

# Heteronuclear 3D NMR Studies of Water Bound to an FK506 Binding Protein/Immunosuppressant Complex<sup>†</sup>

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Received November 9, 1992; Revised Manuscript Received December 21, 1992

**ABSTRACT:** From a series of <sup>15</sup>N-resolved 3D ROESY-HMQC and <sup>13</sup>C-resolved 3D NOESY-HMQC spectra of the FK506 binding protein (FKBP)/ascomycin complex in H<sub>2</sub>O, the locations of three tightly bound water molecules were identified. These waters are all buried within the interior of the complex and form an integral part of its structure via a network of hydrogen bonds. Water molecules in identical locations exhibiting a similar hydrogen bonding pattern were also observed in the X-ray crystal structures of FKBP/FK506 [Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L., & Clardy, J. (1991) *Science* 252, 839-842] and FKBP/rapamycin [Van Duyne, G. D., Standaert, R. F., Schreiber, S. L., & Clardy, J. (1991) *J. Am. Chem. Soc.* 113, 7433-7434]. However, none of the surface waters observed in the X-ray structures were detected in the NMR experiments due to their fast exchange with bulk water. In order to examine the effects of the three internal water molecules on NMR structure determinations of the FKBP/ascomycin complex, two sets of NMR structures were calculated either with or without the waters. By including the three internal waters in the structure calculations, a decrease in the root mean square deviation and improved angular order parameters was observed for FKBP residues in the vicinity of the water molecules. In addition, subtle conformational differences were observed between NMR structures generated either with or without the waters. These differences not only are caused by the added distance restraints involving water but also may be due to the bulk of the water and ability of the water to bridge otherwise distant regions of the protein through water-mediated hydrogen bonds. These results suggest that in some cases internal water molecules are important to include in three-dimensional structure determinations of proteins by NMR.

Water molecules play an important role in enzyme mechanisms, protein/ligand interactions, and protein folding and stability. High-resolution X-ray crystal structures of proteins typically include the coordinates of water oxygens and have greatly contributed to our understanding of the protein/water structural interface. However, water molecules found in X-ray structures are mainly located on the surface of proteins. Their locations may differ in the crystal and solution, since these waters could be influenced by neighboring proteins in the crystal or may be missing due to protein/protein contacts (Otting et al., 1991). Although fewer in number, water molecules located in the interior of proteins are often an integral part of the protein structure and are usually well-ordered in X-ray crystallographic studies.

Recently, NMR spectroscopy has been used to determine the locations of protein-bound water molecules in solution and to characterize the lifetime of bound waters (Otting & Wüthrich, 1989; Clore et al., 1990; Otting et al., 1991; Forman-Kay et al., 1991). In the three cases where protein-bound waters have been studied by NMR, only a small number of water molecules located in the interior of the protein were detected. The water molecules observed in NMR experiments of BPTI (Otting & Wüthrich, 1989; Otting et al., 1991) and interleukin-1β (Clore et al., 1990) were found in locations similar to those of the internal waters in the crystal structures of these proteins. None of the surface waters observed by X-ray crystallography were detected in the NMR experiments (Otting & Wüthrich, 1989; Clore et al., 1990; Otting et al., 1991; Forman-Kay et al., 1991). Additional NMR studies

involving different proteins will be important for further characterizing protein hydration in solution.

In this paper, we report on the locations of protein-bound water molecules in the complex formed between the FK506 binding protein (FKBP)<sup>1</sup> (Sekierka et al., 1989; Harding et al., 1989) and the immunosuppressant ascomycin (Hatanka et al., 1988; Morisaki & Arai, 1992) (Figure 1). These results were obtained from <sup>15</sup>N-resolved 3D ROESY-HMQC spectra of [U-<sup>15</sup>N]FKBP/ascomycin (Clore et al., 1990) and <sup>13</sup>C-resolved 3D NOESY-HMQC spectra (Ikura et al., 1990; Zuiderweg et al., 1990) of [U-<sup>13</sup>C, <sup>15</sup>N]FKBP/ascomycin and FKBP/[U-<sup>13</sup>C]ascomycin. The locations of the water molecules detected by NMR are compared to those of the waters observed in the X-ray crystal structures of FKBP/immunosuppressant complexes (Van Duyne et al., 1991a,b). Finally, we describe the effects of including the bound water molecules on the precision and accuracy of FKBP/ascomycin structures (Meadows et al., 1993) determined by NMR.

## EXPERIMENTAL PROCEDURES

**Sample Preparation.** Recombinant human FKBP was cloned from a Jurkat T cell cDNA library and expressed at high levels in *Escherichia coli* using the translational coupling to the 5' end of the *E. coli* kds B gene (manuscript in preparation). [U-<sup>15</sup>N]FKBP and [U-<sup>13</sup>C, <sup>15</sup>N]FKBP were

<sup>†</sup> This research was supported in part by the National Institute of General Medical Sciences (GM45351, awarded to S.W.F.).

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<sup>1</sup> Abbreviations: FKBP, FK506 binding protein; 3D ROESY-HMQC, three-dimensional rotating frame Overhauser effect spectroscopy-heteronuclear multiple quantum correlation; 3D NOESY-HMQC, three-dimensional nuclear Overhauser effect spectroscopy-heteronuclear multiple quantum correlation; NOE, nuclear Overhauser effect; ROE, rotating frame Overhauser effect; RMSD, root mean square deviation; AOP, angular order parameter.

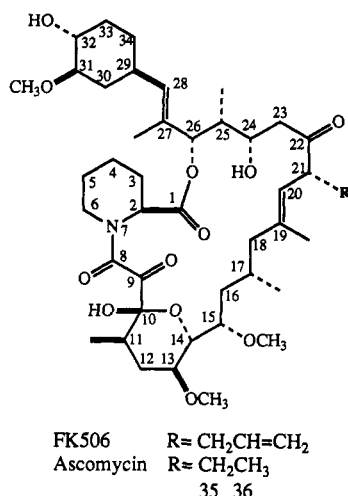


FIGURE 1: Structures of the immunosuppressants FK506 and ascomycin.

purified from cells grown on a minimal medium containing [<sup>15</sup>N]ammonium chloride alone or in combination with [U-<sup>13</sup>C]-acetate, using ion-exchange and size-exclusion chromatography (Edalji et al., 1992). [U-<sup>13</sup>C]Ascomycin was prepared as previously described (Petros et al., 1991) from *Streptomyces hygroscopicus* subsp. *ascomyceticus* UV125 grown in a chemically defined fermentation medium containing uniformly <sup>13</sup>C-labeled glucose.

FKBP/ascomycin (1/1) complexes (3 mM) were prepared in H<sub>2</sub>O solution (pH = 6.5) containing potassium phosphate (50 mM), sodium chloride (100 mM), and dithiothreitol-*d*<sub>10</sub> (5 mM) by incubating FKBP with an excess amount of ascomycin for 24–48 h at room temperature. The excess ascomycin, which is insoluble in water, was removed by centrifugation.

**NMR Spectroscopy.** All NMR spectra were collected on a Bruker AMX500 (500-MHz) NMR spectrometer. NMR spectra were processed and analyzed using in-house written software on Silicon Graphics computers.

<sup>15</sup>N-Resolved 3D ROESY-HMQC spectra were acquired in eight scans per experiment at either 10 or 30 °C using mixing times of 10 or 33 ms. A relatively long relaxation delay (2.4 s) between scans was used to minimize the saturation of the slowly relaxing water signal (Clare et al., 1990). The data set contained 96 (*t*<sub>1</sub>) × 37 (*t*<sub>2</sub>) × 1024 (*t*<sub>3</sub>) complex points with spectral widths of 6024 Hz (*ω*<sub>1</sub>, <sup>1</sup>H), 1773 Hz (*ω*<sub>2</sub>, <sup>15</sup>N), and 8333 Hz (*ω*<sub>3</sub>, <sup>1</sup>H). Water suppression was accomplished with a 2.5-ms spinlock pulse using the method of Wüthrich and co-workers (Messerle et al., 1989). The total accumulation time for each 3D data set was 3.3 days.

The <sup>13</sup>C-resolved 3D NOESY-HMQC spectra of [U-<sup>13</sup>C, <sup>15</sup>N]FKBP/ascomycin and FKBP/[U-<sup>13</sup>C]ascomycin were acquired at 30 °C in eight scans per increment using a mixing time of 50 ms. Two additional data sets were acquired for the [U-<sup>13</sup>C, <sup>15</sup>N]FKBP/ascomycin complex at 10 °C using mixing times of 15 and 50 ms. Data sets consisted of 96 (*t*<sub>1</sub>) × 48 (*t*<sub>2</sub>) × 1024 (*t*<sub>3</sub>) complex points with spectral widths of 6024 Hz (*ω*<sub>1</sub>, <sup>1</sup>H), 5747 Hz (*ω*<sub>2</sub>, <sup>13</sup>C), and 8333 Hz (*ω*<sub>3</sub>, <sup>1</sup>H). The relaxation delay between scans was 1.8 s, and the total accumulation time for each data set was 3.3 days.

**Structure Calculations.** Structure calculations employed a distance geometry/dynamical simulated annealing procedure (Nilges et al., 1988) and were carried out with the program XPLOR/DG (Brünger, 1990; Kuszewski et al., 1992) on a Silicon Graphics 4D-360 computer as previously described (Meadows et al., 1993). A total of 1713 NOE-derived

Table I: Interatomic Distance Restraints Applied to the Three Water Molecules in the FKBP/Ascomycin Complex Observed in the NMR Experiments

FKBP	Hydrogen Bonds <sup>a</sup>			
	water	FKBP	water	
92 O	1	95 O		2
83 O	1	60 OE2		4
95 N	1	49 O		4
82 N	2	54 N		4

Distance Restraints from NOE/ROE Cross-Peaks					
FKBP	water	upper bound <sup>b</sup>	FKBP	water	upper bound <sup>b</sup>
86 HN	1	4.50	55 HG2 # <sup>c</sup>	3	5.50
94 HN	1	4.50	52 HN	3	4.50
95 HN	1	4.50	54 HN	3	4.50
82 HN	2	4.50	48 CG	3	6.50
82 CG	2	6.50	54 HG # <sup>c</sup>	3	5.50
51 HN	3	4.50			

<sup>a</sup> All hydrogen bonds were given bounds of 1.8–2.7 Å (H→O) and 2.4–3.3 Å (N or O→O). In addition to those listed, a hydrogen bond was applied between waters 1 and 2 on the basis of their close proximity.

<sup>b</sup> Upper bounds were applied as 4.50 Å plus an additional allowance for center averaging when necessary (Wüthrich et al., 1983). <sup>c</sup> # indicates center averaging.

distances, 104 hydrogen bond restraints, 66  $\chi$ <sub>1</sub> and 46  $\phi$  angular restraints were included in the calculations of the structures that did not contain water molecules. Another set of structures was generated that included the three waters and an additional 29 restraints. Included in the 29 restraints were 18 restraints for 9 hydrogen bonds (H→O, <2.7 Å; N or O→O, 2.4–3.3 Å) and 11 ROE/NOE-derived distance restraints. The distance restraints were given bounds of 1.8–4.5 Å, and when appropriate, standard corrections were made for center averaging (Wüthrich et al., 1983). Since the distance restraints were to the oxygen atoms vs protons of the water molecules, the distance bounds contain a 1.0 Å pseudoatom correction. The additional restraints involving the water molecules are given in Table I.

## RESULTS AND DISCUSSION

**Identification of Internal Water Molecules.** Figure 2 depicts <sup>1</sup>H(*ω*<sub>1</sub>), <sup>1</sup>H(*ω*<sub>3</sub>) planes extracted at different <sup>15</sup>N(*ω*<sub>2</sub>) chemical shifts from an <sup>15</sup>N-resolved 3D ROESY-HMQC spectrum of the [U-<sup>15</sup>N]FKBP/ascomycin complex acquired at 30 °C. A ROESY versus a NOESY experiment was chosen in order to distinguish between cross-peaks due to chemical exchange and ROEs between nearby protons. Indeed, as shown in Figure 2, two types of cross-peaks were detected at the water frequency (arrow). One set, corresponding to the chemical exchange between the amides and water, appears as negative peaks (denoted as EX in Figure 2, gray contours). Chemical exchange cross-peaks were observed at 30 °C for the amides of FKBP residues V2, T6, R18, K35, R42, N43, and G89. These data agree with our earlier NMR studies of deuterium exchange rates in which these solvent-exposed amides exhibited fast hydrogen exchange (<0.3/h) (Xu et al., 1993). The other set of peaks, appearing as positive cross-peaks in Figure 2, correspond to rotating frame Overhauser effects (ROE) between the amide protons and either nearby (<4 Å) water molecules or exchangeable protons of FKBP.

In order to interpret the ROE data, a high-resolution structure of the FKBP/ascomycin complex calculated without water was employed. ROEs involving FKBP amides in close proximity (<4 Å) to exchangeable protons were assigned as such using the NMR structure. ROEs were mainly observed between FKBP amides (T14, T21, R40, D41, T75, S77, T85)

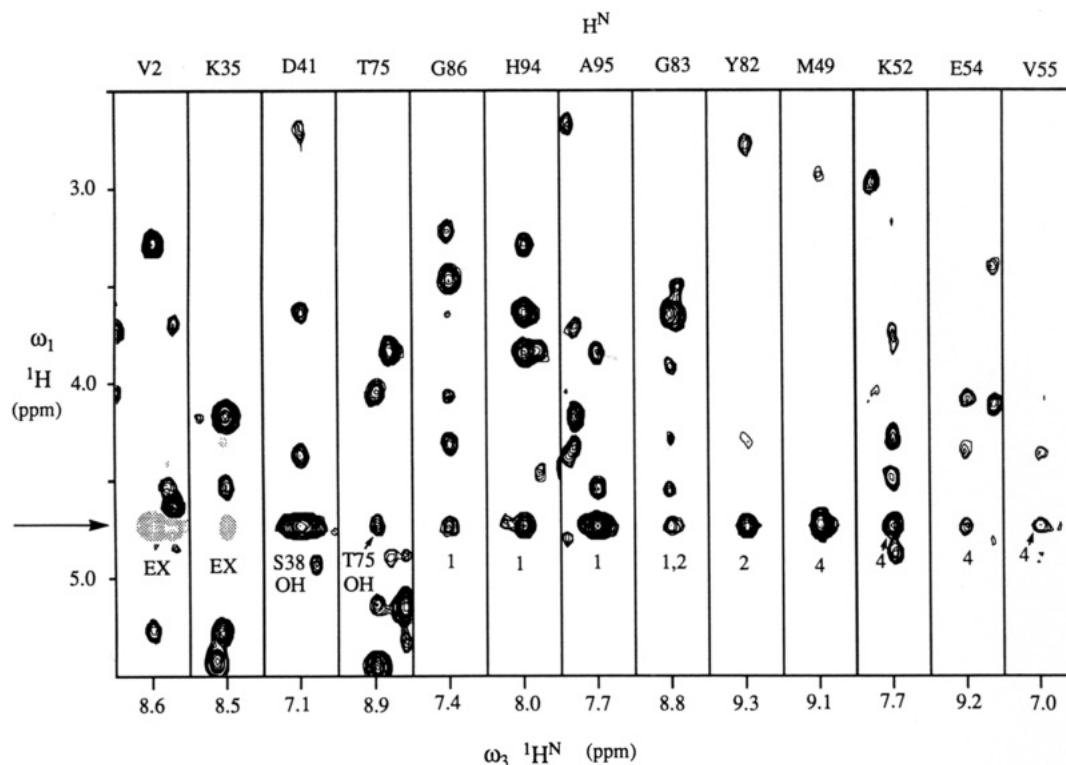


FIGURE 2:  ${}^1\text{H}(\omega_1), {}^1\text{H}(\omega_3)$  planes extracted at different  ${}^{15}\text{N}(\omega_2)$  chemical shifts from a  ${}^{15}\text{N}$ -resolved 3D ROESY-HMQC spectrum of the  $[\text{U-}^{15}\text{N}]$ FKBP/ascomycin complex acquired at 30 °C. The arrow indicates the water frequency. Negative contours are plotted with dotted lines and appear gray. Exchange cross-peaks are labeled EX, and ROE peaks to the individual waters (1, 2, 4) are indicated.

and nearby side-chain hydroxyl protons of Thr and Ser residues which display a minimum exchange rate at the pH (6.5) of the NMR experiments (Wüthrich, 1986; Liepinsh et al., 1992).

ROEs that could not be explained by an interaction with exchangeable FKBP protons must therefore involve bound water molecules. In order to interpret these data, we used a high-resolution NMR structure of the FKBP/ascomycin complex and an X-ray structure of FKBP/FK506 (Van Duyne et al., 1991a, 1993) that contained 134 water molecules. The NMR and X-ray structures, which are very similar (RMSD = 0.8 Å for backbone heavy atoms), were superimposed, and a hybrid of the NMR structure and water molecules from X-ray diffraction was used for assigning Overhauser effects to specific water molecules. Hydrogen bonds involving the water molecules were identified by searching for hydrogen bond donors and acceptors in close proximity to the bound waters whose location was supported by the ROE/NOE data.

The NMR data acquired at 30 °C were consistent with three water molecules buried within the interior of the complex. In Figure 3A, two of these water molecules (1 and 2) are shown along with the ROEs that support their location. Water 1 shows ROEs to the amides of G86, H94, and A95 and appears to be stabilized by hydrogen bonds involving G83-CO, P92-CO, and A95- $\text{H}^{\text{N}}$  of FKBP. Water 2 has ROEs to the amide of Y82 and appears to hydrogen bond to Y82- $\text{H}^{\text{N}}$  and A95-CO. These two water molecules serve to stabilize the structure of the large loop (residues 78–96) located between the second and third  $\beta$ -strands of FKBP by linking together the two sides of the loop through water-mediated hydrogen bonds. The other water molecule detected by the NMR experiments is shown in Figure 3B. It is labeled as 4 to be consistent with the numbering scheme used in the X-ray structure of the FKBP/FK506 complex (Van Duyne et al., 1991a, 1993). As shown in Figure 3B, the location of water 4 is supported by several ROEs and is consistent with hydrogen bonds between the water and M49-CO, E54- $\text{H}^{\text{N}}$ , and the side chain carboxyl

group of E60. This water molecule is an integral part of the structure of the complex and stabilizes the structure of the polypeptide chain between the fifth  $\beta$ -strand and  $\alpha$ -helix that constitutes part of the ligand binding site.

The intensity of the ROE peaks is dependent not only on proton-proton distances but also on the lifetimes of the bound water or exchangeable protons. Thus, additional peaks may be observed by increasing the lifetime of the bound waters by lowering the temperature (Otting & Wüthrich, 1989). Indeed, seven additional ROESY peaks were observed at the water frequency involving the amides of E5, E31, K47, M49, Q53, S77, and Y80 in the  ${}^{15}\text{N}$ -resolved 3D ROESY-HMQC spectrum acquired at 10 °C. Although most of these additional ROEs involve exchangeable protons, two of the cross-peaks were assigned as ROEs between FKBP amides and water. One of these has been tentatively assigned to an ROE between K47- $\text{H}^{\text{N}}$  and water 11 and the other has been assigned between E31- $\text{H}^{\text{N}}$  and water 13. In the hybrid NMR structure containing the waters from X-ray crystallography, both amide protons are very close to the water molecules which may compensate for the magnetization losses incurred by their relatively fast exchange rate. No other ROEs or NOEs support the presence of these two water molecules. However, this is not unexpected, since all other protons are further away from these waters compared to the amide protons.

In an attempt to observe additional ROE peaks involving rapidly exchanging water molecules, 3D ROESY-HMQC spectra were acquired using a shorter mixing time which serves to lessen the magnetization loss due to chemical exchange. However, no additional ROE peaks were observed in a  ${}^{15}\text{N}$ -resolved 3D ROESY-HMQC spectrum of the  $[\text{U-}^{15}\text{N}]$ FKBP/ascomycin complex acquired using a mixing time of 10 ms. In fact, all of the ROEs that were observed were less intense than those in the data set acquired using a longer mixing time (33 ms).

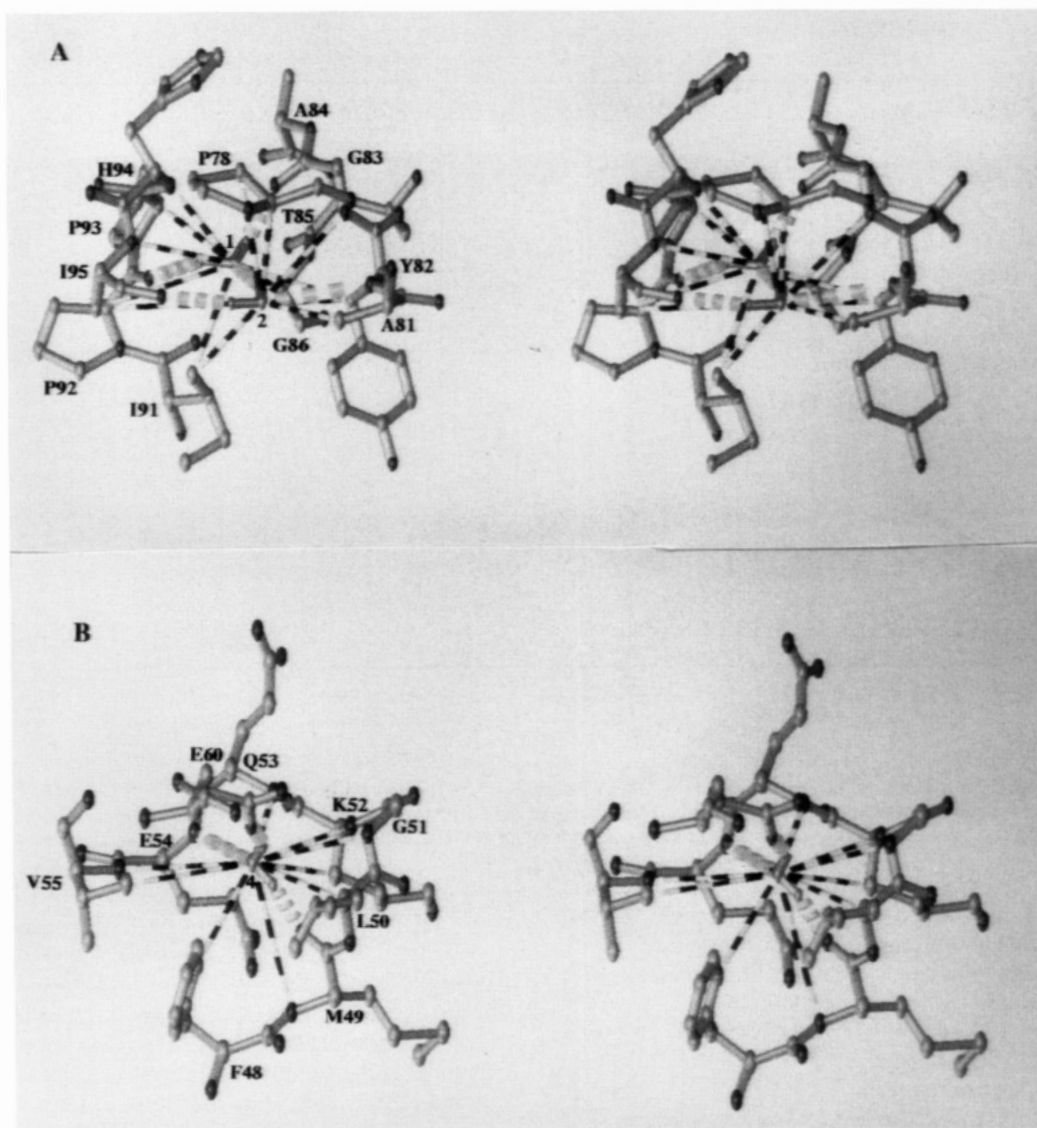


FIGURE 3: Stereoplots of portions of the FKBP/ascomycin NMR structure that bind to (A) waters 1 and 2 and (B) water 4. The illustrations were prepared with the program RIBBONS (Carson, 1987). ROEs and NOEs between FKBP and the water molecules are indicated by thin dashed lines, and hydrogen bonds are shown as cylinders.

In order to precisely determine the location of bound waters by NMR, it is important to include ROEs and NOEs not only from the backbone but also from the side chain protons as well. To that end,  $^{13}\text{C}$ -resolved 3D NOESY-HMQC spectra of the  $[\text{U-}^{15}\text{N}, ^{13}\text{C}]$ FKBP/ascomycin complex were acquired at 10 and 30 °C using a mixing time of 50 ms and at 10 °C using a mixing time of 15 ms. Figure 4 depicts  $^1\text{H}(\omega_1)$ ,  $^1\text{H}(\omega_3)$  planes extracted at different  $^{13}\text{C}(\omega_2)$  chemical shifts from the  $^{13}\text{C}$ -resolved 3D NOESY-HMQC spectrum of the  $[\text{U-}^{15}\text{N}, ^{13}\text{C}]$ FKBP/ascomycin complex acquired at 30 °C. Using a mixing time of 50 ms, a total of 82 peaks at 30 °C and 80 peaks at 10 °C were observed at the water frequency. As shown in Figure 4, some of these NOEs were between FKBP side chain protons and water and were helpful in defining the location of the bound water molecules (Figure 3). However, most of the observed NOEs involved rapidly exchanging protons of FKBP rather than bound waters. In contrast to a recent NMR study of protein hydroxyl groups (Liepinsh et al., 1992), all of the exchangeable protons were found to be in the fast exchange regime and appeared at the chemical shift of the water resonance. As pointed out by Liepinsh et al. (1992), such rapid exchange between the Ser/Thr hydroxyl groups and water may be due to exchange

catalysis by the phosphate (50 mM) in our NMR samples.

In an attempt to identify bound water molecules in the vicinity of the ligand, a  $^{13}\text{C}$ -resolved 3D NOESY-HMQC spectrum was recorded of uniformly  $^{13}\text{C}$ -labeled ascomycin bound to FKBP. Figure 5 depicts  $^1\text{H}(\omega_1)$ ,  $^1\text{H}(\omega_3)$  planes extracted at different  $^{13}\text{C}(\omega_2)$  chemical shifts of  $[\text{U-}^{13}\text{C}]$ -ascomycin from the 3D data set. None of the NOEs could be unambiguously assigned to a water molecule. The NOEs that were detected at the water frequency in the 3D experiment involve ascomycin protons in close proximity to exchangeable OH protons of either the ligand or protein.

**Comparison with X-ray Structures of FKBP/Immunosuppressant Complexes.** Two X-ray crystal structures of FKBP/immunosuppressant complexes have been reported (Van Duyne et al., 1991a,b). As part of the X-ray structures of FKBP/FK506 and FKBP/rapamycin, 134 and 133 water molecules were included, respectively (Van Duyne et al., 1993). Of these, 42 were found in similar locations in the two structures and exhibited the same hydrogen bonding patterns (Van Duyne et al., 1993). Of the 134 water molecules observed in the X-ray structure of the FKBP/FK506 complex, only three water molecules were observed in the FKBP/ascomycin complex by NMR at 30 °C. As shown in Figure 3, all three

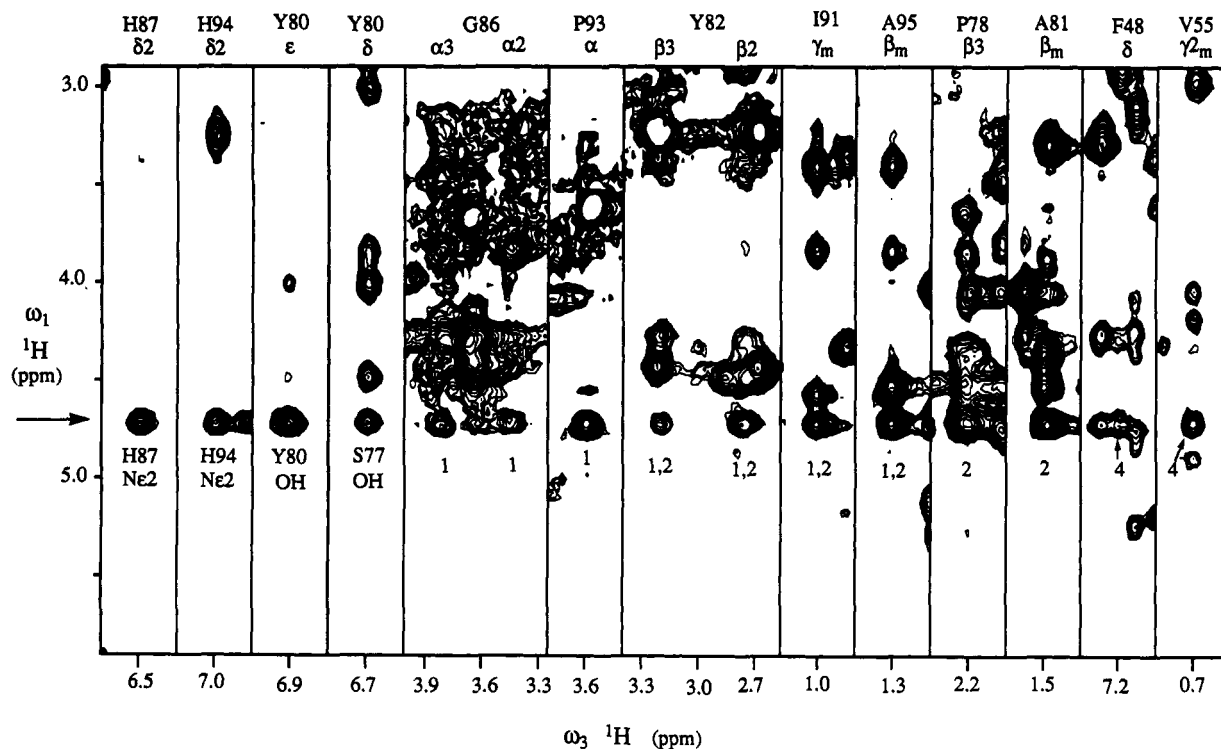


FIGURE 4:  $^1\text{H}(\omega_1)$ ,  $^1\text{H}(\omega_3)$  planes extracted at different  $^{13}\text{C}(\omega_2)$  chemical shifts from the  $^{13}\text{C}$ -resolved 3D NOESY-HMQC spectrum of a  $[\text{U-}^{15}\text{N}, ^{13}\text{C}]$ FKBP/ascomycin complex acquired at 30 °C. The arrow indicates the water frequency. The NOE peaks to the individual waters (1, 2, 4) are indicated.

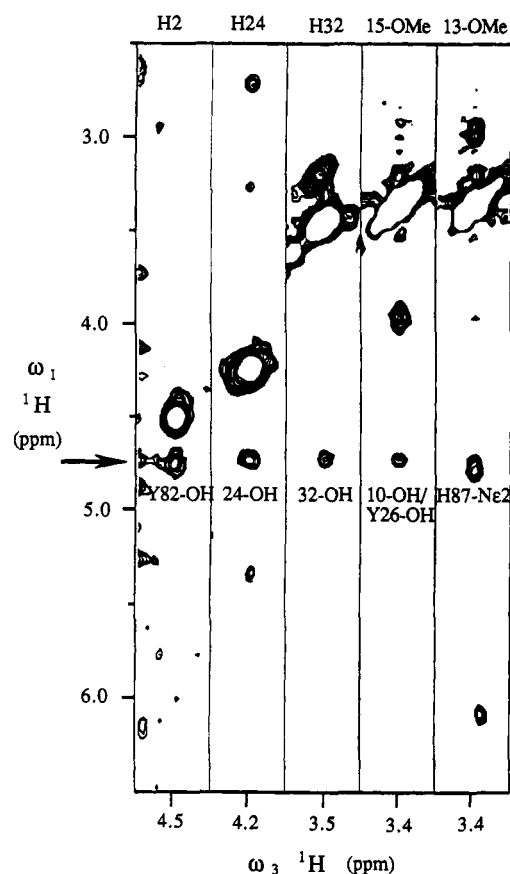


FIGURE 5:  $^1\text{H}(\omega_1)$ ,  $^1\text{H}(\omega_3)$  planes extracted at different  $^{13}\text{C}(\omega_2)$  chemical shifts from a  $^{13}\text{C}$ -resolved 3D NOESY-HMQC spectrum of the FKBP/ $[\text{U-}^{13}\text{C}]$ ascomycin complex acquired at 30 °C. The arrow indicates the water frequency.

waters detected in the NMR experiments are buried in the interior of the protein and are stabilized by the same network

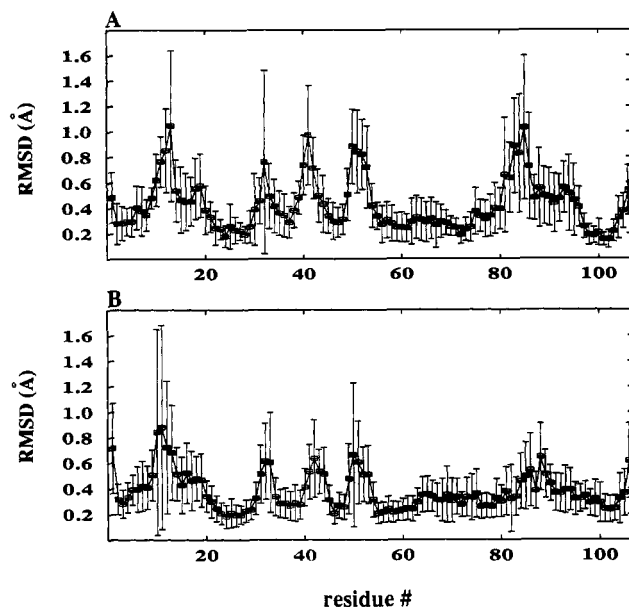


FIGURE 6: Residue-based RMSD to the mean atomic coordinates for protein residues of the FKBP/ascomycin complex generated with (B) or without (A) the three water molecules.

of hydrogen bonds observed in the X-ray structures of the FKBP/immunosuppressant complexes (Van Duyne et al., 1993). Interestingly, these three waters display the highest electron density when compared to the other water molecules in the FKBP/FK506 X-ray structure. The electron densities for water molecules 1, 2, and 4 are 4.3, 4.2, and 3.5  $\text{e}/\text{\AA}^3$ , respectively (Van Duyne et al., 1993). The only other water molecules that could be observed by NMR were waters 11 and 13. These were detected from relatively weak ROEs at 10 °C and have electron densities of 2.5 (water 11) and 2.4 (water 13)  $\text{e}/\text{\AA}^3$  (Van Duyne et al., 1993). Other water molecules with comparable electron densities were not detected

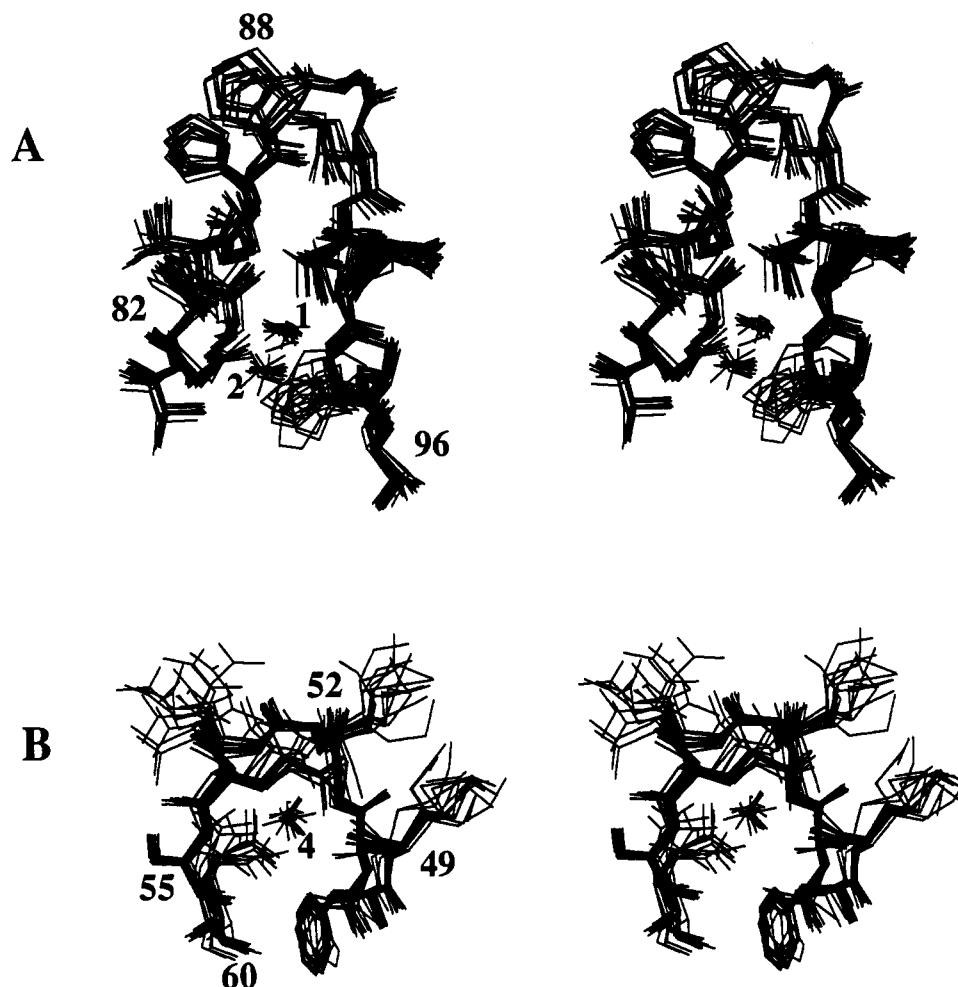


FIGURE 7: Stereoplots for portions of  $^{15}\text{N}$ MR structures of the FKBP/ascomycin complex calculated using distance restraints involving the NMR-observed water molecules (Table I). (A) FKBP residues 82–96 and waters 1 and 2 and (B) FKBP residues 49–55 and 60 and water 4.

by NMR due to their fast exchange with bulk water even though these waters are in close proximity to FKBP protons in the X-ray structures (Van Duyne et al., 1993).

**Effects of Including Bound Waters in the NMR Structure Determination.** In order to study the effects of including bound waters in the structure determinations of the FKBP/ascomycin complex, two sets of structures were calculated and compared. In one set, three water molecules were included in the calculations along with their associated NMR-derived distance restraints and hydrogen bonds. In another set, the waters were excluded from the calculations.

The influence of the waters on the FKBP/ascomycin structures was examined in several ways. First, the ability of the water molecules to affect the precision of the structures was tested by comparing the atomic root mean square deviations (RMSD) for 15 structures from each set of calculations. The atomic root mean square deviations to the mean atomic coordinates for the NMR structures without waters were 0.55 and 0.88 Å for the backbone and all heavy atoms, respectively. For the structures with water, these values decreased slightly to 0.42 and 0.73 Å. Thus, the overall convergence of the two sets of structures was very similar, consistent with the effects of bound waters previously observed in the NMR structure determinations of interleukin-1 $\beta$  (Clare et al., 1991) and thioredoxin (Forman-Kay et al., 1991). However, as shown in Figure 6 in the regions immediately surrounding the three water molecules, the hydrated structures had a significantly lower local RMSD compared to the

nonhydrated structures. Several residues in the loop comprised of residues 78–97 exhibit a substantial decrease in the atomic RMSD upon inclusion of waters 1 and 2 (Figure 6). Likewise, the RMSD for residues 45–57 is significantly influenced by water 4. As shown in Figure 6, the convergence is greater for the NMR structures derived with the three waters. The precision of the NMR structures in the vicinity of the water molecules is illustrated in the stereoviews depicted in Figure 7. The structures of the protein near the water molecules as well as the waters themselves (1, 2, 4) are well-defined by the NMR data except for some of the solvent-exposed side chains (Figure 7B).

An additional measure of the convergence properties of the two sets of structures was obtained by analyzing the angular order parameter (AOP) (Hyberts et al., 1992). Briefly, the angular order parameter reports on the convergence of the dihedral angles within a set of structures. The values can range between 0 and 1 with a value of 0 indicating a random distribution and 1 indicating identical dihedral angles in all of the structures. The  $\phi$  and  $\psi$  backbone angular order parameters for the FKBP/ascomycin complex are given in Figure 8. Significant differences were observed in  $\phi$  and  $\psi$  angle convergence between the unhydrated (Figure 8A,C) and hydrated (Figure 8B,D) structures. Residues 82–84 appear highly divergent in the structures without water yet are nearly identical upon inclusion of waters 1 and 2. Modest backbone torsional angle stabilization also occurs in the region of water 4 (Figure 8). In this region, it is G51 that is primarily



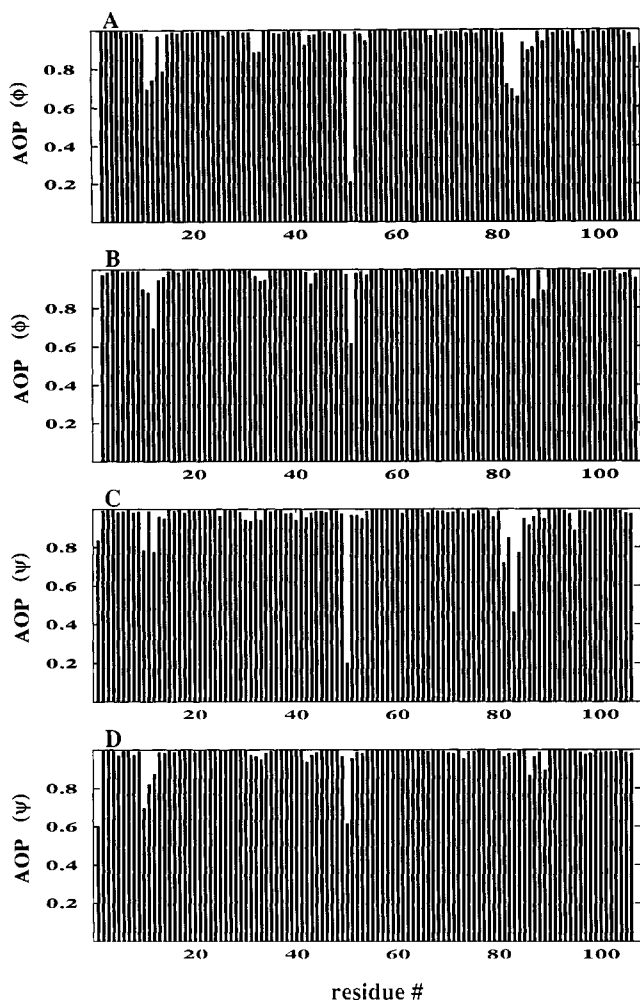


FIGURE 8: Angular order parameters (AOP) (Hyberts et al., 1992) for the (A, B)  $\phi$  and (C, D)  $\psi$  angles of FKBP residues calculated from NMR structures of the FKBP/ascomycin complex generated with (B, D) or without (A, C) the three water molecules.

Table II: Interatomic Distances (Å) in the Two Sets of Structures of the FKBP/Ascomycin Complex

C $\alpha$ -C $\alpha$ atom pairs	without waters	with waters
51-60	8.38 $\pm$ 0.58	7.83 $\pm$ 0.37
52-59	12.00 $\pm$ 0.41	11.50 $\pm$ 0.20
53-56	7.22 $\pm$ 0.37	7.37 $\pm$ 0.22
92-85	7.47 $\pm$ 0.96	8.20 $\pm$ 0.40
94-83	6.01 $\pm$ 0.67	5.46 $\pm$ 0.49
95-80	8.66 $\pm$ 0.49	8.50 $\pm$ 0.25

affected by the addition of waters even though no hydrogen bonds and only 1 NOE to water 4 involve G51.

In addition to the effects on the local precision of the NMR structures caused by including the water molecules, subtle differences were also observed in the protein conformations as indicated by the different interatomic distances in the two sets of structures (Table II). These differences not only are caused by the added distance restraints but also may be due to the bulk of the water molecules which serves to separate relatively tightly packed regions of the protein. Such a role for water has been noted previously and has been used as an argument for the inclusion of waters into molecular dynamics simulations of proteins (Guenot & Kollman, 1992). In addition, the waters can act as a common site for hydrogen bonding, ultimately serving to bring together otherwise distant regions of the protein. As shown in Table II, several interatomic distances appear to change upon inclusion of the

waters. Some of the atoms increase their interatomic separation in order to accommodate the bulk of the added waters, while other distances become smaller as a result of water-mediated hydrogen bonds. In all cases, the standard deviation decreases for the hydrated structures (Table II).

## CONCLUSIONS

From  $^{15}\text{N}$ -resolved 3D ROESY-HMQC and  $^{13}\text{C}$ -resolved 3D NOESY-HMQC spectra of the FKBP/ascomycin complex in  $\text{H}_2\text{O}$ , the locations of three water molecules were identified. These water molecules were found to be buried in the interior of the complex and stabilized by a network of hydrogen bonds. The three waters detected by NMR were only a small fraction of the total number of water molecules observed in the X-ray crystal structures of FKBP/FK506 (Van Duyne et al., 1991a) and FKBP/rapamycin (Van Duyne et al., 1991b). These results are consistent with earlier NMR studies of protein hydration where only a small number of internal water molecules were observed (Otting & Wüthrich, 1989; Clore et al., 1990; Otting et al., 1991; Forman-Kay et al., 1991).

In order to examine the effects of the three water molecules on the NMR structures of the FKBP/ascomycin complex, NMR structures were generated either with or without these waters. Including distance restraints involving these waters in the structure calculations results in a lower RMSD and improved angular order parameters (Hyberts et al., 1992) for the hydrated structures compared to the structures generated without the waters for regions of the protein in the vicinity of the water molecules. This convergence not only results from the inclusion of additional hydrogen bonds and intermolecular NOE-derived distance restraints but also may reflect the influence of the small, but important, van der Waals radii of the waters which may restrict the available local conformational space and force apart tightly packed regions of the protein. In addition, the waters may bring together otherwise distant regions of the protein through water-mediated hydrogen bonds. In fact, slightly different NMR structures were obtained depending on whether or not waters were included in the structure calculations. These results suggest that in some cases more precise and more accurate NMR structures of proteins and molecular complexes can be obtained by including water molecules in the structure calculations. Although more time-consuming, this should become a standard practice in the structure determinations of proteins by NMR.

## ACKNOWLEDGMENT

We thank Harriet Smith, Earl Gubbins, Jean Severin, and Tom Holzman for preparation of  $[\text{U-}^{13}\text{C}, ^{15}\text{N}]$ FKBP and Jill Hochlowski and Marianna Jackson for  $[\text{U-}^{13}\text{C}]$ ascomycin. We also thank J. Clardy for providing a preprint of their manuscript (Van Duyne et al., 1993).

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