

Characterization of Three Components of Human Adenovirus Proteinase Activity *in Vitro**

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Human adenovirus contains a virion-associated proteinase activity essential for the development of infectious virus. Maximal proteinase activity *in vitro* had been shown to require three viral components: the L3 23-kDa protein, an 11-amino acid cofactor (pVIc), and the viral DNA. Here, we present a quantitative purification procedure for a recombinant L3 23-kDa protein (recombinant endoproteinase (rEP)) expressed in *Escherichia coli* and the procedure that led to the purification and identification of pVIc as a cofactor. The cofactors stimulate proteinase activity not by decreasing K_m , which changes by no more than 2-fold, but by increasing k_{cat} . rEP alone had a small amount of activity, the k_{cat} of which increased 355-fold with pVIc and 6072-fold with adenovirus serotype 2 (Ad2) DNA as well. Curves of V_{max} of rEP-pVIc complexes with the substrate (Leu-Arg-Gly-Gly-NH)₂-rhodamine as a function of pH in the absence and presence of Ad2 DNA indicate that the pK_a values of amino acids that affect catalysis are quite different from those that affect catalysis by the cysteine proteinase papain. The pK_a values in the absence of Ad2 DNA are 5.2, 6.4, 6.9, 7.5, and 9.4, and those in its presence are 5.2, 6.5, 7.4, and 8.8.

For many animal and plant viruses, a virus-coded proteinase activity is vital for the synthesis of infectious virus (for review, see Ref. 1). These virus-coded proteinases are appealing targets for antiviral therapy. Human adenoviruses encode a proteinase activity that is required for the maturation of infectious virions. Of the 12 major polypeptides from which adenovirus virions are assembled, six are proteolytically processed. Weber (2) isolated a temperature-sensitive mutant, H2ts-1 (ts-1),¹ of human adenovirus serotype 2 (Ad2) that lacks proteinase activity at the nonpermissive temperature. Virions of ts-1 assemble at the

nonpermissive temperature, but contain precursors in place of the mature components present in wild-type virus. Such immature virions attach to cells, but fail to initiate a productive infection (3, 4). The mutation in ts-1 was identified as a single base pair change in a 204-codon open reading frame (L3 23-kDa protein) at the 3'-end of the L3 family of late messages (5). The nucleotides in the L3 23-kDa open reading frame were cloned into plasmids that permitted efficient expression in *Escherichia coli* (6).

Recently, we developed a specific, sensitive, and quantitative assay for the adenovirus proteinase and used it to characterize the activity in disrupted wild-type virus.² The assay is based upon the observation that the adenovirus proteinase will cleave small peptides with sequences that correspond to the sequences on the amino-terminal side of the cleavage sites in virion precursor proteins. For example, the substrate (Leu-Arg-Gly-Gly-NH)₂-rhodamine is cleaved to Leu-Arg-Gly-Gly-NH-rhodamine by the adenovirus proteinase; this is accompanied by a 3500-fold increase in fluorescence that is proportional to the amount of proteinase.

We had previously shown that when wild-type Ad2 virus was incubated with (Leu-Arg-Gly-Gly-NH)₂-rhodamine, significant hydrolysis of the substrate was observed, and that when ts-1 virus was incubated with (Leu-Arg-Gly-Gly-NH)₂-rhodamine, no hydrolysis of the substrate was observed (7). Little or no hydrolysis was observed with purified recombinant L3 23-kDa protein (recombinant endoproteinase (rEP)) expressed in *E. coli*. However, when ts-1 virus and rEP were incubated together with (Leu-Arg-Gly-Gly-NH)₂-rhodamine, significant hydrolysis of the substrate occurred. This implied that cofactors may be required for maximal activity. The first cofactor we discovered was the viral DNA. If disrupted wild-type virus is treated with DNase, proteinase activity is lost, but can be restored upon addition of Ad2 DNA (7). A second cofactor was shown to be a plasmin-sensitive virion protein (7) that turned out to be the 11-amino acid peptide from the C terminus of the precursor to virion protein VI, pVIc (7, 8).

Here, we present our purification procedure for a recombinant L3 23-kDa protein expressed in *E. coli*. Although others have published purification procedures for a recombinant form of the L3 23-kDa protein from *E. coli* (9, 10) and insect cells (8), none of the purification procedures utilized a quantitative assay, so there is, for example, no report of increases in specific activity or even yields. We also present our procedure for the purification and identification of pVIc as a cofactor for proteinase activity. We then show that the cofactors stimulate proteinase activity not by decreasing K_m , which changes by no more than 2-fold, but by increasing k_{cat} , which increases >6000-fold. By measuring initial velocities as a function of pH, we show that the enzyme activity is clearly different from that of papain.

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¹ The abbreviations used are: ts-1, temperature-sensitive mutant H2ts-1; Ad2, adenovirus serotype 2; rEP, recombinant endoproteinase; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl- β -D-thiogalactopyranoside; DTT, dithiothreitol; TAPS, 3-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid; MES, 4-morpholineethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

² McGrath, W. J., Abola, A. P., Toledo, D. L., Brown, M. T., and Mangel, W. F. (1996) *Virology*, in press.

TABLE I
Purification table

Fraction	Volume	Protein	Activity	Specific activity	Yield
	<i>ml</i>	<i>mg/ml</i>	<i>nmol substrate/s</i>	<i>units/mg^a</i>	<i>%</i>
Lysate	218	21	1.42	22.4	100
Supernatant	208	16	1.85	39.9	130
DEAE FT	260	6.6	1.61	67.7	113
SSEPH pool	50	3.5	1.18	485	83
Zn-IDA ^b pool	25.9	3.2	0.94	815	66

^a One unit of activity is 1 nmol of substrate hydrolyzed per s.

^b Zn-IDA, zinc-iminodiacetic acid column.

Moreover, the pK_a values of the amino acids that affect catalysis are different in the presence and absence of Ad2 DNA.

EXPERIMENTAL PROCEDURES

Materials—Tris, HEPES, dithiothreitol, and 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) were purchased from Sigma. TSK-DEAE was obtained from Supelco Inc. S-Sepharose and chelating Sepharose were from Pharmacia Biotech Inc. Octyl glucoside was from Boehringer Mannheim. The pVlc peptide was obtained from Multiple Peptide Systems (San Diego, CA).

Cells and Viruses—The growth of HeLa cells, infection by wild-type Ad2 and the mutant H2ts-1, and the purification of viruses were carried out as described (6). Twice CsCl-banded virus was dialyzed against 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA and centrifuged at $100,000 \times g$ for 1 h. The pellet was suspended in 10 mM Tris-HCl (pH 6.8) containing 20% (v/v) glycerol and, after three 10-s bursts of sonication, was stored at -20°C . Disrupted virus prepared this way was used as the source of *in vivo* or virion-associated proteinase activity.

Assay for Proteinase Activity—For the purification of rEP, activity assays contained, in 1 ml, 20 mM HEPES (pH 8.0), 10 mM octyl glucoside, saturating amounts of pVlc peptide, 140 ng/ml Ad2 DNA, 2 μM (Leu-Arg-Gly-Gly-NH)₂-rhodamine, and rEP. Saturating levels of pVlc were 100 nM when the rEP concentration was <50 nM and 350 nM for higher concentrations of rEP. The assay mixtures were incubated at 37°C , and the increase in fluorescence was monitored as a function of time. The excitation wavelength was 492 nm, and the emission wavelength was 523 nm, both with a 5-nm slit width. Activity was defined as the change in fluorescence of the assay containing rEP with cofactors minus the change in fluorescence of an identical assay but without rEP.

Protein concentration was determined by the bicinchoninic acid protein assay (Pierce) and/or for rEP with a calculated molar absorbance coefficient at 280 nm of 26,510 (11). The concentration of pVlc was determined by titration of its cysteine residue with Ellman's reagent and confirmed by quantitative amino acid analysis. The cysteine titration was done by adding 10 μl of pVlc stock solution to 0.99 ml of Ellman's buffer (0.1 M NaH₂PO₄ (pH 7.3) and 1 mM EDTA containing 0.33 mM 5,5'-dithiobis(2-nitrobenzoic acid)) and then monitoring the increase in absorbance at 412 nm. The moles SH/mol of pVlc was calculated using a molar extinction coefficient at 412 nm of 14,150 (12) for thionitrobenzoate.

SDS-Polyacrylamide Gel Electrophoresis—SDS-PAGE was performed using the Pharmacia Phast System. Proteins were fractionated on either 10–15 or 8–25% gradient gels. Protein bands were visualized with Coomassie Blue or silver staining.

Expression of the Recombinant L3 23-kDa Protein in *E. coli*—The adenovirus proteinase gene, the gene for the L3 23-kDa protein, had been cloned into the pET expression vector pT7AD23k8 and placed in *E. coli* strain BL21(DE3) under the control of the T7 expression system (6). A glycerol stock was used to seed an overnight culture in TB medium (1% Bacto-Tryptone and 0.5% NaCl) supplemented with 50 $\mu\text{g/ml}$ ampicillin, and this was then used at a 1:200 dilution to seed 4 liters of M9-TBY broth (0.1% NH₄Cl, 0.3% KH₂PO₄, 0.6% Na₂HPO₄, 0.4% glucose, and 1 mM MgSO₄/0.1% yeast, 1% Bacto-Tryptone, and 0.5% NaCl) supplemented with 50 $\mu\text{g/ml}$ ampicillin. The cultures were grown with shaking at 30°C until the absorbance at 600 nm was between 0.5 and 0.6. At that time, the cultures were induced by the addition of IPTG to 0.1 mM and allowed to grow an additional 8–16 h (13). SDS-PAGE of the cells at various times after induction revealed that by 8 h, the major protein in the cells was rEP (see Fig. 1). The cells were harvested by centrifugation at $5000 \times g$ for 10 min. Cell pellets were stored at -20°C .

Purification of the Recombinant L3 23-kDa Protein Expressed in *E. coli*—A bacterial cell pellet from 3 liters of cells was suspended in 150

ml (0.05 volume) of 50 mM Tris (pH 8.0), 0.05% (v/v) Triton X-100, 15 mM NaCl, and 5 mM β -mercaptoethanol. The cells were lysed by incubation for 45 min at 4°C with 100 $\mu\text{g/ml}$ egg white lysozyme followed by three cycles of freeze/thaw. DNA was digested upon adding 50 $\mu\text{g/ml}$ DNase and 5 mM MgCl₂ and incubating for 45 min at 4°C . The resultant suspension was then subjected to three 30-s bursts of sonication. This fraction was named the lysate.

The lysate was clarified by centrifugation at $10,000 \times g$ for 10 min. This fraction, named the supernatant, was loaded onto a $2.5 \times 25\text{-cm}$ TSK-DEAE column equilibrated in 50 mM Tris (pH 8.0), 15 mM NaCl, and 5 mM β -mercaptoethanol. The column was washed with 300 ml of the equilibration buffer. rEP was located in the flow-through fractions by SDS-PAGE and by activity assays. The fractions with rEP were pooled and named DEAE FT.

The DEAE FT pool was fractionated on a $1.6 \times 15\text{-cm}$ S-Sepharose Fast Flow column equilibrated in 50 mM Tris (pH 8.0), 15 mM NaCl, and 1 mM DTT. After loading, the column was washed with 200 ml of 50 mM Tris (pH 8.0), 15 mM NaCl, and 1 mM DTT to remove the Triton X-100. Then, a 150-ml linear gradient of 15–400 mM NaCl in 50 mM Tris (pH 8.0) was applied to the column. The fractions containing rEP were identified and pooled and named SSEPH. rEP consistently eluted from this column at an ionic strength of 0.1 M NaCl.

The SSEPH pool was applied to a $1.6 \times 5\text{-cm}$ chelating Sepharose column charged with zinc. Charging with zinc was accomplished by a thorough washing of the resin with 0.05 M EDTA and 1 M NaCl, followed by washing with water to remove the EDTA, and then followed by washing with 0.2 M ZnCl₂ in 5 mM HCl. Next, the column was equilibrated in 25 mM HEPES (pH 8.0) and 0.1 M NaCl. The column was loaded with the SSEPH pool and washed with the equilibration buffer until the absorbance at 280 nm was <0.02 . Then, a series of solutions were applied in steps, each step containing 25 mM HEPES (pH 8.0) and the indicated constituents: 1 M NaCl; 0.1 M NaCl; 35 mM imidazole and 0.1 M NaCl; 0.1 M NaCl; 35 mM imidazole and 0.1 M NaCl; 0.1 M NaCl; 35 mM imidazole and 0.1 M NaCl; and 0.1 M NaCl. rEP eluted in 25 mM HEPES (pH 8.0), 0.1 M NaCl, and 0.01 M EDTA. After dialysis against 20 mM HEPES (pH 8.0), 5 mM NaCl, and 0.1 mM EDTA, the purified enzyme was stored at -70°C .

Purification of the Protein Cofactor pVlc—Twice density gradient-purified wild-type Ad2 virus (5.8×10^{12} virions) was suspended in 0.8 ml of 10 mM Tris-HCl (pH 8), 1 mM EDTA, and 10% pyridine. After 1 h at 25°C , the solution was centrifuged at $12,000 \times g$ for 6 min. The pellet was resuspended in 0.8 ml of 0.01 M HEPES (pH 8) and centrifuged at $12,000 \times g$ for 6 min. The pellet was resuspended in 0.8 ml of 0.01 M HEPES (pH 8), 0.01 M octyl glucoside, and 0.001 M EDTA and then centrifuged at $5000 \times g$ in a Centricon-30 until 90% of the liquid flowed through the membrane. The volume of the liquid that was retained was increased to 0.8 ml with ammonium acetate such that its concentration was 1 M. The solution in the Centricon-30 was again centrifuged at $5000 \times g$ until $>90\%$ of the liquid flowed through the membrane. Assays were performed in the presence of the rEP protein and in the absence or presence of Ad2 DNA.

The flow-through fraction from the second Centricon-30 centrifugation was evaporated to dryness, dissolved in 0.1% trifluoroacetic acid, and applied to a C₁₈ column (Aquapore OD-300 7 μ , 2.1×100 mm). The activity was eluted by a linear gradient of 0–30% acetonitrile in 0.1% trifluoroacetic acid at a rate of 1%/min. Assays of fractions from each peak were performed in the presence of Ad2 DNA and rEP.

RESULTS

Comments on the Purification of rEP—A description of the purification of rEP is given under "Experimental Procedures," and the purification table is Table I. Proteinase activity was measured using the fluorogenic substrate (Leu-Arg-Gly-Gly-

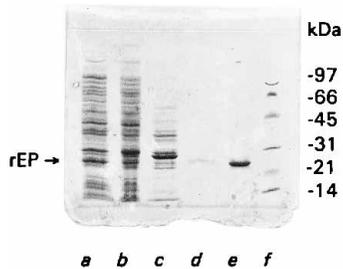


FIG. 1. **Purification of adenovirus rEP.** Samples of lysates from bacteria induced with IPTG to express rEP (lane a), the 10,000 \times g supernatant of the lysate (lane b), the flow-through fraction from the DEAE chromatography step (lane c), the rEP activity pool from the S-Sepharose chromatography step (lane d), rEP purified by passage over a zinc-iminodiacetic acid-Sepharose column (lane e), and molecular mass markers (lane f) were separated by SDS-PAGE and stained with Coomassie Brilliant Blue.

NH)₂-rhodamine.² An SDS-PAGE analysis of the various fractions during the purification procedure is shown in Fig. 1. After clarification of the lysate by centrifugation, the supernatant appeared to contain as much of the mass migrating with rEP as was seen in the lysate. Also, the supernatant contained 130% of the lysate activity. For these two reasons, rEP was probably expressed in a soluble extractable form within *E. coli*. After passing the supernatant through a DEAE column, the specific activity increased 3-fold with an 87% recovery. S-Sepharose chromatography resulted in a 7-fold increase in specific activity with a 73% recovery. The enzyme eluted at low ionic strength. Zinc column chromatography removed the remaining contaminants through successive salt-imidazole steps. Once the contaminants were eluted, rEP was eluted by applying EDTA to the column. The overall yield of rEP was 66%, and the specific activity increased 36-fold. SDS-PAGE analysis of the zinc-iminodiacetic acid column pool indicated that rEP is homogeneous (Fig. 1, lane e).

Complementation of the ts-1 Mutation by rEP—To determine whether rEP was active with *in vivo* substrates and to determine whether it is the protein whose inactivation gives rise to the ts-1 phenotype, we investigated whether we could observe processing of ts-1 precursor proteins upon incubation of rEP with ts-1 virions. Disrupted ts-1 virions were incubated with purified rEP under conditions optimal for assaying proteinase activity in disrupted wild-type virus with our fluorogenic substrates.² As a function of time, the reaction was monitored for processing of the precursor proteins by SDS-PAGE (Fig. 2). After 30 min, the profile (lane f) resembled that of wild-type virus (lanes a and h) more than that of ts-1 virus (lane g). pVI, pVII, and 11K were extensively processed, while pVIII and pIIIa were less extensively processed. After 6 h, ts-1 virus incubated with rEP (lane b) was identical to wild-type virus (lanes a and h), except for a band that is either pVIII or an intermediate in pVI processing. An identical experiment but using heat-inactivated rEP showed no processing, even after an 18-h incubation (data not shown). If the same experiment was done but after pretreatment of disrupted ts-1 virus with DNase and MgCl₂, no processing occurred, whereas processing was observed in the presence of DNase and EDTA (data not shown). We could not see the processing of the precursor to terminal protein because too few copies are present in virions to be visualized even by silver staining.

Comments on the Purification and Identification of the Second Cofactor, pVIc—A description of the purification of the second cofactor is given under "Experimental Procedures." Virions disrupted by 10% pyridine were centrifuged, and the pellet was resuspended in octyl glucoside (Fig. 3). The solubilized cofactor activity was placed in a Centricon-30 (30,000-Da cut-

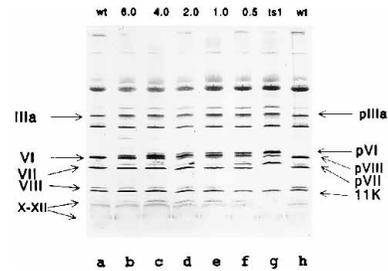


FIG. 2. **Processing of Ad2 ts-1 precursor proteins by rEP.** Twice banded Ad2 ts-1 virions were disrupted by two cycles of freeze/thaw followed by heat treatment. Reactions of 0.18 ml contained 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM octyl glucoside, 3×10^{11} virions, and 0.25 μ M rEP. After the indicated times at 37 °C, aliquots were removed from the reactions, and the proteins were fractionated by SDS-PAGE on 8–25% gradient gels. Lanes a and h, wild-type Ad2 virions; lane g, Ad2 ts-1 virions; lanes b–f, Ad2 ts-1 virions incubated with rEP for 6, 4, 2, 1, and 0.5 h, respectively. The precursor proteins of the ts-1 virion and their mature counterparts in the wild-type (wt) Ad2 virion are labeled. The proteins were visualized by silver staining.

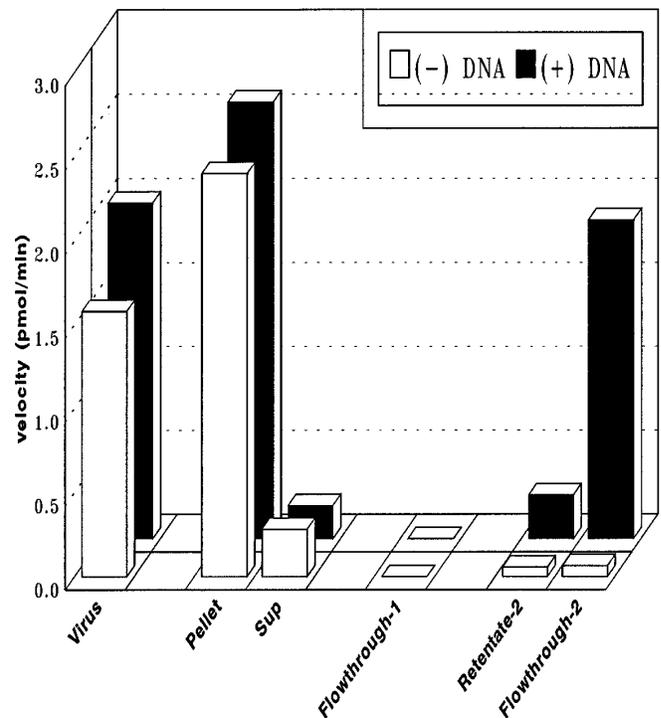
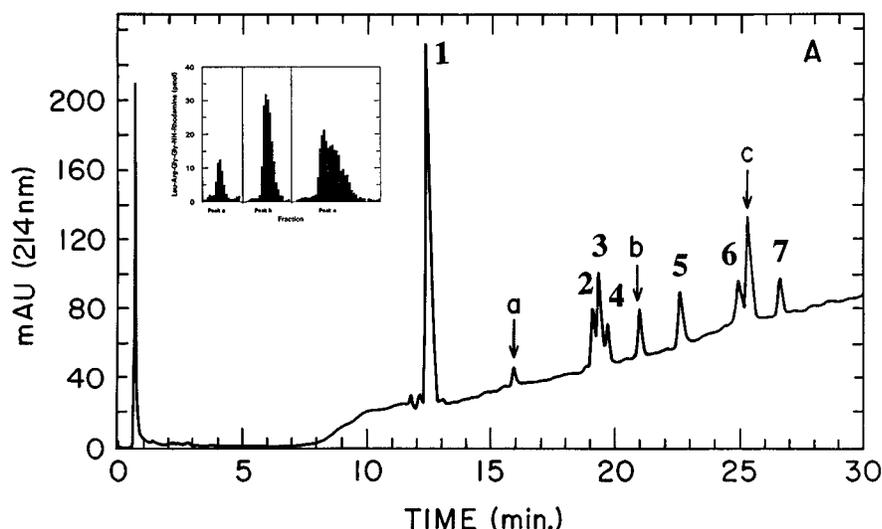


FIG. 3. **Initial purification of the second cofactor activity.** Virus was disrupted by treatment with 10% pyridine. The pellet and supernatant (Sup) were obtained after centrifuging disrupted virus. The pellet was solubilized, placed in a Centricon-30, and centrifuged to yield retentate-1 and flow-through-1. Ammonium acetate to 1 M was added to retentate-1, and the solution was recentrifuged to yield retentate-2 and flow-through-2. Assays were performed in the presence of 3 nM rEP and in the absence or presence of 778 ng/ml Ad2 DNA.

off) and centrifuged until >90% of the liquid passed through the membrane. The salt concentration in the liquid that was retained was increased to 1 M, and the solution in the Centricon-30 was again centrifuged until >90% of the liquid passed through the membrane. This time, the second cofactor activity passed through the membrane.

The final step in the purification of the second cofactor was reverse-phase chromatography. The flow-through fraction from the second Centricon-30 centrifugation was applied to a C₁₈ column, and the cofactor activity was eluted with a linear gradient of 0–30% acetonitrile (Fig. 4). Each peak from the column was assayed for proteinase activity. Only the three peaks marked with arrows showed proteinase activity in the

FIG. 4. Final step in the purification of the second cofactor activity. The flow-through fraction from the second Centricon-30 centrifugation was applied to a C_{18} column, and the activity was eluted by a linear gradient of 0–30% acetonitrile in 0.1% trifluoroacetic acid. *Inset*, assays of fractions from peaks a, b, and c were performed in the presence of 778 ng/ml Ad2 DNA and 2.2 nM rEP. mAU, milli-absorbance units.



Peak a	1	GVQSL?RRR? F
Peak b	1	GVQSLKRRR? F
Peak c	1	GVQSL?RRR? F

presence of rEP and Ad2 DNA. A profile of the proteinase activity in each of the three peaks is shown in the *inset* in Fig. 4. Of the total cofactor activity applied to the C_{18} column, 7% was recovered in peak a, 22% in peak b, and 14% in peak c. The final yield was >49%.

Identification of the Second Cofactor as pVIc—The amino acid sequences of the proteins in each of the three peaks that contained cofactor activity were determined in a gas-phase sequencer. The results (Fig. 5) indicated that the polypeptides in the three peaks were homologous. The differences were in the yield of lysine at position 6. In peak a, 5 pmol of lysine were detected, whereas 15–30 pmol were expected. No amino acid was detected at position 6 in peak b, and in peak c, 35 pmol of lysine were detected where 40–80 pmol were expected. No amino acid was detected at position 10, where a cysteine was expected. This 11-amino acid polypeptide originated from the carboxyl terminus of the precursor to band VI, pVI. That sequence is also shown in Fig. 5. There are two proteinase consensus cleavage sequences in pVI, one beginning at residue 29 (MSGG) and the other at residue 236 (IVGL). Cleavage of the latter sequence at the Leu–Gly bond would liberate the 11-amino acid cofactor.

The proteins in peaks a–c of Fig. 4 were also subjected to time-of-flight mass spectrometry. In peak a, there was one major species with an M_r of 1350. Thus, there was mostly pVIc present. In peak b, there were two species present at a ratio of 2:1, one with an M_r of 1350 and the other with an M_r of 2700. Thus, there was mostly pVIc present, but also some pVIc dimer. In peak c, there was one major species with an M_r of 1350.

Identification of the Proteins in the Other Peaks—We also sequenced the proteins in the peaks in Fig. 4 that did not show proteinase activity. Peak 1 contained the sequence MRRAH-HRRRRASHRRMRGG, which is the Mu peptide from the 11-kDa precursor (14). The sequence in peak 2 was FRHRVR-SPGQGITHLKIR, which is the C-terminal fragment of pVIII. The N-terminal sequence of the 11-kDa precursor, ALT-RLR-FPVPGF, was found in peak 3. The sequence in peak 4, GN-PRA-LRP- -G, comes from the C-terminal fragment of pIIIa; and the sequence GLRFPSKMFGG from peak 5 comes from the C-terminal fragment of the N-terminal fragment of pVII.

Reconstitution of Proteinase Activity *in Vitro* with Purified Components—Ad2 DNA and rEP were assayed in the presence of increasing amounts of the second cofactor (Fig. 6A). At low concentrations of the second cofactor, the amount of proteinase activity was proportional to the amount of second cofactor. At

Amino acid sequence to the precursor of Ad2 protein pVI

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1  MEDINFASLA PRHGSRPFG NWQDIGTSNM MSGGAFSWGSL WSGIKNFGST IKNYGSKAWN
61  SSTGQMLRDK LKEQNFQQKV VDGLASGISG VVDLANQAVQ NKINSKLDPR PPVEEPPPAV
121  ETVSPGEGRG KRPRPDREET LVTQIDEPPS YEEALKQGLP TTRPIAPMAT GVLGQHTPTV
181  LDLPPADTQ QKPVLPGPSA VVVTRPSRAS LRRRAASGPRS MRPVASGNWQ STLNSIVGLG
241  VQSLKRRRCF

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FIG. 5. Amino acid sequence of the second cofactor and comparison with the amino acid sequence of the precursor to adenovirus protein VI. The amino acid sequences of the proteins in peaks a–c in Fig. 4 (*inset*) were determined in a gas-phase sequencer. The question mark at position 6 indicates a variable yield of lysine, as described under “Results.” The amino acid at position 10 was presumed to be a cysteine. The two adenovirus proteinase consensus cleavage sequences in pVI are *underlined*, and the location of the second cofactor sequence beginning at position 240 is in *boldface*. The amino acid sequence of pVI was from Roberts *et al.* (14).

higher concentrations, saturation was approached. If the amount of rEP was doubled, the amount of proteinase activity was doubled, and the saturation limit appeared to double. Proteinase activity of rEP-pVIc complexes was stimulated by the presence of Ad2 DNA (Fig. 6B). As the Ad2 DNA concentration was increased in the presence of a constant amount of rEP and second cofactor, proteinase activity rose, reached a plateau, and eventually began to decrease.

Optimization of Assay Conditions—The assay conditions we used to monitor the purification of rEP and pVIc were those we obtained from optimizing proteinase activity *in vivo*, in disrupted wild-type virus.² With purified components, rEP and pVIc, and the substrate (Leu-Arg-Gly-Gly-NH)₂-rhodamine, we varied the assay parameters to determine the optimal conditions for maximal activity *in vitro* of rEP-pVIc complexes in the absence and presence of Ad2 DNA (Fig. 7). The optimal temperature was 45 °C independent of the presence of Ad2 DNA (Fig. 7A). Proteinase activity was unusually sensitive to ionic strength (Fig. 7B). Half the activity was lost at NaCl concentrations of 10 mM in the absence of Ad2 DNA and 45 mM in the presence of Ad2 DNA. Addition of 10 mM octyl glucoside resulted in a 3-fold increase in activity in the absence of Ad2 DNA; at 20 mM octyl glucoside, the activity decreased to its level in the absence of octyl glucoside (Fig. 7C). In the presence

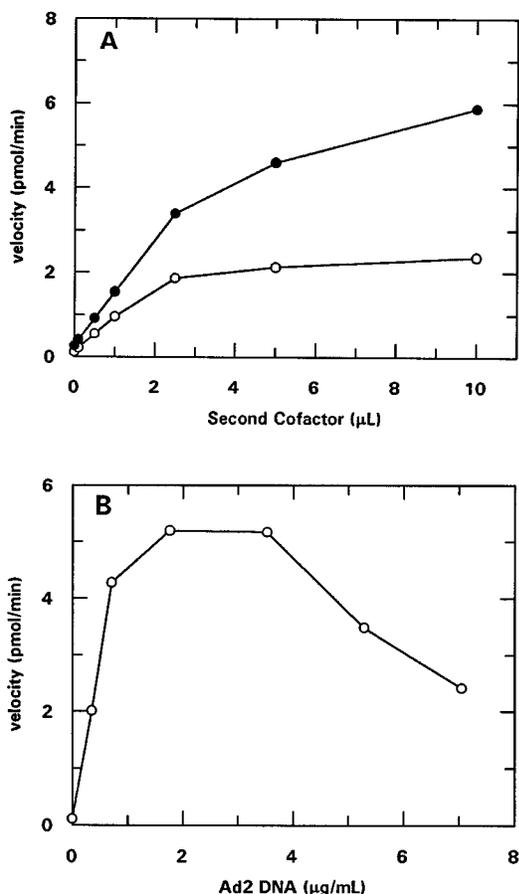


FIG. 6. Reconstitution of proteinase activity *in vitro* with purified components and titration with the second cofactor (A) and with Ad2 DNA (B). A, assays in 400 μ l contained 1.42 μ g/ml Ad2 DNA, either 2 nM (open circles) or 4 nM (closed circles) rEP, and the indicated volumes of cofactor activity purified as described in the legend to Fig. 3. B, assays in 400 μ l contained 2 nM rEP, 10 μ l of cofactor activity purified as described in the legend to Fig. 3, and the indicated concentrations of Ad2 DNA. Rates are expressed as the difference in rates between assays in the presence and absence of the cofactor (A) and Ad2 DNA (B).

of Ad2 DNA, 6–8 mM octyl glucoside increased activity slightly more than 50%. At higher concentrations of octyl glucoside, activity decreased such that at 18 mM it was half-maximal activity. With dithiothreitol, 5 mM increased the activity in the presence of Ad2 DNA by slightly more than 50% (Fig. 7D). In the absence of Ad2 DNA, DTT was only inhibitory, with half-maximal activity at 1 mM.

Based upon these and other observations, standard assay conditions were adopted that included 37 $^{\circ}$ C, 10 mM buffer at pH 8.5 for the absence of DNA and at pH 8.0 for the presence of DNA, and 10 mM octyl glucoside. Occasionally, a preparation of rEP was stimulated by DTT and/or EDTA, in which case, assays also included 0.5 mM DTT and/or 1 mM EDTA. EDTA at higher concentrations was inhibitory; with rEP-pVIc complexes, half-maximal activity was lost at 50 mM EDTA (data not shown). Under standard assay conditions, the increase in fluorescence as a function of time with (Leu-Arg-Gly-Gly-NH)₂-rhodamine as the substrate was linear for >30 min.

K_m and k_{cat} of rEP with the Cofactors—Once we had purified components and optimal assay conditions, we determined the effects of the cofactors on the macroscopic kinetic constants of the interaction of rEP with the substrate (Leu-Arg-Gly-Gly-NH)₂-rhodamine (Table II). rEP alone had a small amount of activity. By incubating Ad2 DNA with rEP, the *K_m* increased 2-fold and the *k_{cat}* 3-fold relative to those with rEP alone. By

incubating pVIc with rEP, the *K_m* increased 2-fold and the *k_{cat}* 355-fold relative to those with rEP alone. With all three components together, rEP plus Ad2 DNA plus pVIc, the *K_m* increased 2-fold and the *k_{cat}* 6072-fold relative to those with rEP alone. Thus, the cofactors increase proteinase activity by increasing the catalytic rate constant, *k_{cat}*.

V_{max} as a Function of pH—Measurement of *V_{max}* as a function of pH can reveal the *pK_a* values of the amino acids involved in catalysis. With rEP-pVIc complexes, in the absence and presence of Ad2 DNA, we measured the rate of hydrolysis of (Leu-Arg-Gly-Gly-NH)₂-rhodamine in the pH range from 4.5 to 10 under conditions in which the substrate concentration was at least 5-fold greater than the *K_m*. The data are shown in Fig. 8. In the absence of Ad2 DNA, the data can best be characterized as the sum of four gaussian peaks with a correlation coefficient of 98.1% (Fig. 8A). In the presence of Ad2 DNA, the data can best be characterized as the sum of three gaussian peaks with a correlation coefficient of 96.9% (Fig. 8B). The curves of *V_{max}* as a function of pH implied that, in the absence of Ad2 DNA, there are at least five amino acids with *pK_a* values that can affect catalysis. The *pK_a* values are 5.2, 6.4, 6.9, 7.5, and 9.4. In the presence of Ad2 DNA, there are four amino acids with critical *pK_a* values: 5.2, 6.5, 7.4, and 8.8. In the absence and presence of Ad2 DNA, the two gaussian curves between pH 5 and 6 appear to be the same; the large, high pH gaussian curve in the absence of Ad2 DNA appears in the presence of Ad2 DNA to have shifted 0.5 pH units to the left. The gaussian curve in the presence of Ad2 DNA with a *pK_a* of 6.5 appears to have split into two gaussian curves with *pK_a* values of 6.4 and 6.9.

DISCUSSION

The rEP protein was purified to apparent homogeneity using three chromatographic steps with an overall yield of 66%. About 125 mg of rEP were induced by IPTG in a 4-liter culture of cells grown to an absorbance at 600 nm between 0.5 and 0.6 before induction by IPTG. rEP does not bind to DEAE probably because of its high isoelectric point, which was calculated to be 8.68. Also, the DEAE step was used to remove nucleic acids because they bind to the column at NaCl concentrations <0.3 M (15). The S-Sepharose anion-exchange column gave the largest increase in specific activity, from 67.7 to 485 units/mg. A chelating Sepharose column charged with zinc was used because rEP contains eight free cysteines³ and three histidines. rEP bound quite tightly to this column as it had to be eluted with EDTA. The final step in the purification of rEP was dialysis against 0.5 mM EDTA. This was done to remove all traces of zinc, which is an inhibitor of enzyme activity. With (Leu-Arg-Gly-Gly-NH)₂-rhodamine as the substrate, the specific activity increased from 22.4 to 815 nmol of substrate hydrolyzed per s/mg of protein.

We were able to observe the processing of most of the virion precursor proteins by adding purified rEP to disrupted ts-1 virus. This established that rEP can find its cofactors and become activated and that activated rEP can cleave *in vivo* substrates. Furthermore, this indicated that rEP need not be post-translationally modified, *e.g.* glycosylated, to become activated and cleave *in vivo* substrates, unless such modifying enzymes and their substrates are in the virion. These data also allow one to conclude that the gene coding for the L3 23-kDa protein is indeed the gene whose inactivation gives rise to the ts-1 phenotype. The viral DNA was implicated as a cofactor because pretreatment of disrupted ts-1 virus with DNase prevented processing after the addition of rEP.

The purification of the second cofactor, pVIc, was very diffi-

³ W. J. McGrath and W. F. Mangel, unpublished observations.

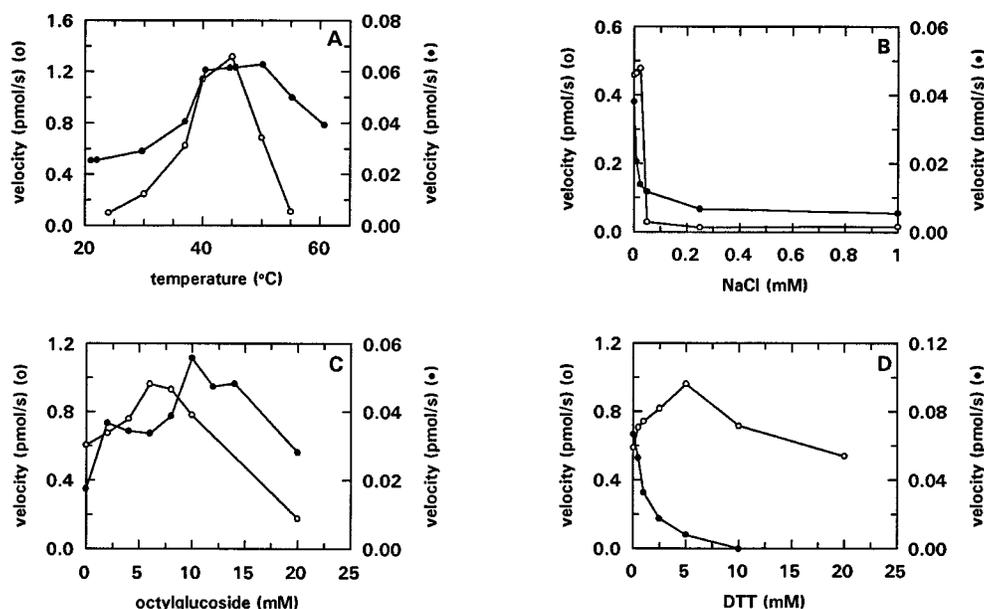


FIG. 7. Optimization of assay conditions in the absence (closed circles) and presence (open circles) of Ad2 DNA: temperature (A), ionic strength (B), octyl glucoside (C), and dithiothreitol (D). In A, complexes between rEP and pVlc were formed by incubating 70 nM rEP and 208 nM pVlc in 0.9 ml of 0.1 mM TAPS (pH 8.5), 10 mM octyl glucoside, 1 mM EDTA, and 0.5 mM DTT for 5 min at 37 °C. The rEP-pVlc complexes were then assayed at pH 8.5 using TAPS buffer. Complexes between rEP, pVlc, and Ad2 DNA were formed the same way, except that the reactions contained 14 nM rEP, 200 nM pVlc, and 140 ng/ml Ad2 DNA. The rEP-pVlc-Ad2 DNA complexes were assayed at pH 8.0 using Tris buffer. After the preincubations, 0.1 ml of 0.1 M buffer, 30 μ M (Leu-Arg-Gly-Gly-NH)₂-rhodamine, 10 mM octyl glucoside, 1 mM EDTA, and 0.5 mM DTT was added; the reactions were incubated at the indicated temperatures; and after 10 min, the increase in fluorescence was determined. In B-D, complexes were formed in 0.9 ml as described for A, except for the absence of the indicated variable. Then, 0.1 ml was added as described for A, except that it contained 10 times the final concentration of the indicated variable, and the increase in fluorescence at 37 °C was monitored as a function of time.

TABLE II

Kinetic constants for stimulation of proteinase activity by cofactors

Assays were performed in 1 ml of 10 mM Tris (pH 8.0), 10 mM octyl glucoside, and concentrations of (Leu-Arg-Gly-Gly-NH)₂-rhodamine from 0.2 to 5 times the K_m value. rEP was assayed at 628 nM in the absence or presence of Ad2 DNA and in the presence of 100 μ g/ml bovine serum albumin. In assays containing the pVlc peptide, rEP was present at 31.4 nM. The Ad2 DNA concentration was 12.5 μ M, and the pVlc concentration was 220 nM. Reactions minus substrate were incubated at 37 °C for 3 min prior to the addition of (Leu-Arg-Gly-Gly-NH)₂-rhodamine. Results are normalized to rates obtained in assays using 31.4 nM rEP.

Condition	K_m	k_{cat} ($\times 10^3$)	Stimulation
	μ M	s^{-1}	
rEP	1.83 \pm 0.70	0.055 \pm 0.006	1
rEP + Ad2 DNA	4.66 \pm 2.33	0.172 \pm 0.0553	3.13
rEP + pVlc	3.83 \pm 0.83	19.5 \pm 2.21	355
rEP + pVlc + Ad2 DNA	4.03 \pm 0.53	334 \pm 23.53	6072

cult, and, with hindsight, we now know why. We lost activity upon column chromatography because it was so basic, it stuck to glass. We lost activity upon dialysis because it was so small, it passed through the pores in the tubing. Consistent with the second cofactor being a small protein are the observations that boiling for 5 min did not irreversibly denature it; at high ionic strength, it passed through a Centricon-3 (3000-Da cutoff); and when incubated in 5 M urea, it rapidly regained activity upon dilution of the urea (data not shown). The amino acid sequence of the second cofactor is consistent with the biochemical data. The second cofactor was sensitive to plasmin because it contains one lysine and three arginines.

The purification data in Fig. 3 implied that pVlc was bound to the viral DNA. At each step before the second Centricon-30 centrifugation, cofactor activity was not greatly stimulated by the addition of Ad2 DNA, but after, enzyme activity was greatly stimulated by the addition of Ad2 DNA. The second cofactor

was not in the flow-through fraction after the first Centricon-30 centrifugation because it is a very basic protein that was probably bound to the viral DNA. High ionic strength would dissociate it from the viral DNA, and thus after the second Centricon-30 centrifugation, it was in the flow-through fraction. Cofactor activity in the flow-through fraction was greatly stimulated by the addition of Ad2 DNA.

The flow-through fraction from the Centricon-30 centrifugation gave numerous peaks on a reverse-phase C₁₈ column. Three of the peaks contained cofactor activity. Sequencing of the three peaks indicated that there was a variable yield in lysine at position 6 and no amino acid was detected at position 10, where we expected a cysteine. Time-of-flight mass spectrometry analysis indicated that the major species in each peak had an M_r of 1350, consistent with the presence of a monomer of pVlc. Webster *et al.* (8) purified the cofactor by solubilizing virions in 4 M guanidine HCl and fractionating by fast protein liquid chromatography on a Superdex S-75 gel filtration column. Two peaks of complementing activity were detected by subsequent reverse-phase high pressure liquid chromatography. One peak was the monomer of pVlc, and the other peak was the disulfide dimer of pVlc.

We were able to reconstitute maximal proteinase activity with purified components: rEP, pVlc purified from wild-type virus, and Ad2 DNA. The cofactors stimulated proteinase activity by increasing k_{cat} >6000-fold. The K_m changed by less than a factor of 3. Previous *in vitro* assays for the Ad2 proteinase activity were successful because they utilized Ad2 precursor proteins in an extract from ts-1-infected cells as substrate and disrupted wild-type virus (16) or rEP as the source of proteinase (6) or synthetic peptides as substrate and disrupted wild-type virus as the proteinase (17, 18). Hence, in those assays, both cofactors were present.

Other proteinases require cofactors for activity, but none, so far, exhibits the requirements of the Ad2 proteinase. Several

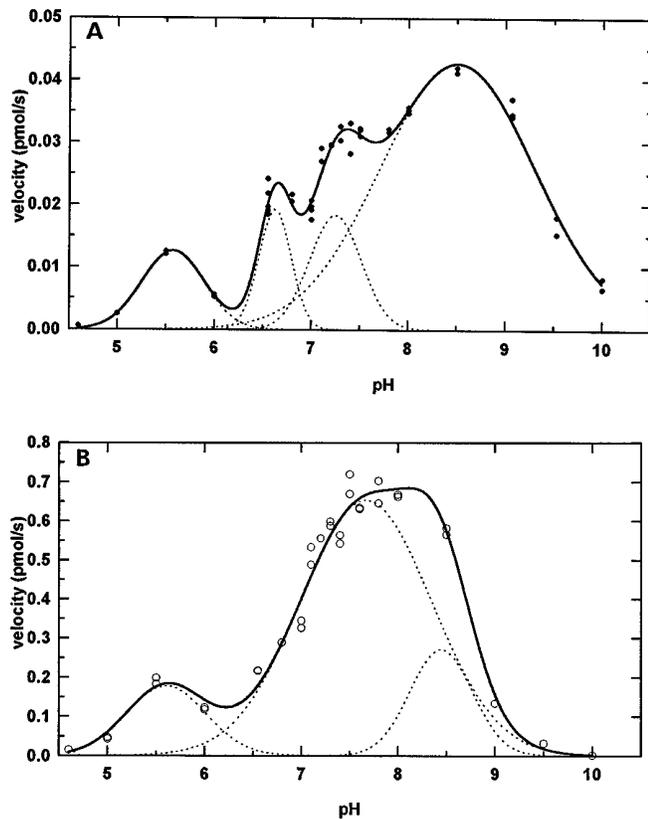


FIG. 8. Maximal velocity as a function of pH for rEP-pVlc complexes (A) and for rEP-pVlc-Ad2 DNA complexes (B). Complexes were formed by incubating 63 nM rEP and 200 nM pVlc (A) or 15.75 nM rEP, 200 nM pVlc, and 5.6 pM Ad2 DNA (B) for 5 min at 37 °C in 0.9 ml of 0.1 mM TAPS (pH 8.5) containing 1.1 mM EDTA, 0.55 mM DTT, and 11 mM octyl glucoside. Then, 0.1 ml was added containing components such that the final concentration of buffer was 10 mM and that of (Leu-Arg-Gly-Gly-NH)₂-rhodamine was 3 μM, and the ionic strength was 16 mM. The increase in fluorescence was then measured as a function of time. The buffers used were sodium citrate (pH 4.0 and 4.6), sodium acetate (pH 5.0 and 5.5), MES (pH 6), sodium cacodylate (pH 6.0–6.8), HEPES (pH 7–7.8), Tris (pH 8.0 and 8.5), CHES (pH 9.0 and 9.5), and CAPS (pH 10.0 and 10.5). The curves were fitted using the program Peakfit (Jandel Scientific) assuming merged gaussian peaks (dotted lines).

neutral proteinases need Ca²⁺ for activity (19). Some proteinases utilize ATP (20). A serine proteinase anchored to the membrane of *Plasmodium falciparum* by a covalently attached glycosylphosphatidylinositol moiety is activated by phosphatidylinositol-specific phospholipase C (21). Many proteinases are synthesized as zymogens and must be activated by proteolytic cleavage, e.g. the activation of trypsinogen to trypsin by enterokinase or trypsin (22) or the activation of plasminogen to plasmin by urokinase (23). The assembly and activation of some of the proteins of the blood coagulation system require a negatively charged surface (24). The virus-coded proteinases from the human immunodeficiency virus and avian sarcoma/leukosis viruses require themselves as cofactors as homodimers are the active form (25–27). Although the *E. coli* RecA protein can facilitate the cleavage of the LexA protein bound to DNA, RecA apparently does so as an allosteric effector and not as a proteinase with an active-site nucleophile (28).

The experiments on optimizing the assay conditions for the proteinase activity of rEP-pVlc complexes in the absence and presence of the viral DNA revealed an unusual sensitivity to ionic strength. In the absence of the DNA, 10 mM NaCl inhibited 50% of the enzyme activity. In the presence of Ad2 DNA, 45 mM NaCl inhibited 50% of the enzyme activity. In contrast to these results, in disrupted virions, 300 mM NaCl was required

for 50% inhibition of activity.² The latter experiment implied that direct inhibition either of the binding of substrate to the active site or, once bound, of the rate of catalysis probably occurs at NaCl concentrations closer to 300 mM than to 30 mM. Thus, NaCl concentrations of 10–45 mM must inhibit enzyme activity by interfering with formation of an active complex, a complex already formed in a disrupted virus particle.

Although we settled upon standard assay conditions, occasionally they must be altered. We have found (data not shown) that with aged disrupted virus, when compared with newly isolated, disrupted virus, the degree of stimulation by low concentrations of DTT varied from zero with newly isolated, disrupted virus to 4–5-fold with aged disrupted virus. Purified rEP exhibits a similar pattern in that as it ages during storage, 0.5 mM DTT will stimulate more and more activity. This result can be interpreted as signifying the importance of the oxidation states of certain cysteine residues. Similarly, the presence of EDTA at concentrations <2 mM sometimes stimulated proteinase activity (data not shown). Perhaps some zinc, which is a potent inhibitor of enzyme activity, remained with the rEP after chromatography on a chelating Sepharose column charged with zinc.

The nature of the active site of the Ad2 proteinase is unclear. The inhibitor profile of wild-type virus does not correspond to profiles exhibited by classical serine or cysteine proteinases (17, 18, 29, 30).² Examination of the L3 23-kDa gene sequence led Webster *et al.* (17, 18) to propose that the Ad2 proteinase may be a member of a new subclass of cysteine proteinases described by Brenner (31), by Bazan and Fletterick (32), and by Gorbalenya *et al.* (33). Based upon site-directed mutagenesis studies, two groups have argued that the enzyme is a cysteine proteinase and that Cys-104 is the active-site nucleophile (34, 35).

The requirement for DNA as a cofactor for a proteinase activity is unprecedented. It is clearly required in the Ad2 virion because proteinase activity is lost upon treatment with DNase and restored upon addition of Ad2 DNA. In addition, the precursor proteins in disrupted ts-1 virus are processed upon incubation with rEP. However, no processing occurs if disrupted virions are pretreated with DNase. Reconstitution of proteinase activity *in vitro* with purified components indicates that Ad2 DNA affects k_{cat} and not K_m . Webster *et al.* (36) found no stimulation of rEP-pVlc complex activity by Ad2 DNA.

The experiments on V_{max} as a function of pH with rEP-pVlc complexes in the absence and presence of Ad2 DNA indicated that the enzyme is quite different from the cysteine proteinase papain. Papain contains an active-site thiolate-imidazolium ion pair between His-159 and Cys-25 (37). The second-order acylation rate constant (k_{cat}/K_m) as a function of pH conforms to a bell-shaped curve. The two ionizing groups with pK_a values near 4 and 8.5 probably correspond to His-159 and Cys-25, respectively, more appropriately to the formation and decomposition of the ion pair. An active-site thiolate-imidazolium ion pair in the adenovirus proteinase could have pK_a values in the absence of Ad2 DNA of 5.17 and 9.43, whereas in the presence of Ad2 DNA, the pK_a values could be 5.15 and 8.78. These pK_a values are similar to the normal pK_a values of 6.0 for histidine and 8.3 for cysteine. Our thiol protection experiment at pH 5.0 *in vivo* with disrupted virus is consistent with the presence of a thiolate-imidazolium ion pair.²

The experiments on V_{max} as a function of pH implied that rEP-pVlc complexes bind to Ad2 DNA. The profiles of rEP-pVlc complexes in the absence and presence of Ad2 DNA are different: pK_a values of 5.2, 6.4, 6.9, 7.5, and 9.4 versus 5.2, 6.5, 7.4, and 8.8, respectively. This indicates that Ad2 DNA does affect the pK_a values of some of the amino acids involved in catalysis

and therefore implies that the rEP-pVIc complexes bind to Ad2 DNA. In addition, the results of measuring the V_{\max} of rEP-pVIc complexes in the presence of Ad2 DNA are similar to those obtained with enzyme activity in disrupted virus. The pK_a values for disrupted virus are 5.2, 6.2, 7.2, and 8.4. This implied that in the virion, rEP-pVIc complexes are bound to the viral DNA.

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