Activation of the protease from human adenovirus type 2 is accompanied by a conformational change that is dependent on cysteine-104

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Adenovirus codes for a protease the activity of which can be regulated in vitro by an 11 residue peptide (GVQSLKRRRCF) derived from another viral protein, pVI. Three cysteine residues, one in the activating peptide and two in the protease (C104 and C122), play a central role in both activation and catalysis. Expression of protease mutants in insect cells has shown that C104 is not essential for proteolytic activity. GVQSLKRRRCF also caused a concentration-dependent increase in tryptophan fluorescence of protease expressed in Escherichia coli that paralleled the increase in proteolytic activity, indicating that activation was accompanied by a conformational change. Tryptophan fluorescence of C104S was not increased by the addition of GVQSLKRRRCF, nor was the fluorescence of wild-type protease increased by the addition of the peptide analogues where cysteine is replaced by aspartic acid or serine, suggesting that C104 is involved in activation and C122 in catalysis.

Proteolysis by a virus-encoded protease plays a key role in the development of infectivity by adenovirus (Weber, 1976). The protease is a 23 kDa protein produced late in infection that is known to cleave six virus structural proteins, namely: pVI, pVII, pVIII, preterminal protein (pTP) IIIa and the I1K protein (Anderson et al., 1973) plus at least one cellular protein (Chen et al., 1993) and possibly one non-structural virus protein, L1-52K (Hasson et al., 1992). Studies of the protease itself have shown it to be a thiol protease which is very unusual in that it does not require proteolytic activation (Webster & Kemp, 1993) but is synthesized in an essentially inactive form that requires the participation of an 11 residue peptide in order to cleave protein or synthetic peptide substrates in vitro (Webster et al., 1993; Mangel et al., 1993). The 11 residue peptide cofactor is derived from the viral protein pVI and has the sequence GVQSLKRRRCF, in which the cysteine residue is known to be essential (Webster et al., 1993).

The mechanism by which the activating peptide operates is not clear, but there is evidence to suggest that it does not participate directly in catalysis. If insect cells are simultaneously infected with separate recombinant baculoviruses expressing the protease and pTP then digestion of pTP to the 62 kDa intermediate (iTP) is observed in the infected cells, although such digestion will not occur with the purified protease and iTP unless activating peptide is added (Webster et al., 1993).

The protease genes from 11 adenovirus serotypes have been sequenced and alignments of the derived protein sequences indicate that two cysteine residues, at positions 104 and 122 of the human adenovirus serotype 2 (Ad2) sequence, are conserved (Houde & Weber, 1990; Grierson et al., 1994). Mutagenesis studies have shown that changing either of these cysteines to alanine or serine results in an inactive protease (Rancourt et al., 1994; Grierson et al., 1994) leading to the conclusion that one of these is involved in the activation mechanism and the other in catalysis (Grierson et al., 1994). We report here on further studies of the protease activity in insect cells and on the influence of the activating peptide on protease conformation which indicate that C122 provides the active site nucleophile, while C104 is necessary for peptide-mediated activation.

The preparation of the protease mutants H54S, C104A and C122A and their expression in BL21(DE3) cells via the pET11c vector has been described previously (Grierson et al., 1994). These mutants were all found to be inactive when tested against synthetic peptide substrates, but it is not clear whether these mutants lack an essential catalytic residue or whether they fail to interact with activating peptide. In order to test this latter possibility these mutants were expressed in the baculovirus insect cell system along with pTP.

Fragments encoding the mutant protease genes were purified by agarose gel electrophoresis and Qiaex gel extraction (Qiagen) after being cut from the pET11c expression vector by digestion with BamHI and XbaI. The resultant
fragments were then ligated into the transfer vector pVL1392 (Invitrogen) that had been cut and purified in the same way. Positive clones were selected by their resistance to carbenicillin and the presence of insert DNA was confirmed by restriction digest of the resultant plasmids. Baculovirus DNA (AcRAK6 lacZ) linearized with the restriction enzyme Bsu36I and the transfer vector were then cotransfected into monolayer cultures of SF9 cells using lipofectin (Gibco-BRL). The resultant recombinant baculoviruses were then purified by plaque assay and the expression of protease was confirmed by analysis of infected SF9 cell lysates by SDS–PAGE and Western blotting. The construction of the recombinant baculovirus expressing Ad2 pTP has been previously described (Temperley & Hay, 1992).

Recombinant baculovirus DNA was purified and the sequence of the mutant protease genes was confirmed by the following method. Two oligonucleotides (Forward: 5' GGATTATTTCATACCGTCCCACCA 3' ; Reverse: 5' TTCTTGCCGGTCCCCAGGAAAGGAT 3') were used to amplify the entire protease gene and its flanking regions from the recombinant baculovirus DNA by PCR (94 °C, 1.5 min; 60 °C, 1.5 min; 72 °C, 2.0 min for 35 cycles and finally 72 °C for 7.0 min) using Vent DNA polymerase (New England Biolabs). PCR products of the correct size were gel purified and extracted by Qiaex (Qiagen) and then cut with BamHI and Xhel. The cut fragments were then ligated back into pET11c, cut and purified in the same way, and sequenced as previously described (Grierson et al., 1994). Two positive clones from each ligation were sequenced.

Coinfections of SF9 cells with recombinant baculoviruses expressing protease, mutant protease and pTP were carried out in 24-well plates seeded with 5 x 10^6 cells per well. Each well was infected with a total volume of 200 μl of varying ratios of virus stocks. Virus was allowed to adsorb for 90 min at 28 °C. 1 ml of TC-100 7% FCS was then added to each well and the infection allowed to proceed for 72 h. Cells were then harvested and rinsed with PBS prior to analysis by SDS–PAGE and Western blotting as previously described (Webster et al., 1993) except that a monoclonal antibody against pTP (generously supplied by Dr A. Webster) was used. Expression of the protease was verified by Western blotting (results not shown).

Digestion of pTP to iTP occurred when pTP was coexpressed with wild-type protease or with the C104A mutant (Fig. 1). No digestion was apparent when pTP was coexpressed with C122A or with H54S. The absence of proteolysis when H54 and C122 are mutated to other residues strongly suggests that these two residues are part of the catalytic mechanism. The fact that activity is retained by the C104 mutant in a system that is operative in the absence of the activating peptide (whereas previous studies have indicated that it is necessary for activity in systems where the activating peptide is essential (Rancourt et al., 1994; Grierson et al., 1994)) indicates that this residue has a role in the peptide-mediated activation process.

The role of C104 in the activation process is supported by studies of the effect of activating peptide on the conformation of the protease as measured by tryptophan fluorescence. In order to investigate this change in fluorescence, protease was expressed in Escherichia coli as previously described (Webster & Kemp, 1993) and purified by chromatography successively on DE-Sepharose, and a mixed bed column of CM-Sepharose, DNA-cellulose and heparin–Sepharose. Activating peptide and variants were synthesized as previously described (Webster et al., 1993). Protease activity was determined as previously described (Grierson et al., 1994) and in accord with previous observations (Mangel et al., 1993) the initial reaction velocity was shown to reach a maximum at an approximately 100-fold molar excess of activating peptide over protease (Fig. 2a). The effect of increasing activating peptide concentration on the conformation of the protease was determined by measuring the fluorescence emission at 345 nm with excitation at 280 nm using a Perkin-Elmer LS50B fluorimeter. The results shown in Fig. 2(b) indicate that the increase in protease activity shown in Fig. 2(a) was accompanied by a corresponding increase in emission at 345 nm, indicating that there is a change in conformation leading to a perturbation of the environment around one or more of the three tryptophan residues in the molecule.

As can also be seen from Fig. 2(b), no increase in tryptophan fluorescence was observed when the C104S mutant was incubated with increasing concentrations of activating peptide. Such an increase in fluorescence did not occur when the activating peptide was first treated with iodoacetamide (results not shown) nor when the cysteine residue in the activating peptide was replaced with either aspartic acid or serine. In contrast, an increase in tryptophan fluorescence was observed on incubation of activating peptide with the mutant C122S, although this was more apparent at lower concentrations of the activating peptide. The results from the tryptophan fluorescence studies show that the cysteine at position 104 is necessary for interaction with the activating peptide and this in turn leads to the conformational change in the protease which
accompanies the development of proteolytic activity. However, the decrease in fluorescence of the C122S mutant that was observed at higher concentrations of activating peptide suggests that the mechanisms responsible for the confor-

mational change accompanying activation are complex and may involve contributions from both C104 and C122.

The conclusion that C104 is primarily involved in activation is at variance with that of Rancourt et al. (1994) who reported that replacement of cysteine-122 with glycine diminishes (but does not abolish) protease activity, leading these authors to suggest that the catalytic thiol must be associated with C104. We have constructed this mutant by site-directed mutagenesis utilizing two successive PCR reactions using two flanking primers and one mutant oligo (Landt et al., 1990). The purified PCR products containing the mutant protease gene were cloned into the pET11c expression vector (Rosenberg et al., 1987) and transformed into XLI-Blue cells. Plasmid DNA was purified from XLI-Blue cells and the sequence of the complete gene was confirmed as previously described. The mutant protease was expressed from transformed BL21(DE3) cells by induction with IPTG and the protein was extracted, purified and tested for protease activity as previously described (Grierson et al., 1994). The activity displayed by the C122G mutant was 0.15 nmol product/nmol protease/min compared to the activity of wild-type which was 45 nmol product/nmol protease/min. In other words, the C122G mutant displayed a specific activity < 0.5% that of wild-type. The C122G mutant was cloned and expressed in insect cells as described above and when insect cells were co-infected with C122G protease-expressing baculovirus and pTP-expressing baculovirus, no digestion of pTP was observed (results not shown). Interestingly, the levels of expression of C122G in E. coli were much higher (approximately 5 mg/l) than were observed with wild-type protease (approximately 1 mg/l). C104A and C122A, both inactive mutants, were expressed at levels comparable to C122G while active mutants such as C126A, C127A and the double mutant C126A/C127A were expressed at levels similar to, or lower than, the wild-type protease.

In a recent review Rawlings & Barrett (1994) identified a group of cysteine proteases, all viral in origin and including the adenovirus protease, in which the catalytic histidine precedes the catalytic cysteine in the primary structure in contrast to the arrangement in papain and the majority of the members of this class. Also included within this group is the cysteine protease from the NS2 region of the hepatitis C virus where site-directed mutagenesis has identified H952 and C993 as the active site residues (Grakoui et al., 1993). The alignments shown in Fig. 3 indicate that the primary structure context of

### Ad2 (100–107):

\[ TPDRCITL \]

### Hepatitis C virus (989–996):

\[ DTACGDl \]

### Ad2 (118–125):

\[ NSAACGFL \]
C122 bears a much greater resemblance to that of the hepatitis C virus active site cysteine than does the context of C104.

The results presented here contribute to the understanding of the mechanisms of activation and catalysis in the adenovirus protease by presenting evidence which indicates that C122 provides the active site nucleophile and that the other highly conserved cysteine, C104, is the focus of the activation mechanism. In addition it is clear that activation was accompanied by a conformational change in the molecule which was caused by a change in the environment of one or more of the tryptophan residues. Given that H54, shown by this and a previous study (Rancourt et al., 1994) to be part of the catalytic mechanism, is next to a tryptophan residue (W55), it is reasonable to speculate that this conformational change results in the exposure or creation of the active site.

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References


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