Different modes of inhibition of human adenovirus proteinase, probably a cysteine proteinase, by bovine pancreatic trypsin inhibitor

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Abstract The type of proteinase and the nature of the active site of the human adenovirus proteinase are unknown. For these reasons we produced an inhibitor profile of the enzyme. Enzyme activity in disrupted virions was inhibited by several serine-specific as well as cysteine-specific proteinase inhibitors. Of the inhibitors that worked, the most useful potentially in illuminating the nature of the active site was bovine pancreatic trypsin inhibitor (BPTI), and for this reason we extensively characterized the interaction with BPTI. In disrupted virions, the enzyme is irreversibly inhibited by BPTI with a K_i of 35 nM and a k_i of 6.2×10^{-4} s^{-1}. One reason enzyme activity is inhibited is that BPTI, a basic protein, precipitates the viral DNA, a cofactor of enzyme activity. In vitro with purified components, BPTI acts as a competitive inhibitor (K_i 2 μM) of the recombinant proteinase complexed with its 11-amino-acid cofactor pVIc. The recombinant endoproteinase is heat labile whereas its 11-amino-acid cofactor is heat stable. We estimate there are about 50 molecules of proteinase per virus particle.

Key words: Antiviral therapy; Bovine pancreatic trypsin inhibitor; Cysteine proteinase; Virus-coded proteinase

1. Introduction

Many animal and plant viruses contain a gene for a proteinase whose activity is vital for the synthesis of infectious virus (see [1] for a review). These virus-coded proteinases are appealing targets for antiviral therapy. Human adenoviruses encode a proteinase whose activity is required for the maturation of infectious virions. Of the 12 major polypeptides from which adenovirus virions are assembled, 6 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 precursor proteins 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) 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 [7,8]. 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 compound (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine is cleaved to Leu-Arg-Gly-Gly-NH-rhodamine by the proteinase in disrupted wild-type virus; this is accompanied by a 3500-fold increase in fluorescence that is proportional to the amount of proteinase. We used this assay to characterize proteinase activity in disrupted wild-type virus. We not only showed that when wild-type Ad2 virus was incubated with the fluorogenic substrate (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine, significant hydrolysis was observed, but when ts-1 virus was incubated with (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine, no hydrolysis was observed. Surprisingly, little or no hydrolysis was observed with purified recombinant endoproteinase (rEP) obtained from expression of the L3 23-kDa gene in *E. coli* [8]. 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. A second cofactor was shown to be a plasmin-sensitive virion protein which turned out to be the 11-amino-acid peptide from the C-terminus of the precursor to virion protein VI, pVIc [8,9].

Although we know the gene sequence for the proteinase activity [10] and have identified two cofactors that allowed us to reconstitute full proteinase activity in vitro with purified components [8], we do not yet know the type of proteinase or the nature of its active site. The DNA sequence of the gene for the Ad2 proteinase is not related to DNA sequences from any proteinase or protein in the data bases [10]. The inhibitor profile of the Ad2 proteinase does not correspond to profiles exhibited by classical serine or cysteine proteinases [7,11–16]. The adenovirus proteinase was originally thought to be a serine proteinase based upon inhibition by diisopropyl fluorophosphate [11,14,17], but more recent data, also based upon inhibitor studies, imply it is probably an unusual cysteine proteinase [15,18]. Of the inhibitors we tested that worked, the most useful potentially in illuminating the nature of the active site of the proteinase is bovine pancreatic trypsin inhib-

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; Cbz, benzyloxycarbonyl; dTdP, 2,2′-dithiodipyridine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DNS-GGACK, dansyl-L-glutamylglycyl-L-arginyl chloromethyl ketone; DTT, dithiothreitol; E-64, L-*trans*-epoxysuccinylleucylamido(4-guanidino)butane; HOAc, acetic acid; IAA, iodoacetic acid; pVIc, 11-amino-acid cofactor from the C-terminus of virion precursor protein pVI; PAGE, polyacrylamide gel electrophoresis; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethanesulfonyl fluoride; <Glu, pyroglutamic acid; rEP, recombinant endoproteinase; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; TLCK, L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone HCl; TPCK, L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone; ts-1, temperature-sensitive mutant H2ts-1

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itor (BPTI). Also, we had plans to convert the reactive-site sequence of BPTI from one recognized by trypsin to one recognized only by the adenovirus proteinase and to use this mutant BPTI as an antiviral agent. For these reasons, we undertook a more detailed characterization of the interaction of the adenovirus proteinase activity with BPTI.

2. Materials and methods

2.1. Materials

Tris, HEPES, dithiothreitol (DTT), 2,2′-dithiodipyridine, bovine pancreatic trypsin inhibitor, 5,5′-dithiobis(2-nitrobenzoate) or Ellman's reagent (DTNB), and iodoacetate were purchased from Sigma (St. Louis, MO). Sephadex G-10 was bought from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). All other proteinase inhibitors as well as octylglucoside were from Boehringer Mannheim (Indianapolis, IN). The pVIc peptide was obtained from Multiple Peptide Systems (San Diego, CA).

2.2. 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 in Anderson [6]. To prepare disrupted virus, we dialyzed twice CsCl banded virions against 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and then centrifuged the suspension at 100 000×g for 1 h. The pellet was then either 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 or the pellet was suspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and placed in a 56°C water bath for 5 min before being stored at −20°C. Virus disrupted by low pH and sonication or by heat exhibited similar amounts of proteinase activity.

2.3. Assay of disrupted virions

The standard assay was performed at 37°C in 0.8 ml assay buffer which contained 0.01 M HEPES (pH 7.5), 0.01 M octylglucoside, 2.5 mM DTT, 2–5 μM (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine as substrate, and the indicated amount of disrupted virus. The increase in fluorescence was 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, ΔF, 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 ΔF, the number of pmol of substrate hydrolyzed was calculated using a molar fluorescence coefficient obtained from a mono-substituted rhodamine derivative of known concentration. No more than 5% of the substrate was hydrolyzed in any assay.

2.4. 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 E-64 in 50% ethanol. Iodoacetic acid was freshly prepared in 50 mM sodium bicarbonate (pH 9.0). PCMB was dissolved in 10 mM NaOH to a concentration of 20 mM. TLCK was dissolved in 1 mM HCl and TPCK in absolute ethanol. PMSF was added as a solid. Inhibitor at the indicated concentration and disrupted virus, 10^{10} particles, were incubated in 30 μl of inhibitor buffer for 10 min at 37°C. Then, 10 μl of 20 μM (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine was added. After 30 min, 30 μl of the reaction were added to 670 μl 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.

2.5. Protein concentration

Protein concentrations were determined by the BCA protein assay from Pierce (Rockford, IL). The concentration of rEP was also obtained by absorbance with a calculated molar absorbance coefficient at 280 nm of 26 510 [19]. 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 carried out by adding 10 μl of stock pVIc solution to 0.99 ml of Ellman's buffer, 0.1 M NaH_2PO_4 (pH 7.3) and 1 mM EDTA, containing 0.33 mM DTNB and then monitoring the increase in absorbance at 412 nm. The mol SH/mol pVIc was calculated using a molar extinction coefficient at 412 nm of 14 150 for thionitrobenzoate [20].

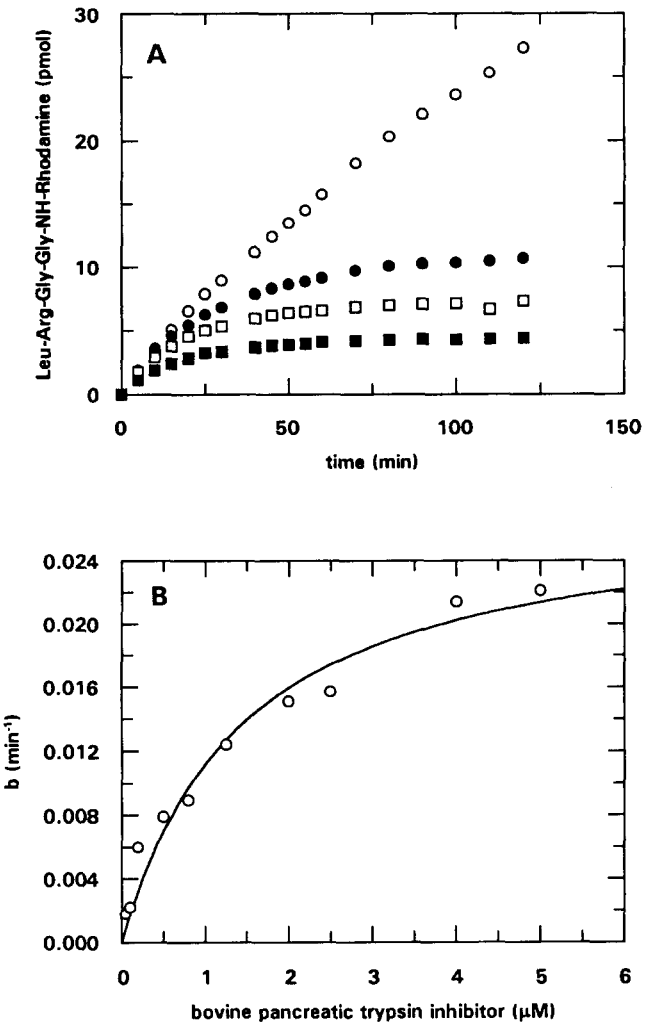

Fig. 1. Competition between bovine pancreatic trypsin inhibitor and (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine for the proteinase of disrupted wild-type Ad2 virus (A) and determination of the macroscopic (K_i) and microscopic (k_i) inhibitory constants (B). In (A) reaction mixtures contained 0.8 ml assay buffer, 5 μM (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine and the following concentrations in μM of BPTI: 0 (○), 1 (●), 2 (□) and 4 (■). Reactions were initiated at 25°C by the addition of 0.1 ml solutions containing 9.2×10^{10} freshly disrupted virus. The increase in fluorescence, ΔF, was determined at the indicated times. In (B) the data in (A) plus additional data obtained at the indicated concentrations of BPTI were analyzed according to the method of Leytus et al. [23]. Tangents to the presteady-state portions of the curves were calculated, and the natural logarithm of the tangents when plotted versus time yielded a straight line whose slope, b, was a pseudo first-order rate constant. A plot of b versus the inhibitor concentration yielded the rectangular hyperbola from which the inhibitory constants of the Ad2 proteinase listed in Table 2 were derived.

2.6. Reduction and carboxyamidomethylation of BPTI

BPTI, 5 mg in 3 ml of 0.1 M ammonium bicarbonate (pH 9.0) containing 8 M urea and 10 mM DTT, was incubated at 37°C for 1.5 h to reduce the 3 disulfide bonds. Then, iodoacetamide to 50 mM was added followed by incubation for 2 h at room temperature in the dark. The resultant reduced, carboxyamidomethylated BPTI was pur-

ified from excess reagents by passage through a Sephadex G-10 column equilibrated in 0.3 M acetic acid. The flowthrough was pooled, protein concentration determined by the BCA protein assay, and the reduced, carboxyamidomethylated BPTI stored at −20°C.

3. Results and discussion

3.1. 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 (Table 1). Disrupted virus and inhibitor were preincubated for 10 min at 37°C and then (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine was added and the amount of proteinase activity determined. Benzamidine, bestatin, EDTA, leupeptin and phosphoramidon had no effect. Several classic serine proteinase inhibitors were effective: from most efficient to least were DNS-GGACK, PMSF, TPCK, TLCK, SBTI and BPTI. However, some of these compounds can also inhibit cysteine proteinases [21]. Furthermore, we observed 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, dithiodipyridine, and PCMB. Much less inhibition was observed with E-64. Similar inhibitor profiles have been obtained using Ad2 immature proteins as substrates [11–14] or using synthetic polypeptides as substrates [15,16].

3.2. Bovine pancreatic trypsin inhbitor

The interaction between BPTI and certain serine proteinases is well characterized. The inhibitory specificity is mainly restricted to enzymes with trypsin-like substrate specificity and trypsin-like tertiary structures in the vicinity of the active site [22]. Chymotrypsin is an exception in that while its substrate specificity is different from that of trypsin, its active-site configuration is similar. Complex formation between BPTI and trypsin can be characterized by a perfect fit of the reactive-site

Table 1
Effect of proteinase inhibitors on the proteinase activity of disrupted Ad2 virions with the substrate (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine[a]

Inhibitor	Relative activity
None	1.00
1 mM 2,2′-dithiodipyridine	0.04
3 μM E-64	0.32
1 mM iodoacetate	0.03
0.5 mM PCMB	0.04
1 mM DNS-GGACK	0.02
135 μM TLCK	0.41
284 μM TPCK	0.24
1 mM benzamidine	1.00
2.2 μM leupeptin	1.00
5 mM PMSF	0.19
85 μM SBTI	0.68
22 μM BPTI	0.69
1.3 mM bestatin	0.96
5 mM EDTA	1.10
5.2 mM phosphoramidon	0.88

[a][7].

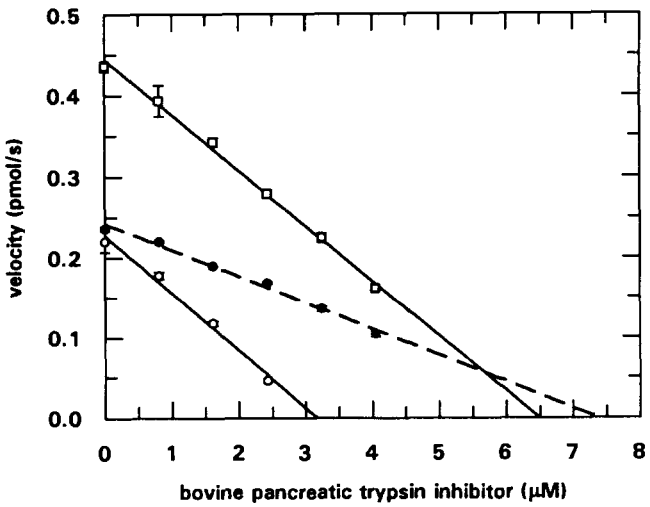

Fig. 2. Titration of Ad2 virion proteinase by BPTI. Heat-disrupted virus, 4.75×10^{10} (○) or 9.5×10^{10} virions (□), were incubated with the indicated concentrations of BPTI in 50 μl of 0.01 M HEPES (pH 7.5), 0.01 M octylglucoside, and 2.5 mM DTT. After 40 min at 37°C, 40 μl of each reaction was diluted into 1 ml of the same buffer but containing 5 μM (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine and the increase in fluorescence monitored as a function of time. A control was assays (● - - ●) of a mixture of 4.75×10^{10} disrupted wild-type virus particles and 4.75×10^{10} wild-type virus particles whose proteinase activity had been inactivated by heating the virions at 80°C for 90 s.

inhibitor residue (a lysine or arginine) in the specificity pocket of the enzyme. This results in a stable complex that is in an intermediate state between the initial Michaelis-Menten complex and the tetrahedral transition state. Cleavage of the scissile bond is hindered by the tight packing of the catalytic residues, especially His^{57}, so that proton transfer, a necessary step for acyl-enzyme formation, cannot take place. Because the interaction between BPTI and trysin is so well characterized, we decided to characterize further the interaction of BPTI with the adenovirus proteinase. Our hope was that the studies would reveal the type of proteinase and the nature of the active site.

3.3. Inhibition by BPTI of the virion enzyme

We quantitatively characterized the interaction of BPTI with the Ad2 proteinase activity in the virus particle. Disrupted virus was incubated with (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine and three different concentrations of inhibitor (Fig. 1A). In the presence of BPTI, the initial change in fluorescence with time was not linear which implied that BPTI was not a reversible, competitive inhibitor. After about 40 min, the change in fluorescence reached a plateau. Thus BPTI acted as an irreversible inhibitor of the Ad2 proteinase.

We used a theory and experimental method we devised for determining individual kinetic constants of fast-acting, irreversible proteinase inhibitors to characterize the interaction between BPTI and the adenovirus proteinase [23]. The theory is based upon formal analysis of the case of an irreversible inhibitor competing with a substrate for the active site of a proteinase. From the theory, an experimental method was devised by which the individual microscopic kinetic constants for the interaction can be determined. Using the theory, we analyzed the data in Fig. 1A and that from two other inhibitor concentrations. Tangents to the presteady-state portions

of the curves were calculated, and the natural logarithm of the tangents when plotted versus time yielded a straight line whose slope was a pseudo first-order rate constant, b. A plot of b versus the inhibitor concentration is shown in Fig. 1B.

The curve in Fig. 1B is a rectangular hyperbola which indicates inhibition by BPTI obeys the following reaction sequence:

$$E + I \overset{K_i}{\leftrightarrow} E{\cdot}I \overset{k_i}{\rightarrow} EI'$$

where E is the Ad2 proteinase, I is BPTI, E·I is an adsorptive enzyme-inhibitor complex, EI′ is a stable, enzyme-inhibitor complex, K_i is an equilibrium dissociation constant, and k_i is a first-order rate constant. From the rectangular hyperbola in Fig. 1B, values for the inhibitor constants were calculated and are shown in the Table 2. The K_i of BPTI with trypsin is 6 orders of magnitude lower than with the Ad2 proteinase activity in the virion.

3.4. Titration of the amount of proteinase per virion

Since BPTI is an irreversible inhibitor of the virion proteinase activity, we should be able to use BPTI to titrate the number of active proteinase molecules per virion. To do this, we incubated three concentrations of disrupted virus particles with various concentrations of BPTI and, after 40 min, assayed for residual enzyme activity (Fig. 2). Extrapolation to the abscissa of the data for the reactions with 4.75×10^{10} virus particles occurred at 3.2 μM BPTI. Since this corresponds to 9.64×10^{13} molecules of BPTI for total inhibition, this meant that there were $9.64\times10^{13}/4.75\times10^{10}$ or about 2000 molecules of enzyme per virion. Doubling the amount of virus in the assay doubled the value extrapolated to on the abscissa, to 6.4 μM BPTI.

This number for the amount of enzyme per virion is considerably higher than the one other estimate. In an attempt at quantitative immunoblotting with known standards, the number of L3 23-kDa protein molecules per virion was estimated to be between 5 and 13 [6]. Therefore, we decided to estimate the amount of L3 23-kDa protein indirectly by estimating how much free pVIc there is per virion. There are 340 molecules of pVI per virion [24]. To determine how much free pVIc there is per virion, we measured the amount of proteinase activity in 1 and 2×10^{10} virions (Fig. 3A,B) and then measured the amount of proteinase activity in the same number of virions but after the addition of 2.2 nM rEP. Cofactor activity was in excess over the L3 23-kDa protein activity by about 5-fold. If one assumes that all of the L3 23-kDa protein in the virion are complexed with pVIc in a 1:1 ratio [25,26] and that after the addition of rEP all pVI molecules become processed to pVIc [27], then there are 340/5 = 68 molecules of the L3 23-kDa protein per virion.

Because the titration by BPTI of the amount of proteinase per virion was much higher than that obtained by two other methods, we performed an additional control experiment. We repeated the titration experiment but with a mixture of 4.75×10^{10} wild-type virus particles and an equal number of wild-type virus particles whose proteinase activity had been inactivated by heating at 80°C for 90 s. The L3 23-kDa protein in the virus particle is heat sensitive (Fig. 3D), although pVIc in the virus particle can survive for 5 min in a boiling water bath (Fig. 3C). We had expected to see a curve identical to that with 4.75×10^{10} wild-type virus particles alone. Although the line extrapolated to the same point on the ordinate, it extrapolated to slightly more than twice the BPTI concentration on the abscissa (Fig. 2). That the line extrapolated to the same point on the ordinate indicated the heat-inactivated enzyme was indeed inactivated. That the line extrapolated to double the point on the abscissa indicated that BPTI was binding to something other than fully functional active sites in the enzyme.

So far, the experiments with BPTI had yielded results that were difficult to interpret. On the one hand, inhibition appeared to be irreversible with a K_i of 35 nM and a k_i of 6.2×10^{-4} s^{-1}. This implied that BPTI was binding to the active site. On the other hand, in the experiments with heat-inactivated proteinase in disrupted virions, the results implied that BPTI was binding to a component in virions other than the active-site of the proteinase. Of course, the results could be interpreted to indicate that BPTI can bind irreversibly to the active-site of heat-inactivated proteinase.

3.5. Inhibition by BPTI of the enzyme in vitro

Because we had been able to reconstitute proteinase activity in vitro with purified components, we decided to characterize the interaction of BPTI with the enzyme activity in vitro. So, we repeated the experiments we had performed with disrupted virions, but with rEP, pVIc and the viral DNA. We again observed irreversible inhibition of proteinase activity (data not shown). However, we noticed that some of the assay solu-

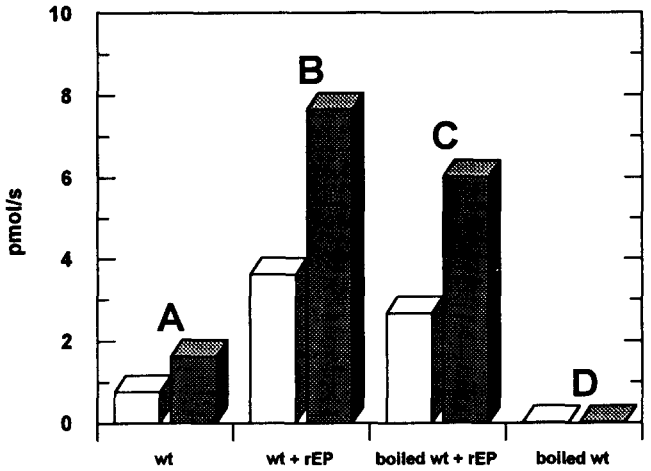

Fig. 3. Identification of the limiting cofactor for proteinase activity in wild-type Ad2 virus and the factor most heat labile. In (A) 1 and 2×10^{10} virus particles were assayed. In (B) 1 and 2×10^{10} virus particles were assayed in the presence of 2.2 nM rEP. In (C) 1 and 2×10^{10} wild-type virus particles in 10 μl of assay buffer were incubated in a boiling water bath for 5 min prior to the addition of rEP (to 2.2 nM) with the substrate. In (D) 1 and 2×10^{10} virus particles were assayed for proteinase activity after the virus was incubated in a boiling water bath for 5 min. 1×10^{10} virus particles (clear bar); 2×10^{10} virus particles (solid bar).

Table 2
Comparison of the interaction of bovine pancreatic trypsin inhibitor with the Ad2 proteinase and bovine trypsin

	K_i (M)	k_i (s^{-1})
Ad2 proteinase	3.5×10^{-8}	6.2×10^{-4}
Bovine trypsin[a]	8.7×10^{-14}	8.7×10^{-10}

[a][23].

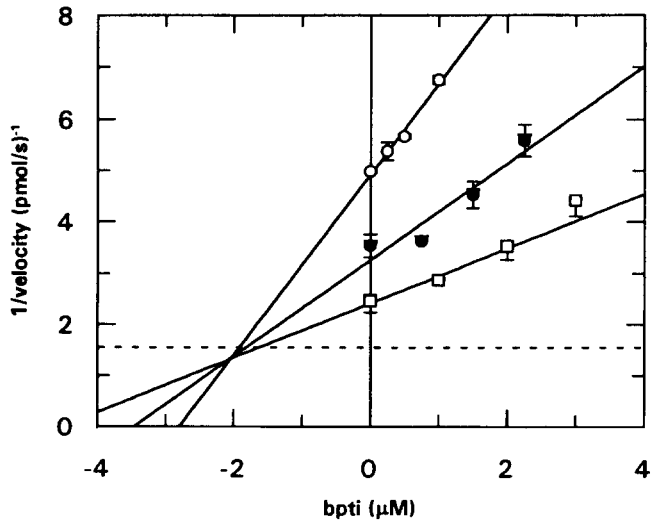

Fig. 4. Competitive inhibition by BPTI. In 0.01 M HEPES (pH 7.5) and 5 mM octylglucoside were incubated 1 μM rEP and 2 μM pVIc. After 5 min at 37°C, the reactions were diluted 40-fold into 1 ml of 10 mM HEPES (pH 7.5), 5 mM octylglucoside, and either 0.5 μM (○), 1.0 μM (●) or 2.0 μM (□) (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine, and the indicated concentrations of BPTI, and the increase in fluorescence monitored as a function of time. The dashed line parallel to the abscissa is $1/V_{max}$.

tions were cloudy. After centrifugation, the assay solutions became clear. The precipitate contained much of the viral DNA. In hindsight this was to be expected. BPTI is a basic protein; its p*I* is 10.5 [28]. Addition of BPTI to 2 μM to a solution of rEP, pVIc or Ad2 DNA resulted in cloudiness only with the solution of Ad2 DNA. These observations opened the possibility that BPTI inhibits proteinase activity by removing the cofactor, the viral DNA, from solution. If this were the case, the type of inhibition would indeed appear irreversible.

If BPTI inhibits the adenovirus proteinase because it precipitates the DNA cofactor, then it should not inhibit the activity of rEP-pVIc complexes in the absence of DNA. However, it did, and in this case the type of inhibition was not irreversible (data not shown). Therefore, we varied substrate and inhibitor concentrations. The data are presented in a Dixon plot (Fig. 4). Because the three lines intersected above the negative region of the abscissa, the mode of inhibition is competitive. Thus, with rEP-pVIc complexes, BPTI is a competitive inhibitor with a K_i of 2 μM. As a control, we reduced and carboxyamidomethylated BPTI. Although reduced and carboxyamidomethylated BPTI no longer inhibited trypsin, it was still a competitive inhibitor of the enzyme activity of rEP-pVIc complexes (data not shown).

How is BPTI acting as a competitive inhibitor? A systematic study of the cleavage of octapeptides by the virion enzyme and molecular modeling indicated that the main determinants of the adenovirus proteinase specificity were the amino acids at the P4 position, either Ile, Leu or Met, the P2 position, Gly, and the P_1' position where the side chain of the amino-acid residue must be orientated away from the scissile bond [15,16]. Analysis of the sequence of BPTI reveals two potential adenovirus proteinase sequence sites: Val^{34}-Try^{35}-Gly^{36}-Gly^{37} and Thr^{54}-Cys^{55}-Gly^{56}-Gly^{57} [22]. Inspection of the structure of BPTI reveals one site is just below the reactive-site loop and the other at the C-terminus. In both regions, the Gly-Gly residues are on the surface of the molecule. If the adenovirus proteinase binds to either of these sites, that would render BPTI a competitive inhibitor of enzyme activity. We see no evidence that BPTI is cleaved upon incubatation with the adenovirus proteinase. This was to be expected as one outstanding property of BPTI is its uncommon stability to proteolytic degradation by other proteinsase [22]. BPTI is known to have antimicrobial and antiviral activities [29]; the observations reported here may be relevent as to how BPTI may accomplish this.

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