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Identification of active-site residues of the adenovirus endopeptidase

(cysteine proteinase/mutants)

CLAUDINE RANCOURT, KAROLY TIHANYI, MARTIN BOURBONNIERE, AND JOSEPH M. WEBER*

Department of Microbiology, Faculty of Medicine, University of Sherbrooke, Sherbrooke, PQ, Canada J1H 5N4

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ABSTRACT **Multiple sequence alignment of the 12 adenovirus endopeptidases known to date identified a number of conserved residues which might be important for enzyme activity. Eleven mutants were created in the cloned gene by site-directed mutagenesis to identify the active site of this thiol endopeptidase. Analysis of the proteolytic activity in a crude system using viral precursor proteins, as well as in a purified system with activated proteinases using a new chromophoric octapeptide substrate, yielded results consistent with Cys-104 and His-54 being two members of the active site. This result was confirmed by the carboxymethylation of the reactive Cys-104 and its prevention by the active-thiol-specific agent E64. Although Cys-122 and Cys-126 were also reactive cysteines, mutation of these residues did not affect enzyme activity. Replacement of the active-site Cys-104 by serine converted the enzyme into a serine-like proteinase, sensitive to serine proteinase inhibitors. The absence of homology to other proteinases, particularly at the active-site cysteine, coupled with the requirement for activation by a substrate cleavage fragment, indicates that the adenovirus endoproteinase may represent a new subclass of cysteine proteinases.**

Biological control through sequence-specific proteolytic cleavages is a commonly encountered mechanism in biology. Adenoviruses, like many complex viruses, encode an endopeptidase which is required for virion maturation and infectivity. This was initially demonstrated by the isolation of the ts1 temperature-sensitive mutant of adenovirus type 2; this mutant is defective for proteolytic cleavages at the nonpermissive temperature, and the mutation maps to the L3 23-kDa protein (1, 2). Formal proof of the proteinase came with the demonstration of the *in vitro* cleavage of viral precursor proteins with enzyme purified after expression in *Escherichia coli* (3, 4). The gene has been sequenced in 12 different adenovirus serotypes, and although the amino acid sequence is highly conserved, it lacks typical proteinase motifs and shows no homology to known proteins (5). The enzyme is a monomer and it shows no changes at its N terminus or in its molecular weight, such as many other proteinases show during activation (4, 6). The substrate specificity of the enzyme has been defined as (M,L,I)XGG-X or (M,L,I)XGX-G (4, 7, 8), and inhibitor studies suggest it to be a member of the cysteine family of proteinases (4, 6, 7). Recently it was shown that full enzyme activity requires a peptide cofactor which may be an 11-amino acid cleavage product from the C terminus of the viral PVI protein (9, 10). One report also suggested that DNA and other negatively charged polymers enhance enzyme activity (10).

The adenovirus proteinase is encapsidated during virus assembly, and it is thought that the precursor proteins are cleaved during or after virus assembly. All of the six currently known substrate proteins—pIIIa, pTP, p11K (also known as pmu), PVI, PVII, and PVIII—are present in virions. The requirement for the peptide cofactor may be a critical step in delaying enzyme activity until virus assembly. Although the precise function of these maturation cleavages remains unknown, studies with ts1 have shown that cleavage is not required for virus assembly, but such virions are not infectious because they fail to uncoat (1, 11). Therefore one possible function of maturation cleavages is the preparation of virions for uncoating in a subsequent infection.

This paper attempts to examine the nature of the active-site residues by genetic and biochemical approaches. Identification of the active-site residues is crucial not only for the classification of the enzyme but also for the identification of potential inhibitors which might be appropriate for chemotherapy of adenovirus infections.

MATERIALS AND METHODS

Plasmids and Bacterial Strains. The endopeptidase coding sequence was subcloned in pLAM, a modified pRIT2T expression vector under the control of the P_L promoter (6). Recombinant plasmids were transfected and selected on *E. coli* N99CI$^+$ cells, which express the wild-type λ CI repressor constitutively. The selected plasmids were then transferred into *E. coli* AR120 cells for expression. AR120 is derived from N99 and is CI$^+$ (12). Induction through the inactivation of CI$^+$ can be promoted with nalidixic acid (80 μg/ml) or via the SOS pathway when cells attain high density (12). The pELF expression vector was constructed by fusion of the 808-bp *Bgl* II–*Pvu* II fragment of pLAM containing the pRIT2T inducible expression system to the 2518-bp *Bam*HI–*Pvu* II fragment of pSVK3 (Pharmacia) which contains the phage f1 origin of replication.

DNA Manipulations and Cloning Procedures. DNA preparation, enzyme reactions, and bacterial transformations were as described (13). Restriction endonucleases were purchased from Pharmacia LKB.

Site-Directed Mutagenesis. Single base changes were introduced into the endopeptidase coding region by oligonucleotide-directed mutagenesis. The mutagenic oligonucleotide was annealed to single-stranded M13mp18 DNA containing the *Hin*cII-J fragment of adenovirus type 2 (nucleotides 21,453–22,835). Mutant candidates were selected by dot blot hybridization using the mutagenic oligonucleotide as probe. All mutants were verified by sequencing the region of the mutation, and the entire endopeptidase open reading frame for those mutants which were inactive. To facilitate this task, all mutants were subcloned in the pELF expression vector.

Proteinase Assays. *Protein substrate assay.* Proteinase activity was measured by the cleavage of core protein PVII to VII as detected by SDS/PAGE followed by autoradiography (6, 14). Quantitation was by densitometry of appropri-

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*To whom reprint requests should be addressed.

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                                                    26
H40         ......MGSS EQELvAIVRE LGCGPYFLGT FDKRFPGFma PHKLACAIVN
H41         ......MGSS EQELvAIaRD LGCGsYFLGT FDKRFPGFma PnKLACAIVN
H12         ......MGSS EQELtAIVRD LGCGPYFLGT FDKRFPGFVS rdrLsCAIVN
H2          ......MGSS EQELkAIVkD LGCGPYFLGT YDKRFPGFVS PHKLACAIVN
H3          mtcgsgnGSS EQELkAIVRD LGCGPYFLGT FDKRFPGFma PdKLACAIVN
H4          ....maaGSg EQELRAIIRD LGCGPYFLGT FDKRFPGFma PHKvACAIVN
Bav3        ......MGSr EeELRfIlhD LGvGPYFLGT FDKhFPGFIS kdrMsCAIVN
Cav1        ...maegGSS EeELRAIVRD LavtPFFLGT FDKRFPGFIS sqritCAVVN
Mav1        ......MGSS EtELRqlVaD LGiGs.FLGi FDKhFPGFIS vnKpACAIVN
Aav1        .....MsGtt EtqLRdllss MhlrhrFLGv FDKsFPGFld PHvpAsAIVN
Bav7        .....MsGlS EkEvflllss LqCthgFLGT FDcRFPGFIn kvKvqtAIIN
Consensus         MGSS EQELRAIVRD LGCGPYFLGT FDKRFPGFIS PHKLACAIVN

                  54                  67
H40         TAGRETGGVH WLALAWNPkn rTCYLFDPFG FSDeRLKQIY QFEYEGLLkR
H41         TAGRETGGVH WLALAWNPkS hTCYLFDPFG FSDeRLKQIY QFEYEGLLkR
H12         TAGRETGGVH WLAFgWNPkS hTCYLFDPFG FSDQRLKQIY QFEYEsLLRR
H2          TAGRETGGVH WMAFAWNPrS kTCYLFEPFG FSDQRLKQVY QFEYEsLLRR
H3          TAGRETGGeH WLAFgWNPry nTCYLFDPFG FSDeRLKQIY QFEYEGLLRR
H4          TAGRETGGeH WLAFAWNPrS nTCYLFDPFG FSDQRLKQIY QFEYEGLLRR
Bav3        TAGRETGGVH WLAMAWhPaS qTfYMFDPFG FSDQkLKQIY nFEYqGLLkR
Cav1        TAGRETGGVH WLAMAWNPrS kTfYMFDPFG FSDskLKQVY sFEYEGLLRR
Mav1        TAsRETGGVH WLAMAWyPtS sTfYLFDPFG FSDrkLqQVY kFEYErLLkR
Aav1        TgsRasGGmH WigFAFdPaa grCYMFDPFG WSDQkLwelY rvkYnaFMRR
Bav7        TgpREqGGIH WiALAWdPkS yqmFiFDPLG WkndqLmkyY kFsYsnLikR
Consensus   TAGRETGGVH WLAFAWNP-S -TCYLFDPFG FSDQRLKQIY QFEYEGLLRR

                104                 122                 ↓ts1
H40         SALASTPDHC ITLIKSTQT. ....VQGPFS AACGLFCCMF LHAFVnWPts
H41         SALASTPDHC ITLVKSTQT. ....VQGPFS AACGLFCCMF LHAFIhWPsN
H12         SALAaTkDRC VTLeKSTQT. ....VQGPFS AACGLFCCMF LHAFthWPdh
H2          SAiASsPDRC ITLeKSTQs. ....VQGPnS AACGLFCCMF LHAFanWPqt
H3          SALA.TkDRC ITLeKSTQs. ....VQGPrS AACGLFCCMF LHAFVhWPdr
H4          SALA.TkDRC VTw.KShQTc rvrvgrcgFS AACstaCa.. ......WPt.
Bav3        SALtSTaDRC lTLIqSTQs. ....VQGPnS AACGLFCCMF LHAFVrWPlr
Cav1        SAiASTPDRC VTLaKSneT. ....IQGPnS AACGLFCCMF LHAFVnWPdN
Mav1        SAvsSssskC VTLVKShQT. ....VQGPhS AACGLFCvLF LaAFgkYPqN
Aav1        tgL.rqPDRC fTLVrSTea. ....VQcPcS AACGLFsaLF ivsFdrYrsk
Bav7        SAL.SsPDkC VkvIKnsQs. ....VQctca gsCGLFCvFF LycFykYksN
Consensus   SALASTPDRC VTL-KSTQT- ----VQGPFS AACGLFCCMF LHAFV-WP-N

                        160
H40         PMErNPTMDL lTGVPNSMLQ SPQVvPTLRh NQERLYRFLa qrSPYFqrHc
H41         PMEqNPTMDL lTGVPNSMLQ SPQVePTLRR NQERLYRFLt qHSPYFRrHR
H12         PMDkNPTMDL lTGVPNcMLQ SPQVvgTLqR NQneLYkFLN slSPYFRhnR
H2          PMDhNPTMnL iTGVPNSMLn SPQVQPTLRR NQEqLYsFLe rHSPYFRSHs
H3          PMDgNPTMkL vTGVsNSMLQ SPQVQPTLRR NQEvLYRFLN tHSsYFRSHR
H4          PMDkNPTMnL lTGVPNgMLQ SPQVePTLRR NQEaLYRFLN sHSaYFRSHR
Bav3        aMDNNPTMnL ihGVPNnMLe SPssQnvFlR NQqnLYRFLr rHSPhFvkHa
Cav1        PfnhNPTMgp lksVPNykLy dPtVQhvLWe NQEkLYkFLe KnSaYFRaHa
Mav1        PMnNNPiMgp ieGVPNdqMf nPcytkTLYR NQqWvYsYLN KnSlYFRlHv
Aav1        PMDgNPviDt vvGVkhenMn SPpyrdiLhR NQERtYyWWt KnSaYFRaHq
Bav7        afkNclfqsL ygsIPs...l tPpnptnLhk NQDFLYkFFk ekSlYFRqne
Consensus   PMDNNPTMDL -TGVPNSMLQ SPQVQPTLRR NQERLYRFLN KHSPYFRSHR

H40         ERIkKATAFD qMKNNM.... .....   205
H41         ERIEKATAFD qMKNaqVlfh nkify   214
H12         ERIEKATsFt KMqNgLk... .....   206
H2          aqIrsATsFc hLKNm..... .....   204
H3          aRIErATAFD rMdmq..... .....   209
H4          aRIEKATAFD rMnqdM.... .....   201
Bav3        aqIEadTAFD KMltN..... .....   204
Cav1        aaIktrTAFn KLKq...... .....   206
Mav1        ElIkKnTAFD KLlvrk.... .....   204
Aav1        EelrreTALn aLpeNhV... .....   206
Bav7        EyIvsnTkig liKshi.... .....   202
Consensus   ERIEKATAFD KMKNNMV--- -----  202
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FIG. 1. Multiple sequence alignment of the human (H), bovine (Bav), canine (Cav), murine (Mav), and avian (Aav) endopeptidases. The alignment was constructed by the PILEUP program and displayed with PRETTY (University of Wisconsin Genetics Computer Group package). Numbering is according to the adenovirus type 2 (H2) sequence. As adenovirus type 5 differs from adenovirus type 2 only in having a histidine instead of an arginine position 63, it is not included. Conserved residues are shown in uppercase letters, nonconserved in lowercase letters. For easier reference, some of the mutagenized positions are numbered. The two active-site residues His-54 and Cys-104 are boxed in. The location of the adenovirus type 2 ts1 mutation (P137L) is indicated.

ately exposed autoradiograms. The substrate consisted of [^{35}S]methionine-labeled purified disrupted adenovirus 2 ts1-39°C virions, as described before (14). The reaction mixture (40 μl) contained 20 μl of substrate, various amounts of enzyme, and buffer (0.1 M Tris·HCl, pH 7.5).

Peptide substrate assay. The reaction mixture contained 5–40 nM purified proteinase in 0.1 M phosphate buffer at pH 6.5. The proteinase was activated by preincubating the enzyme with a 100- to 200-fold molar excess of dimerized peptide pVI-c (GVQSLKRRRCF) for 60 min. At this time 1 mM dithiothreitol and 1 mM octapeptide substrate [LAGG-(4-nitrophenylalanine)-RHR] were added to start the reaction. The decrease in absorbance was monitored at 310 nm (15). The reaction was linear for 15 min. The K_m was 0.17 mM for the purified wild-type enzyme.

Western Blotting. Proteins separated by SDS/PAGE gels were electroblotted onto nitrocellulose Hybond C-extra membranes (Amersham) as described (16), and antigen–antibody complexes were detected with protein A labeled with ^{125}I by the chloramine-T method.

Purification of the Endopeptidase. Bacteria (AR120) bearing the pLPV expression vector were harvested from 1 liter of medium and suspended in 3 volumes of 20 mM Tris·HCl buffer (pH 8.5) containing 10% (vol/vol) glycerol, 10 mM 2-mercaptoethanol, and 0.5 mM EDTA (buffer A) with lysozyme added at a final concentration of 30 μg/ml. Inclusion

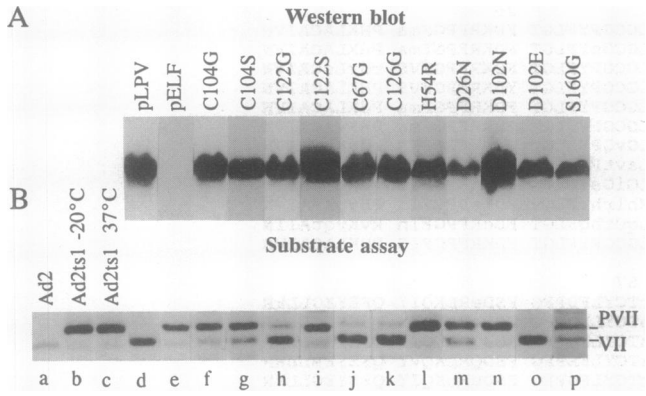

FIG. 2. Expression and proteolytic activity of mutant endopeptidases. (*A*) The proteinase mutants were expressed in the pLPV vector in *E. coli*, and the supernatants of cell lysates were assayed for expression by Western blotting and staining with a polyclonal antiserum to the endoproteinase. (*B*) The same supernatants were also tested for enzyme activity by SDS/PAGE monitoring of the cleavage of viral precursor protein PVII to VII of ts1 virions after incubation at 37°C for 18 hr. An autoradiogram of the SDS/polyacrylamide gel is shown. Lanes: a, protein VII of wild-type adenovirus type 2; b and c, ts1 substrate before and after incubation; d–p, ts1 substrate incubated with the wild-type enzyme (pLPV), the control lysate of *E. coli* bearing the vector without insert (pELF), and the mutant lysates.

bodies were sedimented by centrifugation at 10,000 × *g* for 15 min and the supernatant was kept. The sedimented inclusion bodies were dissolved in buffer A (2 volumes) saturated with urea and then diluted slowly with the supernatant to promote refolding.

DEAE-Sephacel batch treatment. The above solution was batch treated with 10 ml of DEAE-Sephacel equilibrated with buffer A. The support was washed twice with 20 ml of buffer A and the wash was combined with the batch-treated solution.

Hydroxyapatite batch treatment. The DEAE-Sephacel-treated solution was added to 15 ml of hydroxyapatite equilibrated with 20 mM phosphate buffer (pH 6.5) containing 10% glycerol, 10 mM 2-mercaptoethanol, and 0.5 mM EDTA (buffer B). After the hydroxyapatite was washed three times with buffer B, the proteins were eluted by stepwise washing with increasing concentrations of phosphate in buffer B. The protease was eluted at 150–200 mM phosphate.

Carboxymethylcellulose batch treatment. The protease-containing solution was added to 20 ml of carboxymethylcellulose equilibrated with buffer B. After 1 hr of shaking at 4°C, the resin was washed three times with buffer B. The protease was eluted with 0.1 M Tris·HCl buffer (pH 8.5) containing 0.1 mM dithiothreitol and 0.5 mM EDTA. After the eluted pure protease was concentrated with a Centricon 10 microconcentrator, 10% glycerol was added to the protease solution. Purity was estimated at >98%.

SDS/PAGE. Electrophoresis was carried out in 10% or 12.5% polyacrylamide gels. The stacking gel concentration was 3%. The sample treatment and buffer composition were those of Laemmli (17).

Affinity Labeling. Iodo[2-^{14}C]acetate (2.5 μCi; 50–60 mCi/mmol; 1 Ci = 37 GBq) was added to various amounts of proteinase and incubated for 60 min at room temperature in the dark under nitrogen. The reaction was stopped by adding 100 mM 2-mercaptoethanol. The amount of incorporated radioactivity was determined in two ways. (*i*) The proteinase was precipitated with 10% (wt/vol) trichloroacetic acid, centrifuged, and washed 3× with 10% trichloroacetic acid. Finally the washed proteins were dissolved in 0.1 M Tris·HCl buffer (pH 8.0) containing 1% SDS and 10 mM 2-mercaptoethanol, boiled, and mixed with scintillation fluid. (*ii*) Cold acetone/1 M NaOH (98:2, vol/vol) was added to the reaction mixture and centrifuged for 10 min at 4°C. This procedure was repeated three times and the pellets were dissolved and added to scintillation fluid as described above.

Inhibitors. The following stock solutions of inhibitors were made: diisopropyl fluorophosphate, diluted to 5.4 mM with 2-propanol; phenylmethylsulfonyl fluoride, 50 mM in methanol; *p*-chloromercuribenzoate, 20 mM in 10 mM NaOH diluted with bicarbonate buffer at pH 9.5; iodoacetate, 10 mM

Table 1. Enzyme activity of mutants in conserved residues

Mutant	Conservation of residue*	Relative enzyme activity,† % A	B
Wild type	12	100	100
H54R	12	0	ND
C67G	8	88	ND
C104G	12	0	0
C104S	12	16	31
C122G	12	81	67
C122S	12	0	0
C126G	11	89	91
D26N	12	26	ND
D102E	11	100	ND
D102N	11	0	ND
S160G	9	50	ND

*No. of the 12 aligned proteinase sequences with the identical residue at the same position.

†Enzyme activity was determined by means of two assay methods: A, conversion of protein pre-VII to VII of ts1 virions, using recombinant enzyme extracts; B, octapeptide substrate assay, using highly purified proteinase. Values are means of three determinations (standard deviation, ±15%). The activity of the wild type in assay B was 12.9 nmol/min per nmol of proteinase.

Table 2. Effect of inhibitors on the activity of the wild-type and C104S mutant proteinases

Inhibitors	Relative enzyme activity, % Wild type	C104S
Serine proteinase		
Diisopropyl fluorophosphate		
0.11 mM	100	5
0.54 mM	100	0
Phenylmethylsulfonyl fluoride (1 mM)	100	10
Cysteine proteinase		
Iodoacetate (0.1 mM)	0	0
4,4′-Dihydrodipyridine (1 mM)	0	100
p-Chloromercuribenzoate (0.1 mM)	0	100
No inhibitor	100	100

The conversion of protein pre-VII to VII of ts1 virions by recombinant enzyme extracts was assayed.

Table 3. Carboxymethylation of the purified mutant proteinases

Mutant	E64 inhibition of iodoacetate incorporation, %
Wild type	62.2 ± 5.1 (25)
C104G/S	95.3 ± 6.8 (16)
C122G/S	77.3 ± 15.3 (16)
C126G	42.7 ± 21.4 (3)

The prevention of IAA incorporation by E64 in the purified proteinase is given as mean ± SE. The number of experiments for each set, done in parallel, is in parentheses. The data for G/S mutants were combined and shown together. The proteinase was preincubated for 30 min with 0.5 mM E64 or E64-tyrosine before the addition of iodoacetate.

in distilled water, freshly prepared and pH set at 7.5 with Tris; 4,4'-dithiodipyridine, 10 mM in phosphate buffer (pH 7.2).

RESULTS AND DISCUSSION

Endopeptidase Sequences. Fig. 1 shows an alignment of the known adenovirus endopeptidase sequences. The avian adenovirus sequence was from Cai and Weber (5). All of the sequences are translations from DNA sequences. Direct verification by protein sequencing has been partially accomplished only for adenovirus type 2 (4). The amino acid homology, as defined by the percent similarity, among the 12 sequences varies from 93.6% for the closest pair (H40 and H41) to 54.5% for the most distant pair of enzymes (H4 and Bav7). Only 17.6% of the residues are identical across the 12 sequences. The active site of thiol proteinases is generally considered to be composed of a cysteine, a histidine, and an aspartate residue, although in the well-characterized papain family, only cysteine and histidine are involved directly in catalysis (18). Among the adenovirus proteinases His-54 is the only conserved histidine residue. With the newly determined avian sequence the number of conserved cysteine residues drops to two, namely, Cys-104 and Cys-122. These two residues appear to be separated by a short loop structure. Candidates for a possible third residue in the active site are Asp-26, Asp-71, Glu-5, Asn-44, and Asn-170. Homology searches of the Swiss-Prot and GenBank databanks (May 1993) with the BLAST and TFASTA programs have failed to identify any relationship of the adenovirus 2 sequence to other known sequences.

Proteolytic Activity of Mutants. Proteolytic activity in the *E. coli* expression system was tested in a previously characterized assay system using as substrate disrupted ts1 virions grown at the nonpermissive temperature (3, 6). The level of expression of all of the mutants was verified by Western blotting (Fig. 2*A*). Table 1 shows the results obtained with 11 site-specific mutants. His-54 is the only conserved histidine residue among 12 proteinase sequences, and all of the several independently isolated His-54 → Arg (H54R) mutants were negative for enzyme activity. His-54 must therefore be considered part of the catalytic site. Of the two conserved cysteines, the C104G mutant had no activity, whereas the C122G mutant retained 81% activity. This points to Cys-104 as the catalytic cysteine. This conclusion is supported by the fact that mutants C67G and C126G, affecting two partially conserved cysteines, retained most of their activity. These mutants also suggest that Cys-67, Cys-122, and Cys-126 are not involved in disulfide bond formation that is strictly dependent on these residues. A possible disulfide exchange reaction involving either Cys-122 or Cys-126 in the course of enzyme activation by the 11-mer pre-VI-derived peptide, as suggested by Webster *et al.* (9), cannot be ruled out. The validity of these results was tested in the more defined chromogenic substrate peptide assay system using highly purified recombinant enzyme (see *Materials and Methods* for details). The above results were confirmed in this assay (Table 1, column B). This enzyme assay also permitted the measurement of the specific activity of the recombinant wild-type enzyme as 12.9 nmol/min per nmol of proteinase.

Trypsin and poliovirus 3C proteinases are capable of accepting cysteine or serine as their cognate active-site residue without undue decrease in activity (19–22). To test this property we constructed the C104S mutant. The partial restoration of enzyme activity (16% and 31% in the crude and in the purified system, respectively; Table 1) indicates that indeed the adenovirus enzyme can also function with serine at the active site. Furthermore, this enzyme now exhibited properties of a serine proteinase in that its activity was inhibited by inhibitors specific for serine proteinases, but not those specific for cysteine proteinases (Table 2). The inhibition of both enzymes by iodoacetate most likely occurs via interference with the enzyme activation step. Substitution of serine for Cys-122 abolished enzyme activity completely. These data provide further support for Cys-104 as the active thiol.

The carboxymethylation of the reactive cysteines also provided supporting evidence that the active site is C104. The presence of more than one reactive cysteine is suggested by the finding that the inhibitor E64 did not completely prevent carboxymethylation of the purified enzyme (Table 3 and ref. 6). In the C104G or C104S mutant the preincubation of the proteinase with E64 has hardly any effect on the carboxymethyl incorporation, as would be expected if C104 were the only target of E64.

The environment of C104 does not resemble the active-site environment of other cysteine or serine proteinases (23–26). Determination of the atomic structure of the adenovirus proteinase will be necessary to permit structural comparisons with other proteinases. Such modeling studies may finally answer the question of whether this proteinase indeed represents a new subclass of cysteine proteinases.

The search for a possible third member of the catalytic site is more difficult. However, if such a residue does exist it is unlikely to be Asp-26 or Asp-102 because the former retains activity when mutated to an asparagine and the latter because it is not conserved in the avian adenovirus gene.

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