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Identification and Localization of Bound Internal Water in the Solution Structure of Interleukin 1β by Heteronuclear Three-Dimensional ^{1}H Rotating-Frame Overhauser ^{15}N–^{1}H Multiple Quantum Coherence NMR Spectroscopy†

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ABSTRACT: The presence and location of bound internal water molecules in the solution structure of interleukin 1β have been investigated by means of three-dimensional ^{1}H rotating-frame Overhauser ^{1}H–^{15}N multiple quantum coherence spectroscopy (ROESY-HMQC). In this experiment through-space rotating-frame Overhauser (ROE) interactions between NH protons and bound water separated by $\leq$3.5 Å are clearly distinguished from chemical exchange effects, as the cross-peaks for these two processes are of opposite sign. The identification of ROEs between NH protons and water is rendered simple by spreading out the spectrum into a third dimension according to the ^{15}N chemical shift of the directly bonded nitrogen atoms. By this means, the problems that prevent, in all but a very few limited cases, the interpretation, identification, and assignment of ROE peaks between NH protons and water in a 2D ^{1}H–^{1}H ROESY spectrum of a large protein such as interleukin 1β, namely, extensive NH chemical shift degeneracy and ROE peaks obscured by much stronger chemical exchange peaks, are completely circumvented. We demonstrate the existence of 15 NH protons that are close to bound water molecules. From an examination of the crystal structure of interleukin 1β [Finzel, B. C., Clancy, L. L., Holland, D. R., Muchmore, S. W., Watenpaugh, K. D., & Einspahr, H. M. (1989) *J. Mol. Biol. 209*, 779–791], the results can be attributed to 11 water molecules that are involved in interactions bridging hydrogen-bonding interactions with backbone amide and carbonyl groups which stabilize the 3-fold pseudosymmetric topology of interleukin 1β and thus constitute an integral part of the protein structure in solution.

Bound water molecules are invariably present in high-resolution X-ray structures where they may be found either within the interior of the protein or on the surface (Deisenhofer & Steigemann, 1975; Blundell & James, 1976; Finney, 1979; James & Sielecki, 1983; Edsall & McKenzie, 1983; Baker & Hubbard, 1984; Teeter, 1984; Wlodawer et al., 1987). Internal water molecules often play a role in stabilizing the protein structure through bridging hydrogen-bonding interactions and additionally may be involved in catalysis (Bode & Schwager,

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1975; Branden et al., 1975; Rees et al., 1983). Ordered surface waters, on the other hand, tend to be clustered around polar groups (Carter et al., 1974; Rose, 1978; Brünger et al., 1985). Evidence for water molecules bound to proteins in solution has come from a number of sources, which include the demonstration of a layer of hydration through magnetic relaxation experiments (Koenig et al., 1978; Halle et al., 1981; Shirley & Bryant, 1982) and the measurement of bound water exchange with ^{18}O tracer techniques (Tüchsen et al., 1987). It is only very recently, however, that bound internal water has been directly observed in a small protein in solution, namely, bovine pancreatic trypsin inhibitor, by means of 2D[1] rotating-frame Overhauser (ROESY) experiments (Otting & Wüthrich, 1989). The rational behind the ROESY experiment lies in its ability to distinguish interactions between protein protons and water arising from through-space (<4 Å) nuclear Overhauser effects and chemical exchange, as these two processes give rise to cross-peaks of opposite sign (Bothner-By et al., 1984; Bax & Davis, 1985; Davis & Bax, 1985). This contrasts to conventional 2D NOESY experiments in the laboratory frame where NOE and chemical exchange peaks are of the same sign and hence indistinguishable (Jeener et al., 1979). Unfortunately, the application of 2D methods to the identification of bound water molecules is very limited, because all such interactions give rise to cross-peaks on a single trace of the 2D spectrum, parallel to the F_2 axis at the F_1 frequency of the H_2O resonance. This particular trace often shows severe base-line distortion caused by the intense H_2O resonance. Moreover, extensive resonance overlap often occurs on this trace due to the multitude of exchange, ROE, and ROE–exchange (vide infra) interactions. Consequently, many of the ROEs between NH protons and water may be obscured by the presence of much stronger chemical exchange peaks located at the same frequency, effectively wiping out any intensity from the ROE. This is particularly so for ROEs that involve NH protons resonating in the vicinity of side-chain amide and guanidino groups, which invariably give rise to large chemical exchange peaks with water.

In a series of recent papers we have presented the complete assignment of the backbone and side-chain ^1H, ^{15}N, and ^{13}C resonances of interleukin 1β, a protein of 153 residues and molecular mass of 17.4 kDa, together with an analysis of its secondary structure and molecular topology using a range of 3D double- and triple-resonance heteronuclear NMR experiments (Marion et al., 1989a; Driscoll et al., 1990a,b; Clore et al., 1990a). IL-1β is itself of great interest as it is a member of the cytokine family of proteins, which play a central role in the immune and inflammatory responses [see Oppenheim et al. (1986), Dinarello (1988), and Moore (1989) for reviews]. Recently, two refined X-ray structures of IL-1β at 2-Å resolution have been published (Finzel et al., 1989; Priestle et al., 1989), and a number of internal water molecules participating in stabilizing backbone interactions, particularly at the ends of β-sheets, have been identified (Finzel et al., 1989). In this paper, we make use of a novel 3D heteronuclear NMR experiment, 3D ^1H rotating-frame Overhauser ^1H–^{15}N multiple quantum coherence spectroscopy (ROESY-HMQC), to specifically identify NH protons in close proximity to bound water. By separating out the ROE and chemical exchange peaks into a third dimension on the basis of the ^{15}N chemical shift of nitrogens directly bonded to protons, the limitations of the 2D ROESY experiment are completely circumvented.

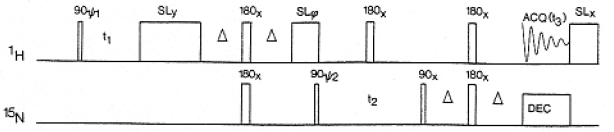

FIGURE 1: Pulse sequence for the ^1H–^{15}N ROESY-HMQC experiment. SL is a spin lock. ROESY mixing is achieved by a spin lock applied for 33 ms along the y axis (SL_y). The two other spin-lock pulses (SL_ϕ and SL_x) are applied for only 2 ms. The delay Δ is set to 2.25 ms, slightly less than $1/(4J_{NH})$. ^{15}N decoupling during the acquisition period is achieved with WALTZ modulation (Shaka et al., 1983). The phase cycling is as follows: $\phi = 4(x), 4(-x)$ and Acq $= 2(x,-x,-x,x)$. Quadrature in F_1 and F_2 is obtained with the TPPI–States method (Marion et al., 1989b), using $\psi_1 = 4(x,-x)$, $4(y,-y)$ and $\psi_2 = 2(x,x,-x,-x)$, $2(y,y,-y,-y)$ and storing data for $\pm x$ and $\pm y$ components of ψ_1 and ψ_2 separately to be processed as complex data. Each time t_1 is incremented, ψ_1 and the receiver phase are increased by 180°, and similarly, each time t_2 is incremented, ψ_2 and the receiver phase are increased by 180°.

Experimental Procedures

Uniformly labeled (>95%) [^{15}N]IL-1β was prepared and purified as described previously (Wingfield et al., 1986; Driscoll et al., 1990a). Samples for NMR contained 2 mM [^{15}N]IL-1β in 100 mM sodium acetate-d_3, pH 5.4, dissolved in 90% H_2O/10% D_2O. NMR spectra were recorded at 36 °C on a Bruker AM600 spectrometer, operating in the reverse mode. The 3D ^1H–^{15}N ROESY-HMQC spectrum was processed on a Sun Sparc station using an in-house routine for the F_2 Fourier transform, together with the commercially available 2D software package NMR2 (New Methods Research Inc., Syracuse, NY) for processing the F_1–F_3 planes, as described previously (Kay et al., 1989).

Results and Discussion

The pulse scheme used for the 3D ^1H–^{15}N ROESY-HMQC experiment is given in Figure 1. After the evolution of ^1H chemical shifts during the period t_1, a 33-ms spin lock applied along the y axis causes mixing of ^1H magnetization through chemical exchange and ROE effects (as well as some Hartmann–Hahn effects). At this stage, proton magnetization is aligned along the effective field, in the yz plane, close to the y axis. After dephasing caused by the ^{15}N–^1H J coupling during the time period $2\Delta \sim 1/(2J_{NH})$, all magnetization residing on ^{15}N-bound protons is subsequently converted into ^1H–^{15}N multiple quantum coherence by the application of a ^{15}N 90° pulse (Mueller, 1979). Note that magnetization from protons not attached to ^{15}N, particularly H_2O magnetization, remains aligned along the y axis at the end of the interval 2Δ. The short 2-ms trim pulse applied along the x axis then effectively removes this magnetization by randomization (Messerle et al., 1989), avoiding the necessity for presaturation of the H_2O resonance. In this manner, water suppression is achieved without perturbing the observation of ROE effects between water and ^{15}N-bound protons. During the evolution period t_2, ^1H offset effects are suppressed by the 180° ^1H pulse, and multiple quantum coherence evolves with the ^{15}N chemical shift frequency. At the end of the t_2 period, multiple quantum coherence is converted back to observable magnetization in the standard manner (Mueller, 1979; Bax et al., 1983). To ensure identical amounts of z magnetization at the start of every sequence, a trim pulse is applied along the x axis immediately after data acquisition. It is important to note that in this type of experiment the water magnetization is randomized and that recovery during the delay time between scans

[1] Abbreviations: IL-1β, interleukin 1β; NOE, nuclear Overhauser effect; ROE, rotating-frame Overhauser effect; 2D, two dimensional; 3D, three dimensional; ^{15}N–^1H ROESY-HMQC, three-dimensional ^1H rotating-frame Overhauser ^1H–^{15}N multiple quantum coherence spectroscopy.

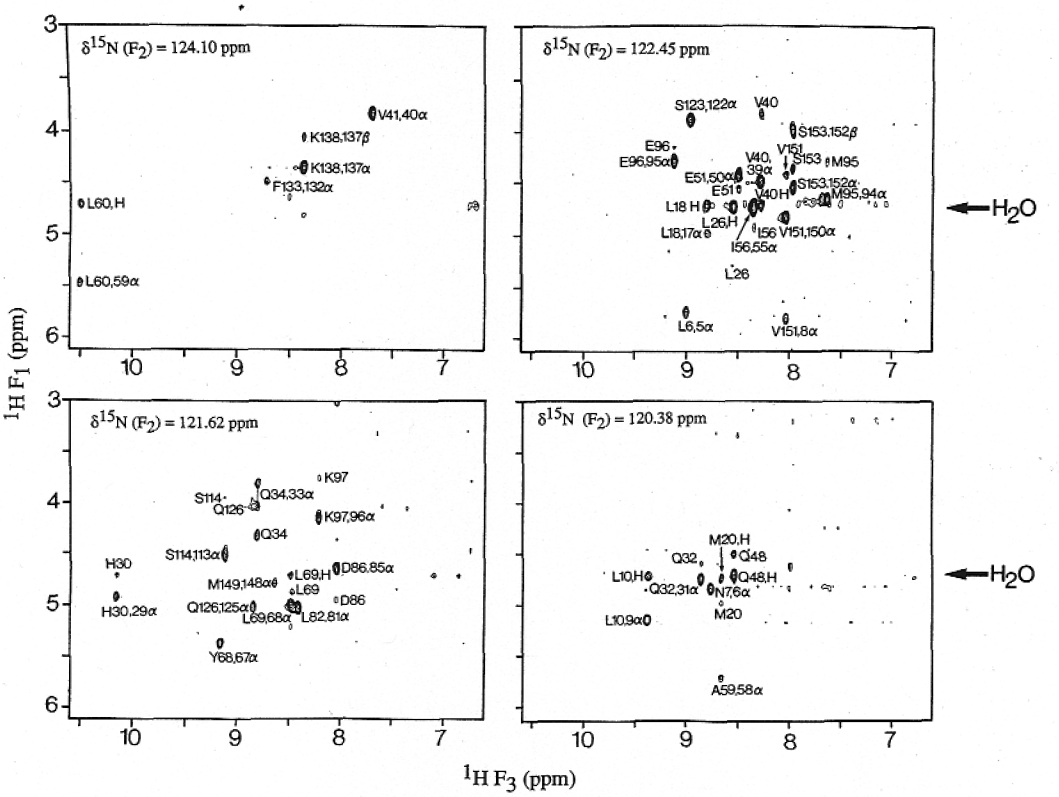

FIGURE 2: Four out of 64 (F_1,F_3) slices of the 3D ^1H–^{15}N ROESY-HMQC spectrum of 1.7 mM uniformly ^{15}N-labeled IL-1β in 90% H_2O at pH 5.4 and 36 °C. Peaks are labeled i,j, where i refers to the NH proton (with the residue name and number indicated) and j refers either to water (indicated by the letter H) or to an aliphatic proton (indicated by the residue number and the proton type). The water frequency is at 4.67 ppm. The 3D spectrum results from a (128 complex) × (32 complex) × (512 real) data matrix, zero-filled to yield a final absorptive spectrum of 256 (F_1) × 64 (F_2) × 512 (F_3) data points. The total measuring time was ~48 h.

occurs with the longitudinal relaxation time T_1, which is often substantially longer than the T_1 of protein protons. In our particular case, using a delay time of 1.1 s between scans, protein protons have recovered ~65% of their equilibrium magnetization, whereas water protons have recovered only ~30% of their equilibrium value at the start of each scan. Thus, ROEs between water and protein protons are attenuated by a factor of about 2 relative to interactions among protein protons.

In the 3D spectrum each cross-peak is labeled by three chemical shifts: ^1H in F_1, ^{15}N in F_2, and ^{15}N-bound ^1H in F_3. Each slice at a particular ^{15}N (F_2) frequency thus represents a 2D ROESY spectrum edited by ^{15}N chemical shifts.

The ROE mixing time (33 ms) was chosen to be about equal to the spin-locked relaxation time $T_{1\rho}$ of the amide protons, thus maximizing the signal-to-noise ratio of the ROE cross-peaks (Bothner-By et al., 1984; Bax & Davis, 1985). It is interesting to note that, even at this relatively long mixing period for a protein the size of IL-1β, the ROE intensities provide nearly quantitative distance information. For example, within the antiparallel β-sheets, the average sequential $C^\alpha H$-(i)–NH(i+1) ROE is about 6 times more intense than the average $C^\alpha H(i)$–NH(i) ROE, in agreement with a factor of 5–7 predicted on the basis of a r^{-6} dependence of the ROE intensity. As discussed previously (Bax et al., 1986), the good quantitative nature of the ROE intensity stems from the fact that the NOE under spin-locked conditions is positive, thereby strongly attenuating spin-diffusion effects.

Cross-peaks in the ^1H–^{15}N ROESY-HMQC spectrum may arise from one of three sources. Negative cross-peaks (of opposite sign to the diagonal peaks at $\delta F_1 = \delta F_3$) arise from NOEs in the rotating frame, known as ROEs. Chemical exchange, on the other hand, gives rise to positive cross-peaks. Hartmann–Hahn effects may also give rise to positive cross-peaks but will only be evident when the matching for homonuclear Hartmann–Hahn transfer is near perfect, that is to say when the two ^1H spins have opposite but approximately equal offsets from the transmitter (Bax, 1988). In considering the origin of negative cross-peaks between NH protons and water, two possible mechanisms must be considered. The first involves solely an ROE and is due to the close proximity of the NH proton and the bound water molecule. The second involves an indirect pathway whereby magnetization is transferred between an NH proton and a rapidly exchanging side-chain proton (i.e., hydroxyl group in Ser, Thr, and Tyr, the side-chain amide group in Asn, Gln, and Lys, and the guanidinium group in Arg), followed by chemical exchange between the latter proton and water. Providing the three-dimensional structure of the protein under consideration is known, the possible contribution of the latter magnetization-transfer pathway can be readily assessed.

Four slices at different ^{15}N chemical shifts of the 3D ^1H–^{15}N ROESY-HMQC spectrum are shown in Figure 2, and it can be clearly seen that there are a number of ROE cross-peaks between NH protons in the F_3 dimension and the water resonance frequency in the F_1 dimension. These cross-peaks involve Leu-60 NH at $\delta^{15}N$ = 124.10 ppm, Leu-18 NH, Leu-26 NH, and Val-40 NH at $\delta^{15}N$ = 122.45 ppm, Leu-69 NH at $\delta^{15}N$ = 121.62 ppm, and Leu-10 NH and Met-20 NH at $\delta^{15}N$ = 120.38 ppm. We were able to identify 17 such cross-peaks in the complete 3D data set, which could not be attributed to ROEs between NH protons and $C^\alpha H$ protons degenerate with the water resonance. (An example of the latter is the cross-peak seen in Figure 2 between Ile-56 NH and Lys-55 $C^\alpha H$ at $\delta^{15}N$ = 122.45 ppm.) The residues corresponding to these 17 cross-peaks are Leu-10, Leu-18,

Met-20, Leu-26, Ala-28, Leu-31, Val-40, Gln-48, Glu-50, Leu-60, Leu-69, Phe-112, Ala-115, Gln-116, Ser-125, Asn-129, and Met-130. Examination of the crystal structure of IL-1β (Finzel et al., 1989) indicates that for 15 of these 17 NH protons there are no labile side-chain protons in close proximity (i.e., <3.5 Å) so that these effects can be attributed directly to ROEs involving bound water. In the case of Ala-115 and Gln-116, however, the NH protons are ~2.5 Å away from the hydroxyl group of Ser-114 so that an alternative pathway involving an ROE to the $O^{\gamma}H$ of Ser-114 followed by chemical exchange of the hydroxyl proton with water is possible.

The intensity of a cross-peak between an NH proton and bound water depends both on the proximity of the NH proton to the water protons and on the lifetime of the bound water molecule. A rough estimate on the lower limit of the bound water lifetime, however, can be obtained as follows. The intensities of the ROE cross-peaks with bound water vary from 3 to 8 times lower than the average intensity of the $C^{\alpha}H(i)$–$NH(i+1)$ sequential ROE peaks within a β-sheet, which corresponds to a distance of ~2.2 Å. As discussed above, the ratios of ROE cross-peak intensities at the mixing time (33 ms) employed still provide a good estimate of interproton distance ratios. Hence, the intensity of a ROESY peak between two protons is a direct reflection of the corresponding dipole–dipole relaxation rate in the rotating frame. This rate is in turn directly proportional to the inverse sixth power of the distance and $f(\tau_{eff}) = 4\tau_{eff} + 6\tau_{eff}/(1 + \omega^2\tau_{eff}^2)$, where τ_{eff} is the effective correlation time of the interproton vector and ω the spectrometer frequency. Ignoring internal motion, τ_{eff} is given approximately by $\tau_{eff}^{-1} \sim \tau_R^{-1} + \tau_{ex}^{-1}$, where τ_R and τ_{ex} are the rotational correlation time of the protein and the lifetime of the bound water molecule, respectively. If the residence time of the bound water molecule is much longer than τ_R, the ROE intensity, for a given interproton distance, is determined solely by the overall tumbling time of the protein, which in this case is 8.3 ns (Clore et al., 1990b). If, on the other hand, the lifetime τ_{ex} is much shorter than τ_R, the ROE will be determined by τ_{ex}. If we assume that we could detect a cross-peak up to 20 times less intense than the sequential $C^{\alpha}H(i)$–$NH(i+1)$ cross peaks within an antiparallel β-sheet, then an ROE between an NH proton ~2, ~2.5, and ~3 Å away from a water proton (taking into account the 2-fold attenuation of ROEs involving water protons relative to those involving only protein protons, mentioned above) could still be observed at a mixing time of ~30 ms providing the lifetime of the bound water molecule would be greater than about 0.2, 2, and 8 ns, respectively.

Because the bound water resonances are degenerate with water, it is clear that the results can only be interpreted in structural terms in light of a high-resolution structure of IL-1β. Examination of the X-ray structure of IL-1β reveals the presence of 84 bound water molecules (Finzel et al., 1989). We, however, only observe ROEs involving a small proportion of these, and in each case, the water molecule involved takes part in bridging backbone hydrogen bonds of the type NH---OH---CO. In the case of 12 of the 15 NH protons, the NH proton is itself hydrogen bonded to water, whereas in the other three cases (Val-40, Leu-69, and Asn-129) it is within close proximity (<3 Å) of a bound water molecule. These interactions are summarized in Figure 3.

The overall topology of IL-1β displays internal pseudo-3-fold symmetry (Priestle et al., 1988; Finzel et al., 1989; Driscoll et al., 1990b). Each topological unit is composed of five antiparallel β-strands (Figure 3A–C), which we shall denote as A–E. These form three antiparallel β-sheets in a triangular

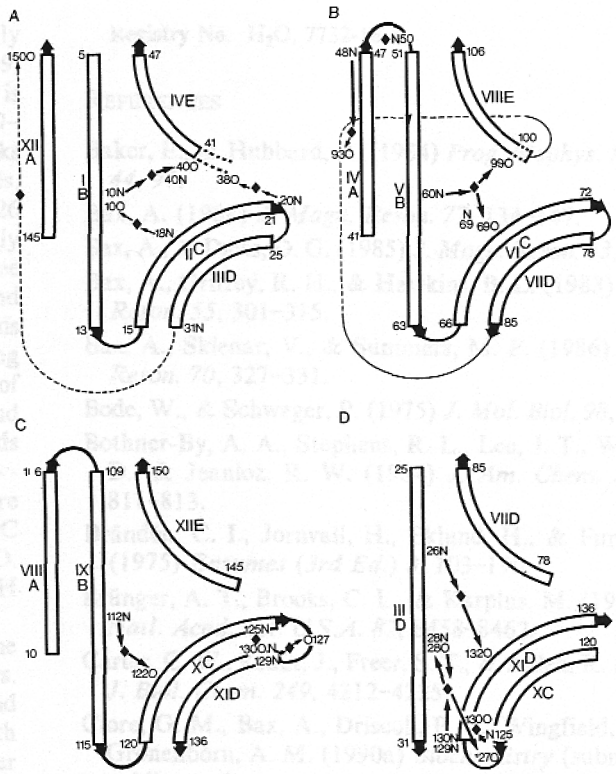

FIGURE 3: Location of bound water molecules in IL-1β deduced from the presence of ROESY cross-peaks between NH protons and water in the 3D ^1H–^{15}N ROESY-HMQC experiment. The molecular topology of IL-1β, derived from both NMR (Driscoll et al., 1990b) and crystallographic (Finzel et al., 1989) data, is depicted schematically. The three repeating topological units, each composed of five antiparallel β-strands, are shown in (A)–(C), and the interface of the three units is shown in (D). The NH protons that are involved in ROESY cross-peaks with water are indicated by residue name and number, and the water molecules are depicted as diamonds. The hydrogen-bonding interactions of these water molecules, as seen in the crystal structure, are indicated by the arrows.

arrangement. Thus, one sheet is formed by strands A and B and the C-terminal end of E, another comprises strands D and C and the C-terminal end of strand B, while the third is made up from strands C and D and the N-terminal end of strand E. Strands B and C are connected by a tight turn, while strands C and D are connected by either a turn or a longer loop. There are a total of 12 β-strands (I–XII), and strand A is equivalent to strands XII (Figure 3A), IV (Figure 2B), and VIII (Figure 3C) in the first, second, and third topological unit, respectively, strand B to strands I, V, and IX, strand C to strands II, VI, and X, strand D to strands III, VII, and XI, and finally strand E to strands IV, VIII, and XII. Note that strand XII is strand A in the first unit and strand E in the third, strand IV is strand A in the second unit and strand E in the first, and strand VIII is strand A in the third unit and strand E in the second. The three units are linked together by three antiparallel β-sheets formed by the D strands of each topological unit (Figure 3D).

Three of the internal molecules that we observe occur in approximately the same location, although not necessarily in the same orientation, within each of the three topological units and form a bridge between strands B and C (Figure 3A–C). Thus, a water molecule bridges Leu-18 NH (strand II/C) and Leu-10 CO (strand I/B) in the first unit (Figure 3A), Leu-60 NH (strand V/B) and Leu-69 CO (strand VI/C) in the second unit (Figure 3B) with the NH of Leu-69 in close proximity to the water molecule, and Phe-112 NH (strand XI/B) and Ile-122 CO (strand X/C) in the third unit (Figure 3C). The