Caged HIV-1 Protease: Dimerization Is Independent of the Ionization State of the Active Site Aspartates

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Human immunodeficiency virus type-1 (HIV-1) protease catalyzes the cleavage of several viral polypeptides yielding mature proteins of both structural and catalytic importance to the virus.[1] HIV-1 protease is crucial for normal viral replication,[2] and is the therapeutic target for several anti-HIV agents.[3]

HIV-1 protease exists as a 22 kDa homodimer that self-assembles from two identical polypeptides to resemble monomeric aspartyl proteases.[4] The active site is formed at the interface of the homodimer and consists of two aspartyl residues, Asp25 and Asp125, one contributed by each subunit.[4c] Both aspartates are necessary for proteolysis.[5] Given the close spatial proximity between the two aspartates, as well as differing protonation states,[6] hydrogen bonding across the dimer interface could well help direct the protease monomers during self-assembly.[7]

To further define the nature of active site involvement in dimer assembly, we have prepared HIV-1 protease analogues having altered hydrogen bonding properties at position 25. In vitro suppression of a UAG codon[9] introduced into position 25 of HIV-1 protease was effected by using misacylated suppressor tRNAs (Figure 1),[10] affording proteins in which Asp25 was replaced with analogues (Figure 2). Presently we demonstrate (i) the elaboration of HIV-1 proteases that can be activated by using a water-soluble palladium catalyst or light, (ii) that deprotection of a caged mutant[11] of HIV-1 protease can restore proteolytic function, and (iii) that HIV-1 protease dimer assembly proceeds even if the active site aspartates cannot ionize.

Four HIV-1 protease genes were synthesized *de novo*: a monomer (PR25) and tethered dimer (PR25PR),[12] as well as the respective wild-type genes. The wild-type tethered dimer of HIV-1 protease prepared in an S-30 translation reaction yielded a full length 23 kDa protein (Figure 3, lane 1) that folded into an active protease and could be assayed directly from the translation mixture. The wild-type monomer of HIV-1 protease, while yielding a full length 13.1 kDa protein product, failed to produce proteolysis after in vitro translation, due presumably to the insolubility of the monomer as well as unproductive aggregation.[13]

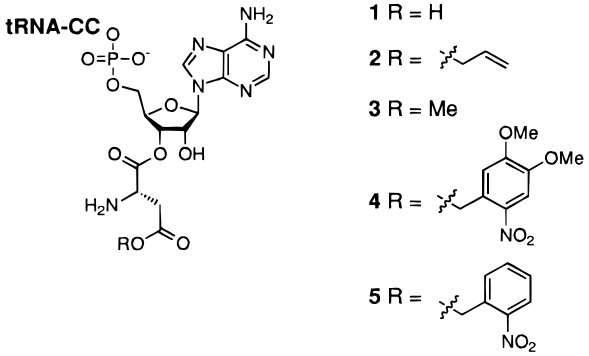

Figure 1. Misacylated suppressor tRNAs used for the introduction of aspartate derivatives into position 25 of HIV-1 protease.

As shown in Figure 3, it was possible to obtain analogues of HIV-1 protease dimer having allyl aspartate, methyl aspartate, or nitrobenzyl aspartate in position 25. The same amino acids were incorporated into position 25 of the monomer (Supporting Information, Figure 1). The tethered dimer of HIV-1 protease prepared in an S-30 cell-free translation system with $tRNA^{Phe}_{CUA}$ misacylated with aspartic acid[9h] had the same specific activity as wild-type protease in a fluorescence-based protease assay.[14] In their blocked forms, HIV-1 protease analogues having allyl, methyl, and nitrobenzyl aspartates at position 25 had minimal proteolytic activity. Irradiation of the dimeric protease containing nitrobenzyl aspartate afforded a deprotected species that was 97% as active as its wild-type counterpart (Supporting Information, Figure 2).[15] Likewise, a water-soluble palladium catalyst, $Pd[PPh_2(m\text{-}SO_3NaPh)]_3$,[16] catalyzed the removal of the allyl ester from analogue **2**, affording an active protease.[17,18]

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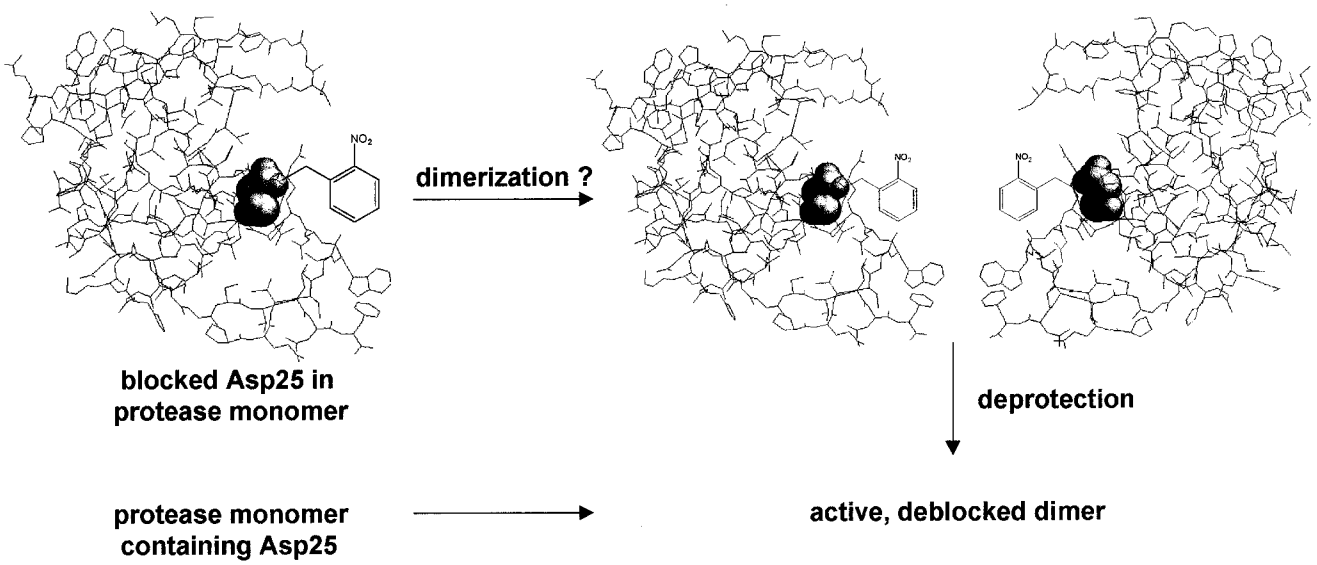

Figure 2. Scheme demonstrating deprotection of a caged (inactive) analogue of HIV-1 protease to yield catalytically active protein. The nitrobenzylated HIV-1 protease was compared to wild-type HIV-1 PR to study the extent of active site involvement in facilitating dimerization.

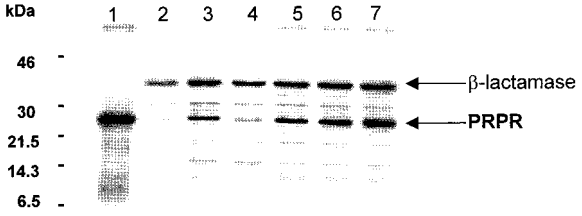

Figure 3. Autoradiogram of a 15% SDS-polyacrylamide gel illustrating the in vitro synthesis of HIV-1 protease tethered dimer analogues. Protein synthesis was carried out in vitro in the presence of [^{35}S]methionine mRNA containing a UAG stop codon at position 25 and an unacylated suppressor tRNA (lane 2) or a misacylated suppressor tRNA (lanes 3−7). Suppression efficiencies are noted in parentheses. Lane 1, HIV-1 protease dimer elaborated from wild-type mRNA; lane 2, full length unacylated $tRNA^{Phe}_{CUA}$; lane 3, aspartic acid (**1**) (16%); lane 4, nitroveratryl aspartate (**4**) (1%); lane 5, nitrobenzyl aspartate (**5**) (38%); lane 6, allyl aspartate (**2**) (44%); lane 7, methyl aspartate (**3**) (35%).

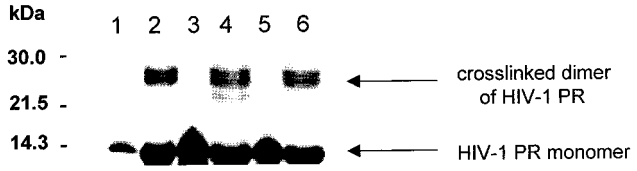

Figure 4. Chemical cross-linking of HIV-1 protease monomers by dimethyl suberimidate. HIV-1 protease having nitrobenzyl aspartate-25 was synthesized in vitro, purified (nickel affinity chromatography), and subsequently refolded by dialysis. Lane 1, wild-type protease monomer; lane 2, wild-type protease monomer + dimethyl suberimidate; lane 3, protease containing nitrobenzyl aspartate (**5**); lane 4, protease containing nitrobenzyl aspartate + dimethyl suberimidate; lane 5, deprotected protease; lane 6, deprotected protease + dimethyl suberimidate.

Hydrogen bonding within the active site of HIV-1 protease may arise between differentially protonated active site aspartates or through the coordination of a water molecule.[5b,6] Upon replacing the active site residues with asparagine, protease monomers were found to self-associate although the stability of the protein dimer decreased.[7,8] This mutation prevents H-bonding through a coordinated water; however, it does not exclude the possibility that one asparagine side chain may H-bond with the other, facilitating dimerization. To eliminate the possibility of H-bonding, an HIV-1 protease monomer was synthesized bearing nitrobenzyl aspartate **5** at position 25 (Supporting Information, Figure 1, lane 5). After purification, the protease was dialyzed to promote dimerization. To assay for protease dimers, the protein was chemically cross-linked with dimethyl suberimidate,[4b] permitting discrimination of monomer from dimer by SDS-PAGE. HIV-1 protease dimerizes even with a nitrobenzyl ester on the aspartate side chain (Figure 4). Thus H-bonding, while important to dimer stability, is not a prerequisite for association.[19]

The HIV-1 proteases are the first caged analogues of this enzyme. They are of potential utility for the study of time-dependent processes mediated by HIV-1 protease, such as autoprocessing.[1] Because HIV-1 protease undergoes facile self-proteolysis,[20] the chemically protected species described here should also facilitate structural and kinetic analysis by precluding degradation.

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Supporting Information Available: Figures illustrating the in vitro synthesis of analogues of HIV-1 protease monomer, the enzymatic activity of the monomer following deprotection of allyl aspartate, and dimerization and the enzymatic activity of HIV-1 protease dimer following photochemical removal of the nitrobenzyl protecting group (4 pages, PDF). See any current masthead page for Web access instructions.

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(17) Under denaturing conditions, in the presence of 6 M urea and 2 mM catalyst, Pd-catalyzed deblocking afforded an active protease monomer having a specific activity 85% that of wild type (16 h incubation, 23 °C), whereas the caged enzyme was inactive (Supporting Information, Figure 3).

(18) It may be possible to employ this protecting group under native conditions using well-solvated aspartates on the surface of enzymes. See: Pollit, S. K.; Schultz, P. G. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 2104.

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