With the availability of a large number of high-resolution X-ray structures, 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). This subnanomolar inhibitor was developed in a series of steps, starting from the linear C9-symmetric diol P9941 (2), that were based on careful inspection of the high-resolution X-ray structures available for HIV protease-inhibitor complexes. 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

two strands of the protease homodimer and donates two hydrogen bonds to carbonyl oxygens of the inhibitor. 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 apurinic complexes. The rationale underlying the development of DMP323 was to place 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. 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 techniques and has provided valuable insights into the role of structural water in proteins, DNA, and protein–DNA complexes. 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, 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. 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 13C and/or by using the fact that the spin–lock relaxed time, T1, for water is typically more than 2 orders of magnitude longer than that for protein protons.

Incomplete 13C enrichment or the presence of unlabelled 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–H spin flips with off-resonance protein protons. Hence, in the "control spectrum" all interactions with water or with protons that rapidly exchange with water are attenuated.

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
been replaced. Note also that the IleSO-HN 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 HN T1, T2, and T1 values.

For the complex with 2, such an analysis shows Ile50-HN magnetization exchange rates of -6 ± 2 s⁻¹ for the ROE experiment and +4.5 ± 0.7 s⁻¹ for the NOE experiment. A rotational correlation time, τc, of 9 ns has been derived for the protease-P9941 complex from 1H T1 and T2 measurements (Nicholson et al., unpublished). For this 9-ns τc value, the 4.5-s⁻¹ magnetization exchange rate in the NOE experiment corresponds to an interproton distance of 2.2 Å, 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-HN/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 Val12-HN 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 Val12-HN is caused by its proximity to Thr40-OH. Indeed, in the 3D 15N-separated NOESY, an intense cross peak from Val12-HN to a non-drug/non-13C-attached proton is observed at 4.61 ppm. The fact that the NOE/ROE to Val12-HN is not affected by H2O presaturation indicates that in the protease–DMP323 complex, the rate at which Thr40-OH 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 Kᵢ of 1 for HIV protease (0.27 M) is 30-fold lower than that of 2, whereas the Kᵢ of 1 for human pepsin and renin remains larger than 10 μM. 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 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.