

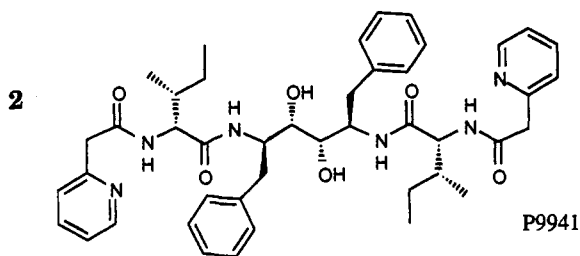
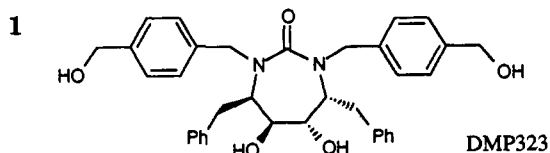
NMR Evidence for the Displacement of a Conserved Interior Water Molecule in HIV Protease by a Non-Peptide Cyclic Urea-Based Inhibitor

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With the availability of a large number of high-resolution X-ray structures,¹ the HIV protease has become the target of a massive effort to design potential anti-AIDS drugs. Recently, the DuPont Merck group has succeeded in the development of a potent nonpeptide orally bioavailable HIV protease inhibitor, DMP323 (1).² This subnanomolar inhibitor was developed in a series of steps, starting from the linear C₂-symmetric diol P9941 (2),³ that were based on careful inspection of the high-resolution X-ray structures available for HIV protease-inhibitor complexes.² A



common feature in all of these complexes is the presence of a structural water molecule which accepts two hydrogen bonds from the backbone amide hydrogens of the Ile⁵⁰ residues of the

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two strands of the protease homodimer and donates two hydrogen bonds to carbonyl oxygens of the inhibitor.¹ This water molecule is considered to play a critical role in closing the "flaps" over the protease inhibitor, and its presence has not been observed in mammalian aspartic proteases. The rationale underlying the development of DMP323 was to displace the water molecule by the cyclic urea carbonyl oxygen, and indeed the conserved water molecule was not seen in the HIV protease-DMP323 crystal structure.² No crystal structure has been obtained for the complex between the protease and 2. The present communication describes the application of a new and sensitive 2D NMR experiment to the study of tightly bound water molecules in the complexes between the HIV protease and the inhibitors 1 and 2.

The short-distance NOE interaction between individual water molecules and specific protein protons can be probed by a variety of NMR techniques⁴⁻¹⁰ and has provided valuable insights into the role of structural water in proteins,^{5,6,11} DNA,^{12,13} and protein-DNA complexes.⁷ Because of the complexity of the 1D protein NMR spectrum, NOE interactions between protein and water have most commonly been studied by 3D techniques, where the information of interest is present in a single cross section through the 3D spectrum. As demonstrated recently for the study of amide hydrogen exchange,⁸ such 3D experiments can be replaced by more sensitive and convenient 2D experiments, using a set of low-power ¹H pulses for selective inversion of the H₂O resonance. These 2D analogs offer significantly better sensitivity than the 3D versions because of their lower dimensionality and because the slowly relaxing water is never saturated.^{8,9} For uniformly ¹³C-enriched proteins, the NOE interaction between amide protons and water is separated from interactions with protein protons resonating in the vicinity of H₂O by using purge pulses that select protons not attached to ¹³C¹⁴ and/or by using the fact that the spin-locked relaxation time, T_{1ρ}, for water is typically more than 2 orders of magnitude longer than that for protein protons.⁸

Incomplete ¹³C enrichment or the presence of unlabeled ligands with protons resonating in the vicinity of H₂O can also give rise to NOE/ROE interactions in the 2D H₂O NOESY- or H₂O ROESY-HSQC spectra. As shown below, such interactions with non-water protons can be identified by a "control experiment", where very weak (~10 Hz) H₂O presaturation is used until ~200 ms prior to the selective H₂O inversion sequence. After the short 200-ms delay, H₂O magnetization remains attenuated about 20-fold relative to its equilibrium value. In contrast, protein protons resonating under the H₂O resonance have returned to their equilibrium value due to ¹H-¹H spin flips with off-resonance protein protons.¹⁵ Hence, in the "control spectrum", all interactions with water or with protons that rapidly exchange with water are attenuated ca. 20-fold, whereas interactions to nonexchanging or slowly exchanging protons remain nearly unaffected.

Figure 1 shows small regions of the selective H₂O ROESY-HSQC spectra obtained for 0.7 mM (dimer) samples of HIV protease complexed with the inhibitors 1 and 2. Resonance

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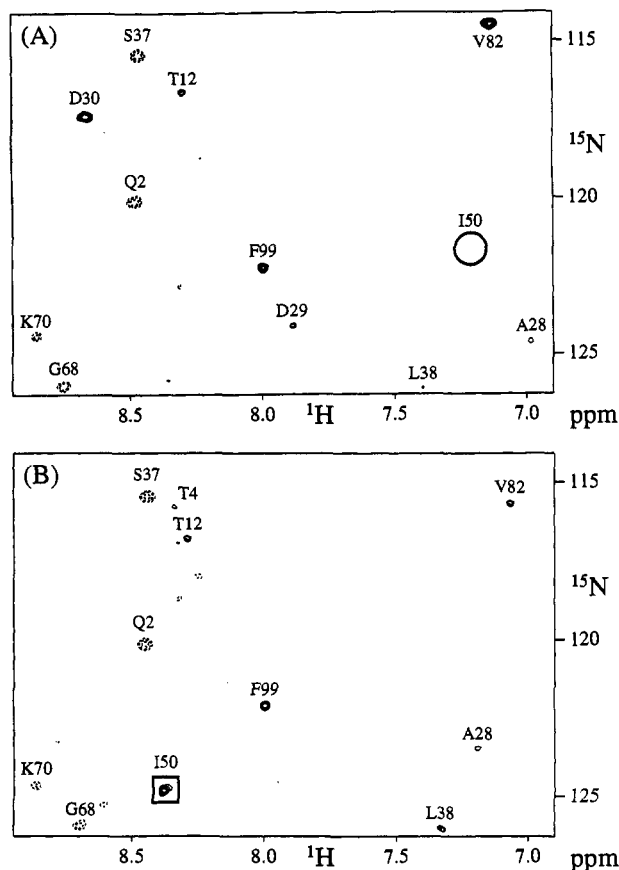


Figure 1. Small regions of the 2D H₂O ROESY-HSQC difference spectra, recorded for complexes between [U-¹³C/¹⁵N]HIV protease and inhibitors **1** (A) and **2** (B). Spectra are recorded on a Bruker AMX-600 equipped with a triple resonance pulsed field gradient probehead, using a sample concentration of 0.7 mM (dimer) in a 200 μ L of Shigemi microcell, pH 5.2, 34 $^{\circ}$ C. The ROE mixing time was 30 ms, and the total measuring time was 12 h. Dashed contours correspond to negative resonances (amide hydrogen exchange); solid contours are positive and correspond to (indirect) ROE interactions to H₂O. The bold circle in A marks the position of the Ile⁵⁰-H^N-¹⁵N correlation. Absence of cross peak intensity at this position indicates the absence of tightly bound water in the vicinity of the Ile⁵⁰-H^N. The square in B highlights the Ile⁵⁰-H^N-¹⁵N NOE cross peak with water.

assignments for the complex between **1** and HIV protease are taken from Yamazaki et al.¹⁶ Chemical shifts for the complex with **2** are substantially different and were made on the basis of two triple resonance 3D spectra, CBCANH¹⁷ and CBCA(CO)-NH;¹⁸ these are presented in the supplementary material. Although all crystal structures of HIV protease show small differences between the conformations of the two chains of the homodimer, a single resonance is observed for each backbone amide, indicating that any conformational difference is averaged out on the NMR time scale. In the ROESY version of the H₂O HSQC difference experiment, correlations for amides that are in rapid exchange with water are of opposite sign relative to amides that show a dipolar interaction with water, distinguishing exchange and ROE interactions.⁴ The complex with **2** (Figure 2B) shows an intense ROE cross peak with water for Ile⁵⁰ (at $F_1/F_2 = 125/8.4$ ppm), as expected for a water hydrogen-bonded to Ile⁵⁰-H^N. In contrast, this cross peak with water is absent for the complex with **1** (Figure 2A), indicating that the H₂O molecule indeed has

been replaced. Note also that the Ile⁵⁰-H^N resonance shifts upfield by more than 1 ppm.

The magnetization exchange rates can be quantified on the basis of the intensities in the H₂O NOESY-HSQC (supplementary material) and H₂O ROESY-HSQC difference spectra and on measurement of the applicable H^N $T_{1\rho}$ and selective T_1 values.⁸ For the complex with **2**, such an analysis shows Ile⁵⁰-H^N magnetization exchange rates of -6 ± 2 s⁻¹ for the ROE experiment and $+4.5 \pm 0.7$ s⁻¹ for the NOE experiment. A rotational correlation time, τ_c , of 9 ns has been derived for the protease-P9941 complex from ¹⁵N T_1 and T_2 measurements (Nicholson et al., unpublished). For this 9-ns τ_c value, the 4.5-s⁻¹ magnetization exchange rate in the NOE experiment corresponds to an interproton distance of 2.2 \AA , which is in good agreement with the distance expected on the basis of X-ray crystal structures for protease-inhibitor complexes. Therefore, the lifetime of the bound water is at least 9 ns. As the Ile⁵⁰-H^N/H₂O NOE is not observed in the presaturation control experiment, the lifetime must be shorter than ~ 100 ms.

The spectra shown in Figure 1 display a number of additional ROE and exchange interactions with water. Most of the ROEs are due to short distances between amide protons and protein or drug hydroxyl protons. All but one of these disappear (or are very strongly attenuated) in the control spectra (supplementary material), indicating that they correspond to ROE interactions to protons that are in fast exchange with bulk water. Interestingly, for the protease-DMP323 complex, the ROE interaction observed for Val⁸²-H^N is not affected by water presaturation, but it is obliterated in the complex with P9941 (supplementary material). Based on the X-ray structures for complexes between protease and inhibitors (including DMP323), the ROE interaction observed for Val⁸²-H^N is caused by its proximity to Thr⁸⁰-O γ H. Indeed, in the 3D ¹⁵N-separated NOESY, an intense cross peak from Val⁸²-H^N to a non-drug/non-¹³C-attached proton is observed at 4.61 ppm. The fact that the NOE/ROE to Val⁸²-H^N is not affected by H₂O presaturation indicates that in the protease-DMP323 complex, the rate at which Thr⁸⁰-O γ H exchanges with solvent is exceptionally slow (< 1 s⁻¹).

Water molecules can play a critical structural role in proteins. Identification of the presence of such a water in the immediate vicinity of the substrate binding site of HIV protease has led to the design of a powerful and highly selective inhibitor. The K_i of **1** for HIV protease (0.27 nM) is 30-fold lower than that of **2**, whereas the K_i of **1** for human pepsin and renin remains larger than 10 μ M. Although no crystal structure for the complex between HIV protease and **2** has been obtained, our NMR results show the presence of a long-lived water molecule in the expected position and its displacement in the complex with the cyclic urea derivative. The isotope-assisted NMR methods used in the present study provide a powerful and rapid method for identifying both the presence of such water molecules and the upper and lower bounds for their lifetimes.

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Supplementary Material Available: NOE difference spectra for the complexes between HIV protease and the inhibitors **1** and **2** and the corresponding control spectra, recorded with water presaturation; one table with ¹H^N and ¹⁵N resonance assignments for the HIV protease backbone when complexed with **2** (4 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.

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