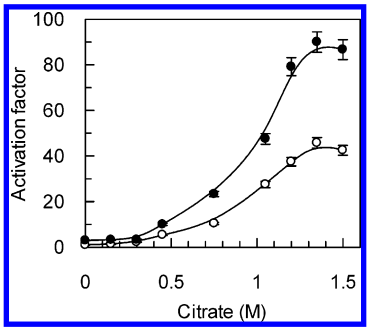

FIGURE 6: Solvent isotopic effect in PV3C protease salt activation. Activation factors (v/v_0) were determined by measuring the pseudo-first-order rate constant of hydrolysis of Abz-EALFQGPLQ-EDDnp by PV3C protease in varying concentrations of Na citrate either in water (○) or in deuterium oxide (●). The assay conditions are described in Materials and Methods.

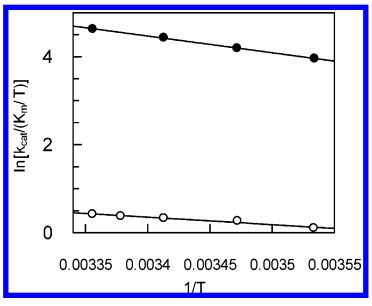

FIGURE 7: Eyring plots for PV3C protease. Measurements were carried out in the absence (○) and presence (●) of 1 M Na citrate. Assay conditions are described in Materials and Methods.

base-catalyzed mechanism (*37*, *38*) in D_2O is precluded even in the activated enzyme.

Temperature Dependence of PV3C Activity. Figure 7 shows the Eyring plot using the k_{cat}/K_m values of the hydrolysis of Abz-EALFQGPLQ-EDDnp by PV3C protease in the presence and absence of 1 M Na citrate over the temperature range 10−25 °C in which a linear relation was observed. This is in accordance with the earlier reported data with the same enzyme (*7*) for the temperature range used.

Table 2: Activation Parameters for the Hydrolysis of Abz-EALFQGPLQ-EDDnp by PV3C Protease[a]

	ΔS^* ($J\ mol^{-1}\ K^{-1}$)	ΔH^* ($kJ\ mol^{-1}$)	ΔG^* ($kJ\ mol^{-1}$)
without salt	-146 ± 14	14 ± 1.3	58 ± 5.1
1 M Na citrate	-53 ± 0.5	31 ± 0.6	47 ± 0.7

[a] Assay conditions are described in Materials and Methods. The standard deviations are indicated, which were obtained from the variation of k_{cat}/K_m values in each temperature, and the propagation error was taken into account.

The linear Eyring plots permitted the calculation of the following thermodynamic parameters: entropy of activation (ΔS^*), enthalpy of activation (ΔH^*), and Gibbs free energy of activation (ΔG^*), which are shown in Table 2. In presence or absence of salt, ΔS^* has negative values that are consistent with the gain of order during ES complex formation. The entropy in the presence of Na citrate ($\Delta S^* = -53 \pm 0.5$ J Mol^{-1} K^{-1}) is lower than that in its absence ($\Delta S^* = -147 \pm 14$ J Mol^{-1} K^{-1}). This suggests that high Na citrate concentration favors a higher structural organization of PV3C protease.

DISCUSSION

The activation of PV3C protease by salts followed the Hofmeister series, citrate> SO_4^{2-} > HPO_4^{2-} > Cl^-, with a remarkable enhancement of 100 times in the hydrolysis rate of the FRET peptide Abz-EALFQGPLQ-EDDnp in the presence of 1.5 M Na citrate (Figure 1). Similar peptidase activations by salts were described earlier for the human glandular kallikrein 3 (hK3), which corresponds to human prostate-specific antigen (*39*), HRV-14 3C protease (*12*), human cytomegalovirus (hCMV), and herpes simplex virus (HSV) proteases. The last two proteases are activated by forming homodimers in the presence of salt (*25*−*28*), whereas hK3 remains a monomer in the activated form. Kosmotropic salts, like sodium citrate, Na_2SO_4, and Na_2HPO_4, are preferentially excluded from the solvation shell

of proteins because they have higher affinity for water than for the protein surface. The interaction of these salts with protein surfaces is unfavorable, and the tendency of the protein is to change its conformation, reducing the exposition of the surfaces (*39*−*43*).

The activation of PV3C protease by Na citrate was associated with conformational changes as demonstrated by ANS binding and circular dichroism and without oligomerization, as shown by gel filtration. The 3D structure of the enzyme shows a flexible loop in the region between residues 140−146, which could stabilize the substrate in its transition state form (*32*). Similar activation by salts with changes in the protein conformation was reported for human prostate-specific antigen (*39*). Human kallikrein 6 (hK6) was also shown to be strongly activated by Na citrate but without detectable conformation changes (*44*). All these salt-activated serine peptidases belong to the trypsin family (S1 family). It is noteworthy that activation by salt is one of three characteristics that the PV3C protease shares with trypsin-like peptidases besides the 3D structure and the operation of a His40-imidazole group as a general base.

In addition to the activation by Na citrate, the pH-activity profile of PV3C protease was also affected, the pK_1 value being shifted to basic pH, possibly due to modifications in the microenvironment of catalytic His40. The rates of hydrolysis of peptide Abz-EALFQGPLQ-EDDnp and its Ala substituted derivatives (Table 1), which were susceptible to PV3C protease, were increased to similar extents in the presence of Na citrate without change to the cleavage site. The high selectivity of PV3C protease to Gln at S_1 is retained in the presence of Na citrate. This result suggests that the conformational modification of the protease induced by Na citrate, which leads to activation, influences the catalytic site groups more than the peptide binding site and relates well with the increase in k_{cat} rather than K_m. It is also consistent with the notion that in the evolution of enzymes, it is more important to improve catalysis than binding (*45*−*47*). That the structure of enzyme catalytic sites, as that of PV3C protease, can be modulated by environmental conditions such as salt concentration exemplifies a further fine-tuning mechanism for biological control and adaptation. Such fine-tuning properties could be particularly relevant for infective viruses that occupy, as does poliovirus, special complexes of vesicles formed by the virus inside the cells (*16*−*20*) in which the immediate environmental composition can be regulated.

ACKNOWLEDGMENT

We thank to Dr. Peter Shoolingin-Jordan from Southampton University, U.K. for his assistance in editing the text.

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BI060793N