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

(Received for publication, August 17, 1995, and in revised form, September 29, 1995)

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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 N0, the NH2-terminal 247 amino acid catalytic domain of the UL26 gene product, also expressed as a GST fusion protein. Upon cleavage with N0, 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 Na2SO4 and KPO4 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 Na2SO4 or KPO4, 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) 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 in two distinct sites, both contained within the protease poly peptide 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) 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 N0, an NH2-terminal, 247-residue polypeptide that contains the proteinolytic 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, DiIannis et al. (16) surveyed several peptide substrates and reported kcat and Km values of 0.2 min−1 and 190 μM, respectively, for the cleavage of ALVNASSAHVDV (M site peptide mp5-P8). In a subsequent study, Darke et al. (17) reported kcat and Km values of 2.0 min−1 and 0.88 mM, respectively, for the cleavage of the R site peptide HTYLFASEKFMKW-amide (rP6-P7) and about twice the activity as DiIannis et al. (16) for mp5P8. 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 Na2SO4 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.

3 Peptides spanning these regions, such as the M site 8-mer LVNAHTYLFaseKFMKW-amide (rP6-P7), and about twice the activity as DiIannis et al. (16) for mp5P8. 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 Na2SO4 result in changes to both the protease and substrate and stimulate activity over 100-fold.

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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 NH2-terminal 247-residue domain of the UL26 gene product. The fusion protease (GST-N) and the thrombin-released form (N) have been described (12, 16). The homologous protease from human CMV (HCMV N) 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% saturation of (NH4)2SO4, 1.7 M KCl, and chromatographed over DEAE Sephacel (Pharmacia Biotech Inc.). [35S]ICP35 was prepared from bacteria metabolically labeled with [35S]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 mM [35S]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 [35S]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 pTHHSV (6) by the polymerase chain reaction. A fragment spanning amino acids 580–635 (mp31-P25) was generated with flanking BamHI and EcoRI restriction enzyme sites and cloned into pGEX-2T (Pharmacia). The homologous HCMV fusion protein substrate (GST-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. Radio labeling in E. coli with [35S]methionine was carried out as described for [35S]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-Cl, 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) and eluted 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. When analyzed on a 14% SDS gel, it migrated as a diffuse 7000-dalton protein with specific immunoreactivity. When analyzed on a 14% SDS gel, it migrated as a diffuse 7000-dalton protein with specific immunoreactivity. When analyzed on a 14% SDS gel, it migrated as a diffuse 7000-dalton protein with specific immunoreactivity.

Protease Assays—Protease activity was measured in a 30-μl reaction containing 50 mM Tricine, pH 8.0, 10 mM DTT, 100 μCi/ml bovine serum albumin, 1.25 mM Na2SO4, 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 Vivo Transcription and Translation—UL26 RNA was transcribed in vitro using the plasmid p18000 (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 [35S]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 okadaic phosphate detection. Reverse phase HPLC analysis of peptides was performed 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 (Cambridge). Fluorescence emission spectra were recorded on a Spex Fluordoc-2 recording spectrophotometer. 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.

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 potentially stimulated by buffers containing high concentrations of Na2SO4 or KPO4 but not NaCl. 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 [35S]ICP35 could be cleaved by GST-N 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, with recovery 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, [35S]GST-56 was obtained at >95% purity with yields of 0.15 mCi of labeled protein/mCi of labeled culture (15-fold greater yield than [35S]ICP35).

Effect of Na2SO4 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. Na2SO4 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. 2A shows a titration of Na2SO4 into an assay for [35S]GST-56 cleavage by GST-N. The activity was virtually undetectable in the absence of Na2SO4 and increased only slightly upon the addition of up to 750 mM. However, at 1.0 M and 1.25 M Na2SO4, the activity was about 75- and 150-fold higher, respectively. The highest activity was at 300 mM Na2SO4 and decreased at higher concentrations of Na2SO4. This activation of protease activity by Na2SO4 was specific to the cleavage reaction because control trypsin digestion of ICP35 was not stimulated by Na2SO4. In contrast, the control trypsin digestion of ICP35 was not stimulated by Na2SO4. In contrast, the control trypsin digestion of ICP35 was not stimulated by Na2SO4.
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 [35S]GST-56 polypeptide corresponded to the appearance of a single cleavage product (Fig. 2B). Furthermore, when these bands were quantitated on a two-dimensional radioactivity scanner, the decrease in [35S]GST-56 radioactivity was closely proportional to the increase in that of its cleavage product (GST-31, Fig. 2C). A similar salt dependence was seen when the assay was performed using [35S]ICP35 as substrate or when N0 was used in place of GST-N0 (data not shown). Due to the limited solubility of the proteins at high concentrations in 1.25 M Na2SO4, reliable kinetic analysis was not possible. Initial studies indicated a Km for Na2SO4, an attempt was made to see whether the more highly soluble antichaeotropic salts, (NH4)2SO4 or KPO4, 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 KPO4, which produced twice the peak activity level of either Na2SO4 or (NH4)2SO4.

HSV-1 Protease Is Stimulated by Antichaeotropic Agents—The initial finding that Na2SO4 and KPO4 but not KCl stimulated protease activity in the translation assay was reproduced using purified N0 in the in vitro TCA precipitation assay (Fig. 3, note replacement of KCl by NaCl). Due to the limited solubility of Na2SO4, an attempt was made to see whether the more weakly antichaeotropic salts, (NH4)2SO4 or KPO4, 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 KPO4, which produced twice the peak activity level of either Na2SO4 or (NH4)2SO4.

Given the lack of activity by NaCl, additional antichaeotropic 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 lyotropic series of anions, the most potent being the phosphates, followed by the sulfates and the more weakly antichaeotropic acetates. KPO4 and (NH4)PO4 were about 75% more potent than Na2SO4. (NH4)SO4 and MgSO4 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, enzyme was about an order of magnitude more active that the HSV-1 enzyme.

The stimulation of HSV-1 N0 activity by Na2SO4 was further examined using a peptide substrate. [35S]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 NH2 terminus, was purified under nondenaturing conditions. When assayed with HSV-1 N0 in the presence of 0.5, and 1.25 M Na2SO4 and analyzed by HPLC, cleavage was seen only in the presence of Na2SO4 (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 KPO4, 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 Na2SO4.

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due to the limited solubility of the proteins at high concentrations in 1.25 M Na2SO4, reliable kinetic analysis was not possible. Initial studies indicated a Km > 10 μM and kcat = 1 min⁻¹.

In parallel studies using the homologous protease from HCMV, a similar stimulation was seen (Table I). HSV-1 N0 and HCMV N0 were stimulated 60- and 130-fold, respectively, by 1.25 M Na2SO4 when using saturating levels of radiolabeled fusion protein substrates. Under these conditions, the HCMV

| Table I: HSV-1 Protease Stimulation | |
|-------------------------|--------------------------|
| Protease | Without Na2SO4 | With Na2SO4 | Stimulation |
| HSV-1 N0 | <0.05 | 2.0 | >60 |
| HCMV N0 | 0.28 | 35 | 130 |

Protease assays were incubated at 30 °C ± 1.25 M Na2SO4 in reaction mixtures containing 130 mM Tricine, 10 mM DTT, 100 μg/ml bovine serum albumin. HSV-1 N0 (2 μM no salt; 50–100 nM Na2SO4) was assayed at pH 7.7 with 18 μM [35S]GST-56 and HCMV N0 (1–3 μM, no salt; 5–20 μM Na2SO4) was assayed at pH 7.5 with 18 μM [35S]cGST-51. Reaction products were separated by SDS gel electrophoresis and quantitated on a Betascope (triplicate determinations).
polyethylene glycols of average molecular weights 600-3350 were used in place of No, and (ii) it was not present when No and salt because (i) it was not seen when carbonic anhydrase produced as light stimulation when present at 10–20% (results not shown).

Fluorescence Emission Analysis of HSV-1 No—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-naphthalenesulfonate (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, shows the fluorescence emission spectra of No 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 Na₂SO₄, the addition of ANS produced a second fluorescent peak, centered at 465 nm (Fig. 4B). This second peak was not due to No itself but rather to ANS. It was not present when ANS alone was examined in Na₂SO₄ and was not a general phenomenon of protein and salt because (i) it was not seen when carbonic anhydrase was used in place of No and (ii) it was not present when No and chromophore were examined in 1.25 M or 2.2 M NaCl (Fig. 4C). However, when No was examined in the presence of ANS in the antichaeotrophic salt KPO₄, the 465 nm fluorescence peak was again present (not shown).

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 [³⁵S]GST-56 cleavage (IC₅₀ = 3–10 μM), was used as a competitor. In Fig. 4D, the addition of rP4-P8 to a solution of No and ANS in 1.25 M Na₂SO₄ 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, ASNAEALVNAS (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.
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$_2$SO$_4$ 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 antichaeotropic salt; little effect was seen at physiological ionic strength. In addition, NaCl did not affect activity between 0 and 2.5 $\text{m}$. These studies were extended by surveying a variety of salts (Table III), spanning the lyotropic series of anions and cations. The effects of cations were 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$_2$SO$_4$. 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-S6, 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$_2$SO$_4$ or KPO$_4$ required for optimal cleavage by the protease. Nevertheless, these studies suggested that one role of an antichaeotropic 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$_2$SO$_4$. 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 $10^{-5}$ 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$_p$ ANS bound protein only in the presence of 1.25 m Na$_2$SO$_4$ or KPO$_4$ but not NaCl (Fig. 4). These conditions paralleled those 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 antichaeotropic 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 antichaeotropic salt.
