

The Encephalomyocarditis Virus 3C Protease Is Rapidly Degraded by an ATP-Dependent Proteolytic System in Reticulocyte Lysate

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The encephalomyocarditis virus 3C protease has been observed to undergo rapid degradation, both *in vivo* in mouse cells and *in vitro* in reticulocyte lysate. Experiments were carried out to characterize the turnover of the 3C protease in reticulocyte lysate. 3C protease prepared in reticulocyte lysate by *in vitro* translation and processing of a precursor polyprotein could be separated from the proteolytic activity responsible for its degradation. This implies the 3C protease is not directly involved in its own proteolysis. Active 3C protease flanked by only a few amino acids was degraded at a rate identical to that of a similar protein containing an inactivated catalytic site. This indicates that 3C protease activity is not indirectly required for the proteolytic process. Other viral proteins, including the 3D polymerase and capsid proteins, were relatively stable in the lysate. In addition, polyprotein precursors containing 3C protease with an inactive catalytic site and various flanking proteins displayed distinctly different stabilities. These results suggest that the reticulocyte proteolytic system functions in a selective manner toward the viral proteins. The effects of several proteolytic inhibitors on the lysate proteolytic system were evaluated. The results of these experiments indicate that the rapid degradation of the EMC virus 3C protease requires the hydrolysis of ATP. © 1993 Academic Press, Inc.

INTRODUCTION

Like all picornaviruses, the encephalomyocarditis (EMC)² virus contains a single, positive-stranded RNA which codes for a large polyprotein (Rueckert, 1985). Virus replication and virion assembly require that this polyprotein be processed by specific cleavages into individual, functional proteins (Krausslich *et al.*, 1988; Palmenberg, 1990). Most of these cleavages are catalyzed by a proteolytic activity which maps in the 3C region of the genome (Palmenberg *et al.*, 1979, 1984). The 3C protease cleaves the polyprotein both intra- and intermolecularly at certain glutamine-glycine and glutamine-serine junctions (Palmenberg *et al.*, 1984; Parks and Palmenberg, 1987). This leads to the generation of smaller precursors, followed by the appearance of individual proteins, including the mature 22-kDa 3C protease protein.

It was recently observed that the EMC virus 3C protease is rapidly degraded, both in infected mouse cells and in mouse cells transformed with an inducible expression vector containing 3C protease coding sequences (Lawson *et al.*, 1989). Pulse-chase experi-

ments and 3C protease activity assays revealed that the 3C protein was degraded in these cells with a half-life of about 1 hr. In the EMC virus-infected cells, mature 3C protease protein and 3C protease activity reached their greatest levels about 6 hr before cell lysis. Both the 3C protease protein and 3C protease activity levels then quickly declined. Mature 3C protease, as well as a polyprotein precursor containing the 3C protease attached to the 3B and part of the 3A proteins, were also found to quickly disappear in reticulocyte lysate (Lawson *et al.*, 1989). Evidence has been reported which suggests that the 3C-type proteases of related viruses are also very unstable in both infected cells and in cell lysates (Aschauer *et al.*, 1991; Jia *et al.*, 1991; Gauss-Muller *et al.*, 1991; Thomas *et al.*, 1983).

These observations raise the possibility that the rapid rate of degradation of the 3C protease proteins may play a role in determining their concentration in infected cells. No information has been reported to date, however, concerning the processes by which these proteins are degraded. We have examined the turnover of the EMC virus 3C protease and 3C protease-containing polyproteins in reticulocyte lysate, with the goal of obtaining information that will facilitate future studies with infected cells. This *in vitro* system was selected because it has been employed as a model system for examinations of cellular proteins which are rapidly degraded (Ciechanover *et al.*, 1991; Etlinger and Goldberg, 1980; Ganoth *et al.*, 1988;

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² Abbreviations: EMC, encephalomyocarditis; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; DEAE, diethylamino ethyl; AMP-PNP, 5'-adenylylimidodiphosphate; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane.

Hershko *et al.*, 1983; Hough *et al.*, 1987), and it has been used extensively in studies of the synthesis and processing of the polyproteins of EMC virus and other picornaviruses (Jackson, 1986; Jia *et al.*, 1991; Kean *et al.*, 1991; Palmenberg *et al.*, 1979; Palmenberg and Rueckert, 1982; Parks and Palmenberg, 1987; Parks *et al.*, 1986, 1989; Shih *et al.*, 1979; Ypma-Wong *et al.*, 1988a). Here we present evidence showing that the EMC virus 3C protease, as well as some polyproteins containing a mutated, inactive 3C protease catalytic site, are rapidly and selectively degraded by an ATP-dependent proteolytic system present in reticulocyte lysate.

MATERIALS AND METHODS

Construction of plasmids

Several *in vitro* transcription plasmids were prepared by inserting segments of the cloned EMC viral genome (Palmenberg *et al.*, 1984) into pGEM-3Z (Promega). pE3A'BCD (Fig. 1A) was constructed by first treating the previously described pE3A'BCD' (Lawson *et al.*, 1989) with *Hind*III, *Sal*I, *E. coli* DNA polymerase I Klenow fragment, and T4 DNA ligase to remove an upstream out of frame translation initiation codon. The 435 base pair *Bgl*II-*Eco*RI fragment containing the 3' portion of the 3C coding sequence and the first 16 codons of the 3D coding sequence was then replaced with an 1801-base pair *Bgl*II-*Eco*RI fragment containing the entire 3D coding sequence, which was obtained from pBMCE3.³ pE3B'CD' (Fig. 1C) was prepared by treating pE3A'BCD' with *Pst*I, T4 DNA polymerase, and T4 DNA ligase. Insertion mutants of both pE3A'BCD and pE3B'CD' were prepared by following the previously described method of Parks *et al.* (1986). This resulted in the insertion of a 12-base pair *Cla*I linker (New England Biolabs) into the *Bgl*II site of the 3C coding region. The resulting plasmids were designated as pE3A'BCiD and pE3B'CiD' (Figs. 1A and 1C). pE3B'CiD (Fig. 1B) was prepared by replacing the 441-base pair *Cla*I-*Eco*RI segment of pE3B'CiD' with the 1807-base pair *Cla*I-*Eco*RI fragment from pE3A'BCiD.

The construction of pE5LVPO, which contains sequences coding for the EMC virus leader (L) protein and the 1A and 1B proteins inserted behind the T7 promoter, has been described previously (Parks *et al.*, 1986). pE5LP1-2A' was prepared by inserting the 1851-base pair *Stu*I fragment from pEM3 (Palmenberg *et al.*, 1984) into pE5LVPO which had been digested with *Xba*I, treated with Klenow fragment, and then digested with *Stu*I. The resulting plasmid contains se-

quences coding for the L protein, the entire P1 polyprotein, and a portion of the 2A protein.

Preparation and partial purification of *in vitro* translation products and assays for 3C protease activity

In vitro transcription reactions to prepare capped RNA were carried out as previously described (Lawson *et al.*, 1989). Prior to transcription, unless otherwise indicated, pE3B'CD', pE3B'CiD', pE3B'CiD, pE3A'BCD, and pE3A'BCiD were linearized by treatment with *Eco*RI. pE5LVPO and pE5LP1-2A' were linearized by treatment with *Xba*I and *Sal*I, respectively. *In vitro* translations were carried out in micrococcal nuclease-treated reticulocyte lysate (Promega, GIBCO-BRL) with conditions based upon those previously described (Lawson *et al.*, 1986). A typical reaction mixture contained 0.05 pmol/ μ l of RNA in 20 mM HEPES-KOH, pH 7.5, 100 mM $K_2H_3O_2$, 1 mM $Mg(C_2H_3O_2)_2$, and 58% (v/v) reticulocyte lysate supplemented with 20 μ M each amino acid except methionine. Labeled proteins were prepared by including 15 to 30 μ Ci [³⁵S]-methionine (Amersham) in the reaction mixtures. The reactions were incubated at 30° for 30 to 60 min and then terminated by the addition of cycloheximide at a final concentration of 100 μ g/ml. Translation products were examined by adding 1 to 1.5 μ l of the reaction mixtures to 20 μ l 1% SDS in 1 mM EDTA, pH 7.5, and 1 mM DTT (stop buffer), and precipitating with five volumes of cold acetone. The precipitates were dissolved in Laemmli sample buffer (Laemmli, 1970) and analyzed by SDS-PAGE (12% polyacrylamide) and autoradiography.

Mature 3C protease, resulting from the self-processing of the 3A'BCD precursor polyprotein, and the 3B'CD' protein were partially purified from *in vitro* translation reaction mixtures as follows. Terminated translation reaction mixtures (36 μ l) were diluted to 100 μ l in 20 mM HEPES-KOH, pH 7.5, 100 mM $K_2H_3O_2$, 1 mM $Mg(C_2H_3O_2)_2$, and 1 mM DTT (buffer S) and sedimented through 2-ml gradients of 5 to 20% (w/w) sucrose in the same buffer, in a TLS-55 rotor at 55,000 rpm for 5 hr at 4°. One-hundred-microliter fractions were collected from the gradients, and labeled proteins were located by SDS-PAGE and autoradiography. In some instances, fractions containing mature 3C or 3B'CD' proteins were then centrifuged through 800 μ l (suspended volume) DEAE-Sephadex (Pharmacia) equilibrated with buffer S in 0.22 μ m Ultrafree-MC filter units (Millipore). The filtrate was concentrated twofold by centrifugation through 10,000 NMWL Ultrafree-MC filter units.

To assay for 3C protease activity, 4 μ l of terminated translation reaction mixtures, prepared in the absence

³ The construction of pBMCE3 will be described elsewhere.

of [^{35}S]methionine, were combined with 1 μl of terminated translation reaction mixture containing ^{35}S -labeled LVP0 polyprotein. Following a 2-hr incubation at 30°, 2.0 μl of the mixtures were precipitated with acetone and analyzed by SDS-PAGE as described above. The generation of the VP0 (1AB) diprotein was taken to be an indication of 3C protease activity.

Assays for protein degradation

For assays carried out in terminated *in vitro* translation reaction mixtures, translations were first carried out for 30 min, and a solution containing cycloheximide in 58% (v/v) reticulocyte lysate (to give a final cycloheximide concentration of 100 $\mu\text{g}/\text{ml}$) was added. The mixtures were then incubated further at 30°. Beginning 10 min after the addition of cycloheximide, 1.5- μl aliquots were removed at the indicated times, added to 20 μl of stop buffer, and precipitated with acetone. The samples were analyzed by SDS-PAGE and autoradiography. For quantitation of the loss of labeled proteins during the incubations, gel slices were cut out, hydrated with 50 μl water, and then incubated for 2 hr at 70° with 250 μl tissue solubilizer TS-1 (Research Products International). Five milliliters 3a20 cocktail (RPI) was then added, and the radioactivity present was measured by liquid scintillation counting. The amount of radioactivity present in the zero time samples was taken to represent 100% of the labeled protein present at the beginning of the incubation. In order to test the stability of the partially purified proteins, mixtures were prepared which contained 5 μl of the labeled protein preparation and 1.5 μl reticulocyte lysate or wheat germ extract (GIBCO-BRL), in a final volume of 7.5 μl containing 20 mM HEPES-KOH, pH 7.5, 100 mM $\text{KC}_2\text{H}_3\text{O}_2$, 1 mM $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2$, and 1 mM DTT. The mixtures were incubated at 30°. At the indicated times the reactions were terminated by adding 7.5 μl 2X stop buffer and precipitating with five volumes of acetone. The quantity of labeled protein remaining in the mixtures was determined by SDS-PAGE and liquid scintillation counting as described above.

Measurements of the effects of protease inhibitors and ATP analogues on the degradation of the 3C protease

All measurements of the effects of protease inhibitors and ATP analogues were made with the same lot of nuclease-treated reticulocyte lysate (Promega). ^{35}S -labeled mature 3C protease, resulting from the self-processing of the 3A'BCD precursor polyprotein was partially purified from *in vitro* translation reactions as described above. In order to test the effects of known protease inhibitors (Sigma) on the degradation of the 3C protease, mixtures were prepared which contained

5 μl of the labeled protein preparation, 1.5 μl reticulocyte lysate, and the indicated concentration of the inhibitor compound in a final volume of 7.5 μl containing 20 mM HEPES-KOH, pH 7.5, 100 mM $\text{KC}_2\text{H}_3\text{O}_2$, 1 mM $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2$, and 1 mM DTT. The mixtures were incubated for 3 hr at 30°. The reactions were terminated by adding 7.5 μl 2X stop buffer and precipitating with five volumes of acetone. The quantity of labeled protein remaining in the mixtures was determined by SDS-PAGE and liquid scintillation counting as described above.

The effects of the ATP analogues 5'-adenylylimidodiphosphate (AMP-PNP) and AMP (both from Sigma) were evaluated with the same procedure employed for the protease inhibitors. The effects of AMP-PNP and AMP were also measured in terminated *in vitro* translation reaction mixtures containing labeled 3C protease produced by the synthesis and self-processing of the 3A'BCD polyprotein precursor. These reaction mixtures included 2.5 μl terminated *in vitro* translation reaction mixture, 4.35 μl fresh reticulocyte lysate (to give a final total lysate concentration of 58% (v/v)), and the indicated concentration of AMP-PNP or AMP in a final volume of 10 μl containing 20 mM HEPES-KOH, pH 7.5, 100 mM $\text{KC}_2\text{H}_3\text{O}_2$, 1 mM $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2$, and 1 mM DTT. The mixtures were incubated at 30°, and 1.5- μl aliquots were removed at the indicated times, added to 20 μl of stop buffer, and precipitated with acetone. The quantity of labeled protein remaining in the mixtures was determined by SDS-PAGE and liquid scintillation counting as described above. For all reaction mixtures containing the analogues the concentration of $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2$ was increased to give additional Mg^{2+} :analogue ratios of 1:1 for AMP-PNP and 0.5:1 for AMP (Johnston and Cohen, 1991).

RESULTS

Labeled active and inactive 3C protease-containing proteins prepared by *in vitro* translation

In order to prepare mature, *in vitro* synthesized EMC virus 3C protease for studies of its stability in reticulocyte lysate, it was necessary to generate a 3C protease-containing polyprotein capable of undergoing reasonably efficient self-processing. The *in vitro* transcription plasmid pE3A'BCD (Fig. 1A) carries the coding sequence for most of the P3 (Palmenberg, 1990) segment of the polyprotein. This same coding sequence has been previously used in *in vitro* studies of the P3 polyprotein self-processing, and the processing products have been identified (Parks *et al.*, 1986). Translation of the RNA transcribed from this plasmid produced the results shown in Fig. 1D, lane 1. The unprocessed 3A'BCD precursor migrates near the top of the gel. The appearance of this polyprotein as a

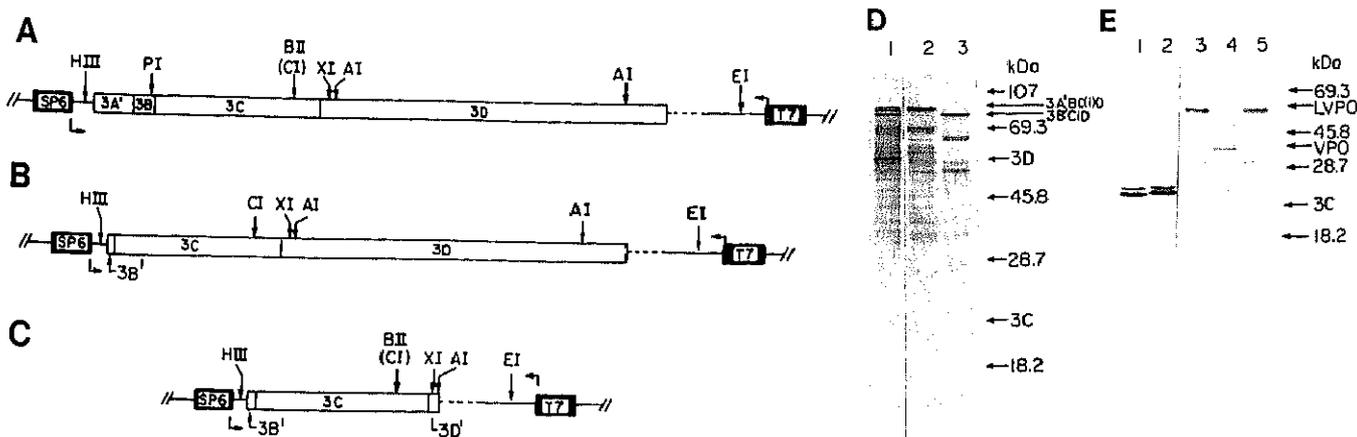


Fig. 1. *In vitro* transcription vectors and *in vitro* translation and processing products. (A) Features of pE3A'BCD and pE3A'BCiD. Open boxes represent the EMC virus coding regions. ("Ci" indicates 3C coding region containing an insertion mutation and the *Cla*I site instead of the *Bgl*II site.) The symbol (') indicates incomplete coding sequences. The dotted line represents the polylinker containing stop codons (Lawson *et al.*, 1989). The indicated restriction sites are: AI, *Apal*; BII, *Bgl*II; CI, *Cla*I; EI, *Eco*RI; HIII, *Hind*III; PI, *Pst*I; XI, *Xho*I. The open reading frame begins with the codons for MRS, followed by the codons for the last 56 amino acids of the 3A sequence, and ends with the viral sequence stop codons downstream of the 3D coding region. (B) Features of pE3B'CiD. Symbols are identical to those used in A. The open reading frame begins with the codons for MP, followed by the last five codons of the 3B sequence, and ends with the viral sequence stop codons downstream of the 3D coding region. (C) Features of pE3B'CD' and pE3B'CiD'. Symbols are identical to those used in A. The open reading frame begins as in B, above, and ends with an in-frame stop codon in the polylinker. The sequence downstream of the 3C region contains first 10 codons of the 3D sequence, followed by codons for SNRGP. (D) Translation and processing products resulting from the transcripts coded for by pE3A'BCD, pE3A'BCiD, and pE3B'CiD. The transcripts were translated in reticulocyte lysate in the presence of [³⁵S]methionine for 1 hr, and aliquots of the reaction mixtures were analyzed by SDS-PAGE and autoradiography. Lane 1, transcript coding for the 3A'BCD polyprotein; lane 2, transcript coding for the 3A'BCiD polyprotein; lane 3, transcript coding for the 3B'CiD polyprotein. Bands corresponding to the 3A'BCD and 3A'BCiD polyproteins (indicated by the label 3A'BC(i)D), the 3B'CiD polyprotein (which comigrates with the 3CD diprotein in lane 1), and the 3C and 3D proteins are indicated. (E) Translation products resulting from the transcripts coded for by pE3B'CD' and pE3B'CiD' and assays for 3C protease activity. Transcripts coding for the 3B'CD' (lane 1) and 3B'CiD' proteins (lane 2) were translated for 1 hr in the presence of [³⁵S]methionine. The transcript coding for the LVPO substrate polyprotein was translated in the presence of [³⁵S]methionine and combined with translation reaction mixtures containing unlabeled 3B'CD' or 3B'CiD' proteins and incubated as described under Materials and Methods. Lane 3, LVPO polyprotein plus a mock translation reaction mixture; lane 4, LVPO polyprotein plus a 3B'CD' translation reaction mixture; lane 5, LVPO polyprotein plus a 3B'CiD' translation reaction mixture. The location at which the 3C protein, if present, would be found is indicated. Bands corresponding to the LVPO and VPO polyproteins are also indicated.

doublet in this polyacrylamide gel system has been observed for some other polyproteins containing the EMC virus 3C protease (Jackson 1986; Lawson *et al.*, 1989; Parks *et al.*, 1986), and in some instances, for the 3C protease itself (Jackson, 1986). No explanation for this behavior has been reported. As expected, this polyprotein was capable of undergoing self-processing to generate the 22-kDa 3C protease and the 52-kDa 3D polymerase (Parks *et al.*, 1986). A protein corresponding to the 3CD diprotein (Parks *et al.*, 1986; compare lanes 1 and 3) is also apparent. Other faint bands are most likely incomplete *in vitro* translation products, the appearance of which are commonplace, especially when long RNA transcripts are involved (Dasso and Jackson, 1989). The level of mature 3C protease which accumulated in these reaction mixtures reached a maximum after about 60 min of total incubation time. This maximum level, however, was usually represented by rather faint 3C protease bands in autoradiograms, presumably the consequence of the instability of the protein. The observed incomplete self-processing of 3C protease-containing polypeptides prepared

by *in vitro* translation is not uncommon (Jackson, 1986; Lawson *et al.*, 1989; Palmenberg and Rueckert, 1982; Parks *et al.*, 1986). To permit the examination of polyprotein precursors incapable of undergoing self-processing, *in vitro* transcription vectors carrying coding sequences for polyproteins with an inactive 3C protease were constructed. The insertion of a 12-base pair *Cla*I linker into the *Bgl*II site in the 3C coding region leads to the insertion of four extra amino acids into the active site of the 3C protease (Parks *et al.*, 1986), resulting in what is referred to here as the 3Ci protein. Translation of the RNA synthesized from p3A'BCiD (Fig. 1A) produced a protein which comigrates with the active 3A'BCD polyprotein (Fig. 1D, lane 2), but the mutant preparation did not undergo self-processing to yield detectable 3Ci protein, 3D polymerase, or 3CiD diprotein. Another inactive 3C protease-containing polypeptide was synthesized from the RNA transcribed from pE3B'CiD (Fig. 1B). The 3B'CiD polyprotein did not undergo detectable self-processing to produce mature 3C protease or 3D polymerase (Fig. 1D, lane 3). Additional proteins were prepared for use in experiments to

directly compare the effects of the active and the inactive, mutated 3C protease catalytic sites on the stability of 3C protease-containing substrates. Because the processing of the P3 polyprotein to cleave out the 22-kDa 3C protease occurs primarily in cis (Hanecak *et al.*, 1984; Palmenberg and Rueckert, 1982), it is difficult to generate mature, inactive 3Ci protein from 3Ci protein-containing precursors. To circumvent this difficulty, the plasmids pE3B'CD' and pE3B'CiD' (Fig. 1C) were constructed. Since the recognition of specific peptide bonds for cleavage by the 3C proteases is based upon structural features in addition to the presence of certain amino acid junctions (Dewalt *et al.*, 1989; Ypma-Wong *et al.*, 1988b), we theorized that the small number of amino acids flanking the normally cleaved glutamine-glycine junctions (7 amino acids on the amino-terminus and 16 amino acids on the carboxyl-terminus) in these polypeptides might produce a context unfavorable for cleavage. The *in vitro* translation products of the RNA transcribed from these vectors are shown in Fig. 1E, lanes 1 and 2. As with the 3A'BCD polyprotein precursor (Fig. 1D, lane 1), both of these proteins were resolved as doublets in the SDS-PAGE system. The lower bands in the 3B'CD' and 3B'CiD' doublets migrated at positions consistent with the expected molecular weights for the proteins of 24.5 and 25.0 kDa, respectively. In contrast to the results with the 3A'BCD polyprotein (Fig. 1D, lane 1), little or no mature 3C protease was observed in these translation reaction mixtures. This suggests that the glutamine-glycine junctions in the 3B'CD' protein do not, in fact, serve as effective substrates for cleavage by the 3C protease catalytic domain. In order to test the possibility that the observed doublets might represent some self-processing of the primary translation products, the RNA transcripts coding for the 3B'CD' and 3B'CiD' proteins were translated in the presence of 2.5 mM Zn²⁺ or 1.5 mM cystatin, both of which have been shown to inhibit picornavirus 3C protease activity (Baum *et al.*, 1991). While both Zn²⁺ and cystatin were found to inhibit the activity of the 3B'CD' protease in the trans activity assay described below, their presence in *in vitro* translation reactions did not reduce or eliminate the appearance of the doublet bands (data not shown).

The 3B'CD' and 3B'CiD' proteins were tested for protease activity by assaying their ability to cleave the LVP0 polyprotein precursor. This polyprotein contains the leader protein (L) attached to the VP0 diprotein, the latter of which is autocatalytically cleaved to generate the 1A and 1B capsid proteins during virion assembly (Palmenberg, 1990; Rossman *et al.*, 1985). Labeled LVP0 was prepared by *in vitro* translation of the transcript synthesized from pE5LVP0 (Parks *et al.*, 1986). As the results in Fig. 1E, lanes 3 through 5, demonstrate, the 3B'CD' preparation was capable of cleaving

the viral leader protein from the LVP0 polyprotein, while the 3B'CiD' preparation could not. Lane 3 shows the LVP0 polyprotein incubated alone. Incubation with added unlabeled 3B'CD' protein resulted in the removal of the very unstable leader protein (Lawson *et al.*, 1989) and the generation of the VP0 diprotein (lane 4), while incubation with added unlabeled 3B'CiD' had no effect (lane 5). The results of this experiment also show that the amino acids flanking the 3C protease in the 3B'CD' protein do not interfere with the folding of the polypeptide into a catalytically active configuration.

3C protease and 3C protease with flanking amino acids are rapidly degraded by a proteolytic system in reticulocyte lysate

Consistent with previous observations (Lawson *et al.*, 1989), the EMC virus 3C protease was found to be unstable in reticulocyte lysate. Mature 3C protease generated from ³⁵S-labeled 3A'BCD polyprotein *in vitro* did not accumulate during incubations, but instead the protein rapidly disappeared. After incubation times of 3 hr or more, little or no mature 3C protease could be detected in these mixtures (see below).

An important question is whether the 3C protease is involved, either directly or indirectly, in its own degradation, or if the responsible proteolytic system resides exclusively in the reticulocyte lysate. One approach to answer this question is to attempt to separate the 3C protease from the activity responsible for its degradation. It was found that 3C protease generated from the *in vitro* synthesis and subsequent processing of the 3A'BCD polyprotein precursor could be rendered stable by sedimentation of the reaction mixtures through sucrose gradients. Most of the unprocessed polyprotein precursors and much of the 3D polymerase which cosedimented with the 3C protease in the gradients were removed by passage of gradient fractions containing labeled 3C protease through DEAE-Sephadex (Fig. 2A, lane 1). This step reduced or eliminated any subsequent generation of additional 3C protease by self-processing of the precursors. This partially purified 3C protease was found to retain catalytic activity, since the preparations could cleave the L protein from the LVP0 polyprotein (data not shown).

Incubation of the partially purified 3C protease for several hours resulted in very little degradation of the protein. Degradation was restored, however, by the addition of reticulocyte lysate. These results are summarized in Fig. 2. Figure 2A, lanes 2 and 3, shows the results of incubating the 3C protease in the presence of either added buffer or micrococcal nuclease-treated reticulocyte lysate (20% by volume). In other experiments, the percentage of 3C protease remaining as a function of time under these conditions was measured (Fig. 2B). In the absence of lysate, a small fraction of

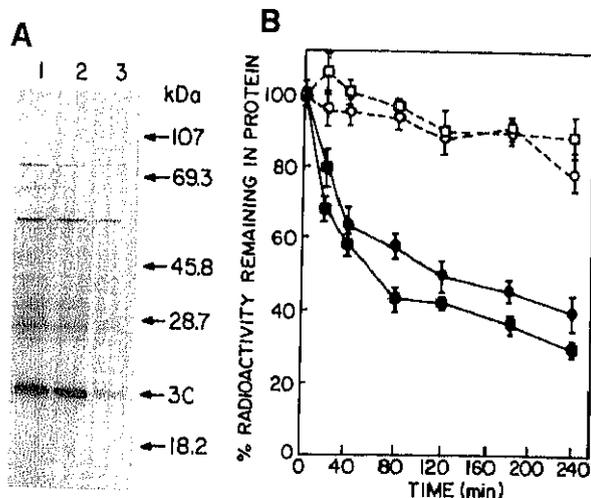


Fig. 2. Stability of partially purified 3C and 3B'CD' proteases in the absence and presence of reticulocyte lysate. (A) Analysis of partially purified 3C protease after incubation in the presence of buffer or reticulocyte lysate. ^{35}S -labeled 3C protease produced by *in vitro* translation and processing of the 3A'BCD polyprotein was partially purified by sucrose gradient sedimentation and passage through DEAE-Sephadex. The preparation was incubated in the presence of either added buffer or nuclease-treated reticulocyte lysate (20% of final volume), and then analyzed by SDS-PAGE and autoradiography. Lane 1, 3C protease prior to incubation; lane 2, 3C protease after incubation for 2 hr in the presence of buffer; lane 3, 3C protease after incubation for 2 hr in the presence of lysate. (B) Results of the stabilities of the partially purified 3C and 3B'CD' proteases in the presence of added buffer or reticulocyte lysate. The reaction mixtures were prepared and analyzed by SDS-PAGE and liquid scintillation counting as described under Materials and Methods. Symbols used: \square , 3C protease plus buffer; \blacksquare , 3C protease plus lysate; \circ , 3B'CD' protease plus buffer; \bullet , 3B'CD' protease plus lysate. Bars represent the standard deviations from three measurements.

the protein was degraded after 4 hr. In the presence of added lysate, however, almost 70% of the 3C protease was degraded. The rate of degradation of the mature 3C protease was compared with that of the active 3B'CD' protease, which was also partially purified from *in vitro* translation reaction mixtures by sucrose gradient sedimentation and passage through DEAE-Sephadex. As Fig. 2B shows, the partially purified 3B'CD' protease was also stable in the absence of reticulocyte lysate, but it was rapidly degraded when lysate was present. The mature 22-kDa 3C protease was degraded at a slightly greater rate than the 3B'CD' protease under these conditions. These results demonstrate that the 3C protease is not directly involved in its own degradation, but rather a proteolytic system present in the reticulocyte lysate is responsible. In addition, the data show that 3C protease with a few additional flanking amino acids also serves as a substrate for degradation by the lysate proteolytic system.

It was still necessary to determine if 3C protease activity is required for the rapid degradation of the 3C

and 3B'CD' proteins. For example, mature 3C protease could cleave one or more cellular proteins, which in turn might lead to the activation of the system which degrades the viral proteins. To examine this possibility, the stability of the catalytically active 3B'CD' and inactive 3B'CD' proteins was compared in terminated *in vitro* translation reaction mixtures. The results are shown in Figs. 3A through 3C. Both of these proteins were rapidly degraded at similar rates. The disappearance of active 3B'CD' was accompanied by a corresponding reduction in the ability of the mixture to cleave the LVP0 polyprotein precursor (data not shown). Not surprisingly, both the rate and extent of disappearance of the labeled proteins was greater in the presence of 58% lysate, as compared with the 20% lysate conditions employed for the assays described in Fig. 2. These results show that 3C protease activity is not required for the rapid degradation of the 3C protein in this system. The data also indicate that any structural changes caused by the insertion of four amino acids into the 3C protease active site do not alter the susceptibility of the inactive protein to degradation in the lysate.

In order to demonstrate that the rapid degradation of the EMC virus 3C and 3B'CD' proteins is not dependent upon the source of reticulocyte lysate, several different lots of lysate from two different suppliers were tested, and both micrococcal nuclease-treated and untreated lysate were evaluated. All lysate preparations tested were found to support the rapid degradation of the 3C and 3B'CD' proteases. Untreated lysate was generally found to be slightly less effective at supporting the degradation when compared with treated lysate. The results of some of these assays are summarized by the data in Table 1. Here, ^{35}S -labeled 3B'CD' protease was partially purified from *in vitro* translation reaction mixtures by sucrose gradient sedimentation. The protein was incubated in the presence of either buffer or the indicated lysate preparation as described under Materials and Methods. The ability of a wheat germ extract to support the degradation was also tested (Table 1). Under these conditions, little or no degradation occurred in the presence of wheat germ extract. It is possible, however, that wheat germ preparations are simply too dilute to rapidly degrade the 3B'CD' protein.

The degradation of the 3C protease in reticulocyte lysate is selective

It was important to determine if the instability of the EMC virus 3C protease is a common characteristic of EMC viral proteins or if it is unique to the protease. To test this, the stability of other EMC virus proteins in reticulocyte lysate was examined. Since the 3A'BCD polyprotein undergoes self-processing to generate

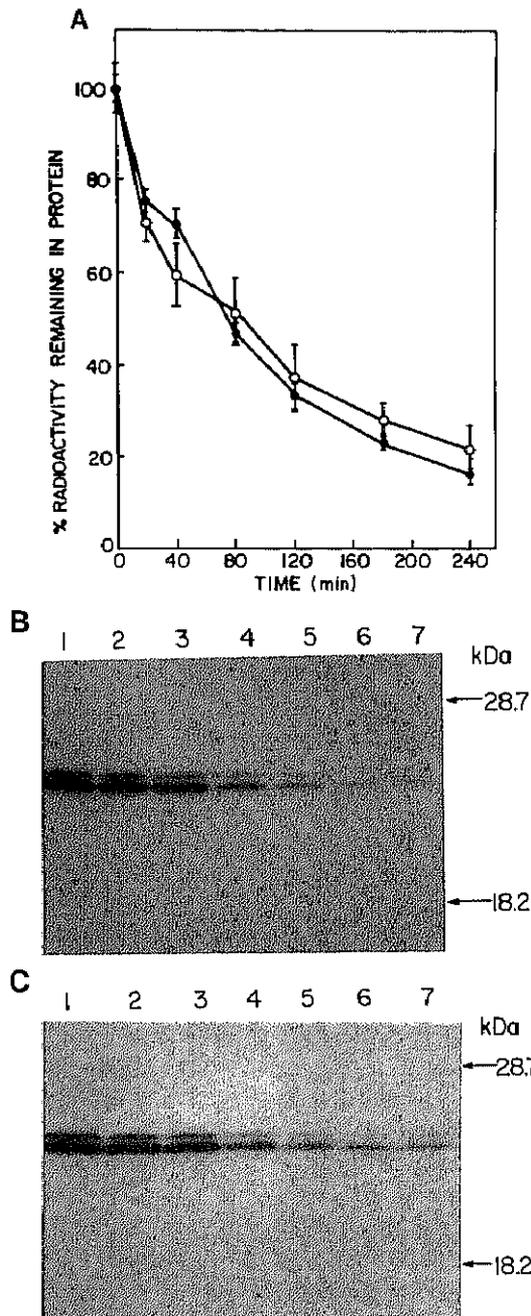


FIG. 3. Comparison of the stabilities of the 3B'CD' and 3B'CiD' proteins in terminated *in vitro* translation reaction mixtures. (A) Results of the analysis of the stabilities of the 3B'CD' and 3B'CiD' proteins. The transcripts coding for the 3B'CD' and 3B'CiD' proteins were translated for 30 min in reticulocyte lysate in the presence of [³⁵S]methionine, and the reactions were terminated by the addition of cycloheximide. Aliquots were removed during the subsequent incubations and analyzed by SDS-PAGE and liquid scintillation counting as described under Materials and Methods. Symbols used: ●, 3B'CD' protein; ○, 3B'CiD' protein. Bars represent the standard deviations from three measurements. (B) Autoradiogram which resulted from an analysis of the stability of the 3B'CD' protein. (C) Autoradiogram which resulted from an analysis of the stability of the 3B'CiD' protein. For both B and C, aliquots were removed at: 0 min, lanes 1; 20 min, lanes 2; 40 min, lanes 3; 80 min, lanes 4; 120 min, lanes 5; 180 min, lanes 6; 240 min, lanes 7.

TABLE 1

MEASUREMENTS OF THE ABILITY OF VARIOUS RETICULOCYTE LYSATE AND WHEAT GERM PREPARATIONS TO SUPPORT THE DEGRADATION OF THE PARTIALLY PURIFIED 3B'CD' PROTEIN

Lysate added ^a	%Radioactivity remaining in protein after 3 hr	
	Experiment 1	Experiment 2
None (buffer only)	97.6	91.3
Reticulocyte lysate ^b	50.2	45.4
Reticulocyte lysate ^c	50.4	47.5
Reticulocyte lysate ^d	54.3	48.9
Wheat germ extract ^e	99.0	88.8

^a To 20% of final reaction mixture volume.

^b Promega, micrococcal nuclease-treated (117 mg total protein/ml).

^c GIBCO-BRL, micrococcal nuclease-treated (131 mg total protein/ml).

^d Promega, untreated (152 mg total protein/ml).

^e GIBCO-BRL (25.1 mg total protein/ml).

both mature 3C protease and 3D polymerase, it was possible to compare the stabilities of the two proteins directly. As Fig. 4A shows, the small amount of 3C protease which accumulated early during the incubation in terminated *in vitro* translation reaction mixtures rapidly disappeared, until after 3 hr, little could be detected (lane 6). In contrast, there appeared to be a more gradual decrease in the amount of accumulated 3D protein. It is possible that the 3A'BCD polyprotein continued to undergo self-processing to produce the 3C and 3D proteins during these incubations, which would result in the replacement of proteins lost to degradation. Even if the 3D protein is degraded in the lysate, the rate of degradation is clearly much slower than that which occurs with the 3C protease. The stabilities of proteins coded for by the P1 coding region of the viral genome were also examined. Figure 4B shows the results of the analysis of the stability of the VP0 capsid protein precursor, which was generated by treating ³⁵S-labeled LVPO polyprotein with unlabeled 3B'CD' protease. Little degradation of the protein occurred, even after a 6 hr incubation. The leader protein was, however, very rapidly degraded, as was observed previously (Lawson *et al.*, 1989). The stabilities of the mature 3C protease, 3D polymerase, and the VP0 protein in these reaction mixtures are quantitatively compared in Fig. 4C. Other capsid proteins were generated by treating the labeled products resulting from the translation of the transcript coded for by pE5LP1-2A' with the 3B'CD' protease. Although the processing of the LP1-2A' polyprotein did not go to completion under these conditions, the processed products all appeared to be at least as stable as the VP0 precursor (data not

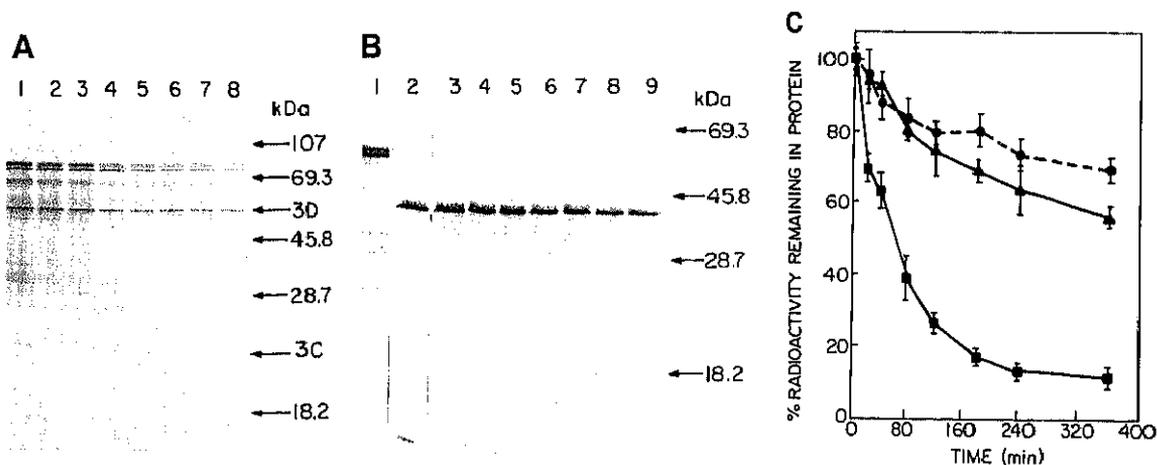


Fig. 4. Analysis of the stabilities of the 3D polymerase and the VP0 capsid precursor in terminated *in vitro* translation reactions. (A) Analysis and comparison of the stabilities of the 3D polymerase and the 3C protease. The transcript coding for the 3A'BCD polyprotein was translated for 40 min in reticulocyte lysate in the presence of [³⁵S]methionine. The reaction was terminated by the addition of cycloheximide and then incubated an additional 20 min before removing aliquots for analysis by SDS-PAGE and autoradiography. The exposure of the autoradiogram was controlled so as to avoid overexposure of the band which represents the 3D polymerase. Aliquots were removed at 0 min, lane 1; 20 min, lane 2; 40 min, lane 3; 80 min, lane 4; 120 min, lane 5; 180 min, lane 6; 240 min, lane 7; and 360 min, lane 8. (B) Analysis of the stability of the VP0 capsid precursor. The transcript coding for the LVP0 polyprotein was translated for 40 min in reticulocyte lysate in the presence of [³⁵S]methionine, and the reaction was terminated by the addition of cycloheximide. A translation reaction mixture containing unlabeled 3B'CD' protease was prepared and added to the labeled LVP0 preparation as described under Materials and Methods. After a 40-min incubation, aliquots were removed for analysis by SDS-PAGE and autoradiography. Lane 1 shows the analysis of the unprocessed LVP0 polyprotein precursor. The remaining lanes show analysis of the mixtures subsequent to incubation with the 3B'CD' protein at 0 min, lane 2; 20 min, lane 3; 40 min, lane 4; 80 min, lane 5; 120 min, lane 6; 180 min, lane 7; 240 min, lane 8; and 360 min, lane 9. (C) Comparison of the stabilities of the 3D polymerase, the 3C protease, and the LVP0 polyprotein precursor. Aliquots removed during incubations of reaction mixtures like those described in A and B above were analyzed by SDS-PAGE and liquid scintillation counting as described under Materials and Methods. Symbols used: ■, 3C protease; ▲, 3D polymerase; ●, LVP0 polyprotein. Bars represent the standard deviations from three measurements.

shown). These results indicate that the 3D polymerase and the proteins generated by the 3C protease-dependent processing of the capsid polyprotein precursors are considerably more stable than the 3C protease or the L protein in reticulocyte lysate. This suggests that the 3C protease, and the L protein as well, is selected for rapid degradation by the reticulocyte proteolytic machinery.

Another consideration is whether the mature 3C protease is targeted for rapid degradation only after it has been processed from polyprotein precursors, or if the polyprotein precursors are themselves rapidly turned over. Results from experiments such as those shown in Fig. 4A indicate that 3C protease-containing precursors disappear as a function of time, but this could be due to self-processing as well as, or instead of, degradation. To examine this question, the stabilities of polyproteins containing the inactive 3Ci protease were compared. Since these molecules are incapable of undergoing self-processing, they can serve as models for the polyprotein precursors. In addition to the ³⁵S-labeled 3B'CiD' protein and the 3A'BCiD and 3B'CiD polyproteins (Fig. 1), a polyprotein containing 56 amino acids from 3A, all of the 3B and 3Ci proteins, and 2 carboxyl-terminal amino acids from the 3D protein was prepared by run-off translation of the RNA

transcript synthesized from pE3A'BCiD linearized with *Xho*I (Fig. 1A). This polyprotein is referred to as 3A'BCiD'. The stabilities of these polypeptides in terminated *in vitro* translation reactions were analyzed. As the results in Table 2 show, these polyproteins exhibited markedly different stabilities. The A'BCiD' polyprotein, for example, was the least stable, while the B'CiD polyprotein was relatively resistant to degradation. It thus appears that some, but not all, of the polyprotein precursors containing the 3C protease are degraded quickly in reticulocyte lysate. These data provide additional support for the selectivity of the reticulocyte proteolytic processes toward the EMC virus proteins.

TABLE 2

COMPARISON OF THE STABILITY OF POLYPEPTIDE PRECURSORS CONTAINING INACTIVE 3C PROTEASE IN TERMINATED *IN VITRO* TRANSLATION REACTION MIXTURES

Precursor	%Radioactivity remaining in protein after 3 hr ^a
3B'CiD'	30.5 ± 3.74
3A'BCiD'	19.2 ± 2.70
3A'BCiD	44.7 ± 3.40
3B'CiD	76.3 ± 7.70

^a *n* = 3 for standard deviation calculations.

Characterization of the proteolytic system which degrades the 3C protease

The ability to separate the mature 3C protease from the activity responsible for its degradation offered the opportunity to carry out experiments to characterize the nature of the lysate proteolytic system. Experiments were performed to determine if individual fractions from sucrose gradients through which reticulocyte lysate had been sedimented contained the 3C protease-degrading activity. None of the fractions, when added to partially purified 3C or 3B'CD' protease, were capable of restoring degradation. This suggests the responsible proteolytic system may consist of more than one component. In order to obtain some specific information about the nature of the proteolytic system, the effects of a number of compounds known to inhibit specific classes of proteases and certain types of proteolytic systems were tested for their effects on the degradation of the 3C protease.

Since the turnover of many cellular proteins has been found to require energy produced by the hydrolysis of ATP (Hershko and Ciechanover, 1982; Tanaka *et al.*, 1983), the effects of AMP-PNP and AMP on the degradation of the 3C protease were tested. AMP-PNP inhibits reactions requiring hydrolysis of the bond between the β and γ phosphates, while AMP inhibits reactions requiring hydrolysis of the bond between the α and β phosphates (Johnston and Cohen, 1991). As the results in Fig. 5A and Table 3 show, both AMP-PNP and AMP inhibited the degradation of partially purified 3C protease by added reticulocyte lysate, with AMP-PNP serving as the more effective inhibitor. These analogues also inhibited the degradation of the 3C protease produced by processing of the 3A'BCD polyprotein in terminated *in vitro* translation reaction mixtures (Fig. 5B, Table 3). The degradation of partially purified 3B'CD' protein by added reticulocytelysate was inhibited to a similar extent by the presence of these compounds. The results shown in Fig. 5B also show that the presence of the AMP-PNP and AMP reduced the rate of disappearance of the large precursor polyprotein molecules in these mixtures. The degradation of both the mature 3C protease and the 3B'CD' protease by added untreated reticulocyte lysate was slightly stimulated by the addition of ATP (data not shown). This is consistent with the fact that the untreated lysate preparations lack an added ATP-generating system. It thus appears that the system which degrades the EMC virus 3C protease requires at least two distinct ATP hydrolysis events.

The effects of a number of protease inhibitors on the degradation of the 3C protease are summarized in Table 4. The inhibitors selected include compounds known to inhibit the activities of the major types of

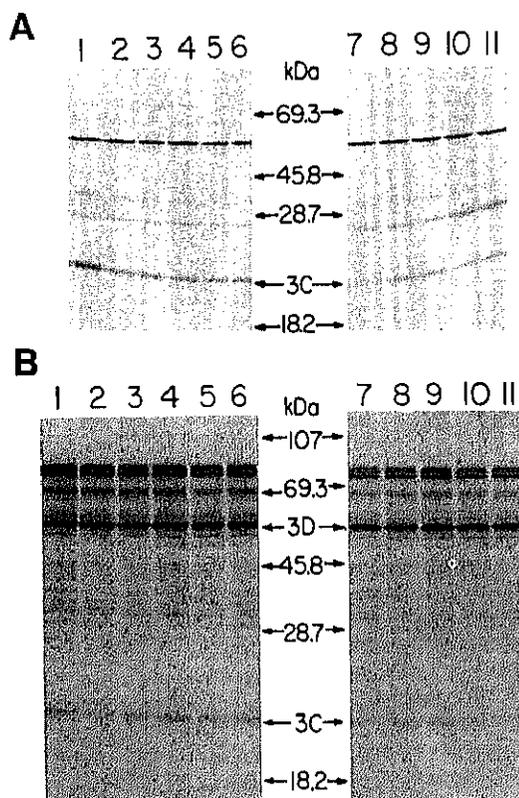


Fig. 5. Effects of ATP analogues on the degradation of the 3C protease. (A) Effects of analogues on the degradation of partially purified 3C protease by added reticulocyte lysate. 35 S-labeled 3C protease produced by *in vitro* translation and processing of the 3A'BCD polyprotein was partially purified by sucrose gradient sedimentation and passage through DEAE-Sephadex. The preparation was incubated for 30 min or 3 hr in the presence of added nuclease-treated reticulocyte lysate (20% of final volume) and the indicated analogues, and the mixtures were analyzed by SDS-PAGE and autoradiography. The 3C preparation used in this experiment contains more of the 52-kDa 3D polymerase than the preparation shown in Fig. 2A. (B) Effects of analogues on the degradation of 3C protease in terminated *in vitro* translation reaction mixtures. The transcript coding for the 3A'BCD polyprotein was translated for 40 min in reticulocyte lysate in the presence of [35 S]methionine. The reaction was terminated by the addition of cycloheximide and incubated an additional 20 min. This preparation was added to reaction mixtures containing fresh nuclease-treated lysate (total lysate, 58% of final volume) and the indicated analogues as described under Materials and Methods. The mixtures were incubated for 30 min or 3 hr and then analyzed by SDS-PAGE and autoradiography. For both A and B, lanes 1 show analyses of the reaction mixtures prior to incubation. Lanes 2 through 6 show analyses of reaction mixtures incubated for 30 min, and lanes 7 through 11 show analyses after incubations for 3 hr. The following analogues were present in the reaction mixtures: Lanes 1, 2, and 7, no added analogue; lanes 3 and 8, 2 mM AMP-PNP; lanes 4 and 9, 4 mM AMP-PNP; lanes 5 and 10, 2 mM AMP; lanes 6 and 11, 4 mM AMP.

known proteases. Each inhibitor has been shown to be effective within the range of the two concentrations employed here (Baum *et al.*, 1991, and references therein; Rivett, 1989, and references therein; Wilkin-

son, 1991, and references therein; and ED, 1991, and references therein) that the reactive site of the 3C protease is an ATP-dependent serine protease. The 3C protease is a member of the serine protease family and the mechanism of its action could be similar to that of other members of the family. The 3C protease is a member of the serine protease family and the mechanism of its action could be similar to that of other members of the family.

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TABLE 3

EFFECTS OF ATP ANALOGUES ON THE DEGRADATION OF THE 3C PROTEASE

Analogue	Concentration (mM)	Ratio % radioactivity remaining in 3C protease after 3 hr ^a [(in presence of analogue)/(in absence of analogue)]	
		Partially purified protease plus lysate	Protease in <i>in vitro</i> translation reaction mixture
AMP-PNP	2	1.69	1.44
	4	1.65	2.01
AMP	2	1.14	1.14
	4	1.51	1.22

^a The ratios were calculated from measurements carried out three or more times. The standard deviations for the average percentage radioactivity remaining under each condition did not exceed $\pm 5.45\%$.

son, 1990, and references therein). The most effective inhibitors were found to be *N*-ethylmaleimide, hemin, and EDTA. The results with *N*-ethylmaleimide imply that the degradation of the 3C protease involves a reactive thiol group. Hemin has been found to inhibit ATP-dependent proteolysis in reticulocyte lysates (Etlinger and Goldberg, 1980; Wilkinson, 1990), although the mode of action is unclear. The inhibition by EDTA could be partially removed by including additional Mg^{2+} in the reactions (Table 4), a result also consistent with a requirement for ATP hydrolysis. Zn^{2+} , an inhibitor of a number of proteolytic enzymes (Baum *et al.*, 1991; Rivett, 1989), and a high concentration of phenylmethylsulfonyl fluoride (PMSF), which inhibits serine proteases, both also slightly reduced the rate of degradation of the 3C protease.

DISCUSSION

The results of the experiments described here have demonstrated that the EMC virus 3C protease is degraded by a proteolytic system present in reticulocyte lysate. 3C protease with 7 additional amino acids flanking the amino-terminus and 16 additional amino acids flanking the carboxyl-terminus (the 3B'CD' protease) was also found to be degraded by the lysate proteolytic system, although at a slightly slower rate than the mature 22-kDa 3C protease. Both proteins disappeared with a half-life of 1 hr or less in terminated *in vitro* translation reaction mixtures (Figs. 3, 4A, and 4C). These rates are very similar to those previously observed to occur *in vivo* in virus-infected mouse cells (Lawson *et al.*, 1989). The 3C protease does not participate directly in its own degradation, since partially purified, catalytically functional 3C and 3B'CD' proteases were stable for several hours. This is not surprising, since studies have shown that the EMC virus 3C pro-

tease, as well as the 3C protease of poliovirus, exhibit great specificity and can recognize and cleave only certain peptide bonds in the viral polyproteins (Krausslich *et al.*, 1988). The fact that the inactive 3B'CD' protein was degraded at the same rate as the active 3B'CD' protein indicates that action by the 3C protease catalytic site is not required to activate or stimulate the reticulocyte proteolytic system.

A significant feature of the reticulocyte proteolytic system is that it functions in a selective manner. While the EMC viral leader protein has been found to be degraded at least as fast as the 3C protease (Lawson *et al.*, 1989), the 3D polymerase, capsid proteins, and capsid protein precursors were shown here to be considerably more stable during long incubations. Model polyprotein precursors containing an inactive 3Ci protease also showed distinctly different susceptibilities to degradation. The stability of polypeptides tested followed the hierarchy B'CiD > A'BCiD > B'CiD' > A'BCiD'. This hierarchy of stability suggests that the presence of the complete 3D polymerase reduces the ability of polyproteins to serve as a substrate for the lysate proteolytic system. It is interesting to note that the 3CD diproteins of both EMC virus and poliovirus are believed to act *in trans* as the functional protease in the processing of capsid polyprotein precursors into

TABLE 4

EFFECTS OF PROTEASE INHIBITORS ON THE DEGRADATION OF PARTIALLY PURIFIED 3C PROTEASE BY ADDED RETICULOCYTE LYSATE

Inhibitor	Concentration (mM)	Ratio % radioactivity remaining in 3C protease after 3 hr ^a [(in presence of inhibitor)/(in absence of inhibitor)]
Cystatin	1.00	1.02
	5.00	1.04
E-64	0.500	1.06
	2.50	1.02
Hemin	0.200	2.62
	1.00	3.12
Leupeptin	0.200	1.04
	1.00	0.963
<i>N</i> -Ethylmaleimide	2.00	1.68
	10.0	2.74
Pepstatin	0.250	0.974
	1.25	1.06
Phenylmethylsulfonyl fluoride	2.00	0.876
	10.0	1.11
Zn^{2+}	0.500	1.14
	2.50	1.19
EDTA	1.00	1.65
	5.00	2.72
EDTA + Mg^{2+}	5.00 EDTA + 10 Mg^{2+}	1.52

^a The ratios were calculated from measurements carried out three or more times. The standard deviations for the average percentage radioactivity remaining under each condition did not exceed $\pm 5.03\%$.

functional capsid proteins (Jore *et al.*, 1988; Parks *et al.*, 1989; Ypma-Wong *et al.*, 1988a). It is possible that an enhanced stability of the EMC virus diprotein allows it to accumulate to levels required for efficient capsid protein processing *in vivo*. It is also noteworthy that studies of the synthesis and processing of the EMC virus polyproteins in infected cells (Jen and Thach, 1982; Parks *et al.*, 1986; Ramabhadran and Thach, 1981) and *in vitro* (Jackson, 1986; Shih *et al.*, 1979) have produced observations consistent with the results described here. Little or no mature 3C protein is usually apparent in these systems. The P3 polyprotein intermediate (3ABCD) and the 3CD diprotein, however, accumulate to relatively high levels. While the rate at which the individual polyprotein intermediates are produced is certainly a major contributor to the amount of each protein which is present at any given time, it appears that the rate at which at least the EMC virus 3C protease and 3C protease-containing proteins are degraded can also play a role in determining their concentrations.

Very little research concerning the proteolytic turnover of mammalian viral proteins has been carried out to date. Research into cellular protein turnover has shown that nonviral proteins are degraded, both *in vivo* and *in vitro*, with half-lives ranging from a few minutes to several hours (Hershko and Ciechanover, 1982; Schimke, 1983). The EMC virus 3C protease can therefore be categorized as a relatively unstable protein. Observations have been reported which suggest that 3C-type proteases produced by other viruses are also quickly degraded, although no examinations of the proteolytic processes which might be responsible have been carried out. Poliovirus 3C protease has been found to be unstable in lysates prepared from infected HeLa cells (Thomas *et al.*, 1983). Measurements of rhinovirus 3C protease levels in infected cells suggests very little of this protein accumulates *in vivo* (Aschauer *et al.*, 1991). Indirect evidence has been obtained for the rapid turnover of the hepatitis A virus 3C protease in infected cells (Gauss-Muller *et al.*, 1991), and certain other hepatitis A proteins from the P3 segment of the polyprotein appear to be unstable in reticulocyte lysate (Jia *et al.*, 1991). If all of these proteins are indeed subject to rapid degradation, then it must be considered whether the degradation of the 3C-type proteases may be a significant event in the infectious cycles of these viruses.

Complete understanding of the mechanism by which the EMC virus 3C protease is rapidly degraded in reticulocyte lysate will require that the components of the responsible proteolytic system be identified and characterized. The results of the experiments carried out here to begin this characterization have revealed a requirement for ATP hydrolysis. Inhibition of the degra-

ation of the 3C protease by both AMP-PNP and AMP indicate the existence of two hydrolysis events at separate pyrophosphate positions. The observed requirement for Mg^{2+} and the inhibition of the degradation by hemin (Etlinger and Goldberg, 1980) also implicate ATP involvement. These data are consistent with characteristics exhibited by other ATP-dependent proteolytic systems, the most well studied being the ubiquitin-mediated system (Hershko and Ciechanover, 1992). This selective proteolytic system is known to be involved in the rapid degradation of a number of cellular proteins, as well as in the partial breakdown of antigens for presentation to T-lymphocytes (Goldberg and Rock, 1992). In addition, the ubiquitin-mediated system has been shown to rapidly degrade the adenovirus E1A protein (Ciechanover *et al.*, 1991), and it may be involved in the turnover of the Sindbis virus RNA polymerase in reticulocyte lysate (De Grot *et al.*, 1991).

The data which resulted from measurements of the effects of specific protease inhibitors on the degradation of the 3C protease do not support the involvement of many cysteine or aspartate proteases, but they do leave open the possibility of the participation of one or more serine-type proteases. The proteolytic mechanism requires a reactive thiol group, as is shown by the results with *N*-ethylmaleimide. It is interesting to note that the pattern of inhibition revealed by the data in Table 4 is similar to that observed for the multicatalytic protease (Rivett, 1989), which is suspected of being involved in the degradation of many rapidly turned over cellular proteins by both ubiquitin-mediated and ubiquitin-independent processes (Matthews *et al.*, 1989; Rivett, 1989). One notable exception is the effect of leupeptin, which did not inhibit the turnover of the 3C protease but is known to be an inhibitor of the multicatalytic protease (Rivett, 1989).

Further work will be required to positively identify the proteolytic system responsible for the rapid and selective degradation of the EMC virus 3C protease. It is too early to say whether a similar proteolytic system degrades the 3C protease in mouse cells, although the data from both the study described here and from others concerning turnover rate (Lawson *et al.*, 1989) and the accumulation of certain polyprotein processing intermediates (Jackson, 1986; Jen and Thach, 1982; Parks *et al.*, 1986; Ramabhadran and Thach, 1981; Shih *et al.*, 1979) suggest the two systems share common characteristics. It will also be interesting to determine if the 3C proteases of other picornaviruses are degraded as rapidly as the EMC virus 3C protease and if the same cellular proteolytic system might be responsible. The results of the study described here will provide a foundation for the exploration of these questions.

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