NMR and X-ray Evidence That the HIV Protease Catalytic Aspartyl Groups Are Protonated in the Complex Formed by the Protease and a Non-Peptide Cyclic Urea-Based Inhibitor

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There is intense interest in developing effective inhibitors of the HIV protease, because active enzyme is required for the production of infectious virus particles.[2] High-resolution crystal structures of HIV protease and HIV protease/inhibitor complexes have stimulated structure-based design and synthesis of potent protease inhibitors.[3] One such molecule, DMP323,[1] which incorporates protease binding substituents onto a novel seven-membered ring, possesses high affinity and specificity for the viral protease.

The structure of the protease/DMP323 complex reveals many stabilizing protein−ligand interactions.[1] In particular, the crystal structure shows that the carboxyl oxygens of the catalytic aspartic acid residues, Asp25/25′, and the diol oxygens of the inhibitor are positioned to form a network of hydrogen bonds. However, the pK_a values of the Asp25/25′ residues have not been determined when the protease is bound to DMP323 or to any other high-affinity inhibitor. Recent molecular dynamics simulations[4] predict that ionization states of the catalytic Asp residues strongly depend upon the local environment of the protease/inhibitor complex. Hence, measurement of Asp25/25′ pK_a values is fundamental to understanding the interactions that stabilize the DMP323/protease complex. The present communication reports the use of chemical shift titrations, H/D isotope shift measurements, and the X-ray crystal structure to determine the pK_a values of all Asp and Glu residues in the protease/DMP323 complex. Taken together the data provide strong evidence that the Asp25/25′ carboxyl groups are protonated over the pD range 2−7 and form a network of strong hydrogen bonds with the diol hydroxyl groups of the inhibitor, which stabilize the structure of the complex. In contrast with the catalytic Asp residues, the salt-bridging Asp29/29′ side chains have a pK_a of 2.0.

The titration of the protease Asp and Glu side-chain carboxyl groups was followed by measurement of the chemical shifts of all Asp (C^β,H^β,C^γ) and Glu (C^γ,H^γ,C^δ) signals as a function of pD. All chemical shifts were determined at each pD value by recording a modified CT-HCACO 3D triple resonance spectrum[5] (supplementary material) on a fully ^{13}C enriched sample of the protease bound to DMP323. Side-chain carboxyl carbons were assigned at pD = 5.2 by correlation of the $H^\beta C^\beta C^\gamma$ and $H^\gamma C^\gamma C^\delta$ signals observed in the 3D spectrum (supplementary material), with the reported assignments[6] of the Asp and Glu methylene ^1H and ^{13}C signals. The monomer units of the protease homodimer have identical chemical shifts.

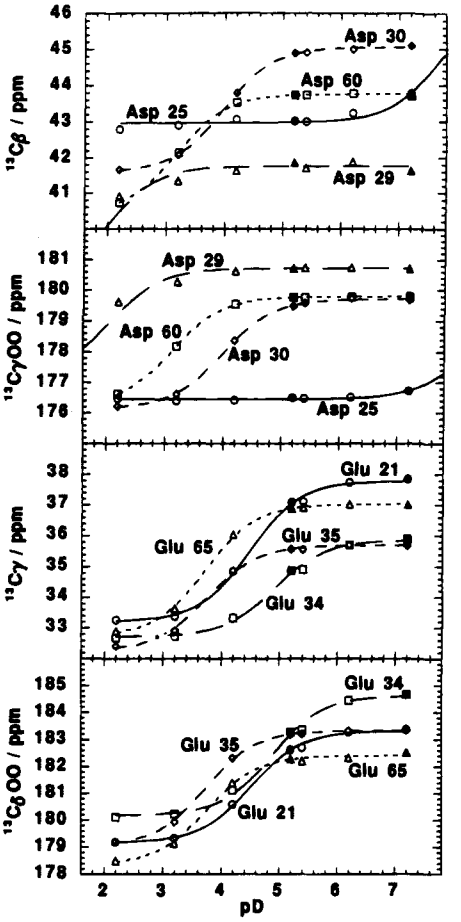

Figure 1. The Asp and Glu chemical shifts plotted as a function of pD (glass electrode reading, uncorrected for isotope effects) and the best fits obtained using the function given in Table 1. Data at pD 5.2 and 7.2 were obtained on the Cys95Ala single site mutant, while data at other pD values were obtained using the Cys67,95Ala double mutant, to circumvent the problem of formation of interprotein disulfide bonds. Because nearly all chemical shifts of the single and double mutant proteins are the same, the signal assignments of the double mutant at pD 5.2 were readily obtained from a 3D HBHA(CO)NH[17] experiment together with known assignments of the single site mutant. Chemical shifts are referenced as described previously.[18]

The protease Asp(C^β,C^γ) and Glu(C^γ,C^δ) chemical shifts are plotted as a function of pD in Figure 1. Table 1 shows that all Glu side chains have pK_a values in the range 3.7−4.9, close to the value of 4.2 obtained in a solvated model peptide.[7] This observation agrees with the X-ray structure of the protease/DMP323 complex in which all four Glu carboxyl groups are solvent accessible. The side chains of Asp29/29′, Asp30/30′, and Asp60/60′ are also solvent exposed in the crystal structure, and the observed pK_a values of the two latter Asp residues, Table

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(1) Lam, P. Y. S.; Jadhav, P. K.; Eyermann, C. J.; Hodge, C. N.; Ru, Y.; Bacheler, L. T.; Meek, J. L.; Otto, M. J.; Rayner, M. M.; Wong, Y. N.; Chang, C.-H.; Weber, P. C.; Jackson, D. A.; Sharpe, T. R.; Erickson-Viitanen, S. *Science (Washington, D. C.)* **1994**, *263*, 380−384.

(2) Seelmeier, S.; Schmidt, H.; Turk, V.; von der Helm, K. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 6612−6616.

(3) Wlodawer, A.; Erickson, J. W. *Annu. Rev. Biochem.* **1993**, *62*, 543−585.

(4) Harte, W. E., Jr.; Beveridge, D. L. *J. Am. Chem. Soc.* **1993**, *115*, 3883−3886.

(5) Grzesiek, S.; Bax, A. *J. Magn. Reson., Ser. B* **1993**, *102*, 103−106.

(6) Yamazaki, T.; Nicholson, L. K.; Torchia, D. A.; Stahl, S. J.; Kaufman, J. D.; Wingfield, P. T.; Domaille, P. J.; Campbell-Burk, S. *Eur. J. Biochem.* **1994**, *219*, 707−712.

(7) Richarz, R.; Wuethrich, K. *Biopolymers* **1978**, *17*, 2133−2141.

0002-7863/94/1516-10791$04.50/0

Table 1. pK_a Values of HIV-1 Protease Asp and Glu Residues[a]

residue	pK_a[b]	pK_a[c]	residue	pK_a[b]	pK_a[c]
Asp25[d]	8.19	7.70	Glu21	4.52	4.48
Asp29[d]	2.06	1.97	Glu34	4.88	4.91
Asp30	3.99	3.96	Glu35	3.73	3.75
Asp60	3.11	3.15	Glu65	3.76	3.72

[a] Determined by fitting the pD dependent chemical shifts, δ, with the function $\delta = \delta_{A^-} - (\delta_{A^-} - \delta_{AH})/(1 + 10^{(pD-pK_a)})$, where pK_a, δ_{A^-}, and δ_{AH} are parameters obtained from the fit, and the latter quantities are the respective chemical shifts in either the absence or presence of a proton on the carboxyl group. [b] From fits of carboxyl carbon data. [c] From fits of β-carbon (Asp) and γ-carbon (Glu) data. [d] Determined assuming that $\delta_{A^-} - \delta_{AH} = 3.5$ ppm and $\delta_{A^-} - \delta_{AH} = 3.1$ ppm for carboxyl and β-carbons, respectively.[7]

1, differ by less than 1 pD unit from the peptide Asp pK_a value of 3.9.[7] The markedly smaller pK_a value of the Asp29/29′ residues, ca. 2.0, is ascribed to salt bridges between Asp29/29′ and Arg8,87/8′,87′ side chains,[1] an observation consistent with the pK_a depression previously reported for salt-bridging Asp side chains.[8,9]

Although the pK_a of Asp 29 is not known in the free protease, the unusually low pK_a found in the protease/DMP323 complex suggests that the Asp29/Arg8′,87 salt bridges stabilize the folded structure of the protein. This idea is supported by the observation that the protease/DMP323 complex unfolds at a pD only slightly below the pK_a of Asp29/29′. In addition, the observation that mutating Asp29 to Glu, Asn, Ala, Gly, or Val inactivates the recombinant protease in *Escherichia coli*[10] is further evidence that the Asp29/29′–Arg8,87/8′,87′ salt bridges stabilize the protease.

In contrast with all other Asp and Glu side chains, the carboxyl groups of Asp25/25′ are completely buried. The observation that their C^β and C^γ chemical shifts are independent of pD shows that Asp25/25′ carboxyls do not titrate in the pD range 2–7. The carboxyl carbon signal position of these residues, 176.4 ppm, indicates that both Asp side chains are protonated. This conclusion is strongly supported by the observation that the Asp25/25′ C^γ signals shift downfield by 0.26 ± 0.02 ppm, when the solvent is changed from D_2O to H_2O, at pD = pH = 5.2. This value is in agreement with the isotope shift of 0.225 ± 0.015 obtained from model compound studies.[11,12] The Asp25/25′ isotope shift was halved in 50% D_2O/50% H_2O. As expected, a negligible isotope shift, −0.01 ± 0.02 ppm, was observed for the C^γ signals of the nonprotonated Asp29/29′ carboxyl side chains.

The observation that the Asp25/25′ side chains are buried in a region of the protein crystal structure that is devoid of (a) internal water molecules and (b) positively charged groups also indicates that both Asp side chains are uncharged. In addition, when two Asp side chains share a proton, as found in the structures of uninhibited proteases, the side chains are typically coplanar,[13,14] while in the protease/DMP323 complex the planes of the Asp25/25′ carboxyls make an angle of ca. 45°.

In the crystal structure, the Asp25/25′ carboxyl side chains and inhibitor diol groups are oriented so that a hydrogen bond network having nearly ideal donor/acceptor bond angles and internuclear oxygen distances can be formed when the Asp residues are protonated, Figure 2. A minor rearrangement of

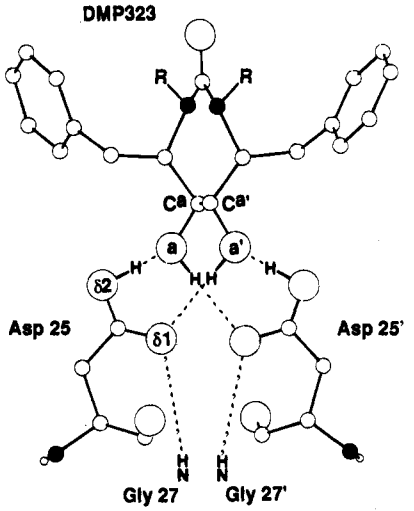

Figure 2. Proposed network of the hydrogen bonds formed by protonated Asp25/25′ carboxyl side chains and diol groups of DMP323. The hydrogen bonds shown have nearly ideal geometry on the basis of the oxygen internuclear distances (3.06 Å [$d(O^{\delta 2}O^{a})$] and 3.09 Å [$d(O^{\delta 1}O^{a'})$]) and the carbon–oxygen bond angles (103.4° [$C^{\gamma}O^{\delta 2}O^{a}$], 112.8° [$C^{\gamma}O^{\delta 1}O^{a'}$], 110.8° [$O^{\delta 2}O^{a}C^{a}$], and 110.3° [$O^{\delta 1}O^{a'}C^{a'}$]), obtained from the crystal structure. The large, small, and filled circles represent oxygen, carbon, and nitrogen atoms, respectively. For purposes of clarity, the *p*-(hydroxymethyl)benzyl nitrogen substituents of the DMP323 ring are represented as R.

the hydrogen-bonding protons yields an alternative model (supplementary material), with a slightly higher energy, as calculated using the CHARMm force field (supplementary material). Two nearly isoenergetic networks, in rapid equilibrium on the NMR time scale, would make a favorable entropic contribution to complex formation.

An analysis[15] of protease pH rate studies of model substrates has shown that, in the active enzyme, the catalytic Asp carboxyl groups have distinct pK_a values of ca. 3.5 and 5.7. In addition, the uninhibited protein unfolds[16] at a pH of <4. Hence, binding to DMP323 significantly increases the Asp25/25′ pK_a's and stabilizes the protease against acid denaturation. These results support the conclusion that interactions involving protonated Asp25/25′ side chains significantly stabilize the protease/DMP323 complex.

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Supplementary Material Available: Modified CT-HCACO pulse sequence used to record the 3D spectra of the protease/DMP323 complex, strips taken from the modified CT-HCACO spectrum showing the $H^\beta C^\beta C^\gamma$ and $H^\gamma C^\gamma C^\delta$ correlations of all Asp and Glu residues at pD = 5.2, drawing of alternate hydrogen bond network formed by DMP323 diol groups and Asp25/25′ carboxyl groups, and results of CHARMm energy calculations of the two proposed H-bond networks (9 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(8) Anderson, D. E.; Lu, J.; McIntosh, L.; Dahlquist, F. W. In *NMR of Proteins*; Clore, G. M., Gronenborn, A. M., Eds.; Macmillan: London, 1993.
(9) Oda, Y.; Yamazaki, T.; Nagayama, K.; Kanaya, S.; Kuroda, Y.; Nakaumra, H. *Biochemistry* **1993**, *33*, 5275–5284.
(10) Loeb, D. D.; Swanstrom, R.; Everitt, L.; Manchester, M.; Stamper, S. E.; Hutchison, III, C. A. *Nature (London)* **1989**, *340*, 397–400.
(11) Ladner, H. K.; Led, J. J.; Grant, D. M. *J. Magn. Reson.* **1975**, *20*, 530–534.
(12) Led, J. J.; Petersen, S. B. *J. Magn. Reson.* **1979**, *33*, 603–617.
(13) Wlodawer, A.; Miller, M.; Iaskolski, M.; Sathyanarayana, B. K.; Baldwin, E.; Weber, I. T.; Selk, L. M.; Clawson, M.; Schneider, J.; Kent, S. B. H. *Science (Washington, D.C.)* **1989**, *245*, 616–621.
(14) Davies, D. R. *Annu. Rev. Biophys. Biophys. Chem.* **1990**, *19*, 189–215.
(15) Hyland, L. J.; Tomaszek, T. A., Jr.; Meek, T. D. *Biochemistry* **1991**, *30*, 8454–8463.
(16) Grant, S. K.; Deckman, I. C.; Culp, J. S.; Minnich, M. D.; Brooks, I. S.; Hensley, P.; Debouck, C.; Meek, T. D. *Biochemistry* **1992**, *31*, 9491–9501.
(17) Grzesiek, S.; Bax, A. *J. Magn. Reson.* **1992**, *99*, 201–207.
(18) Ikura, M.; Kay, L. E.; Bax, A. *Biochemistry* **1990**, *29*, 4659–4667.
(19) Bax, A.; Ikura, M.; Kay, L. E.; Torchia, D. A.; Tschudin, R. *J. Magn. Reson.* **1990**, *86*, 304–318.