Kinetic Analysis of the Conjugation of Ubiquitin to Picornavirus 3C Proteases Catalyzed by the Mammalian Ubiquitin-protein Ligase E3α*

The 3C proteases of the encephalomyocarditis virus and the hepatitis A virus are both type III substrates for the mammalian ubiquitin-protein ligase E3α. The conjugation of ubiquitin to these proteins requires internal ten-amino acid-long protein destruction signal sequences. To evaluate how these destruction signals modulate interactions that must occur between E3α and the 3C proteases, we have kinetically analyzed the formation of ubiquitin-3C protease conjugates in a reconstructed system with E3α, HsUbc2b/E2α, and human E3α. Our measurements show that the encephalomyocarditis virus 3C protease is ubiquitinated in this system with $K_m = 42 \pm 11 \mu M$ and $V_{max} = 0.051 \pm 0.01 \text{ pmol/min}$ whereas the parameters for the ubiquitination of the hepatitis A virus 3C protease are $K_m = 20 \pm 5 \mu M$ and $V_{max} = 0.018 \pm 0.003 \text{ pmol/min}$. Mutations in the destruction signal sequences resulted in changes in the rate at which E3α conjugates ubiquitin to the altered 3C protease proteins. The $K_m$ and $V_{max}$ values for these reactions change proportionally in the same direction. These results suggest differences in rates of conjugation of ubiquitin to 3C proteases are primarily a $k_{cat}$ effect. Replacing specific encephalomyocarditis virus 3C protease lysine residues with arginine residues was found to increase, rather than decrease, the rate of ubiquitin conjugation, and the $K_m$ and $V_{max}$ values for these reactions are both higher than for the wild type protein. The ability of E3α to catalyze the conjugation of ubiquitin to both 3C proteases was found to be inhibited by lysylalnine and phenylalanylalnine, demonstrating that the same sites on E3α that bind destabilizing N-terminal amino acids in type I and II substrates also interact with the 3C proteases.

The selection of proteins for destruction by the ubiquitin 26 S/proteasome pathway depends upon specific interactions that occur between the targeted substrates and enzymes involved in the formation of the ubiquitin-target protein conjugates. A hierarchical family of pathways, each composed of at least three enzymes, accomplishes the attachment of ubiquitin to proteins destined to be degraded (1–3). Common to all of these pathways is the ubiquitin-activating enzyme, E1, which recuits free ubiquitin through the ATP-dependent formation of a thiolester bond between a cysteine in the E1 and the C-terminal glycine of the ubiquitin molecule. This ubiquitin is then transferred to one of several members of the E2 family of enzymes that are referred to as ubiquitin carrier proteins or ubiquitin-conjugating enzymes. Finally, the ubiquitin is transferred from the E2 to the target substrate protein through the action of an ubiquitin-protein ligase, or E3. Although each E2 protein appears to function with several specific ubiquitin-protein ligases, each E3 can specifically interact with only a limited number of substrate proteins. Regardless of the E3 involved in the ubiquitination process, following the conjugation of the first ubiquitin molecule to a primary amine on the substrate protein, the E3, or the E3 plus E2 proteins, can catalyze additional conjugating reactions that result in the synthesis of a chain of ubiquitin molecules attached to the substrate (4).

Important unanswered questions remain as to precisely how the E3 ubiquitin-protein ligases recognize and interact with their substrate proteins. It appears that proteins degraded by the ubiquitin/26 S proteasome system contain structural features, often short primary sequence elements (2, 5), that act as protein destruction signals, and presumably it is these structural features that serve as sites for interaction with specific E3 proteins. Very few precisely mapped protein destruction signal structures have been matched with their cognate ubiquitin-protein ligase, however (2). Among the most well studied E3 proteins are mammalian E3α, which functions in conjunction with the ubiquitin carrier protein HsUbc2/E2α (see Refs. 6 and 7), and the yeast homologue of E3α, Ubr1p, which requires the presence of yeast Ubc2p/Rad6 ubiquitin carrier protein (8, 9). E3α and Ubr1p were first shown to recognize proteins with N-terminal basic (type I) or bulky hydrophobic (type II) amino acids as substrates (10–16). Based on the affinity resin binding behavior of E3α (13), measurements of the degradation rates of artificial substrates in reticulocyte lysate and in intact yeast cells (7, 12), and in vitro and in vivo dipeptide competition studies (11, 13, 14), it was proposed that these enzymes contain both type I and type II N-terminal amino acid binding sites (13, 15, 16). These binding sites were assumed to provide the means by which substrate proteins are recognized.

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by E3α and Ubr1p. In recent years it has been discovered that E3α and Ubr1p can catalyze the ubiquitination of proteins lacking destabilizing N-terminal amino acids (type III substrates). The short-lived yeast proteins Gpap and Cup9p, neither of which contains a destabilizing N-terminal amino acid, have been reported to be substrates for Ubr1p (17–19). Ribonuclease S, the subtilisin-derived fragment of ribonuclease A, has a stabilizing serine N terminus, but it is known to be a substrate for mammalian E3α-dependent ubiquitin-protein conjugate synthesis (20). This indicates these E3 enzymes can recognize substrate proteins through associations with other types of structural elements.

E3α has recently been found to catalyze the conjugation of ubiquitin to two additional proteins that, based upon their N-terminal amino acids, would not be predicted to be N-end rule substrates for degradation. The 3C proteases produced by the encephalomyocarditis virus (EMCV) and the hepatitis A virus (HAV), both members of the picornavirus family, have been shown to serve as substrates for E3α-dependent ubiquitination (21–23). The ten-amino acid sequence 34LLVR–GRTLTVV43, located in what is probably a strand-turn-strand structure, has been discovered to function as a protein degradation signal. The 3C proteases produced by the EMCV and HAV 3C protease with an alanine inserted between amino acid positions 37 and 38, and pETHAV3C дер, were prepared using polymerase chain reaction. pHAV3C (21) was employed as a template, and DNA insert fragments containing the mutated HAV 3C protease coding sequence were synthesized using the appropriate mutagenic primer (CTGGACCAGATGTGAGAGGCGCCCGCACCTTGGTG ATGTAAAGT for pETHAV3CA37, GAGGCCGCACCGCGGTAGTAAATTAGACACATG for pETHAV3C A38, and CTCTTGTGAGAGGCGCCCGCACCTTGGTG ATGTAAAGT for pETHAV3C A38) and inserted into pET3d. The inserts were ligated into pET3d at the NcoI and BamHI sites (24). Likewise, the expression plasmids pETHAV3CA37, which contains the sequences coding for the HAV 3C protease with an alanine inserted between amino acid positions 37 and 38, and pETHAV3C дер were prepared using polymerase chain reaction. pHAV3C (21) was employed as a template, and DNA insert fragments containing the mutated HAV 3C protease coding sequence were synthesized using the appropriate mutagenic primer (CTGGACCAGATGTGAGAGGCGCCCGCACCTTGGTG ATGTAAAGT for pETHAV3CA37, GAGGCCGCACCGCGGTAGTAAATTAGACACATG for pETHAV3C A38, and CTCTTGTGAGAGGCGCCCGCACCTTGGTG ATGTAAAGT for pETHAV3C A38) and inserted into pET3d. The inserts were ligated into pET3d at the NcoI and BamHI sites.

**Experimental Procedures**

**Construction of Plasmids Containing 3C Protease Coding Sequences**—The construction of the expression plasmids pETE3B CD*, pETEAV3C, and pETPCD have already been described (21–23). pETE3CA37, which contains the sequences coding for the EMCV 3C protease with an alanine inserted between amino acid positions 38 and 39, pETE3C A34A, pETE3C A38, pETE3C K10,14R, pETE3C K74,77R, and pETE3C K98,101R were prepared using polymerase chain reaction-based oligonucleotide-directed mutagenesis. pE3C (23) was employed as a template, and DNA insert fragments containing the mutated EMCV 3C protease coding sequences were synthesized using the appropriate mutagenic primer (CTGGACCAGATGTGAGAGGCGCCCGCACCTTGGTG ATGTAAAGT for pETHAV3CA37, GAGGCCGCACCGCGGTAGTAAATTAGACACATG for pETHAV3C A38, and CTCTTGTGAGAGGCGCCCGCACCTTGGTG ATGTAAAGT for pETHAV3C A38) and inserted into pET3d. The inserts were ligated into pET3d at the NcoI and BamHI sites.

**Purification of E1, HsUbc2b, E3α, and EMCV and HAV 3C Protease Proteins**—Human erythrocyte fraction II was prepared using procedures described previously (25). Human ubiquitin-activating enzyme E1 was purified from this material using a ubiquitin affinity column and fast protein liquid chromatography methods described previously (26). Human recombinant HsUbc2b (27) was expressed in *Escherichia coli* and purified using methods reported previously (20). Some of this protein was employed in the preparation of an affinity column, which was then used to purify ubiquitin-protein ligase E3α from the fraction II preparation as reported recently (20).

The wild type EMCV 3C and mutated (3C A38), 3C L34A, 3C K10,14R, 3C K74,77R, and 3C K98,101R protease proteins were expressed in *E. coli* from pETE3B CD*, pETE3C A38, pETE3C A34A, and pETE3C K98,101R and were purified from refolded inclusion body material using procedures reported previously (22). Wild type HAV 3C protease was expressed in *E. coli* from pETHAV3C and purified as described previously (21). The mutated HAV 3C (A37* and 3C L34A) proteins were expressed from pETHAV3C A37* and pETE3C L34A. The purified proteins were used in substrate degradation assays. Measurement of the Rates of 125I-Ubiquitin–3C Protein Conjugate Formation and Km and Vmax Determinations—Bovine ubiquitin (Sigma) was purified further to apparent homogeneity (28) and radiodinated.

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2 M. E. Sweep, T. G. Lawson, and A. L. Haas, unpublished results.
3 V. P. Losick, P. E. Schlax, R. E. Emmons, and T. G. Lawson, unpublished results.
4 The HsUbc2b is identical in sequence to its rabbit ortholog (27) and is functionally indistinguishable from the *a* isofrom (28).
by the Chloramine T procedure (14). Some of this material was subjected to reductive methylation (29). The initial rates of 125I-ubiquitin conjugation were measured using an adaptation of methods employed previously (20). The reaction mixtures typically contained, in a final volume of 25 μl, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 10 mM creatine phosphate, 2 mM ATP, 1 mM dithiothreitol, 1 international unit/ml creatine phosphokinase (Sigma), 1 international unit/ml high protein liquid chromatography-purified yeast inorganic pyrophosphate (Sigma), 50 mM purified E1, 500 mM purified HsUbc2b, 2 μg of affinity-purified E3α preparation, 0 to 20 μM exogenous protein substrates, and 4 μM 125I-ubiquitin or 125I-methylated ubiquitin (about 12,000 cpm/ pmol). The mixtures were incubated for 15 min at 37 ° and then boiled for 4 min in the presence of 25 μl of added sample buffer. This incubation time was selected to yield a linear initial rate of monoubiquitination. The samples were analyzed by 12% SDS-PAGE and autoradiography. The amounts of monoubiquitin-substrate protein conjugates formed were determined by cutting slices from the dried gels and subjecting them to β-counting (30). Control experiments confirmed that the initial rate was linear with [E3α]0 and independent of [E1]0 or [HsUbc2b]0 (20). Reaction rate data sets were generated by measuring the initial rates of monoubiquitinated conjugates produced in several simultaneously incubated reaction mixtures containing varying concentrations of 3C protease substrate. Two to four data sets were generated for each substrate protein using the same preparations of E1, HsUbc2b, and E3α. The rate versus substrate concentration data sets were simultaneously fit for each substrate to the Km value using a non-linear least squares regression analysis program (Sigma Plot 5.0). The Vmax values

**FIG. 1.** The kinetic analysis of the E3α-dependent synthesis of ubiquitin-EMCV 3C protease conjugates in the reconstituted reaction system. A, analysis of reaction mixtures containing α-lactalbumin or the wild type EMCV 3C protease as substrates. Reconstituted reaction mixtures containing either 125I-ubiquitin (lanes 1–3) or 125I-methylated ubiquitin (lanes 4 and 5) were incubated for 15 min in the presence of no added substrate (lanes 1 and 4), 10 μM α-lactalbumin (lane 2), 10 μM wild type EMCV 3C protease (lane 3), or 20 μM wild type EMCV 3C protease (lane 5) and then aliquots were removed and analyzed by 12% SDS-PAGE and autoradiography. The mobility of labeled monoubiquitin- and methylated ubiquitin-3C protease (mono Ub-3C and MeUb-3C) and monoubiquitin- and methylated ubiquitin-HsUbc2b (mono Ub-HsUbc2b and MeUb-HsUbc2b) conjugates is indicated. B, analysis of reaction mixtures containing different concentrations of the wild type EMCV 3C protease. Reconstituted reaction mixtures containing the indicated amounts of EMCV 3C protease as the substrate were incubated for 15 min and then aliquots were removed and analyzed by 12% SDS-PAGE and autoradiography. The samples analyzed are as follows: lane 1, no 3C protease added; lane 2, 4 μM 3C protease; lane 3, 8 μM 3C protease; lane 4, 12 μM 3C protease; lane 5, 16 μM 3C protease; lane 6, 20 μM 3C protease. The location of labeled monoubiquitinated 3C protease, monoubiquitinated HsUbc2b, and diubiquitin (diUb) are indicated. C, simultaneously fitted plots of initial reaction velocity versus wild type EMCV 3C protease concentration from four separate experiments. The data were fitted using non-linear least squares regression analysis. D, reciprocal plots of the data sets graphed in panel C. Plots of the residuals versus the theoretical fit of the data in C and D are shown in the lower panels.
for each substrate were calculated by averaging the values derived from the fits for each data set.

Evaluation of the Susceptibility of Poliovirus 3C Protease Proteins toward Conjugation with Ubiquitin in Reticulocyte Lysate—A coupled in vitro transcription-translation rabbit reticulocyte system (Promega) was employed to prepare [35S]-labeled protease 3C and 3C(N33R) proteins encoded within pETP3C and pETP3C_N33R. The ability of these proteins to serve as substrates for ubiquitination was evaluated by incubating 7 μl of transcription-translation reaction mixtures in a final volume of 20 μl containing 20 mM HEPES-KOH, pH 7.5, 1 mM dithiothreitol, 0.1 mM methylated ubiquitin, 0.1 mg/ml cycloheximide, and 60% by volume reticulocyte lysate containing an energy-generating system at 30 °C for 40 min (21-23). Aliquots of the reaction mixtures were analyzed by 12% SDS-PAGE and fluorography.

RESULTS
Kinetic Characterization of E3α-catalyzed Conjugation of Ubiquitin to the 3C Proteases and to 3C Proteases with Protein Destruction Signal Mutations—Under appropriate conditions, kinetic analysis provides a sensitive and accurate means of quantifying enzyme-substrate interactions and the catalytic competence of the resulting Michaelis complex. We employed a biochemically defined, reconstituted N-end rule ubiquitin ligation system, comprised of affinity-purified human E1 and E3α and recombinant human HsUbc2b, to quantitatively evaluate the targeting of the EMCV and HAV 3C proteases for ubiquitin attachment and to assess the effect disrupting their respective destruction signal sequences has on their selection as N-end rule pathway substrates.

A comparison of the labeled products generated by the reconstituted system with either α-lactalbumin or wild type EMCV 3C protease as the substrate is shown in the autoradiogram in Fig. 1A, lanes 1–3. In the absence of a substrate protein, the otherwise complete assay mixture containing E1, HsUbc2b, and E3α catalyzes the synthesis of hyperconjugates to trace protein contaminants in the enzyme preparations, seen at the top of lane 1 (see Fig. 1A and Ref. 20). Monoubiquitin-HsUbc2b conjugates are also generated under these conditions, at the concentration of this enzyme employed to assure E3α-limiting conditions. In reaction mixtures in which α-lactalbumin was the substrate most of the products synthesized during the incubation period were high molecular mass polyubiquitin-α-lactalbumin conjugates, and the characteristic ladder of sequential ubiquitin adducts is apparent (Fig. 1A, lane 2). The conjugation of ubiquitin to the EMCV 3C protease occurred at a markedly lower rate, and the majority of these products consisted of monoubiquitinated 3C protease (Fig. 1A, lane 3). The rate of monoubiquitin-3C protease synthesis was found to be linear for up to 15 min. An increasingly large fraction of the products consisted of polyubiquitinated 3C protease at longer times (data not shown). This suggests that the attachment of the first ubiquitin molecule to the 3C protease occurs more slowly than subsequent polyubiquitinated conjugate synthesis. It should be noted that the synthesis of polyubiquitinated α-lactalbumin conjugates has been shown to also be linear with respect to time and E3α concentration in a reaction system very similar to the one used here (20). Because our goal in this study was to attempt to detect potentially subtle differences in the kinetics with which related substrate proteins are ubiquitinated by the same ubiquitin-protein ligase, we preferred to avoid artifacts that might result from the use of ubiquitin mutants or derivatives that do not support polyubiquitin chain synthesis. In addition, the rate of the first ubiquitin attachment is more likely than subsequent steps to reflect substrate recognition events mediated by E3α. To confirm that the formation of polyubiquitinated 3C protease was not a major event during the incubation time, reactions were carried out in which [125I]-methylated ubiquitin was used in place of [125I]-ubiquitin. Measurements of the fraction of the total labeled 3C protease-dependent products that migrated in SDS-PAGE gels above the methylated ubiquitin-3C protease (Fig. 1A, lanes 4 and 5) were found to be virtually identical to the fraction of analogous material synthesized in reaction mixtures containing [125I]-ubiquitin. This indicates the amount of monoubiquitinated 3C protease present in the reaction mixtures at the end of 15 min is not significantly affected by reactions that lead to the synthesis of polyubiquitinated 3C protease. Identical results were obtained using the HAV 3C protease as a substrate. The rate of formation of monoubiquitinated 3C protease products was therefore taken to be a valid measure of the initial rate at which E3α catalyzes the conjugation of the first ubiquitin to these substrates.

For each kinetic analysis, the quantity of [125I]-ubiquitin incorporated into monoubiquitinated conjugates during the 15-min incubation period was measured as a function of 3C-protease concentration. An example of the SDS-PAGE gel analysis from a set of reaction mixtures used in measuring the rate of conjugation of ubiquitin to the wild type EMCV 3C protease is shown in Fig. 1B. The range of concentrations of some of the 3C protease proteins employed in these measurements did not produce initial velocities that approach saturation, which has the potential to introduce error into the calculations of kinetic parameters. Higher substrate concentrations would, however, have required the use of stock 3C protease preparations at concentrations we have observed to sometimes result in the formation of aggregates during long term storage. Despite this limitation to the reaction conditions, the reproducibility of the velocity versus substrate concentration measurements supports the reliability of the data. Fig. 1C shows the simultaneous fit of initial velocity versus substrate concentration data from four sets of measurements using the EMCV 3C protease. As with all of the 3C proteins employed in this study for which rate measurements could be made, the dependence of the initial ubiquitin attachment rate upon substrate concentration exhibited hyperbolic kinetics. This is confirmed by the linearity of the data in the double reciprocal plots, demonstrated in Fig. 1D. The random distribution of the plotted residuals versus the theoretical fit of the data (Fig. 1, C and D) are consistent with the Michaelis-Menten analysis used here to determine the kinetic parameters. The $K_m$ and $V_{max}$ values for the conjugation of ubiquitin to the substrate proteins were calculated using non-linear least squares fit analysis of the velocity versus substrate concentration data, as described under “Experimental Procedures.”

Both the wild type 3C proteases and 3C proteases containing mutations in the protein destruction signal sequence were evaluated as E3α substrates. The wild type protein destruction signal region sequences and the sequences present in the mutated proteins used in this study are shown in Fig. 2. The L34A and +A38 mutations in the EMCV 3C protease were selected, because proteins carrying these changes have been previously to serve as poor substrates for the ubiquitin/26 S proteasome system in rabbit reticulocytes (23). The leucine residue occupying the first position of the sequence has been shown to be particularly important for signal function, as has the distance separating the hydrophobic amino acid triplets on either end of the signal sequence. The +A37 mutation in the HAV 3C protease was prepared, because we wished to evaluate the kinetics of an HAV 3C protein carrying a mutation we predicted would render the protein a poor E3α substrate by increasing the distance between the distal hydrophobic residues in the destruction signal sequence. The HAV 3C(D37R) mutant was prepared to provide a substrate for testing the effects of replacing one of the two negatively charged amino acids in the HAV 3C protease destruction signal with a positively charged
amino acid. This mutated protein carries an arginine residue in the same relative position as the Arg-39 residue in the wild type EMCV 3C protease signal sequence. To serve as a complement for this substrate, the EMCV 3C(R39D) protein was also prepared.

Measurements of the initial rates for the E3α-dependent conjugation to the wild type and mutated EMCV and HAV 3C proteases were used to calculate the $K_m$ and $V_{\text{max}}$ values for the reactions with each substrate. The values of the kinetic parameters are depicted, for ease of comparison in Fig. 3A (open bars for $K_m$ values, and shaded bars for $V_{\text{max}}$ values), and a comparison of the $V_{\text{max}}/K_m$ ratios is presented in Fig. 3B. Because the same concentration of E3α was used in all of the reaction mixtures, $V_{\text{max}}$ can be assumed to be proportional to $k_{\text{cat}}$, and $V_{\text{max}}/K_m$ can be employed for comparing catalytic efficiencies with which E3α conjugates ubiquitin to the 3C protease substrates. Also for comparison, an SDS-PAGE analysis of the products generated in reactions containing α-lactalbumin and the 3C proteases is shown in Fig. 3C. Again, α-lactalbumin was rapidly incorporated into primarily large polyubiquitinated conjugates, whereas the ubiquitinated wild type 3C protease products were synthesized more slowly and included mostly monoubiquitinated conjugates. Differences in the quantity of monoubiquitinated 3C protease products synthesized during the incubation period are evident.

The $K_m$ and $V_{\text{max}}$ values for attachment of the first ubiquitin to the wild type 3C proteases (42 ± 11 μM and 0.051 ± 0.01 pmol/min, respectively, for the EMCV 3C proteases and 20 ± 5 μM and 0.018 ± 0.003 pmol/min, respectively, for the HAV 3C proteases) are of similar orders of magnitude as those reported for other substrates of mammalian E3α (20). Changes in the protein destruction signal sequences of both 3C proteases were found to have measurable effects on the kinetics with which these proteins are ubiquitinated. Both the $K_m$ and $V_{\text{max}}$ values for the attachment of the first ubiquitin to the EMCV 3C(+A38) and EMCV 3C(L34A) proteases were found to be reduced relative to the values calculated for the reactions with the wild type protease (Fig. 3A). An example of the simultaneous fit of the initial velocity versus substrate concentration data for one of the EMCV 3C protease destruction signal mutants, 3C(+A38), is shown in Fig. 4. In this case, the reaction does approach saturation at the higher concentrations of substrate. The HAV 3C(+A37) protease was such a poor substrate for E3α (evident from the results shown in Fig. 3C) that reliable initial rate measurements were not possible. These results are consistent with the earlier characterization of the 3C protease destruction signal (23). The reactions with the HAV 3C(D37R) protease occurred with $K_m$ and $V_{\text{max}}$ values both severalfold larger than the corresponding wild type substrate parameters (Fig. 3A). Although the data clearly indicate the D37R mutation results in large increases in $K_m$ and $V_{\text{max}}$ for the ubiquitination of the protease, the high parameter values mean that the substrate concentrations in the reaction mixtures were far from saturating. These values therefore have a relatively large associated uncertainty. These results nevertheless indicate that a basic amino acid in this position positively affects interactions between the destruction signal region and E3α. The complementary reverse mutation in the EMCV 3C(R39D) protease did not, however, lead to a reduction in the kinetic parameter values. This substrate was ubiquitinated in the reconstituted system with $K_m$ and $V_{\text{max}}$ values similar to those for the wild type protease (Fig. 3A). As Fig. 3B shows, the $V_{\text{max}}/K_m$ ratios for the ubiquitination of the wild type and mutated 3C proteases are similar.

Although the differences between the $K_m$ and $V_{\text{max}}$ values for the wild type and mutated 3C proteases are not large, they likely reflect genuine differences in the interactions between these proteins and E3α. A previously published demonstration that mutations in the destruction signal region of the EMCV 3C protease can interfere with the E3α-catalyzed ubiquitination of this protein (23) supports the quantitatively derived results obtained here. It is unlikely the kinetic differences are due, for example, to the variable presence of small amounts of nicked or truncated species in the protein preparations that resulted in the exposure of types I or II substrate N-terminal amino acids. The same preparations of E1, HaUbc2b, E3α, and energy system enzymes were used for all of the measurement reported here. Two different preparations of the wild type HAV 3C protease were used in the rate measurements performed for this study, and both were ubiquitinated with very similar kinetic parameters. This indicates that if contamination of the protease preparations occurs, it occurs in a reproducible fashion. If the 3C proteases are nicked by proteases during purification, it seems likely that peptide bond cleavage would occur in the same locations and with similar frequencies in both the wild type and mutated protein preparations. Finally, the dramatic reduction in the rate of ubiquitination of the HAV 3C protease caused by the +A37 mutation was not accompanied by the obvious synthesis of any other ubiquitin-protein conjugates (Fig. 3C), making it appear unlikely that competition with better E3α substrate contaminants is a significant factor.

Attempts were made to measure the rate at which E3α catalyzes the conjugation of ubiquitin to the wild type poliovirus 3C protease, but these were unsuccessful. The poliovirus 3C protease has been shown to be a very poor substrate for the ubiquitin/26 S proteasome system (21). The poliovirus 3C protease contains, in a position analogous to that of the EMCV and HAV 3C protease destruction signal sequences, the sequence LGVHDNVAIL27 (23). Given the results obtained with the HAV 3C(D37R) and EMCV 3C(R39D) proteins, we wondered whether replacing the asparagine residue at position 33 with an arginine would by itself substantially improve the ability of the poliovirus 3C protease to serve as a substrate for the purified E3α. An arginine residue in this position aligns with both the Arg-39 in the wild type EMCV 3C protease and with the arginine residue substituted into the HAV 3C(D37R) protein (see Fig. 2). Attempts to refold the mutated poliovirus protein from inclusion bodies obtained from expressing E. coli cells were unsuccessful. The poliovirus 3C(N33R) protein was, therefore, instead prepared by in vitro translation, and its ability to serve as a substrate for conjugation with ubiquitin was tested using rabbit reticulocyte cell extracts supplemented with methylated ubiquitin (21–23). The results of these experiments showed that the N33R substitution had little or no effect on the susceptibility of the poliovirus 3C protein toward

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**EMCV 3C w.t.:** 34 LLVRGRTLVV

**EMCV 3C (+A38):** 34 LLVRGRTLVV

**EMCV 3C (+L34A):** 34 ALVRGRTLVV

**EMCV 3C (R39D):** 32 LLVRGDLTVV

**HAV 3C w.t.:** 32 LGVKKDWWLV

**HAV 3C (+A37):** 32 LGVKKDWWLV

**HAV 3C (D37R):** 32 LGVKDRWV

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**Fig. 2.** The protein destruction signal sequences in the wild type and mutated EMCV and HAV 3C protease proteins. *w.t.*, wild type.
ubiquitination (data not shown). Although this result does not prove conclusively that E3/H9251 fails to interact easily with the poliovirus 3C(N33R) protein, it does suggest that a basic amino acid in this position does not in itself generate a functional protein destruction signal.

Effects of Selected Lysine to Arginine Substitutions on the Kinetics of E3/H9251-catalyzed Ubiquitination of EMCV 3C Proteases—The EMCV 3C protease contains twelve lysine residues, none of which occur in the destruction signal sequence, and it has been shown that any of these amino acids can serve as the initial ubiquitin-conjugating site (23). It is conceivable that the location of lysine residues in a substrate protein, relative to a protein destruction signal for example, can influence the likelihood with which they are selected by ubiquitin-protein ligases (16, 31). To determine whether eliminating some of the potential ubiquitin attachment sites in the EMCV 3C protease changes the kinetics with which E3/H9251 catalyzes the ubiquitination of this protein, we prepared three EMCV 3C protease proteins that each contain two lysine to arginine substitutions. Based upon sequence alignments with the HAV 3C protease and examinations of the HAV 3C protease three-dimensional structure (32–34), we selected substitution sites likely to exist in separate locations on approximately the same face of the EMCV 3C protein as does the protein destruction signal region. These proteins, EMCV 3C(K10,14R), 3C(K74,77R), and 3C(K98,101R), have been shown previously to retain levels of catalytic activity similar to the wild type 3C protease, indicating these mutations do not induce significant higher order structural alterations (23).

The initial rates at which these mutated proteins are ubiquitinated in the E3/H9251-dependent ubiquitin-conjugating system were greater than for the wild type protein (Fig. 5), leading to increases in both $K_m$ and $V_{max}$ in each case. Although these results provide a clear indication that all three mutations led to a similar effect on the kinetics with which the EMCV 3C protease is ubiquitinated, the calculated $K_m$ and $V_{max}$ values contain much larger standard errors, because the $K_m$ values are beyond the concentration ranges possible for the 3C protease substrates.

E3α Recognizes the 3C Proteases as N-end Rule Substrates—A characteristic feature of E3α ligase is its inhibition by dipeptides containing cognate N-end rule residues in the

**FIG. 3.** The kinetic parameters for the E3α-dependent conjugation of ubiquitin to wild type EMCV (WT) and HAV 3C protease proteins and to 3C proteins containing mutations in the destruction signal regions. A, bar graph of the $K_m$ and $V_{max}$ values calculated from the rate measurements for the ubiquitination of the 3C protease proteins in the reconstituted reaction system. Open bars correspond to the $K_m$ values, and shaded bars correspond to the $V_{max}$ values. B, bar graph of the $V_{max}/K_m$ values for the reactions with each substrate protein. C, analysis of reaction mixtures containing α-lactalbumin, wild type 3C proteases, or mutated 3C protease substrates. Reconstituted reaction mixtures containing 10 μM each substrate protein were incubated for 15 min, and aliquots were removed for analysis by 12% SDS-PAGE and autoradiography. The positions of the monoubiquitinated 3C protease conjugates are indicated by the arrows.
N-terminal position (8, 10, 11). It was demonstrated recently that this effect follows classic non-competitive inhibition (20), suggesting that the association of substrates with E3α involves a site in the ligase distinct from the site that recognizes N-terminal amino acids. We tested whether the EMCV and HAV 3C proteases behave as N-end rule substrates, as defined by the effect dipeptides have on the E3α-dependent synthesis of ubiquitin-3C protease conjugates. As shown by the data in Fig. 6, both lysylalanine and phenylalanylalanine inhibited the conjugation of ubiquitin to both wild type 3C protease proteins. The ubiquitination of the HAV 3C protease is more strongly inhibited by the dipeptides, especially lysylalanine, than is the EMCV 3C protease. This is probably a reflection of the significant differences in the sequences of these two proteins (32, 33), which in turn affects the stability of at least one of the 3C protease-E3α interactions that must occur during the ubiquitin-conjugating mechanism. The inhibitory effects evident when the control dipeptides alanyllysine and alanylphenylalanine were present in the reaction mixtures are because of a reduction in the steady state formation of the activated ubiquitin-E1 thiolester complex (20).

DISCUSSION

We have used a kinetic approach to examine the interaction of affinity-purified human ubiquitin-protein ligase E3α with two naturally occurring N-end rule substrate proteins, the 3C proteases produced by the picornaviruses EMCV and HAV. Both of these proteins behave as type III substrates, because they are substrate-dependent variations in $k_i$ or $k_{cat}$.

Rate measurements in a reconstituted system under E3α-limiting conditions were used to calculate the kinetic parameters for the E3α-catalyzed ubiquitination of the wild type EMCV and HAV 3C proteases and 3C proteases containing mutations in the protein destruction signal regions. Conjugation of wild type EMCV 3C protease shows saturation kinetics with a $K_m$ of $20 \pm 5 \mu M$ and a $V_{max}$ of $0.018 \pm 0.003 \text{ pmol/min}$. That the conjugation of ubiquitin to these proteins occurs with dissimilar kinetics is not surprising, given that these picornavirus protease orthologs do not share a high degree of sequence homology, and their destruction signal sequences are dissimilar (23, 32, 33), presumably reflected in the different kinetics of E3α ligation. The initial rates with which the 3C proteases are ubiquitinated in the reconstituted system were found to be much lower than for the type I N-end rule substrate α-lactalbumin (20), and the major product formed was the monoubiquitinated 3C protease adduct.

Mutations in the destruction signal sequences of both the EMCV and HAV 3C proteases alter the kinetics with which these proteins undergo E3α-dependent ubiquitination. Replacing the first leucine in the EMCV 3C protease destruction signal sequence with alanine or increasing to five the number of amino acids in the central regions of both the EMCV and the HAV 3C protease signal sequences resulted in proteins ubiquitinated by E3α with lower $K_m$ and $V_{max}$ values than the wild type proteins. Such mutations in the destruction signal sequence reduce the ability of these proteins to serve as substrates for ubiquitination in reticulocyte lysate (23). An attempt was made to demonstrate that a basic amino acid located in the third position in the central hydrophilic region of the destruction signal sequence enhances signal function in the E3α reaction system. Although the replacement of the aspartate at position 37 in the HAV 3C protease with an arginine resulted in an increase in the E3α-dependent rate of ubiquitination and increases in both the $K_m$ and $V_{max}$ for the reaction, the results of experiments with the EMCV 3C(R39D) and poliovirus 3C(N33R) proteases do not support the conclusion that a basic amino acid in this position is a required component of a functional destruction signal. Given that the 3C protease destruction signal is located in a stand-turn-strand motif (23, 34), it is conceivable that higher order structure plays a role in the recognition of these proteins by E3α. It is of interest to note that all of the mutations that resulted in reduced $K_m$ and $V_{max}$ values for ubiquitin attachment have also been found to reduce or eliminate the catalytic activities of the EMCV and HAV 3C proteases. Because the destruction signals are located near the catalytic site in both proteins, the effects of the mutations on the reaction kinetics observed here may be due, at least in part, to higher order structural alterations.

The $V_{max}/K_m$ ratios for the ubiquitination of the 3C proteases in the reconstituted reaction system are similar, and this provides insights into the mechanistic behavior of E3α. If it is assumed E3α acts upon these substrates in a non-equilibrium, sequential reaction process that can be described by the classic two-step binding and catalysis mechanism, so that $V_{max} = k_{cat}[E]_{total}[S]$, $K_m = (k_{-1} + k_{cat})/k_{cat}$, and it is assumed $k_{-1} < k_{cat}$, then differences in $k_{cat}$ will affect both the $V_{max}$ and the $K_m$ proportionally and in the same direction, as observed for the 3C protease substrates. Substrate-dependent variations in $k_i$ or $k_{cat}$.

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5 T. G. Lawson, unpublished results.
The simple interpretation of the data is that the differences in the rates at which E3α catalyzes the initial attachment of ubiquitin to the 3C proteases are the results of mechanistic events that occur subsequent to the initial recognition of the substrate proteins by E3α. It is possible that enzymatic steps following the initial ubiquitination reaction monitored here can impact the kinetics with which the first reaction occurs, especially if a significant portion of the available E3α is involved in the synthesis of polyubiquitinated 3C protease conjugates. Because the synthesis of monoubiquitinated 3C protease conjugates is rate-limiting, and the rate measurements were made prior to the formation of significant amounts of polyubiquitinated products, however, these downstream reaction events should not compromise our interpretation of the data.

The low $V_{\text{max}}$, and therefore low $k_{\text{cat}}$, values that characterize the E3α-dependent ubiquitination of the 3C proteases in the reconstituted reaction system are not without precedent. The $V_{\text{max}}$ for the ubiquitination of ribonuclease S, another type III N-end rule substrate, by E3α is similar to those calculated for the 3C proteases (20). These $V_{\text{max}}$ values are relatively low compared with the type I and type II substrates $\alpha$-lactalbumin and $\beta$-lactoglobulin (20). Moreover, the $V_{\text{max}}/K_m$ ratio for the ubiquitination of ribonuclease S is similar to the ratio found to characterize the reactions with the 3C proteases. $\alpha$-Lactalbumin and $\beta$-lactoglobulin are ubiquitinated in reactions characterized by $V_{\text{max}}/K_m$ ratios about 30- and 130-fold greater, respectively (20). This difference in $V_{\text{max}}/K_m$ values must reflect the effect on E3α of the internal position of the recognized destruction signal versus the N terminus of the substrate proteins.
The kinetic analysis of the E3α-dependent ubiquitination of three EMCV 3C protease proteins carrying pairs of lysine to arginine substitutions yielded $K_m$ and $V_{\text{max}}$ values both greater than for the wild type protein. This result is something of a surprise, because it might be expected that a reduction in the number of available lysine residues would lower the $k_{\text{cat}}$ and thereby the $V_{\text{max}}$ with which ubiquitin conjugation occurs. One possible explanation for these results is that the selection of the initial ubiquitin attachment site is a stochastic process (15), and the active site of E3α transiently associates with several available lysine residues until one in a favorable structural context is located. Reducing the availability of less optimal lysine residues could perhaps reduce the time required for E3α to locate favored ubiquitin attachment sites. Alternatively, it may be that surface-exposed basic amino acids are specifically involved in the discriminatory interactions that take place between E3α and substrate proteins and that E3α associates more effectively with arginine residues than with lysines. It can be concluded, at least, that the ten-amino acid protein destruction signal is not the only feature in the EMCV 3C protease capable of modulating E3α activity. It is of interest to note that changes in the basic amino acid composition of substrate proteins has also been shown indirectly to affect the activity of yeast Ubr1p, although it is unclear how these changes modulate Ubr1p-substrate interactions (35).

Our analysis of the kinetics of the ubiquitination of the EMCV and HAV 3C proteases indicate that E3α interacts with these proteins through a complicated mechanism and that substrate selection is not based solely upon a simple recognition of the substrate to form a ligase-substrate complex. Baboshina et al. (20) have proposed a model by which E3α interacts with at least two sites on substrate proteins. Their data suggest that interactions between the N-terminal amino acids of type I or type II N-end rule substrates and a site on E3α promote the formation of a catalytically competent conjugation of the ligase. This model predicts certain features of type III substrates other than N-terminal amino acids also associate with the N-terminal amino acid binding site and induce, though less effectively, the formation of a catalytically active E3α. Our results with the 3C proteases are consistent with this model. In the case of the 3C proteins, the destruction signal motifs appear to regulate how rapidly E3α can catalyze the conjugation of ubiquitin to the protease proteins. The initial recognition of the 3C protease by E3α, as defined by $k_2/k_1$ in the two-step reaction model, does not appear to contribute in a major way to the observed differences in the overall rates of initial ubiquitin conjugation. That the 3C proteases must interact with the same site, or sites, on E3α that binds basic or bulky N-terminal amino acids is indicated by the fact that both lysylalanine and phenylalanylalanine inhibit the ubiquitination of both the EMCV and HAV 3C proteases. It is conceivable that the hydrophobic and basic amino acids in the destruction signals can mimic destabilizing N-terminal amino acids during the association of the substrates with E3α. The 3C protease destruction signals do not appear to be as effective as N-terminal basic or bulky hydrophobic amino acids at stimulating E3α activity, because the $V_{\text{max}}$ values for the E3α-dependent ubiquitination of types I and II substrates are much higher than for the formation of ubiquitin-3C protease conjugates (20).

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