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Stimulation of the Herpes Simplex Virus Type I Protease by Antichaeotrophic Salts*

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The herpes simplex virus type 1 protease is expressed as an 80,000-dalton polypeptide, encoded within the 635-amino acid open reading frame of the UL26 gene. The two known protein substrates for this enzyme are the protease itself and the capsid assembly protein ICP35 (Liu, F., and Roizman, B. (1991) *J. Virol.* 65, 5149–5156). In this report we describe the use of a rapid and quantitative assay for characterizing the protease. The assay uses a glutathione *S*-transferase fusion protein containing the COOH-terminal cleavage site of ICP35 as the substrate (GST-56). The protease consists of N_o, the NH_2-terminal 247 amino acid catalytic domain of the UL26 gene product, also expressed as a GST fusion protein. Upon cleavage with N_o, a single 25-mer peptide is released from GST-56, which is soluble in trichloroacetic acid. Using this assay, the protease displayed a pH optimum between 7 and 9 but most importantly had an absolute requirement for high concentrations of an antichaeotrophic agent. Strong salting out salts such as Na_2SO_4 and KPO_4 (1 M) stimulated activity, whereas NaCl and KCl had no effect. The degree of stimulation by 1.25 M Na_2SO_4 and KPO_4 were 100–150- and 200–300-fold, respectively. Using the fluorescent probe 1-anilino-8-naphthalene sulfonate, the protease was shown to bind the dye in the presence of 1.25 M Na_2SO_4 or KPO_4, but not at low ionic strength or in the presence of 1.25 or 2.2 M NaCl. This binding was most likely at the protease active site because a high affinity cleavage site peptide, but not a control peptide, could displace the dye. In addition to cleaving GST-56, the herpes simplex virus type I protease also cleaved the purified 56-mer peptide. Circular dichroism and NMR spectroscopy showed the peptide to be primarily random coil under physiological conditions, suggesting that antichaeotrophic agents affect the conformation of the substrate as well as the protease.

The existence of a herpesvirus-specific protease was first reported in 1991, when the protein encoded by the UL26 gene of herpes simplex virus I (HSV-1)[1] was shown to process both itself and the precursor form of a capsid scaffolding protein ICP35 (Ref. 1; also known as VP22a, p40). Shortly afterwards, other reports demonstrated the existence of similar proteases in simian (2) and human (3) cytomegaloviruses (CMV). These processing systems are reminiscent of those in bacteriophage, whereby a specific phage protease processes a scaffolding protein during capsid maturation (4, 5).

In early studies involving expression of recombinant protein in *Escherichia coli*, the HSV-1 protease was shown to cleave two distinct sites, both contained within the protease polypeptide itself (6, 7). Both occur between Ala and Ser residues, at positions 247 and 248 and positions 610 and 611 (7). This specificity is shared by the CMV enzyme (2–3). The carboxyl-terminal cleavage occurs at a site common to both the protease and a capsid scaffolding protein ICP35 (M site)[2] and releases a 25-residue, COOH-terminal peptide. ICP35 was recently shown to be a product of the UL26.5 gene and to be in-frame and entirely contained within the carboxyl-terminal 329 amino acids of the protease (9). Functionally, the processing of ICP35 by the protease appears to be an essential viral event, because a *ts* mutant that fails to process ICP35 at the nonpermissive temperature also fails to package DNA (10). Cleavage at the site proximal to the amino terminus (R site) results in the release of N_o, an NH_2-terminal, 247-residue polypeptide that contains the proteolytic activity (11, 12). This processed form of the protease (also known as Prn or VP24) is known to be a constituent of the viral capsid (13) and has been shown to be a serine protease (14, 15).

A major barrier in the study of the HSV-1 protease has been the extremely low activity level of the enzyme when assayed *in vitro*. In one study, DiIanni *et al.* (16) surveyed several peptide substrates and reported k_{cat} and K_m values of 0.2 min^{-1} and 190 μM, respectively, for the cleavage of ALVNASSAAHVDV (M site peptide mP5-P8′). In a subsequent study, Darke *et al.* (17) reported k_{cat} and K_m values of 2.0 min^{-1} and 0.88 mM, respectively, for the cleavage of the R site peptide HTYLQASEKFKMW-amide (rP6-P7′) and about twice the activity as DiIanni *et al.* (16) for mP5′P8′. In the present report we make use of a quick and sensitive assay for the HSV-1 protease to characterize a unique interaction with antichaeotrophic salts. Most significantly, we find that molar concentrations of Na_2SO_4 result in changes to both the protease and substrate and stimulate activity over 100-fold.

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[1] The abbreviations used are: HSV-1, herpes simplex virus type I; CMV, cytomegalovirus(es); HCMV, human CMV; GST, glutathione *S*-transferase; DTT, dithiothreitol; TCA, trichloroacetic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; ANS, 1-anilino-8-naphthalene sulfonate; HPLC, high pressure liquid chromatography.

[2] The M and R cleavage site nomenclature as described in Ref. 8. Peptides spanning these regions, such as the M site 8-mer LVNA-↓-SSAA will be denoted mP4-P4′, etc.

MATERIALS AND METHODS

Protease Purification—An active form of the HSV-1 protease was expressed from pGST247 in *E. coli* BL21(DE3) as a fusion protein of glutathione *S*-transferase coupled to the NH_2-terminal 247-residue domain of the UL26 gene product. The fusion protease (GST-N_o) and the thrombin-released form (N_o) have been described (12, 16). The homologous protease from human CMV (HCMV N_o) has also been described (18).

ICP35 Purification—Recombinant ICP35 was purified from *E. coli* BL21(DE3) transformed with the plasmid pT7ICP35 (6) as described by Weinheimer *et al.* (12). In brief, cells were lysed and then clarified by centrifugation. The supernatant was successively fractionated with 25% of saturation of $(NH_4)_2SO_4$, 1.7 M KCl, and chromatographed over DEAE Sepharose (Pharmacia Biotech Inc.). [^{35}S]ICP35 was prepared from bacteria metabolically labeled with [^{35}S]methionine (DuPont NEN). Cells were grown in minimal salts, supplemented with 0.2% glucose and 2.5% methionine assay medium (Difco Laboratories). Following induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside, cells were washed and incubated with 0.2 mCi/ml [^{35}S]methionine in 1/10 the original volume for 3 h. The labeled protein was purified using the above protocol, except that the chromatography step was omitted. Due to the selective incorporation of the [^{35}S]methionine into ICP35 under the induction conditions used, the protein was ~90% radiochemically pure and ~50% pure by protein weight.

GST Fusion Proteins—The HSV-1 fusion protein substrate GST-56 was constructed by using a double-stranded DNA fragment amplified from pT7HSV (6) by the polymerase chain reaction. A fragment spanning amino acids 580–635 (mP31-P25′) was generated with flanking *Bam*HI and *Eco*RI restriction enzyme sites and cloned into pGEX-2T (Pharmacia). The homologous HCMV fusion protein substrate (cGST-51) was similarly constructed using a fragment spanning amino acids 618–667 of HCMV UL80 (mP26-P25′). Following expression in *E. coli*, the fusion proteins were purified by glutathione affinity chromatography. Cells were lysed and clarified as described (12), with the exception that the initial lysis buffer contained 1 mM phenylmethylsulfonyl fluoride. The supernatant was adsorbed to a column of glutathione agarose (Sigma), washed with 50 mM Tris-HCl, pH 8.0, 1 mM DTT, and eluted in the same buffer containing 5 mM reduced glutathione. Peak fractions were pooled, dialyzed extensively to remove glutathione, and stored at −80 °C in the above buffer containing 40% glycerol. Radiolabeling in *E. coli* with [^{35}S]methionine was carried out as described for [^{35}S]ICP35, and the proteins were purified over glutathione agarose.

Preparation of the HSV-1 ICP35 Cleavage Site 56-mer Peptide—Purified mP31-P25′ 56-mer was obtained from GST-56 following treatment with thrombin. The reaction contained 2 mg/ml GST-56, 150 mM Tris-HCl, pH 8.0, and 8 units/ml bovine thrombin (Sigma). After 2 h at 30 °C, the material was adsorbed to a MonoQ column (Pharmacia) equilibrated in 50 mM Tris-HCl, pH 8.0, and then eluted with a linear gradient of 0–400 mM NaCl. The 56-mer eluted at ~75 mM NaCl, whereas the GST eluted at 225 mM. Purified 56-mer appeared as a single band when analyzed by HPLC. When analyzed on a 14% SDS gel, it migrated as a diffuse 7000-dalton protein with specific immunoreactivity against peptide antibodies raised against UL26 sequences (599–620 and 611–626) flanking the M cleavage site. [^{35}S]56-mer was purified by the same procedure, using [^{35}S]GST-56 as starting material.

Protease Assays—Protease activity was measured in a 30-μl reaction containing 50 mM Tricine, pH 8.0, 10 mM DTT, 100 μg/ml bovine serum albumin, 1.25 M Na_2SO_4, and the indicated radiolabeled substrate. Reactions were initiated by the addition of protease, incubated at 30 °C for 1 h, and then quenched with 100 μl of 10% trichloroacetic acid. Following centrifugation, the supernatant was removed, and the radioactivity was quantitated by liquid scintillation counting. In some assays, the polypeptides were examined directly. Following removal of the supernatant, the pellet was solubilized in SDS sample buffer and analyzed on a 12% SDS gel followed by autoradiography or quantitation on a Betascope 603 Blot Analyzer (Betagen Corp).

In Vitro Transcription and Translation—UL26 RNA was transcribed *in vitro* from pRB4090 (plasmid U in Ref. 1) using SP6 RNA polymerase (Life Technologies, Inc.). This RNA was then purified and translated for 20 min in a reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine. The reaction was quenched by the addition of cycloheximide (100 μg/ml) and then diluted 20-fold into a test buffer (see "Results"). Protease reactions were typically incubated for 6 h at 30 °C and then quenched by the addition of 10% TCA. Pellets were washed once with 100% ethanol, solubilized in SDS sample buffer, and analyzed on 12% SDS gels.

Other Methods—Protein concentration was measured using the method of Bradford (19). SDS gel electrophoresis was performed using 12 or 14% Laemmli gels (20) and then either stained with Coomassie Blue R250 or transferred to nitrocellulose for immunoblot analysis with alkaline phosphatase detection. Reverse phase HPLC analysis of peptides was on a Vydac C4 column, using an acetonitrile gradient in 0.1% trifluoroacetic acid. Analysis was with a Waters 991 Diode Array system and Radiomatic A-250 radioactivity detector (Camberra). Fluorescence emission spectra were recorded on a Spex Fluorolog-2 recording spectrofluorometer. Spectra were recorded at 90 ° in a 200-μl fused silica micro cell using an excitation wavelength of 280 nm and slit settings of 2 nm.

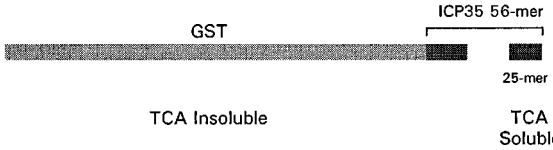

FIG. 1. **Schematic of the fusion protein substrate GST-56, depicting GST (*light box*) and M site 56-mer peptide (*dark box*) domains.** Cleavage of the 56-mer by the HSV-1 protease releases a TCA-soluble 25-mer peptide containing two of the protein's twelve methionines.

RESULTS

Development of an in Vitro Protease Assay—Initial studies on the HSV-1 protease were performed using the coupled *in vitro* translation/cleavage assay described by Liu and Roizman (1). UL26 RNA was translated as described under "Materials and Methods" and then diluted into buffers of varying composition for 6 h to allow cleavage to occur. The protease exhibited maximal activity between pH 7 and 9; it was virtually inactive below pH 6 or above 10. Surprisingly, it was potently stimulated by buffers containing high concentrations of Na_2SO_4 or KPO_4 but not KCl.[3] This last finding was essential for the development of the assay described below.

Following the purification of an active form of the protease (12), a need arose for a rapid and quantitative assay for activity. An acid solubilization assay was originally developed in which metabolically labeled [^{35}S]ICP35 could be cleaved by GST-N_o to release a COOH-terminal, 25-amino acid peptide. This peptide was soluble in 10% trichloroacetic acid. Because it contained two of the protein's seven methionines, it could be easily quantitated by liquid scintillation counting. An adaptation of this assay was to use a fusion protein substrate in which the COOH-terminal 56-residue peptide of ICP35 was fused to the COOH-terminal end of glutathione *S*-transferase (GST-56, Fig. 1). This protein was expressed at high levels in *E. coli* and could be purified in one step by glutathione agarose affinity chromatography in yields of 20–30 mg/liter of culture. The purified protein was free of nonspecific proteolytic activity and was stable to prolonged incubations at 30 °C. When purified from metabolically labeled cells, [^{35}S]IGST-56 was obtained at >95% purity with yields of 0.15 mCi of labeled protein/mCi of labeled culture (15-fold greater yield than [^{35}S]ICP35).

Effect of Na_2SO_4 on HSV-1 Protease Activity—Using the assay described above, the salt stimulation of HSV-1 protease seen in the *in vitro* translation/cleavage assay was re-examined. Na_2SO_4 was initially selected for detailed study because it potently stimulated HSV-1 protease activity in the translation assay and avoided the buffering and chelating properties of phosphate. Fig. 2*A* shows a titration of Na_2SO_4 into an assay for [^{35}S]GST-56 cleavage by GST-N_o. The activity was virtually undetectable in the absence of Na_2SO_4 and increased only slightly upon the addition of up to 750 mM. However, at 1.0 M and 1.25 M Na_2SO_4, the activity was about 75- and 150-fold

[3] G. Yamanaka, C. L. DiIanni, D. R. O'Boyle II, J. Stevens, S. P. Weinheimer, I. C. Deckman, L. Matusick-Kumar, and R. J. Colonno, unpublished data.

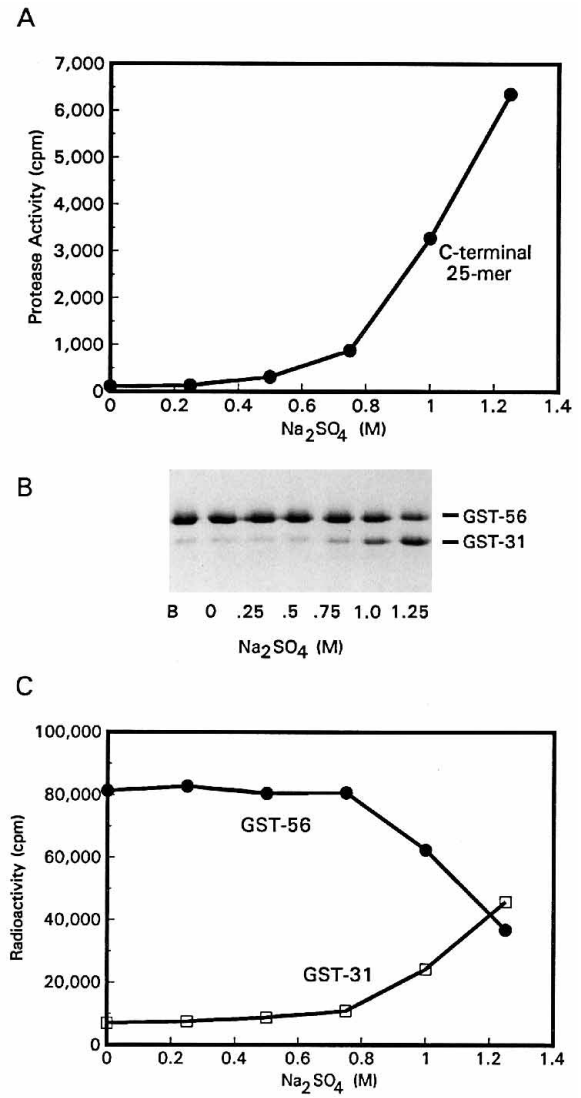

FIG. 2. **HSV-1 protease assay, showing the salt dependence for cleavage of 4 μM [^{35}S]GST-56 by 0.3 μM GST-N$_o$.** *A*, release of the COOH-terminal, 25-mer peptide, as measured by liquid scintillation counting. *B*, analysis of the polypeptide cleavage by SDS gel electrophoresis and autoradiography. *Lane B*, no enzyme was present. *C*, quantitation of the polypeptides shown in *B* by two-dimensional radioactivity detection.

greater than the level seen in the absence of this salt. When the polypeptide products of the above cleavage reactions were analyzed by SDS gel electrophoresis and autoradiography, the disappearance of the [^{35}S]GST-56 polypeptide corresponded to the appearance of a single cleavage product (Fig. 2*B*). Furthermore, when these bands were quantitated on a two-dimensional radioactivity scanner, the decrease in [^{35}S]GST-56 radioactivity was closely proportional to the increase in that of its cleavage product (GST-31, Fig. 2*C*). A similar salt dependence was seen when the assay was performed using [^{35}S]ICP35 as substrate or when N$_o$ was used in place of GST-N$_o$ (data not shown). Due to the limited solubility of the proteins at high concentrations in 1.25 M Na_2SO_4, reliable kinetic analysis was not possible. Initial studies indicated a $K_m > 10$ μM and $k_{cat} = \sim 1$ min^{-1}.

In parallel studies using the homologous protease from HCMV, a similar stimulation was seen (Table I). HSV-1 N$_o$ and HCMV N$_o$ were stimulated 60- and 130-fold, respectively, by 1.25 M Na_2SO_4 when using saturating levels of radiolabeled fusion protein substrates. Under these conditions, the HCMV enzyme was about an order of magnitude more active that the HSV-1 enzyme.

TABLE I
Stimulation of herpes proteases by Na_2SO_4

Protease assays were incubated at 30 °C ± 1.25 M Na_2SO_4 in reaction mixtures containing 130 mM Tricine, 10 mM DTT, 100 μg/ml bovine serum albumin. HSV-1 N$_o$ (2 μM no salt; 50–100 nM Na_2SO_4) was assayed at pH 7.7 with 18 μM [^{35}S]GST-56 and HCMV N$_o$ (1–3 μM, no salt; 5–20 nM Na_2SO_4) was assayed at pH 7.5 with 18 μM [^{35}S]cGST-51. Reaction products were separated by SDS gel electrophoresis and quantitated on a Betascope (triplicate determinations).

Protease	Without Na_2SO_4	With Na_2SO_4	Stimulation
	pmol/min/pmol protease		*-fold*
HSV-1 N$_o$	<0.05	2.8	>60
HCMV N$_o$	0.28	35	130

The stimulation of HSV-1 N$_o$ activity by Na_2SO_4 was further examined using a peptide substrate. [^{35}S]GST-56 from metabolically labeled cells was treated with thrombin to produce GST and a 60-residue peptide. The peptide, containing the 56-mer fused to a thrombin site linker (Gly-Ser-Pro-Met) at its NH_2 terminus, was purified under nondenaturing conditions. When assayed with HSV-1 N$_o$ in the presence of 0, 0.5, and 1.25 M Na_2SO_4 and analyzed by HPLC, cleavage was seen only in the presence of Na_2SO_4 (Table II). The appearance of a single radiolabeled proteolysis product (13.3 min) was consistent with the occurrence of a single cleavage event in a peptide where the only two methionines were at the extreme COOH terminus. However, when the 56-mer was examined for secondary structure, by either circular dichroism (in 100 mM KPO_4, pH 6.7) or NMR spectroscopy (25 mM sodium acetate, pH 5.5), it exhibited spectra characteristic of random coil (results not shown). Spectra were not obtainable in 1.25 M Na_2SO_4.

HSV-1 Protease Is Stimulated by Antichaeotrophic Agents—The initial finding that Na_2SO_4 and KPO_4 but not KCl stimulated protease activity in the translation assay was reproduced using purified N$_o$ in the *in vitro* TCA precipitation assay (Fig. 3, note replacement of KCl by NaCl). Due to the limited solubility of Na_2SO_4, an attempt was made to see whether the more highly soluble antichaeotrophic salts, $(NH_4)_2SO_4$ or KPO_4, would further stimulate the protease at higher concentrations (Fig. 3). Again, both salts greatly stimulated protease activity, with maxima in the 1.25–2.0 M range. The potency was greatest for KPO_4, which produced twice the peak activity level of either Na_2SO_4 or $(NH_4)_2SO_4$.

Given the lack of activity by NaCl, additional antichaeotrophic or salting out agents (21, 22) were examined (Table III). None of the chlorides had any effect, even when added at 2.5 M. However, all of the antichaeotrophs stimulated activity, in parallel with the lyotrophic series of anions, the most potent being the phosphates, followed by the sulfates and the more weakly antichaeotrophic acetates. KPO_4 and $(NH_4)PO_4$ were about 75% more potent than Na_2SO_4. $(NH_4)SO_4$ and $MgSO_4$ were about 3-fold less potent, whereas guanidine sulfate was nearly inactive. Among the acetates, only $Mg(OAc)_2$ showed activity at 1.25 M. However, because this salt contains two acetate anions/mol, the other acetates were assayed at 2.5 M. All showed weak to moderate stimulation.

In addition to salts, several other solutes that stabilize proteins were examined to look for HSV-1 protease stimulation (23). Sucrose at 34% and glycerol at up to 62% produced marginal, if any stimulation, whereas results using larger polymers were inconclusive. Ficoll 400 and dextran sulfate rendered the solutions too viscous to permit pelleting of the substrate in the TCA assay, whereas polyethylene glycols (400–8000 molecular weight range) precipitated everything. However, when activity was measured using the coupled translation/cleavage assay,

TABLE II
Cleavage of [^{35}S]56-mer by HSV-1 N_0

Protease assays were incubated at 30 °C in a reaction mixture containing 50 mM Tricine, pH 8.0, 10 mM DTT, 50 μg/ml bovine serum albumin, purified [^{35}S]56-mer (≈2500 cpm), 100 nM HSV-1 N_0, and the indicated concentration of salt. Reactions were quenched by a 6-fold dilution into 100 mM sodium Acetate, pH 5, then analyzed by reverse phase HPLC. Retention times of 16.3 and 13.3 min correspond to the intact 56-mer and cleaved 25-mer peptides, respectively.

Incubation time	Na_2SO_4 Concentration	HPLC peak area	
		13.3 min	16.3 min
h	*M*	*cpm*	
0	0		1715
1.5	0		842
	0.5		541
	1.25	464	40
16	0		824
	0.5	13	248
	1.25	1152	15

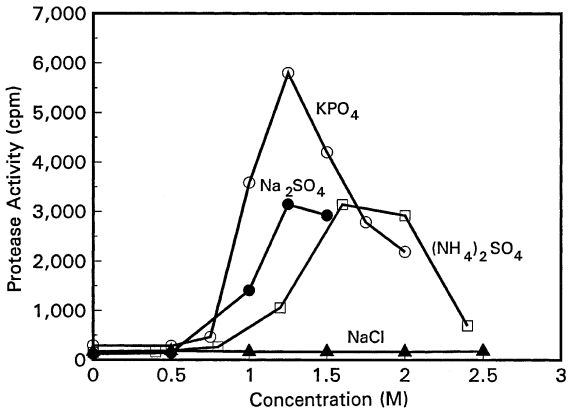

FIG. 3. **Comparison of different salts on the stimulation of the HSV-1 protease.** Assays were performed as described under "Materials and Methods" using 1 μM [^{35}S]GST-56 and 0.1 μM N_o. Indicated salts were present in the assays at the concentrations shown: Na_2SO_4 (●), KPO_4 (○), $(NH_4)_2SO_4$ (□), and NaCl (▲).

polyethylene glycols of average molecular weights 600-3350 produced a slight stimulation when present at 10–20% (results not shown).

Fluorescence Emission Analysis of HSV-1 N_o—To learn whether or not the protease stimulation by antichaeotrophic agents was due to a conformational change in the vicinity of the substrate binding pocket, the chromophore reagent 1-anilino-8-naphthalene sulfonate (ANS) was used to probe the protein's topology. ANS is known to be nearly nonfluorescent in aqueous buffers but to partition into hydrophobic sites within proteins, resulting in the appearance of a strong emission band around 450 nm (24). Fig. 4*A*, shows the fluorescence emission spectra of N_o in 50 mM Tricine, pH 8.0, either in the presence or absence of ANS. In both instances, the major feature was a broad emission peak at ~340 nm, attributable to the protease's two tryptophan residues. When a similar experiment was performed in the same buffer containing 1.25 M Na_2SO_4, the addition of ANS produced a second fluorescent peak, centered at 465 nm (Fig. 4*B*). This second peak was not due to N_o itself but rather to ANS. It was not present when ANS alone was examined in Na_2SO_4, and was not a general phenomenon of protein and salt because (i) it was not seen when carbonic anhydrase was used in place of N_o, and (ii) it was not present when N_o and chromophore were examined in 1.25 M or 2.2 M NaCl (Fig. 4*C*). However, when N_o was examined in the presence of ANS in the antichaeotrophic salt KPO_4, the 465 nm fluorescence peak was again present (not shown).

TABLE III
Effect of salts on HSV-1 protease activity

Protease assays were incubated for 1 h at 30 °C in a reaction mixture containing 50 mM Tricine, pH 8.0, 10 mM DTT, 50 μg/ml bovine serum albumin, 1 μM [^{35}S]GST-56 (60,000 cpm/reaction), 100 nM HSV-1 N_0, and the indicated concentration of salt. Results were normalized to the value for Na_2SO_4 at 1.25 M from two or three determinations. Reproducibility was generally within 20% for values above 500 cpm.

Salt	Protease activity		
	0.5 M	1.25 M	2.5 M
	cpm (% control)		
No salt	50 (1)		
Sulfates			
Na_2SO_4	230 (4)	6300 (100)	
$(NH_4)_2SO_4$	100 (2)	2100 (33)	
$MgSO_4$	150 (2)	2300 (37)	
Guanidine SO_4	60 (1)	240 (4)	
Chlorides			
NaCl	100 (2)	30 (1)	0
KCl	70 (1)	140 (2)	0
$CaCl_2$	0	0	
$MgCl_2$	80 (1)	0	
Guanidine HCl	0	0	
Acetates			
NaOAc	30 (1)	60 (1)	2000 (32)
KOAc	0	60 (1)	770 (12)
(NH_4)OAc	50 (1)	60 (1)	330 (5)
$Mg(OAc)_2$	190 (3)	1300 (20)	
Phosphates			
KPO_4	310 (5)	10900 (173)	
$(NH_4)PO_4$	290 (5)	10800 (171)	

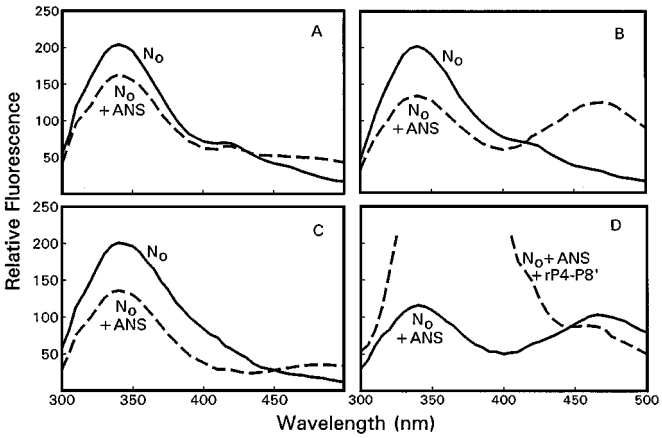

FIG. 4. **Fluorescence emission spectra of 0.5 μM HSV-1 N_o ± 10 μM ANS in buffers of varying composition.** *A*, 50 mM Tricine, pH 8.0. *B*, 50 mM Tricine, pH 8.0, 1.25 M Na_2SO_4. *C*, 50 mM Tricine, pH 8.0, 2.2 M NaCl. *D*, same conditions as *B*. In the experiment denoted by the *dotted curve* 50 μM rP4-P8′ was added to the reaction.

If the 465 nm fluorescence were due to the specific binding of ANS to the substrate binding pocket of the protease, it might be possible to quench this fluorescence through competition for this site by a specific protease ligand. For this purpose, the R cleavage site peptide YLQASEKFKMWG (rP4-P8′), previously shown to inhibit [^{35}S]GST-56 cleavage (IC_{50} = 5–10 μM)[3], was used as a competitor. In Fig. 4*D*, the addition of rP4-P8′ to a solution of N_o and ANS in 1.25 M Na_2SO_4 buffer resulted in a large increase in emission from tryptophan (peptide Trp at P7′) but a decrease of the 465 nm peak. Such an effect was not seen using a control peptide, ASNAEAGALVNAS (mP12-P1′), previously implied to be noncleavable (16) and shown to be noninhibitory.[3] Thus, the presence of an antichaeotrophic salt results in the formation of a hydrophobic area on the protease, possibly the substrate binding site, which is not present at low ionic strength or at high concentrations of a neutral salt.

DISCUSSION

In the initial characterization of the HSV protease, an *in vitro* translation and cleavage assay (1) surprisingly revealed that activity could be greatly stimulated by high concentrations of Na_2SO_4 and KPO_4 but not KCl. This finding was confirmed in a more defined system, using purified proteins for both the protease and substrate. Most striking, however, was the requirement for molar concentrations of an antichaeotrophic salt; little effect was seen at physiological ionic strength. In addition, NaCl did not affect activity between 0 and 2.5 M (Fig. 3). These studies were extended by surveying a variety of salts (Table III), spanning the lyotropic series of anions and cations. Most conclusive was the finding that the potency of stimulation paralleled the order of stabilizing anions, which preferentially hydrate proteins (21, 22): chloride $\ll$ acetate $<$ sulfate $<$ phosphate. The effect of cations was less pronounced. Sodium ion appeared to be slightly more potent than ammonium, but guanidine, which binds tightly to protein (25), was inhibitory. Several other reagents known to stabilize proteins were also examined. Glycerol (26) and sucrose (27), both known to preferentially hydrate proteins, were ineffective. This was somewhat surprising, because glycerol was earlier shown to stimulate the activity of the related HCMV protease (28), which behaved like the HSV-1 enzyme toward Na_2SO_4. Polyethylene glycols, which have a more pronounced effect on water activity, were potent precipitants in the TCA precipitation assay. They did, however, slightly stimulate activity in the *in vitro* translation/cleavage assay.

The above findings led to the question of whether the solvent effect might be on the substrate or the enzyme. The substrate was initially examined using spectroscopic methods to probe secondary structure. The 56-mer domain of GST-56, which parallels authentic ICP35 in its cleavage behavior, was isolated and purified under native conditions and then submitted to analysis by circular dichroism and NMR spectroscopy. In physiological concentrations of salt, the peptide was primarily random coil. We were unable to obtain spectra in the concentrations of Na_2SO_4 or KPO_4 required for optimal cleavage by the protease. Nevertheless, these studies suggested that one role of an antichaeotrophic agent might be to induce some unique feature of secondary structure into the cleavage site region. Earlier studies on peptides suggested a requirement for secondary structure on the P′ side of the scissile bond (16).

The effects of salt on the protease were also examined. Peptide cleavage experiments (not shown) suggested that the K_m for substrates decreased when assays were performed in high concentrations of Na_2SO_4. This led to the notion that the effect might involve changes in the structure at the active site. For this purpose, the fluorescent dye ANS was used to probe protease topology. Stryer (24) showed that it bound to apomyoglobin with a dissociation constant of $\sim 10^{-5}$ M but not to myoglobin. Furthermore, it could be displaced from the apoprotein by the addition of hemin. When added to solutions of HSV-1 N_o, ANS bound protein only in the presence of 1.25 M Na_2SO_4 or KPO_4 but not NaCl (Fig. 4). These conditions paralleled those required for optimal activity, suggesting that a conformational change had occurred upon introduction of these antichaeotrophic salts. In contrast, carbonic anhydrase did not bind ANS under any conditions.

To examine the possibility that ANS was binding to the active site of N_o, a high affinity substrate peptide, rP4-P8′, was used as competing ligand. Addition of this peptide to the ANS bound protein solution resulted in a decrease in ANS fluorescence, consistent with a model that the salt-induced hydrophobic site was indeed the substrate binding site.

A consequence of our above results is the question of just why a protease should be stimulated by unusually high concentrations of antichaeotrophic salts. One explanation involves the local environment of the protease during cleavage. Recent work on the assembly and maturation of the HSV-1 capsid suggests that the uncleaved form of ICP35 (*i.e.* containing the COOH-terminal 25 amino acids) is required for the formation of "sealed" capsids (29–31). Because the processed form of this protein is the predominant form found when immature B capsids are isolated from infected cells (32–35), cleavage by the protease most likely occurs within the capsid. Furthermore, based on the ultrastructure and protein stoichiometry calculations of Brown and colleagues (36, 37), a major fraction of a B capsid volume can be accounted for by ICP35 protein. This suggests that the water activity (38) must be very low, a state that is perhaps approximated in an *in vitro* assay by the addition of molar concentrations of an antichaeotrophic salt.

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