Table I. ^{15}N and ^{1}H Shifts and $^{1}J_{NH}$ Couplings of Imino and Imino-like Sites[a]

d(TCGA)				d(G(AG)$_8$) + d(C(TC)$_8$), 1:2			
base	N	H	$^{1}J_{NH}$	base	N	H	$^{1}J_{HN}$
G	147.2	10.14	-90	G	147.1	12.63	-74
T	158.5	11.44	-88	T	161.6	13.41	-85
				T	161.8	14.05	-85
CH$^+$	168.5	15.09	-47	CH$^+$	149.2	14.82	-74

[a] Couplings are in hertz and are assumed to be negative, ^{15}N shifts are relative to NH_3, and ^{1}H shifts are relative to (trimethylsilyl)-propionic acid, TSP. Assignments are based on ^{1}H NOESY results.

(Table I) also shows a substantial variation between the two systems examined, being an unusually low -47 Hz for the d(TCGA) and -74 Hz for the triple-helix system. Typically $^{1}J_{NH}$ couplings of about -90 Hz[12,16,17] are found for imino nitrogens.

Shifts and couplings can be interpreted in terms of understood effects on ^{15}N.[2-4,17-20] Protonation of a nitrogen nucleus generally causes a large upfield shift. The magnitude of the coupling constant is indicative of the s character in the NH bond.[16] For CH$^+$ in DNA, direct comparison of shifts can be made with reported values for CMP in aqueous solution as a function of pH.[4] The N3 of CMP was found to move upfield 60 ppm from about 202 ppm to 144 ppm relative to NH_3 on protonation. Effects on couplings and shifts of modulating the NH bond have been studied in the context of azo-hydrazone tautomerism,[19,20] and both parameters were shown to reflect the degree to which the proton is associated with the nitrogen. Since the NH couplings could be observed in that case, as they can in the case of CH$^+$ reported here, the exchange regime for the ^{15}N resonances must be slow. Thus the shifts reflect the intrinsic NH bond interaction and are not just a fast weighted average of the limiting shifts.

In the triple helix, the shift of 147.1 for N3 of the CH$^+$ residue resembles the model value of 144 for protonated CMP.[4] This shift approaches the value for the *N*-methoxycytosine where N3 is protonated.[21] The $^{1}J_{NH}$ coupling seen in the triplex is comparable to the value for the other normal imino protons in the complex. (The width of the proton lines and the presentation in absolute value mode may introduce some inaccuracy in measurement of the splitting.) This leads to the conclusion that the N3 is essentially sp^3, leaving the bond between C4 and the exocyclic nitrogen largely double bond in character with the charge on this nitrogen (structure II). NQR data[6] on solid samples of cytosine salts suggests such an arrangement, as does ^{14}N NMR data.[5] Consistent with this analysis is the similarity of the shift between CH$^+$ and G imino ^{15}N resonances. The imino nitrogens of G have a carbonyl group as one neighbor and an sp^2 carbon as the other, while for this CH$^+$ the immediate neighbors are a carbonyl group and an sp^2 carbon with the double bond to the exocyclic nitrogen.

For the CH$^+$ in d(TCGA) the N3 resonance is about 20 ppm further downfield from the triplex, at 168.5 ppm, placing it almost exactly halfway between the positions of the protonated and nonprotonated N3 of aqueous CMP.[4] Further, the coupling is about half of the normal imino value. A weaker NH bond would imply less s bond character to N3 and would at least qualitatively explain both the intermediate coupling and shift for the d(TCGA) case, with analogy to the interpretation of the azo-hydrazone tautomeric system.[19,20] Thus, for the d(TCGA) system the positive charge of CH$^+$ would be delocalized between N3 and the amino nitrogen and their substituents (structure I), as was suggested for methylcytosine.[22]

It would have been informative to observe the amino nitrogens, but nonequivalence of these protons in the complexes and the greater proximity of these resonances to the H_2O resonance complicated matters, and we have not been able to observe them.

Although CH$^+$ bases can be observed by optical spectroscopic techniques,[27] ^{15}N NMR can provide more specific information about the characteristics of the base.

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Novel Method for NMR Spectral Correlation between the Native and the Denatured States of a Protein. Application to Ribonuclease A

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Recently, we have developed a new NMR technique, state-correlated two-dimensional (SC-2D) NMR spectroscopy, in which correlation of NMR spectra can be obtained between two chemically distinct states before and after a sudden temperature jump.[1,2] In the present communication, SC-2D NMR spectroscopy is applied for the first time to obtain a spectral correlation between the native conformer (N) and the denatured conformer (D) of a protein in solution.

Figure 1 shows the pulse sequence used to obtain the SC-2D NMR spectra of ribonuclease A (RNase A) in 0.15 M KCl, pH 1.0 in deuterium oxide. The pulse sequence resembles that of 2D exchange or 2D NOE, but the mixing period is replaced by the transition period during which a microwave pulse is applied for a temperature jump. The solution temperature increased from θ_1 = 30 °C to approximately θ_2 = 45 °C within 150 ms during or after which conformational transition (thermal denaturation) of the protein took place. Since the conformational transition can be complete during the transition period (200 ms) well within proton spin-lattice relaxation times of the protein (about 1 s at 400 MHz),[3] the spin magnetizations keep their amplitudes modulated by Larmor precession in the t_1 domain, even after they have been brought to the t_2 domain in which they precess with different precession frequencies, resulting in a 2D correlation spectrum after double Fourier transformation. The experiment was carried out at 400 MHz on a JEOL GX-400 NMR spectrometer with a homemade probe head.

Figure 2 shows the first successful observation of an SC-2D spectrum between N and D forms of RNase A in the aromatic proton region. Since at pH 1.0 RNase A transforms from an essentially N conformer to a fully D conformer by the temperature

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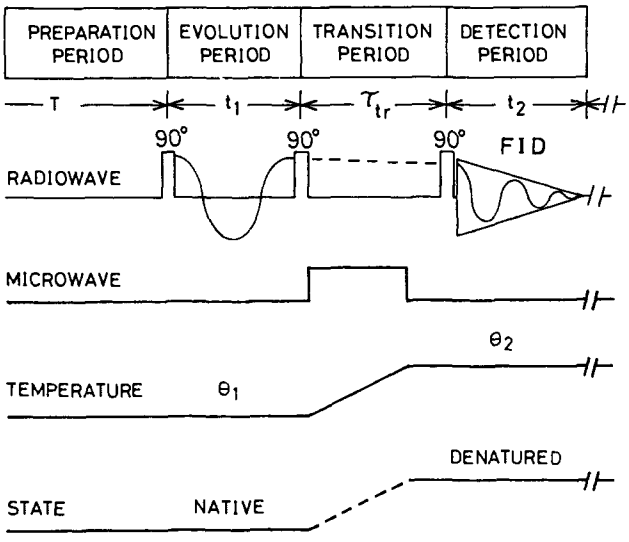

Figure 1. The pulse sequence used to perform state-correlated 2D NMR spectroscopy on RNase A. The protein is in the native state at θ_1, but makes a sudden transition into the denatured state upon a temperature jump to θ_2 during the transition period τ_{tr}. The temperature jump is brought about by a microwave pulse (2.45 GHz) of 150-ms duration from a 1.3-kW pulsed microwave generator (IMG-2502P, IDX Corporation, Tokyo) which is controlled by a pulse programmer of a JEOL GX-400 NMR spectrometer. An additional 50 ms was allowed for equilibration of the D state before acquisition of free induction decay signals, after which $T = 180$ s was allowed for sample cooling and renaturation of the protein. While the initial temperature θ_1 was set and controlled by a JEOL variable-temperature accessory, the final temperature θ_2 after the jump was determined from the chemical shift position of the $^1H^2HO$ signal.

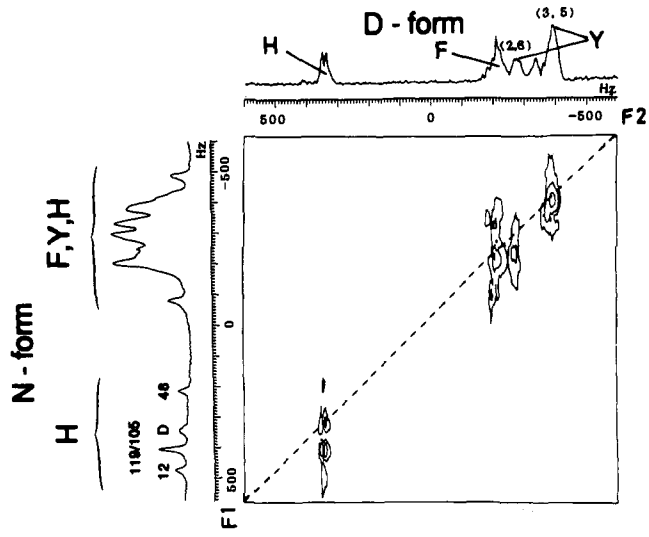

Figure 2. The proton correlation spectrum (absolute mode) between N and D forms of RNase A (10% in ^2H_2O containing 0.15 M KCl, pH 1.0) obtained with the pulse sequence shown in Figure 1 (aromatic proton region). The carrier frequency was set on the middle of the aromatic region to cover the spectral width of 1200 Hz with 32 points in the t_1 domain (digital resolution 37.5 Hz) and to cover the full spectral range (6000 Hz) of RNase A with 2K points in the t_2 domain (digital resolution 5.86 Hz). Eight scans were accumulated for each t_1 point for quadrature detection and phase cycling, making the total number of scans equal to 256 and the total experimental time equal to about 13 h. The t_1 domain signals were zero-filled to 128 points before Fourier transformation. Signals of histidines, phenylalanines, and tyrosines are designated by H, F, and Y, respectively. Specific assignments of His C_2 protons are after ref 5. D denotes the His C_2 proton signal of an irreversibly denatured conformer appearing after repeated heat cycles. The 1D spectrum in the F_1 axis is obtained by a separate measurement. The 1D spectrum in the F_2 axis is a projection spectrum.

jump from 30 °C to approximately 45 °C, a particularly simple correlation spectrum is obtained with no diagonal peaks, except for those of the irreversibly denatured conformer labeled D, in sharp contrast to conventional 2D exchange spectroscopy.

Figure 2 suggests two basic usages of the SC-2D NMR spectroscopy to proteins.

1. Separation of signals of N conformer into amino acid types: Signals of a D conformer are usually grouped into a certain number of peaks by the types of protons of amino acids. Since standard chemical shifts of all the amino acid protons in random coiled polypeptides are well established,[4] these groups of peaks of a D conformer are each readily assigned to one or a few particular types of protons of amino acids. Thus by taking appropriate cross sections along the F_1 axis, one can separate the signals of the N conformer according to types of protons of amino acids. For example, in Figure 2, the signals of the half of the 1D spectrum of the N form at higher field are heavily overlapped; by taking correlation to the D form signals in the SC-2D spectrum, one can readily separate them into three groups of signals, characterized by the types of protons, namely, the 2,6-protons of six Tyr residues, the 3,5-protons of six Tyr residues, and the ring protons of three Phe (plus the C_4 protons of four His). With improved resolution in the F_1 axis, further separation of each of these groups of signals from each amino acid residue in the N conformer will become feasible.

2. Specific signal assignments in the D conformer: At higher resolution, signals of a D conformer may not necessarily converge exactly into "standard" positions as in a random coiled peptide, but may show some dispersion, possibly reflecting residual structure. Then, if the signals in the N conformer have been specifically assigned, the corresponding signals in the spectrum of a D conformer may be specifically assigned in a straightforward manner via SC-2D cross peaks. For example, the C_2 proton signals of four His residues of native RNase A are well dispersed and have been specifically assigned (Figure 2).[5] The corresponding signals in the D conformer are congested into a narrow doublet (Figure 2). Even so, using the four separate cross peaks between the N and D signals, one can identify the signal position of each His C_2 proton in the D conformer; the signals of His 105 and 119 contribute to the narrow doublet peaks, while His 12 and His 48 resonate at positions in the middle of the doublet. Specific assignments of proton NMR signals in D conformers are considered important as they provide clues to structures of proteins in the denatured state.[6-8] So far, these assignments have been made in combination with specific deuteration,[6,7] artificial mutation,[8] or saturation transfer.[9] The SC-2D spectroscopy introduced here provides an alternative and simple technique for the same purpose, provided that reversibility in denaturation is assured.

In addition, some general advantages of SC-2D spectroscopy over exchange spectroscopy may be emphasized. Firstly, while exchange spectroscopy is applicable to an equilibrium system under a rather restricted condition for slow exchange,[9] SC-2D NMR is applicable to any system with slow to fast exchange, depending only on the condition that the transition rate is faster than the spin–lattice relaxation rate. Secondly, SC-2D NMR is applicable to a transient intermediate which might appear right after the temperature jump but disappear after an equilibrium is reached. No such possibility exists in exchange spectroscopy performed on an equilibrium system.

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