

## Characterization of Human Adenovirus Proteinase Activity in Disrupted Virus Particles

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Virus-coded proteinases are attractive targets for antiviral therapy; however, lack of quick, sensitive, quantitative, and selective assays for enzyme activity makes it difficult to characterize these proteinases and to screen large numbers of potential inhibitors. Here we describe new substrates for the adenovirus proteinase, fluorogenic Rhodamine-based substrates containing tetrapeptides corresponding to sequences cleaved in adenovirus precursor proteins. Proteinase activity in as few as  $10^9$  disrupted virions could be quantitatively detected in a 30-min assay. With the substrate (Leu-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine, the  $K_m$  was 1.4  $\mu$ M, and the  $V_{max}$  was 3.24 pmol substrate hydrolyzed/sec/pmol virus. Enzyme activity was stimulated by dithiothreitol and inhibited by several serine-specific as well as cysteine-specific proteinase inhibitors. In a thiol protection experiment, the virion enzyme was shown to have a cysteine residue with an unusually low  $pK_a$ , a  $pK_a$  similar to that of the active-site nucleophile of the cysteine proteinase papain. The curve of  $V_{max}$  as a function of pH is unlike the curve from papain and implied that there are at least three ionizable groups whose protonation state can affect catalysis—one with a  $pK_a$  of 6.2, another with a  $pK_a$  of 7.2, and a third with a  $pK_a$  of 8.3. © 1996 Academic Press, Inc.

### INTRODUCTION

For many animal and plant viruses, proteolysis of precursor proteins at various stages in their replication cycle is an absolute requirement for the generation of new, infectious virus particles. Six of the 12 major virion proteins in human adenovirus are processed to mature virion components by the adenovirus proteinase (Anderson *et al.*, 1973, 1989; Weber and Anderson, 1988). The proteinase itself is assembled into virions (Anderson, 1990; Bhatti and Weber, 1978, 1979a,b) and cleavage of precursor proteins probably occurs in newly assembled virus particles. A temperature-sensitive mutant of adenovirus type 2, H2ts-1, when grown at the nonpermissive temperature contains precursors instead of mature components and lacks proteinase activity (Weber, 1976) because the proteinase was not packaged into the virion (Rancourt *et al.*, 1995). H2ts-1 virions assemble efficiently at the nonpermissive temperature and even attach to cells, but they do not initiate a productive infection (Hanan *et al.*, 1983; Mirza and Weber, 1980). Among the other medically important virus families with members known to encode proteinases are retroviridae, which include HIV, and picornaviridae, which include polio and hepatitis A (for a review, see Krausslich and Wimmer, 1988). Viral proteinases are extremely specific enzymes, and there is an expectation that equally specific inhibitors may be effective antiviral agents.

Most current assays for viral proteinases use precursor virion proteins or oligopeptides as substrates and

require long incubations with enzyme, fractionation of reaction mixtures by SDS-polyacrylamide gel electrophoresis, fast protein liquid chromatography, or thin layer electrophoresis, and quantitation of the results (Anderson *et al.*, 1989; Bhatti and Weber, 1978, 1979a,b; Webster *et al.*, 1989a,b). However, natural substrates are not very suitable for quantitative enzyme studies because they are often impure or contain multiple sites of cleavage. Furthermore, the peptide products are, in many cases, a constantly changing mixture of secondary substrates and inhibitors. Also, assays with proteins or oligopeptides are not very sensitive, and the results are difficult to quantitate. Such assays become quite cumbersome when used to screen for large numbers of potential proteinase inhibitors. Synthetic substrates, on the other hand, can be very useful in assaying proteinases and in determining their specificity. They have a well-defined chemical structure, and the kinetics of hydrolysis are simplified by cleavage at a single, uniform position.

Here we describe the synthesis of several Rhodamine-based substrates containing tetrapeptides corresponding to sequences cleaved in adenovirus precursor proteins. Amino acid derivatives of Rhodamine have been shown to be extremely sensitive, specific, and selective substrates for proteinases (Leytus *et al.*, 1983a,b, 1984; Mangel *et al.*, 1985, 1987). Bis-substituted substrates are virtually nonfluorescent, because the fluorophore is in the lactone state. Upon cleavage by an endoproteinase of one of the two peptide bonds adjacent to the Rhodamine moiety, the Rhodamine moiety in the resultant mono-substituted product switches to the quinone state concomitant with a large increase in fluorescence inten-

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sity. Conversion from the lactone state to the quinone state results in a high degree of conjugation and hence stability, which is one reason why the susceptible bonds in the substrate are so unusually reactive.

The substrates we synthesized were used to develop a quick, sensitive, quantitative, and selective assay for the Ad2 proteinase. Enzyme activity in disrupted virus was optimized. An inhibitor profile of the proteinase activity was obtained. A thiol protection experiment indicated that the enzyme in the virion was papain-like; however, a curve of  $V_{\max}$  as a function of pH indicated that the enzyme in the virus particle was not papain-like.

## MATERIALS AND METHODS

### Materials

Benzamidine, benzyloxycarbonyl (Cbz)-blocked amino acids and pyroglutamic acid (<Glu), 2,2'-dithiodipyridine (dTdP), 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide, dithiothreitol (DTT), EDTA, iodoacetic acid,  $\rho$ -chloromercuribenzoate (PCMB), phenylmethanesulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone-HCl (TLCK), and L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone (TPCK) were purchased from Sigma Chemical Co.; bestatin, L-transepoxy-succinyl-leucylamido-(4-guanidino)-butane (E64), leupeptin, octylglucoside, and phosphoramidon were from Boehringer-Mannheim; Rhodamine 110 was from Exciton Chemical Co. (Dayton, OH); and dansyl-L-glutamyl-glycyl-L-arginyl chloromethyl ketone (DNS-GGACK) was from Calbiochem. Thin-layer chromatography (TLC) was performed on Polygram Sil G/UV<sub>254</sub>, 0.25-mm silica gel with fluorescent indicator 254, from Macherey-Nagel and distributed by Brinckman Instruments, Inc.

### Preparation of virus

The growth of HeLa cells, infection by wild-type Ad2 and the mutant H2ts-1, and the purification of viruses are described by Anderson (1990). Twice CsCl banded virions were dialyzed against 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and centrifuged at 100,000  $g$  for 1 hr. The pellet was suspended in 10 mM Tris-HCl (pH 6.8) containing 20% (v/v) glycerol and, after three 10-sec bursts of sonication, was stored at  $-20^{\circ}$ . Disrupted virions prepared this way (Tremblay *et al.*, 1983; Webster *et al.*, 1989a) were used as the source of virion proteinase activity.

### Synthesis of substrates

The Rhodamine-based substrates were synthesized according to published procedures (Leytus *et al.*, 1983a,b, 1984; Mangel *et al.*, 1985, 1987) with the following modifications: (Cbz-Gly-Gly-NH)<sub>2</sub>-Rhodamine was synthesized in dimethylformamide (DMF):pyridine (1:1,

v/v) at  $4^{\circ}$  by addition of Cbz-Gly-Gly to 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide with the same stoichiometry of components as described for the synthesis of (Cbz-Arg-NH)<sub>2</sub>-Rhodamine (Leytus *et al.*, 1983a,b). After 5 min, Rhodamine 110 was added and the reaction was allowed to proceed for 2 days until all the Rhodamine 110 was bis-substituted as judged by analytical TLC. The product was purified by four rounds of precipitation with ethyl acetate followed by centrifugation and dissolution in DMF. The blocking group was removed by treatment with 4 M HBr/acetic acid (HOAc) (Leytus *et al.*, 1983a,b). (Cbz-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine was synthesized using Cbz-Arg. The Cbz group was removed, and Cbz-Ile, Cbz-Leu, Cbz-Met, or Cbz-Nle was added to (Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine. Nle is norleucine. The tri- and tetrapeptide derivatives were purified as described for the purification of (Cbz-Ile-Pro-Arg-NH)<sub>2</sub>-Rhodamine (Leytus *et al.*, 1984). The identity and purity of each derivative were determined by amino acid analysis after complete acid hydrolysis. Stock solutions of substrate were made in dimethyl sulfoxide (DMSO) and stored at  $-20^{\circ}$ .

### Standard assay

The standard assay was performed in assay buffer which contained 50 mM Tris-HCl (pH 7.4), 25 mM NaCl, 10 mM octylglucoside, and 1 mM DTT. Substrate at a concentration of 5  $\mu$ M was incubated with 10<sup>10</sup> disrupted virions in a volume of 40  $\mu$ l. After 10 to 30 min at  $37^{\circ}$ , 30  $\mu$ l of the reaction was added to 670  $\mu$ l of assay buffer and the increase in fluorescence measured. The excitation wavelength was 492 nm and the emission wavelength was 523 nm, both with a bandwidth of 5 nm. The change in fluorescence,  $\Delta F$ , is the magnitude of the fluorescence from the sample minus the magnitude of the fluorescence of an identical solution but not containing virus. From the  $\Delta F$ , the number of picomoles of substrate hydrolyzed was calculated using a molar fluorescence coefficient obtained from a mono-substituted Rhodamine derivative of known concentration. The standard assay conditions were used for measurement of the macroscopic kinetic constants. No more than 5% of the substrate was hydrolyzed in each assay.

### Assays with inhibitors

Most proteinase inhibitors were dissolved in inhibitor buffer which contained 50 mM Tris-HCl (pH 7.4), 25 mM NaCl, and 10 mM octylglucoside. Bestatin was dissolved in methanol and assayed at 1.3 mM; E64 in 50% ethanol and assayed at 3  $\mu$ M. Iodoacetic acid, freshly prepared in 50 mM sodium bicarbonate (pH 9.0), was assayed at 1 mM. PCMB was dissolved in 10 mM NaOH to a concentration of 20 mM and assayed at 0.5 mM. TLCK was dissolved in 1 mM HCl and assayed at 135  $\mu$ M; TPCK in absolute ethanol was assayed at 284  $\mu$ M. PMSF was added as solid and assayed at 5 mM. The concentra-

tions of other inhibitors were 1 mM for 2,2'-dithiodipyridine, 1 mM for DNS-GGACK, 1 mM for benzamidine, 2.2  $\mu$ M for leupeptin, 85  $\mu$ M for SBTI, 22  $\mu$ M for BPTI, 5 mM for EDTA, and 5.2 mM for phosphoramidon. Inhibitor at the indicated concentration and disrupted virus,  $10^{10}$  particles, were incubated in 30  $\mu$ l of inhibitor buffer for 10 min at 37°. Then, 10  $\mu$ l of 20  $\mu$ M (Leu-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine was added. After 30 min, 30  $\mu$ l of the reaction was added to 670  $\mu$ l of inhibitor buffer and the increase in fluorescence measured. Control assays lacking inhibitor contained the same kind and volume of solvent used to dissolve the inhibitor.

## RESULTS

### Design of substrates

The amino acid sequences chosen for the Rhodamine-based substrates were similar to sequences at the *in vivo* cleavage sites of two of the six Ad2 proteins that are processed by the proteinase. Virion proteins pVI and pVII are cleaved after the second Gly residue in the sequences Met-Ser-Gly-Gly-Ala and Met-Phe-Gly-Gly-Ala, respectively (Akusjärvi and Persson, 1981; Sung *et al.*, 1983). A systematic study of the cleavage of octapeptides by the virion enzyme and molecular modeling indicated that the main determinants of proteinase specificity were the amino acids at the P<sub>4</sub> position, either leucine or methionine, the P<sub>2</sub> position, Gly, and the P<sub>1</sub>' position where the side chain of the amino acid residue must be oriented away from the scissile bond (Webster *et al.*, 1989a,b). For these reasons we synthesized (Cbz-Met-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine, (Cbz-Nle-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine, (Nle-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine, (Ile-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine, and (Leu-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine. An Arg residue was placed in the P<sub>3</sub> position to facilitate substrate purification by acid precipitation. The structure of the bis-substituted substrate (Cbz-Met-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine is shown in Fig. 1 along with its mono-substituted hydrolysis product Cbz-Met-Arg-Gly-Gly-NH-Rhodamine.

### Assay conditions

Ad2 proteinase activity was assayed in purified wild-type virions that had been disrupted by incubation at 37° in pH 6.8 buffer for 30 min followed by sonication. When disrupted virus was incubated with (Cbz-Met-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine, a linear increase in fluorescence with time was observed (data not shown). The rate of increase in fluorescence was directly proportional to the number of disrupted virus particles present. The assay parameters were then varied to determine optimal conditions for enzyme activity (Fig. 2). The optimal temperature was 45° (Fig. 2A). The proteinase was sensitive to ionic strength (Fig. 2B). Half of the activity was lost at an NaCl concentration of 0.3 M. Addition of 10 mM octylglucoside

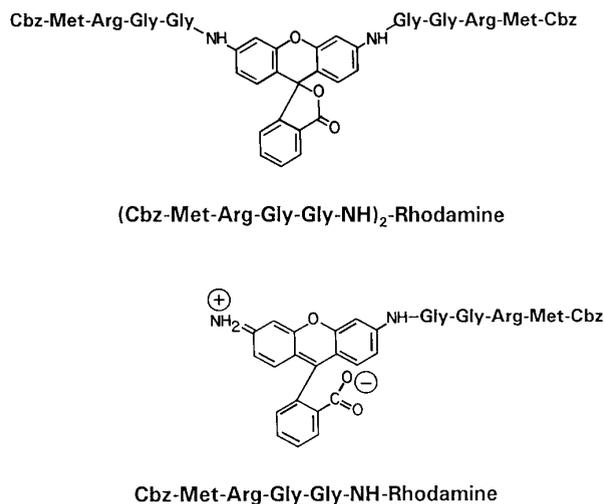


FIG. 1. Structure of the fluorogenic substrate (Cbz-Met-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine and its highly fluorescent hydrolysis product Cbz-Met-Arg-Gly-Gly-NH-Rhodamine. The Rhodamine moiety in the substrate is depicted in the lactone state. Upon cleavage of one of the two Gly-NH-Rhodamine amide bonds in the substrate by the Ad2 proteinase, the Rhodamine moiety in the hydrolysis product converts to the quinone state concomitant with a 3500-fold increase in fluorescence intensity.

resulted in a doubling of activity (Fig. 2C). There was a very narrow range of DTT concentrations that stimulated proteinase activity (Fig. 2D). From 0 to 1 mM the activity increased threefold and then began to decrease such that in 20 mM DTT, proteinase activity was half its maximal value. Based upon these and other observations, standard assay conditions of 50 mM Tris-HCl (pH 7.4), 25 mM NaCl, 10 mM octylglucoside, and 1 mM DTT were adopted. Under these conditions, the increase in fluorescence as a function of time with  $10^9$  disrupted virions was linear for more than 1 hr at 37°.

### Effects of organic solvents and detergents

Because the fluorogenic substrates were dissolved in DMSO and because we wanted to use certain proteinase inhibitors that had to be dissolved in organic solvents, we determined the effect of different organic solvents on enzyme activity (data not shown). DMSO concentrations up to 1.5% had no effect nor did 0.1% dimethylformamide. Ten percent ethanol inhibited 28% of proteinase activity, whereas 1% pyridine inhibited 33% of proteinase activity. Since octylglucoside stimulated proteinase activity, we tested several other detergents. At a concentration of 0.1% (v/v) both NP-40 and Triton X-100 were as effective as 10 mM octylglucoside. However, 0.5% deoxycholate inhibited 98% of enzyme activity and 0.05% SDS totally inhibited the proteinase.

### Substrate specificity

Under standard assay conditions, the Ad2 proteinase exhibited Michaelis-Menten kinetics with five Rhoda-

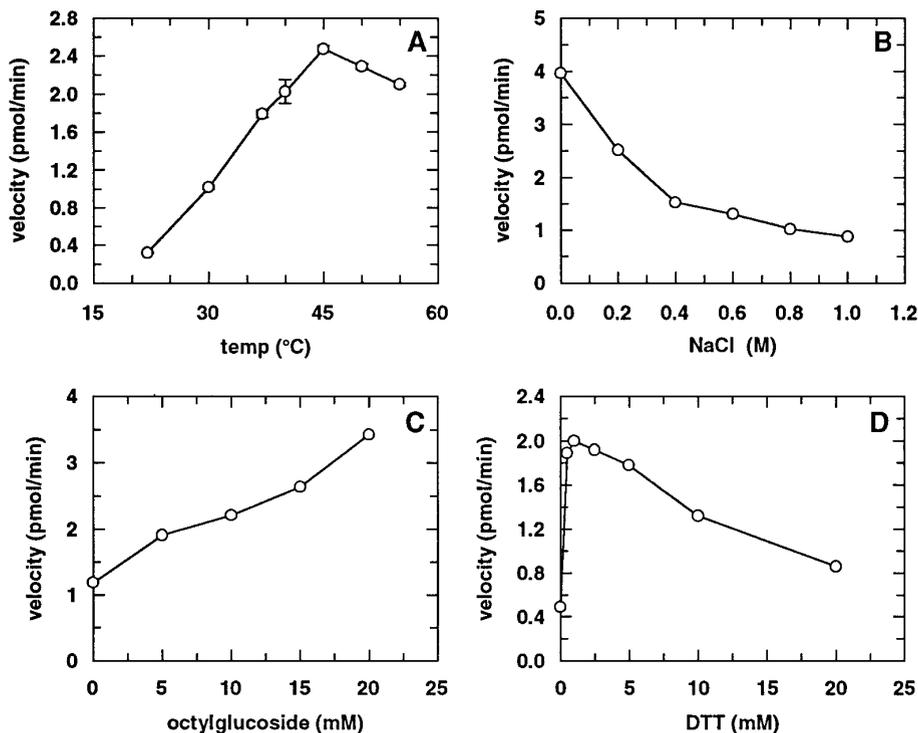


FIG. 2. Optimization of assay conditions for the Ad2 proteinase: (A) temperature, (B) ionic strength, (C) octylglucoside, and (D) DTT. In A, disrupted virus in assay buffer was preincubated at the indicated temperatures for 1 min prior to the addition of (Leu-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine to 5  $\mu$ M and the increase in fluorescence monitored as a function of time. In B–D, disrupted virus was preincubated in assay buffer minus the variable component for 10 min at 37°. Then, the indicated concentrations of the variable and (Leu-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine to 5  $\mu$ M were added and the increase in fluorescence was monitored as a function of time.

mine-based substrates. A direct plot for the hydrolysis of (Cbz-Met-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine is shown in Fig. 3. (Cbz-Nle-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine had the lowest  $K_m$ , 0.758  $\mu$ M, and (Nle-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine had the highest  $V_{max}$ , 7.6 pmol substrate hydrolyzed/sec/pmol virus (1 pmol of virus is  $6.023 \times 10^{11}$  virus particles)

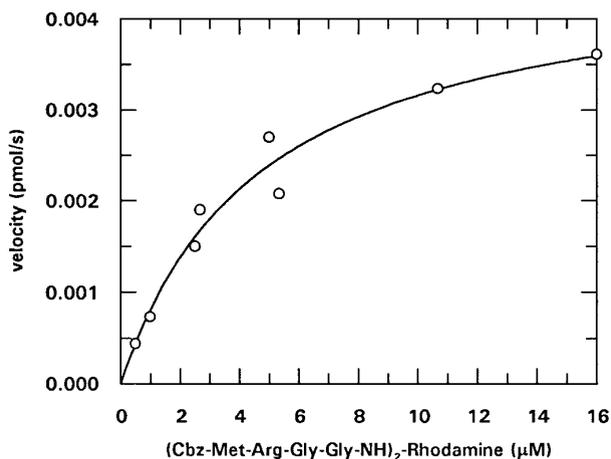


FIG. 3. Michaelis–Menten kinetics for the hydrolysis of (Cbz-Met-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine by the Ad2 proteinase. The standard assay was used with  $3.3 \times 10^{10}$  disrupted virus particles. The  $K_m$  was  $4.74 \pm 1.06 \mu$ M and the  $V_{max}$   $0.0047 \pm 0.0004$  pmol substrate hydrolyzed/sec.

(Table 1). A tetrapeptide of appropriate sequence seemed to be required. Neither (Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine nor (Cbz-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine was hydrolyzed by disrupted virions, even after a 6-hr incubation. Similarly, (Cbz-Ile-Pro-Arg-NH)<sub>2</sub>-Rhodamine and (<Glu-Gly-Arg-NH)<sub>2</sub>-Rhodamine were not hydrolyzed by the proteinase. No hydrolysis of (Leu-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine, (Ile-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine, or (Cbz-Met-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine could be detected during a 6-hr incubation under identical conditions but with substitution of H2ts-1 disrupted virions for wild-type Ad2 disrupted virions.

#### Inhibitor profile

To determine the type of proteinase present in disrupted Ad2 virions, we examined the effect of several proteinase inhibitors under conditions in which both competitive and irreversible inhibition would be observed (data not shown). Benzamidine, bestatin, EDTA, leupeptin, and phosphoramidon had no effect. Several classic serine proteinase inhibitors were effective—from most efficient to least (% activity relative to 100% with no inhibitor present) were DNS-GGACK (2%), PMSF (19%), TPCK (24%), TLCK (41%), SBTI (68%), and BPTI (69%). However, some of these compounds can also inhibit cysteine proteinases (Whittaker and Perez-Villasenor, 1968). Further-

TABLE 1

Macroscopic Kinetic Constants for the Hydrolysis of Rhodamine-Based Substrates by the Ad2 Virion Proteinase

Substrate	$K_m$ ( $\mu M$ )	$V_{max}$ (pmol substrate hydrolyzed/sec/pmol virus) <sup>a</sup>
(Arg-Gly-Gly-NH) <sub>2</sub> -Rhodamine		No hydrolysis
(Cbz-Arg-Gly-Gly-NH) <sub>2</sub> -Rhodamine		No hydrolysis
(Leu-Arg-Gly-Gly-NH) <sub>2</sub> -Rhodamine	1.43 ± 0.77	3.24 ± 0.33
(Ile-Arg-Gly-Gly-NH) <sub>2</sub> -Rhodamine	5.17 ± 0.77	1.28 ± 0.22
(Nle-Arg-Gly-Gly-NH) <sub>2</sub> -Rhodamine	4.24 ± 2.2	7.63 ± 3.5
(Cbz-Nle-Arg-Gly-Gly-NH) <sub>2</sub> -Rhodamine	0.758 ± 0.33	0.322 ± 0.045
(Cbz-Met-Arg-Gly-Gly-NH) <sub>2</sub> -Rhodamine	4.74 ± 1.06	0.846 ± 0.079
(<Glu-Gly-Arg-NH) <sub>2</sub> -Rhodamine		No hydrolysis
(Cbz-Ile-Pro-Arg-NH) <sub>2</sub> -Rhodamine		No hydrolysis

<sup>a</sup> One picomole of virus is  $6.023 \times 10^{11}$  virus particles.

more, we observed that the inhibition was reversible with DNS-GGACK, PMSF, TPCK, and TLCK if 1 mM DTT was added after the incubation with inhibitor but prior to the addition of substrate. Several classic cysteine proteinase inhibitors were also effective, the most efficient being iodoacetate (3%), dithiodipyridine (4%), and PCMB (4%). Much less inhibition was observed with E64 (32%).

### Dithiodipyridine

Both dithiodipyridine and iodoacetate can severely inhibit the virion enzyme. This implies that the active-site nucleophile may be a cysteine residue, although other interpretations are possible. The nucleophilic cysteine of papain is a thiolate anion at pH 5.0, whereas the normal  $pK_a$  for cysteine is 8.3. To see if the virion enzyme has a cysteine with an unusually low  $pK_a$ , we performed a thiol protection assay (Fig. 4A). Disrupted virions were incubated with dithiodipyridine at pH 5.0. The pH was then raised to 8.0, and iodoacetate was added. Enzyme activity was measured in the presence and absence of DTT. If there was an unprotonated cysteine at pH 5.0, then incubation with dithiodipyridine would result in the formation of a cysteine *S*-thiopyridine adduct. Iodoacetate at pH 8.0 will carboxymethylate all the cysteines with normal  $pK_a$  values. Observing full activity in the presence of DTT and none in its absence would imply the presence of a cysteine with an unusually low  $pK_a$ , because incubation of a cysteine *S*-thiopyridine adduct with DTT would result in the regeneration of the free thiolate anion concomitant with the regeneration of enzyme activity. The results (Fig. 4A) implied that the virion enzyme has a cysteine with an unusually low  $pK_a$ . A similar experiment but with papain and the substrate (Cbz-Arg-NH)<sub>2</sub>-Rhodamine (Leytus *et al.*, 1983a,b) gave similar results (Fig. 4B).

### $V_{max}$ as a function of pH

Measurement of  $V_{max}$  as a function of pH can reveal the  $pK_a$  values of amino acids involved in catalysis. With

disrupted wild-type virus we measured  $V_{max}$  from pH 5.5 to 9.0 (Fig. 5). There was about a sixfold variation. At pH 8.0 the  $V_{max}$  was 3.07 pmol substrate hydrolyzed/sec/pmol virus, whereas at pH 6.0 it was 0.49 pmol substrate hydrolyzed/sec/pmol virus. The curve of  $V_{max}$  as a function of pH implied there are at least three  $pK_a$  groups involved in determining enzyme activity.

### DISCUSSION

The assay we describe here for the Ad2 proteinase activity in disrupted virions is quick, sensitive, quantitative, and selective. About  $10^9$  disrupted virions are incubated with a Rhodamine-based substrate for 30 min and the amount of activity is immediately determined by measuring the increase in fluorescence in a fluorometer. The assay should facilitate characterization of the enzyme and a search for inhibitors.

The xanthene dye Rhodamine 110 was used as a reporting group in the substrates described here, because it is one of the most detectable compounds known (Leytus *et al.*, 1983a,b, 1984; Mangel *et al.*, 1985, 1987). In the quinone state Rhodamine exhibits an extremely high molar absorbance coefficient, greater than  $70,000 M^{-1} cm^{-1}$ , and an extremely high quantum yield, greater than 0.9. Its fluorescence intensity is maximal in the neutral to basic pH range. It absorbs maximally at 492 nm where the output from a xenon lamp is relatively high and where interference from most biological compounds is low.

One of the newer assays for a viral proteinase employing a synthetic substrate was designed for the HIV enzyme, an aspartic proteinase (Matayoshi *et al.*, 1990). The assay is based upon intramolecular fluorescence resonance energy transfer. Emission from a donor fluorophore is quenched by the proximity of an acceptor, both covalently connected by a short polypeptide containing a sequence cleaved by the proteinase. Although this substrate enables continuous assays of enzyme activity to be performed, the extinction coefficient of the fluorophore is  $5438 M^{-1} cm^{-1}$  and the quantum yield is 0.13 so that

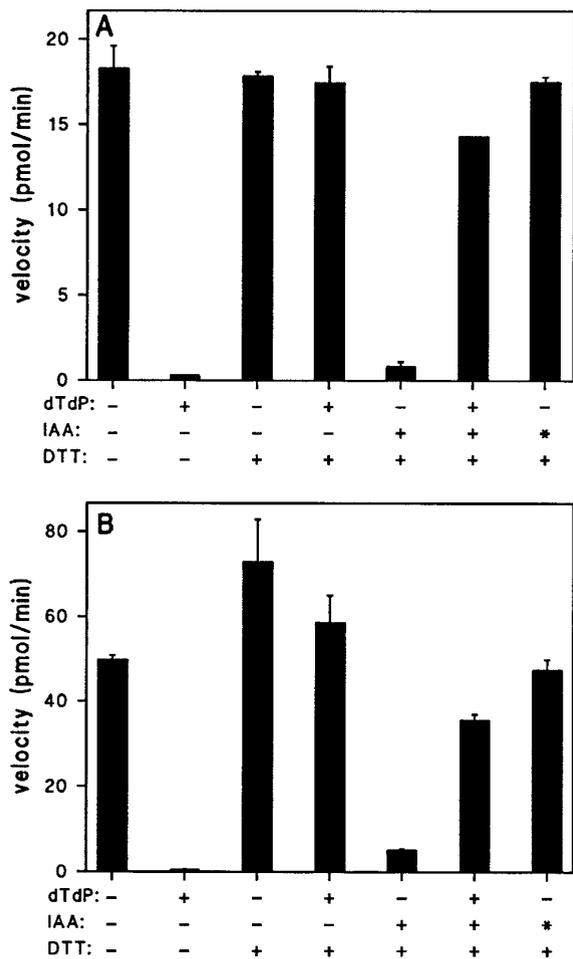


FIG. 4. Thiol protection assay: (A) adenovirus virions and (B) papain. In A, disrupted Ad2 virions ( $9.6 \times 10^{10}$ ) in 25 mM sodium acetate (pH 5.0) were incubated in the absence (-) or presence (+) of 888  $\mu$ M 2,2'-dithiodipyridine in a volume of 30  $\mu$ l. After 20 min, 10  $\mu$ l of 1 M Tris-HCl (pH 8.0) (-) or 16 mM iodoacetate in 1 M Tris-HCl (pH 8.0) (+) was added and the incubation continued for an additional 2 min. The reaction was then diluted 25-fold with 10 mM HEPES (pH 7.5) containing 10 mM octylglucoside, none (-) or 5 mM DTT (+), and 5  $\mu$ M (Leu-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine. The change in fluorescence was monitored as a function of time. In B, papain was treated as described for A for Ad2 virions. Prior to the thiol protection assay, the papain was activated by preincubation on ice with 0.5 mM DTT followed by dilution into 25 mM sodium acetate (pH 5.0) to a concentration of 20 nM. The substrate used to assay papain was (Cbz-Arg-NH)<sub>2</sub>-Rhodamine (Leytus *et al.*, 1983a,b). Disrupted virions or papain assayed in 213  $\mu$ M iodoacetate is signified (\*). All steps were performed at 37°.

the molar fluorescence enhancement is only 40-fold. The molar enhancement upon the hydrolysis of a Rhodamine-based substrate is at least 3500-fold (Leytus *et al.*, 1983a,b).

There is a chromogenic substrate for the adenovirus proteinase, LAGG4-F(NO<sub>2</sub>)RHR, that, upon cleavage after the second glycine residue, shows a decrease in absorbance at 310 nm (Weber and Tihanyi, 1994). This class of substrates exhibits a molar difference absorption coefficient at 310 nm,  $\Delta A_{310}$ , of 1700–2000  $M^{-1}$  in the pH

range 2–5 (Dunn *et al.*, 1984), and it has been stated that measurement of  $K_m$  values in the micromolar range with these substrates is difficult and imprecise, since the change in absorbance becomes too small to permit accurate kinetic measurements to be made (Dunn *et al.*, 1986). In one of our fluorimeters, the  $\Delta F/M$ , the change in fluorescence per molar concentration of mono-substituted Rhodamine, is  $1.3 \times 10^9$  in the pH range 2–10.

There are several indications that five of the Rhodamine-based substrates were cleaved by the Ad2 endoproteinase as opposed to other proteinases. The proteinase activity appeared to be highly specific and selective. Only the substrates that contained a P<sub>4</sub> Ile, Leu, Met, or Nle residue and Gly-Gly residues at the P<sub>2</sub> and P<sub>1</sub> positions were cleaved. If the proteinase activity was due to an exopeptidase, neither (Cbz-Met-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine nor (Cbz-Nle-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine should have been excellent substrates. Most important, no hydrolysis of the substrates was detected when disrupted H2ts-1 virus was used as the source of proteinase. None of the precursor proteins in H2ts-1 virus grown at the nonpermissive temperature is processed nor does processing occur if after assembly at the nonpermissive temperature purified H2ts-1 virus is incubated at the permissive temperature (Weber, 1976). Also, no hydrolysis of our substrates was detected with nondisrupted wild-type Ad2 virus or disrupted wild-type Ad2 virus heated to 90° for 90 sec (data not shown).

The substrates we synthesized based upon the adenovirus proteinase consensus sequence may interact with other proteinases. Lopez-Otin *et al.* (1989) have pointed out that Gly-Gly-X is a novel consensus sequence for the proteolytic processing at the Gly-X bond of several viral and cellular proteins. That sequence is cleaved in three structural proteins of African swine fever virus, a large, DNA-containing virus involved in a highly contagious and frequently fatal disease of domestic pigs. During the conversion of polyubiquitin to ubiquitin in humans, chickens, and yeast, a Gly-Met sequence which links several tandem repeats of the mature protein is cleaved. An interferon-induced protein in Daudi cells is processed from a 17-kDa precursor to a 15-kDa protein by cleavage at a Gly-X bond. Similarly designed Rhodamine-based substrates should be useful in characterizing other viral proteinases and in screening large numbers of potential inhibitors.

The Rhodamine-based substrates described here were used to characterize the adenovirus proteinase *in vitro*, most importantly to identify two cofactors (Mangel *et al.*, 1996). For maximal enzyme activity, three components were shown to be required—the L3 23-kDa protein (Houde and Weber, 1990; Yeh-Kai *et al.*, 1983); pVIc, the 11 amino acids at the C-terminus to the precursor of virion protein VI (Mangel *et al.*, 1993; Webster *et al.*, 1993); and the viral DNA (Mangel *et al.*, 1993).

The adenovirus proteinase has proven difficult to clas-

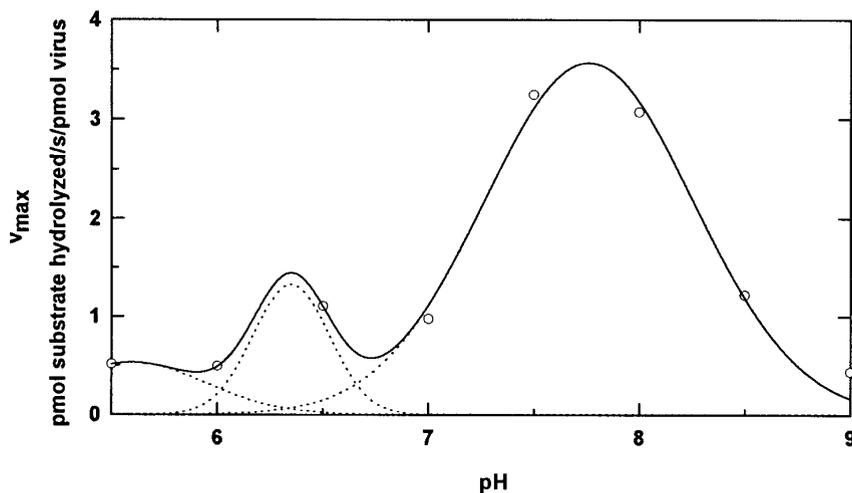


FIG. 5.  $V_{\max}$  as a function of pH. After preincubating for 5 min at  $37^\circ$   $1.23 \times 10^{11}$  disrupted Ad2 virions in 2.5 mM DTT, 10 mM octylglucoside, and 0.1 M of buffer at the indicated pH, (Leu-Arg-Gly-Gly-NH)<sub>2</sub>-Rhodamine in DMSO was added at concentrations ranging from 0.5 to 25  $\mu$ M and the rate of hydrolysis of substrate determined. The data were fitted to the Michaelis-Menten equation using the curve-fitting program in Grafit (Leatherbarrow, 1992). The units of  $V_{\max}$  are pmol of substrate hydrolyzed/sec/pmol virus. The curves were fitted using the program Peakfit (Jandel) assuming merged Gaussian peaks (dashed lines). The buffers used from low to high pH were acetate, 2-(*N*-morpholino)ethanesulfonic acid, HEPES, and Tris.

sify. The gene for the L3 23-kDa protein has been sequenced in 12 different adenovirus serotypes. The amino acid sequence is highly conserved, but it does not contain typical proteinase consensus sequences (Cai and Weber, 1993). The inhibitor profile of the Ad2 proteinase does not correspond to profiles exhibited by classical serine or cysteine proteinases. Similar inhibitor profiles were obtained by Tremblay *et al.* (1983) and Bhatti and Weber (1978, 1979a,b) using Ad2 precursor proteins as substrates and by Webster *et al.* (1989a,b) using synthetic polypeptides as substrates.

The thiol protection experiment showed that at least one of the eight cysteine residues in the L3 23-kDa protein has a low  $pK_a$ , much less than 8.3. This is because incubation with dithiodipyridine at pH 5.0 resulted in the protection at pH 8.0 of a cysteine from iodoacetate. There was no activity at pH 8.0 except in the presence of DTT which presumably converted a cysteine *S*-thiopyridine adduct to a thiolate anion. The control experiment, with similar treatment of papain, also indicated that there is a cysteine residue that is a thiolate anion at about pH 5.0.

The profile of  $V_{\max}$  as a function of pH indicated that the virion-associated enzyme is quite different from papain. Papain contains an active-site thiolate-imidazolium ion pair between His-159 and Cys-25 (Polgar, 1989). The second-order acylation rate constant,  $k_{\text{cat}}/K_m$ , 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 Cys-25 and His-159, respectively, more appropriately to the formation and the decomposition of the ion pair. Measuring the  $V_{\max}$  of the adenovirus proteinase in the virion as a function of pH gave different results.

There appeared to be at least three groups involved, with  $pK_a$  values of 6.2, 7.2, and 8.3. These groups are most likely a part of the enzyme, as opposed to the substrate. The N-terminus on the substrate has a  $pK_a$  of 9.69 and the guanidino group of arginine a  $pK_a$  of 12.48.

On the other hand, the thiol protection assays of both enzymes gave similar results. This may mean that an ion pair exists in the adenovirus proteinase, with the  $pK_a$  values for the ionization of the pair being more basic than those of papain. The differences in  $pK_a$  values for the ion pairs may reflect the different pH optima of the enzymes, pH 5.0 for papain and pH 8.0 for the adenovirus proteinase. Our assays as a function of pH only went as low as pH 5.5; at lower pH levels, enzyme activity was too low to be measured accurately. From the Gaussian fits to the data points in Fig. 5, there appears to be a fourth ionizable group with a possible  $pK_a$  of 5.2. If so, this could be the  $pK_a$  of the active-site nucleophile and the cysteine attacked at pH 5.0 in the thiol protection experiment. Alternatively, the catalytic apparatus and active site may be quite different from that of papain.

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