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 presence and absence 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), 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 in vitro.

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. 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$_2$-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 plasmid-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 $\approx 6000$-fold. By measuring initial velocities as a function of pH, we show that the enzyme activity is clearly different from that of papain.
Moreover, the pH 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 pVIc peptide was obtained from Multiple Peptide Systems (San Diego, CA). Cell culture media and reagents were obtained from Gibco/BRL and Sigma. Tryptic phosphorase from E. coli (Adenovirus Virion Proteinase Activity in Vitro) and Chelex-100 (Bio-Rad) were used to purify the pVIc peptide.

**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). 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 × 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 pVIc peptide, 140 ng/ml Ad2 DNA, 2 μM (Leu-Arg-Gly-Gly-NH2),-chymotrypsin, and 0.5 μM elastase. Saturating levels of pVIc were 100 nM when the rEP concentration was 50 nM and 300 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 420 nm, and the emission wavelength was 525 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 pVIc 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 pVIc stock solution to 0.99 ml of Ellman’s buffer (0.1 × NaH2PO4, pH 7.3) and 1 ml 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 pVIc was calculated using a molar extinction coefficient at 412 nm of 14,150 (12) for thionitrobenzoate.

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific activity</th>
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<tr>
<td>Lysate</td>
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<td>1.42</td>
<td>22.4</td>
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<tr>
<td>Supernatant</td>
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<td>1.85</td>
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<td>SSEPH pool</td>
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<td>1.18</td>
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<td>Zn-IDA pool</td>
<td>25.9</td>
<td>3.2</td>
<td>0.94</td>
<td>815</td>
</tr>
</tbody>
</table>

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

**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-
Adenovirus Virion Proteinase Activity in Vitro

FIG. 1. Purification of adenovirus rEP. Samples of lysates from bacteria induced with IPTG to express rEP (lane a), the 10,000 x 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.

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 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 with ts-1 virions and their mature counterparts in the wild-type (wt) Ad2 virion. The proteins were visualized by silver staining.

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 x 10^11 virions, and 0.25 μM 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 centrifuged 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
Of the total cofactor activity applied to the C18 column, 7% of activity in each of the three peaks is shown in the presence of rEP and Ad2 DNA. A profile of the proteinase activity was determined in the presence of Ad2 DNA and 2.2 nM rEP. mAU, milli-absorbance units.

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 M₉ 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 M₉ of 1350 and the other with an M₉ of 2700. Thus, there was mostly pVIc present, but also some pVIc dimer. In peak c, there was one major species with an M₉ 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-HRRRASHHRMRGG, which is the Mu peptide from the 11-kDa precursor (14). The sequence in peak 2 was FRHRV-SPGOGITHKIR, 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 pVIIia; and the sequence GLRFPF5KMFGG 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 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-NH₂)-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...
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 °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-p VIc 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.

Kₘ and Kₐcat 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 Kₘ increased 2-fold and the Kₐcat 355-fold relative to those with rEP alone. With all three components together, rEP plus Ad2 DNA plus p VIc, the Kₘ increased 2-fold and the Kₐcat 6072-fold relative to those with rEP alone. Thus, the cofactors increase proteinase activity by increasing the catalytic rate constant, Kₐcat.

Vₘax as a Function of pH—Measurement of Vₘax as a function of pH can reveal the pKₐ values of the amino acids involved in catalysis. With rEP-p VIc 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 Kₘ. 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 Vₘax as a function of pH implied that, in the absence of Ad2 DNA, there are at least five amino acids with pKₐ values that can affect catalysis. The pKₐ 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 pKₐ 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 pKₐ of 6.5 appears to have split into two gaussian curves with pKₐ 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 che- lating 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 DNanse prevented processing after the addition of rEP.

The purification of the second cofactor, p VIc, was very diffi-

³W. J. McGrath and W. F. Mangel, unpublished observations.
Adenovirus Virion Proteinase Activity in Vitro

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 pVIc were formed by incubating 70 nm rEP and 208 nm pVIc 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-pVIc complexes were then assayed at pH 8.5 using TAPS buffer. Complexes between rEP, pVIc, and Ad2 DNA were formed the same way, except that the reactions contained 14 nm rEP, 200 nm pVIc, and 140 ng/ml Ad2 DNA. The rEP-pVIc/Ad2 DNA complexes were assayed at pH 8.0 using Tris buffer. After the preincubations, 0.1 ml of 0.1 M buffer, 30 μM (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

<table>
<thead>
<tr>
<th>Condition</th>
<th>Km (μM)</th>
<th>kcat (×10³)</th>
<th>Stimulation</th>
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<tr>
<td>rEP</td>
<td>1.83 ± 0.70</td>
<td>0.055 ± 0.006</td>
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<tr>
<td>rEP + Ad2 DNA</td>
<td>4.66 ± 2.33</td>
<td>0.172 ± 0.0553</td>
<td>3.13</td>
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<tr>
<td>rEP + pVIc</td>
<td>3.83 ± 0.83</td>
<td>19.5 ± 2.21</td>
<td>355</td>
</tr>
<tr>
<td>rEP + pVIc + Ad2 DNA</td>
<td>4.03 ± 0.53</td>
<td>334 ± 23.53</td>
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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+ of 1350, consistent with the presence of a monomer of pVIc. 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 7-5 gel filtration column. Two peaks of complementing activity were detected by subsequent reverse-phase high pressure liquid chromatography. One peak was the monomer of pVIc, and the other peak was the disulfide dimer of pVIc.

We were able to reconstitute maximal proteinase activity with purified components: rEP, pVIc purified from wild-type virus, and Ad2 DNA. The cofactors stimulated proteinase activity by increasing Km > 6000-fold. The Km 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
neutral proteinases need Ca\(^{2+}\) 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 aszymogens 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-pVIc complexes in the absence and presence of 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. 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 EDTA 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 <0.2 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). 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 Gorbelenya 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-pVIc complex activity by Ad2 DNA.

The experiments on \(V_{max}\) as a function of pH with rEP-pVIc 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 adeno virus 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. The experiments on \(V_{max}\) as a function of pH implied that rEP-pVIc complexes bind to Ad2 DNA. The profiles of rEP-pVIc complexes in the absence and presence of Ad2 DNA were 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-pVII complexes bind to Ad2 DNA. In addition, the results of measuring the V_max of rEP-pVII 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-pVII complexes are bound to the viral DNA.

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