Previously it was demonstrated using a model precursor that processing at the N terminus of the HIV-1 protease (PR) precedes processing at its C terminus. We now show the expression, purification, and kinetics of the autoprocessing reaction of a PR precursor linked to 53 amino acids of the native flanking transframe region (ΔTFP-p6pol) of Gag-Pol and containing its two native cleavage sites. The PR contains the two cysteine residues exchanged to alanines, mutations that do not alter the kinetics or the structural stability of the mature PR. ΔTFP-p6pol-PR, which encompasses the known PR inhibitor sequence Gln-Asp-Leu within ΔTFP, undergoes cleavage at the ΔTFP/p6pol and p6pol/PR sites in two consecutive steps to produce the mature PR. Both ΔTFP-p6pol-PR and p6pol/PR exhibit low intrinsic enzymatic activity. The appearance of the mature PR is accompanied by a large increase in catalytic activity. It follows first-order kinetics in protein concentration with a rate constant of 0.13 ± 0.01 min⁻¹ in 0.1 M acetate at pH 4.8. The pH-rate profile for the observed first-order rate constant is bell-shaped with two ionicizable groups of pKᵢ 4.9 and 5.1. The rate constant also exhibits approximately 7-fold higher sensitivity to urea denaturation as compared with that of the mature PR, suggesting that the cleavage at the N terminus of the PR domain from the precursor leads to the stabilization of the dimeric structure.

In HIV-1, the structural and functional proteins are synthesized as two polypeptides, Gag and Gag-Pol, consisting of MA-CA-p2-NC-p1-p6 and MA-CA-p2-NC-TPF-p6pol-PR-RT-IN, respectively (2, 3). A protease (PR) cleavage site separates each of the subdomains of the precursors (Fig. 1). The 99-amino acid HIV-1 PR is encoded in the Pol open reading frame of the large Gag-Pol precursor and is flanked by the p6pol and reverse transcriptase (RT) domains at its N and C termini, respectively (2, 3). The PR is a member of the family of aspartic acid proteases, active only as a homodimer, and is responsible for its own release from the Gag-Pol and the processing of Gag and Gag-Pol to release the mature structural and functional proteins (2). Stage-specific regulation of PR activity in the viral replication cycle is crucial for proper assembly and maturation of the viral polyproteins to produce infectious virion. Maturation of the PR from Gag-Pol is suggested to occur after viral assembly and packaging (4). Molecular alteration of the spatial arrangement or enzymatic activity of PR impairs processes of assembly and maturation leading to the formation of noninfectious virion (5–8). Thus, the PR has proven to be a promising target for anti-retroviral therapy, and various PR inhibitors are now in clinical use (9).

The role of the native transframe region (TFR) sequences that flank the N terminus of PR in the Gag-Pol precursor is not fully understood but may function similar to a prorogen found in zymogen forms of cellular aspartic proteases (see Fig. 1 and Ref. 10). The TFR does not exhibit a stable secondary or tertiary structure except for a small potential for helix formation at its N terminus (11). TFR consists of two domains, a conserved N-terminal transframe octapeptide (TFP) followed by a 48–60 amino acid variable region p6pol. TFP and p6pol are separated by a PR cleavage site (3, 12, 13). The presence of the TFR in fusion with PR has a negative effect on PR processing (14). However, addition of the NC domain to TFR-PR seems to relieve this negative effect possibly by influencing PR dimerization (15). The isolated TFP is a specific competitive inhibitor of the mature PR (3). However, the exact mechanism by which TFR regulates the autocatalytic maturation of the PR from the Gag-Pol precursor is not fully understood.

PR-mediated processing of Gag and Gag-Pol polypeptides and particle maturation are complex events (for a review, see Ref. 16). Our previous studies using a model precursor, which consisted of the PR domain flanked at both ends by short native Gag-Pol sequences and an N-terminal maltose-binding domain (MBP), had shown that it undergoes maturation in two independent sequential steps. The first step involves the cleavage at the N terminus of the protease domain concomitant with a large increase in mature-like enzymatic activity and the appearance of the transient protease intermediate containing the flanking C-terminal RT polypeptide (17). The flanking C-terminal RT sequences, which do not appear to influence the catalytic activity of the PR precursor, are cleaved subsequently...
in a second step via an intermolecular process (18). These observations are consistent with results that blocking the cleavage at the p6pol-PR site leads to much reduced cleavage at the C terminus of PR (19, 20). In contrast to our observation that the model precursor exhibits low catalytic activity prior to the cleavage at the p6pol-PR junction, several reports of mutated PR fusion proteins that either contain a mutation at the p6pol-PR junction or contain short native or non-native sequences flanking the N terminus of PR possess wild type enzymatic activity (16, 21–23). This raised the question whether the MBP in our model construct impeded PR folding/dimerization, catalytic activity, and observed kinetics of the autocatalytic maturation reaction.

In order to clarify these critical steps in the maturation cascade, we expressed and purified PR precursor proteins linked to the native TFR containing the native cleavage sites and characterized the autoprocessing reactions in vitro. To circumvent aggregation of the fusion proteins associated with intermolecular disulfide bond formation that may lead to anomalous kinetic measurements of the autoprocessing reaction, we used PR fusions with Cys residues replaced by Ala, mutations that do not alter the kinetic parameters of the mature enzyme. By using these TFR-PR constructs we examined the mechanism and the pH-rate profile of the autocatalytic maturation reaction, and the stability of the proteins by product analyses and kinetics. In addition and more importantly, we show that two temporally regulated N-terminal cleavages, first at the native TFP/p6pol followed by cleavage at the p6pol-PR, are crucial for PR maturation and enzymatic activity.

MATERIALS AND METHODS

Buffers

The buffers employed in this study are as follows: buffer A, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5 mM benzamidine HCI, 5 mM DTT; buffer B, 50 mM Tris-HCl, pH 7.5, 5 mM guanidine HCl, 1 mM EDTA, 2 mM DTT; buffer C, 50 mM Tris-HCl, pH 8.0, 5 mM DTT, 5 mM EDTA, 5 mM urea; buffer D, 100 mM acetic acid, pH 5.0, 1 mM DTT, 1 mM EDTA, 0.05% Triton X-100.

Expression and Purification of Wild Type and Cys to Ala Mutants of HIV-1 Protease Linked to the TFR

The HIV-1 PR linked to native transframe region was expressed in Escherichia coli as described (24) using the expression vector pET11a and host bacteria, BL21 (DE3 (25)). The native viral sequence spans nucleotide positions 2093–2549 of HIV-1 isolate HIVIX2 (GenBank accession number HIVIX2X292). Cys-63 and Cys-95 in the PR domain was either individually or collectively substituted to Ala using the polymerase chain reaction (26, 27). The cloning strategy that was employed introduces four non-native amino acids, Met-Glu-Phe-Met, linked to the fourth amino acid of TFP from its N terminus beginning with the sequence Glu-Asp-Leu-Ala-Phc-Leu-Gln-Gly-Lys. This precursor is termed ΔTFP-p6pol-PR (see Fig. 1), where Leu-Gln-Gly-Lys is the N terminus of p6pol.

Cells were grown in minimal media at 37 °C, 30% pO2, pH 7.0, in a 2-liter Braun model MD fermentor. When the cell density reached an optical density of 0.5 at 600 nm, protein expression was induced by the sample was applied to a Superdex 200 column (6 × 30 cm, Amersham Pharmacia Biotech) equilibrated with buffer B and run at a flow rate of 4 ml/min. Fractions containing the precursor proteins (ΔTFP-p6pol-PR and p6pol-PR, see Fig. 2) and the smaller molecular weight mature PR that is released by autoprocessing were well separated. Column fractions were analyzed by SDS-polyacrylamide pre-cast gels (Novex) after removing the guanidine HCl from the protein fractions (28). The peak fraction containing the two PR fusion proteins was further fractionated by RP-HPLC on POROS R2 resin (Perspective Biosystems) using a linear gradient of 0–60% acetonitrile in 0.05% trifluoroacetic acid for a period of 12 min to attain nearly homogeneous proteins. Protein fractions were dialyzed (Slide-A-Lyzer 10K dialysis cassettes, Pierce) in 1000-fold excess of 25 mM formic acid, pH 2.8, at 4 °C for 1.5–2 h, concentrated on microcentrifuge 10 concentrators (Amicon, MA), and stored at 4 °C. Alternatively, protein fractions after RP-HPLC separation were lyophilized, dissolved to a concentration of 20–30 μM in buffer C, and stored frozen in aliquots at −70 °C. Protein concentrations for kinetic studies were determined as described previously (22).

Protein Sequencing

Proteins were subjected to SDS-PAGE and electroblotted onto ProBlott membranes (Perkin-Elmer). The bands were excised and applied to a Blott cartridge (Perkin-Elmer), and each sample was subjected to 20 cycles of N-terminal sequencing using an Applied Biosystems model 477A protein sequencer (Perkin-Elmer) to confirm the N-terminal residues of ΔTFP-p6pol-PR and p6pol-PR.

Protein Folding

Two methods were utilized for protein renaturation of the precursor proteins ΔTFP-p6pol-PR and p6pol-PR. 2.5 μl of protein in 25 mM formic acid was diluted in 40-fold excess of appropriate buffer (formate, acetate, or phosphate) to attain final pH values between 4 and 6.5. Alternatively 10 μl of protein in buffer C was diluted with 90 μl of buffer D to reconstitute the first-order reaction for the autoprocessing reaction of p6pol-PR with that of the model precursor reported previously (17). The wild type and mutant mature proteases (17–18 μl in buffer B) were renatured by 33-fold dilution in buffer D or 50-fold from 50 mM formic acid, pH 2.6.

Kinetics

Mature Proteases—The kinetic parameters of the mature wild type PR and its mutants were measured using the substrate Lys-Ala-Arg-Val-Nle-Phe(N03)3-Glu-Ala-Nle-NH2 (where Phe(N03)3 is 4-nitrophenylalanine) (California Peptide Research) at 25 °C as described previously (17, 18).

Precursor Proteins—Reactions were carried out at 25 °C, and aliquots were drawn at various time intervals and assayed for enzymatic activity or subjected to SDS-PAGE followed by immunoblotting and densitometry as described previously (17, 18). The first-order rate constant is calculated by one of two methods. A plot of initial rate versus protein concentration is linear (see inset of Fig. 5), and the slope of the line is the observed first-order rate constant. Alternatively, the first-order rate constant is calculated by fitting the first-order equation to data using the program Enzfitter (published by Biosoft, Cambridge, UK). Sodium acetate buffers (100 mM) containing 100 mM NaCl and the same additives as that of buffer D, but without Triton X-100, were used to obtain the pH-rate profile. The pH values were calculated using the program Enzfitter and the equation (Kc1/Ke1[H]=Ke1/[1+([H]/Kc1+Ke1)]) (see Ref. 29).

RESULTS AND DISCUSSION

Comparison of the Catalytic Activity and Stability of Wild Type and Cys to Ala Mutants of the Mature Protease—To circumvent any problems associated with the oxidation of Cys thiol groups in wild type PR which leads to protein aggregation and complicates kinetic experiments, PR mutants with either Cys-67 or Cys-95 or both residues substituted with Ala were constructed fused to the transframe region (ΔTFP-p6pol-PR, see Fig. 1). Expression of the wild type and mutant constructs in E. coli results in the efficient processing of the precursors to release mature PR. The mature wild type and Cys to Ala mutants were purified. Similar to the wild type enzyme (18, 22), the Cys to Ala mutants exhibit >95% of their enzymatic activity instantaneously with no lag period at pH 5.0 when a denatured solution of the precursor in buffer B is folded by 33-fold dilution in buffer D. The kinetic parameters for the wild type and Cys mutants of PR-catalyzed hydrolysis of a chromogenic substrate under the same conditions are nearly identical (Table I). The dimer stability of the G67A/C95A mutant mature PR examined by urea denaturation shows 50% loss in enzymatic
activity at 1.8 M urea unchanged from that of the wild type PR (see below). Thus, mutating the Cys residues to Ala does not appear to alter the environment of the active site of the mature HIV-1 PR or the overall structure of the enzyme (30).

Precursor Proteins of the HIV-1 Protease—In all constructs the induced protein accumulates in the form of inclusion bodies. Analysis of the inclusion bodies by SDS-PAGE verified the presence of a major band that corresponds in mobility to the mature PR released via autoprocessing of the induced protein accumulates in the form of inclusion bodies (30).

To the mature PR. Although detailed kinetics of the conversion of ΔTFP-p6<sup>pol</sup>-PR to the intermediate p6<sup>pol</sup>-PR was not carried out due to limited availability of the purified protein, the half-life (t<sub>1/2</sub>) of the reaction was estimated by comparing the relative band intensities of the starting material and the intermediate assuming first-order kinetics (see Fig. 3A). The rate of formation of p6<sup>pol</sup>-PR from the ΔTFP-p6<sup>pol</sup>-PR precursor (t<sub>1/2</sub> ~ 20 min, k<sub>1</sub> ~ 0.035 min<sup>-1</sup>) is about three times slower than that of the disappearance of p6<sup>pol</sup>-PR and release of PR (t<sub>1/2</sub> ~ 6 min, k<sub>2</sub> ~ 0.12 min<sup>-1</sup>) at pH 5.4 (Scheme 1).

A plot of the rate of appearance of enzymatic activity versus time is characterized by a lag period followed by a first-order process (Fig. 3B) indicating that cleavage at the N terminus of the PR (p6<sup>pol</sup>/PR site) is required for the activity of PR. Thus, the rate of appearance of mature-like catalytic activity is concomitant with the cleavage at the p6<sup>pol</sup>/PR site (Step 2) and not of the cleavage at the TFP/p6<sup>pol</sup> site (Step 1). The hydrophilic ΔTFP, which is known to inhibit the mature PR in a pH-dependent manner (3), may play a role in determining this stepwise cleavage. Further investigation with the PR precursor having the entire TFP domain is required to assess fully its regulatory role.

Maturation of p6<sup>pol</sup>-PR Precursor—Similar to the wild type...
mature PR and its Cys mutants, the denatured p6<sub>pol</sub>-PR folds instantaneously as indicated by the lack of observable time-dependent change in the intrinsic protein fluorescence (17). The folded p6<sub>pol</sub>-PR undergoes a time-dependent maturation reaction concomitant with increase in enzymatic activity. At pH $\geq$5.0, the reaction proceeds in a single step to produce the mature enzyme, whereas at pH < 5.0 it is characterized by the appearance and disappearance of a single protein intermediate that migrates between p6<sub>pol</sub>-PR and PR (see Fig. 4). This intermediate (termed $\Delta p6<sub>pol</sub>$-PR), which is generated by cleavage at the Leu-24<sup>2</sup>Gln-25 site within p6 pol, was confirmed by subjecting an aliquot of the reaction mixture to RP-HPLC linked to an electrospray-mass spectrometer (data not shown; see Fig. 1). This observation is in agreement with a previous report by Zybarth et al. (21).

The maturation reaction of p6<sub>pol</sub>-PR displays good first-order kinetics between pH 4.0 and 6.5 as indicated by a linear relation between the rate of increase in mature-like catalytic activity and protein concentration (inset, Fig. 5). Plots of the measured densities corresponding to the starting material and the product (mature PR) and the rate of increase in mature-like catalytic activity versus time are shown superimposed on each other (Fig. 5). The first-order rate constants for the maturation reaction under different conditions are shown in Table II. The first-order rate constant displays a bell-shaped dependence on pH with two ionizable groups having $pK_a$ values of 4.9 and 5.1 (Fig. 6). The complete pH profile for the maturation of p6<sub>pol</sub>-PR is in agreement with studies of the time-dependent maturation of a mini precursor in which a mutant HIV-1 PR (A28S) was linked to 25 amino acids of the flanking p6 pol sequence (32, 33). Also, the higher $pK_a$ of 5.1 is in agreement with the $pK_a$ values of 4.8 and 5.2 obtained for the mature PR (34) and PR-$\Delta$Pol (18), respectively, and other fusion proteins (22). However, the lower $pK_a$ of 4.9 is about 1.8 pH units higher than that observed for the mature PR.

Our results for the processing of p6<sub>pol</sub>-PR to mature PR are consistent with the mechanism proposed for the maturation of PR from the model precursor MBP-$\Delta$TF-PR-$\Delta$Pol (17). The pH-rate profile (Fig. 6) and the inhibition constant (17) for the maturation reaction are similar to that reported for the mature PR. These studies indicate that the active site of the p6<sub>pol</sub>-PR precursor resembles that of the mature PR. The concomitant increase in mature-like enzymatic activity and appearance of the mature PR product (Figs. 4 and 5) are consistent with a single rate-limiting step involving the cleavage of the scissile bond at the p6<sub>pol</sub>/PR junction. The first-order kinetics for the maturation of the PR from the native precursor p6<sub>pol</sub>-PR indicate that this N-terminally truncated p6<sub>pol</sub>-PR fused to the PR denoted as $\Delta p6<sub>pol</sub>$-PR. This intermediate is generated via cleavage at the Leu-24<sup>↓</sup>Gln-25 site within the p6<sub>pol</sub>-PR domain (21).
Optimal conditions for the autoprocessing reaction is similar to that observed for the conversion of zymogen form of the gastric protease pepsinogen, which unlike retroviral proteases is a monomeric enzyme (10). Pepsinogen differs from mature pepsin by a 44-amino acid long positively charged N-terminal proregion. Below pH 2.0, pepsinogen is converted in a single step through an intramolecular maturation process to pepsin with a homogenous N terminus, whereas at pH 4.0 the activation product is heterogeneous with multiple N-terminal products (35).

Catalytic Activity and Structural Stability of p6pol-PR—Comparisons of the first-order rate constants for the autoprocessing of the model precursor MBP-DTF-PR-ΔPol and the native precursor p6pol-PR are similar indicating that the MBP domain, which mimics nearly the size of the Gag domain of Gag-Pol, does not perturb the maturation reaction in any significant manner. Thus, the maturation of PR from the native TFR-PR precursor appears to be a sequential multi-step ordered process that involves at least two peptide bond cleavages upstream to the PR domain prior to the generation of optimal enzymatic activity. In the first step, DTFP-p6pol-PR is converted to p6pol-PR with the same low catalytic activity as that of DTFP-p6pol-PR. It is most likely that cleavage at the DTFP/p6pol site also occurs via an intramolecular mechanism as an increase in mature-like catalytic activity is concomitant only with cleavage at the p6pol-PR site (Step 2).

The dimeric form of p6 pol-PR, which is mandatory for the formation of an active site capable of supporting a hydrolytic reaction, is highly sensitive to urea denaturation compared with the mature enzyme (Fig. 7). Loss in enzymatic activity relates to loss of stable tertiary structure (18, 22), suggesting that p6pol-PR is structurally less stable than the mature PR. This result complements our previous studies showing that the PR domain when fused to 19 amino acids of the flanking C-
terminal reverse transcriptase sequence (PR-D Pol) or short native or non-native sequences at its N terminus is also less stable toward urea denaturation (18, 22). The above results are also consistent with results showing that the mature PR is largely dimeric above 10 nM (18), whereas inactive N-terminally extended forms of the PR linked to the TFR fail to dimerize in a qualitative assay (15).

The fact that the maturation reaction of the native p6pol-PR is monitored by following the increase in enzymatic activity (see Fig. 3B and 5) is clear evidence that there is a large difference between the catalytic activity of the mature PR and that of p6pol-PR. The low catalytic activity seems to be intrinsic to the PR when linked to the native TFR having the native cleavage sites. This low catalytic activity could be either due to a conformational difference of the dimeric precursor or can be an apparent effect of the equilibrium that largely favors the unfolded or partially folded form of the protein relative to the folded enzymatically active dimer.

In contrast to our results that indicate that native TFR sequences perturb the structure and PR activity, other PR precursors containing truncated p6pol or short non-native regions flanking the N terminus of PR exhibited catalytic activities that are comparable with that of the mature PR (21–23, 33). These results emphasize the fact that the full-length TFR with its native cleavage sites is critical for the regulated processing of PR from the Gag-Pol and optimal catalytic activity. In accordance with this interpretation, a blocking mutation at the regions flanking the N terminus of PR exhibited catalytic activities that are comparable with that of the native PR (21–23, 33). These results emphasize the fact that the full-length TFR with its native cleavage sites is critical for the regulated processing of PR from the Gag-Pol and optimal catalytic activity. In accordance with this interpretation, a blocking mutation at the regions flanking the N terminus of PR exhibited catalytic activities that are comparable with that of the native PR (21–23, 33).

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