Elucidation of the Origin of NOEs And ROEs that Show the Hydration in the Minor and Major Grooves of DNA Duplex with ATTAAT Tract by a Combination of NOESY and ROESY Experiments

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Abstract. A combination of NOESY and ROESY experiments (using ammonia as a catalyst across the pH range of 5 to 8.6) has given us a clear understanding regarding the origin of nOes that are attributed to the stereochemical location and the residence time of water in the major and the minor grooves of $d5'(^1C^2C^3A^4T^5T^6A^7A^8T^9G^{10}G)_2 3'$ duplex Our conclusions are the following: (i) In the major groove, the presence of ammonia in the buffer does not influence on the process of exchange between bound and bulk water. (ii) It has been found that the observation of the bound water in the minor groove is a result of straight dipole-dipole effect at the physiological pH. (iii) The residence time of water near H2 of adenine (H2A) in the minor groove has been estimated to be in the range of 0.3 - 0.5ns, which is closer to the residence time of the bound water found on the surface of protein. (iv) The hydration pattern in the minor groove in the physiological pH, under our NMR measurement condition, is similar to the ones found in the X-ray structure. (v) It has been shown that at pH > 8.0 the nOe/rOe intensities of the water-H2A crosspeaks dramatically increase due to dipole-dipole and/or relayed magnetization transfer from H2A to water through ammonia catalyst.

The nuclear Overhauser effect based NMR experiments have proved to be sensitive and efficient tool to identify sequentially assigned protein and DNA/RNA protons that have nOe/rOe interactions with nearby water protons[1-3,8,23]. It has been shown[1,2] that the cross-relaxation rate in the ROESY experiment, $\mathbf{R}_{rOe}$, is always positive with all frequencies and correlation time (τ_c). In contrast, in the NOESY experiment, $\mathbf{R}_{nOe}$, vanishes when $\omega_o\tau_c = \sqrt{5}/2$ ($\tau_c \sim$ 360ps at 500 MHz) and then becomes negative, reaching the value of $-\mathbf{R}_{rOe}/2$ in the diffusion limits. The sign of

nOes with water presents a qualitative criterion to determine whether the residence time of the bound-water is shorter or longer than 0.1 - 1ns[1h]. It has been pointed out[3] that water can be considered to be bound if the effective correlation time is significantly longer than ~ 0.5 ns. Thus for protein it has been found[1a-c,4] that there are water molecules that reside close to nonexchangeable proton for a considerable time (> 1ns) before exchanging with another water molecule and rapidly diffusing away[1a-c].

It is a considerable challenge to detect bound-water molecule playing the structural role in DNA with residence time of about 1ns that would be comparable to the behavior of the interior water in globular proteins. It has been proposed from the crystal structures[5,6] of DNA that the zig-zag spine of hydration in the minor groove is responsible for the stabilization of the B-form of the DNA structure. The water molecule found in the spine of hydration in the narrowed minor groove of AT tract in the crystal structure has been assumed to be the same as NMR visible water molecules that are relatively slow exchanging from the neighborhood of H2A[1d,g,i]. Zhou and Bryant[7] have recently measured the lifetime of the bound-water in d(CGCGAATTCGCG)$_2$ using the water spin-lattice relaxation rate as a function of the magnetic field strength. These relaxation experiments have suggested[7] that the numbers of DNA bound-water molecules are fewer if the lifetime of the water molecules at DNA sites is longer than the rotation correlation time of the duplex; alternatively, when the number of water molecules bound to the DNA is nearly as many as those found in X-ray structure, then the average lifetime of water molecule should be shorter than the rotational correlation time of the duplex.

Recently, the water contacts with nonexchangeable protons of d5'(^{1}G^{2}C^{3}A^{4}T^{5}T^{6}A^{7}A^{8}T^{9}G^{10}C)$_2$3' have been investigated by NMR[1i], which show (a) negative nOe and rOe for water-H2(^{3}A) crosspeak, indicating a rapid bound-water exchange on a sub-nanosecond timescale, and (b) positive nOes with water-H2(^{6}A) and -H2(^{7}A), suggesting that the bound-water exchanges at the nanosecond time scale. The nOe for water-H2(^{7}A) was however significantly weaker then the corresponding rOe. These data indicate that the residence time of water located near H2A at the core part was higher than at the termini. Basing on this data, it has been assumed[1i] that a kinetically restrained spine of hydration exists around the core TTAA segment.

In order to interpret the nature of nOe crosspeak between water and nonexchangeable proton of a macromolecule, three different mechanisms[1h] have been considered: (A) A direct intermolecular nOe/rOe between non-exchangeable proton and the proton of hydrated water. (B) A direct nOe/rOe between two protons of the same molecule located within 5Å, so that one of the protons is labile and in rapid exchange with the bulk water (chemically relayed nOe). (C) Chemical exchange between a labile

proton of the molecule and the bulk water, which has opposite sign in the ROESY spectra compared to direct intermolecular rOe (*i.e* mechanism A).

The crosspeaks arising from a rOe/nOe transfer followed by chemical exchange will have the same sign as the crosspeaks arising from a direct rOe/nOe transfer[9]. Earlier, "three-site model" has been used to explain the "chemically relayed nOe"[10,11]. It has been proposed[11] that at higher chemical exchange rate of imino protons in DNA $(k_{ex} \geq 10s^{-1})$ the strong peaks observed between non-exchangeable proton and water are mainly due to chemically relayed nOe.

It is important that the non-exchangeable protons of DNA are indeed spatially separated from its rapidly exchanging protons[1d] in order to discriminate between the *direct nOe/rOe* resulting from H2A and bound-water $(\tau_c > 1ns)$ and the *relay effect*, which originates from the direct intermolecular nOe/rOe between non-exchangeable and exchangeable protons followed by magnetization transfer to the bulk water by rapid chemical exchange.

There are mainly two ways, change of temperature and buffer composition, that can be employed[1h,1d,7,1e] to alter the kinetics of exchangeable protons with bulk water. In this work, we have used both of these tools to evaluate the relative role of the straight dipole-dipole process versus the relay process between H2A-water by performing the NOESY and ROESY experiments when only k_{ex} has been carefully changed.

We here report that (1) at the physiological pH, the bound water in the minor groove has a short residence time of ~0.3-0.5ns for ATTAAT tract, where the dipole-dipole interaction between bound water and H2A protons is dominant. (2) With gradual addition of an exchange catalyst, such as ammonia, or changing of pH, the role of transfer magnetization through ammonia increases without involving the exchangeable imino protons at high pH. (3) We also present evidence that this process becomes dominant without altering the exchange process of the bound-water with the bulk water in the major groove or without changing the correlation time of DNA at high pH. (4) The hydration pattern remains unchanged at the physiological pH upto 0.1M salt concentration.

Results

All nonexchangeable and exchangeable protons of $d5'(^1C^2C^3A^4T^5T^6A^7A^8T^9G^{10}G)_2 3'$ duplex were assigned using a combination of NOESY and DQF-COSY spectra in D_2O solution by conventional assignment procedure[12] adopted for B-DNA type conformation. The ^1H chemical shift assignments of the duplex are presented in Table 1.

TABLE 1: [1]H assignments* (ppm) in d(CCATTAATGG)$_2$.

Base	H8/H6	H5/Me/H2	H1'	H2'	H2"	H3'	H4'	H5'/H5"	NH	NH$_2$
^{1}C	7.76	5.93	5.98	2.11	2.53	4.68	4.12	3.78/3.76	-	7.92/6.95
^{2}C	7.63	5.74	5.47	2.20	2.48	4.89	4.16	4.12/4.06	-	8.55/6.93
^{3}A	8.43	7.79	6.36	2.83	3.01	5.08	4.50	4.21/4.13	-	7.70/6.47
^{4}T	7.23	1.47	5.93	1.99	2.54	4.85	4.22	4.38/4.19	13.67	-
^{5}T	7.38	1.66	5.74	2.13	2.49	4.92	4.16	4.21/4.12	13.46	-
^{6}A	8.28	6.69	5.97	2.76	2.94	5.07	4.44	4.18/4.14	-	7.38/6.27
^{7}A	8.15	7.59	6.12	2.53	2.87	5.01	4.46	4.29/4.26	-	7.34/6.09
^{8}T	7.01	1.34	5.65	1.76	2.18	4.82	4.10	4.28/4.10	13.62	-
^{9}G	7.80	-	5.63	2.64	2.70	4.96	4.35	4.09/4.03	12.97	¥
^{10}G	7.80	-	6.16	2.52	2.37	4.66	4.24	4.24/4.21	13.13	¥

* Proton assignments are given for 21°C, except for exchangeable protons which are given for 15°C.
- No such proton exists;
¥ Shifts are not determined.

(I) Effect of pH on the hydration in the major groove in d(CCATTAATGG)$_2$ duplex

Intermolecular water to methyl (MeT) crosspeaks of ^{4}T, ^{5}T and ^{8}T represent direct nOes between the DNA and water protons because (i) these methyl protons are far away (~ 5Å in a B-DNA) from the rapidly exchanging imino protons of the DNA. (ii) Its normalized intensities [Fig. 1A(iii)] linearly increase upto 100 ms in ROESY experiment which is expected (eq. 4, experimentals) for straight dipole-dipole relaxation mechanism. (iii) They show clearly a reduction (Table. 3) in the intensities with the increasing of temperature in the same way as found for the normalized intensities of the reference crosspeaks, which, as expected, are owing to the decrease of correlation time with the increasing of temperature[1d] (eq. 4). (iv) In NOESY [Fig. 2A(ii)] and ROESY spectra [Fig. 2A(iv)], the nOe crosspeaks for water and methyls of ^{4}T, ^{5}T and ^{8}T have opposite sign with respect to diagonal peaks, which indicate that R_{nOe} and R_{rOe} are positive (Note that in Figs. 2A(ii) and 2A(iv), the negative cross-relaxation rate $R_{nOe} < 0$ gives rise to the positive NOESY crosspeaks and vice versa[1h]). This corresponds to the water residence time of < 0.1 ns[1a-c,h] in the major groove. It should be however pointed out that the intensities of these peaks in the NOESY experiment with two times longer mixing time compared to ROESY experiment are not the same as expected from the short residence time of the bound-water, but they are much weaker (Table 2). This means that the lower limit for the residence time of the bound- water in the major groove is closer to 0.1ns, which is in agreement with current representation of the solvation of major groove of DNA[1d,g,i,9b]. It is noteworthy that the intensities of the crosspeaks of methyl protons with water over the pH range of 5 - 8.6 *have not been*

FIG. 1: Comparison of the cross-sections through the NOESY [panels: B(i), B(ii)] and ROESY [panels: A(i), A(ii), B(iii) and B(iv)] spectra of d(CCATTAATGG)$_2$ taken along the F2 at the F1 frequency of water signal showing nOe/rOe projection of crosspeaks between protons of the DNA and water at different mixing times (marked at the rightside of the panel) at 15°C and pH 6.5 for aromatic [A(i)] and methyl proton [A(ii)] regions. The panel (B) shows the same spectra, but at different temperatures, for aromatic [panels: B(i) and (iii)], methyl [panels: B(ii) and (iv)] proton regions. The assignments of the water-DNA crosspeaks are shown at the top. Panel [A(iii)] is a plot of the nOe or rOe normalized volumes of water-H2A [(■)] ^{3}A(H2), (□) ^{7}A(H2), (●) ^{6}A(H2) solid line] and water-MeT [(○) ^{5}T(CH$_3$), (◆) ^{4}T(CH$_3$), (◊) ^{8}T(CH$_3$) dotted line] crosspeaks as a function of mixing time.
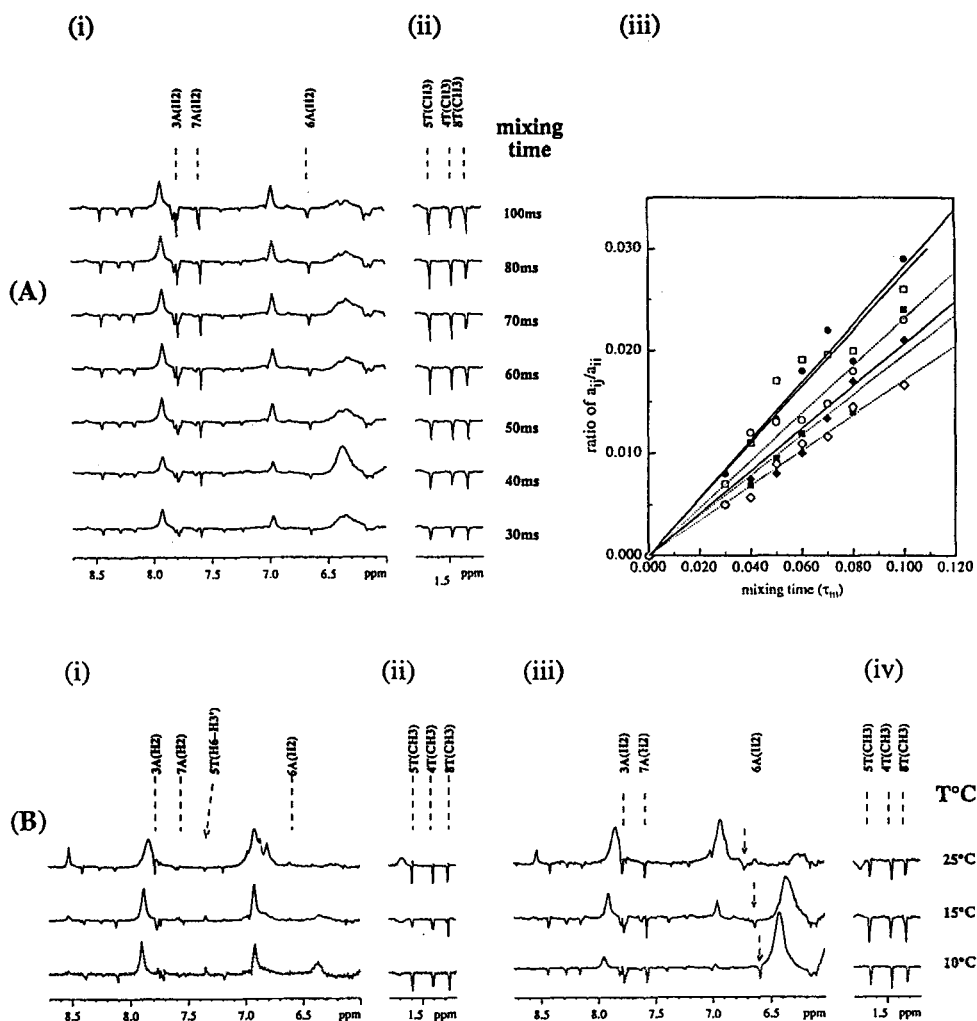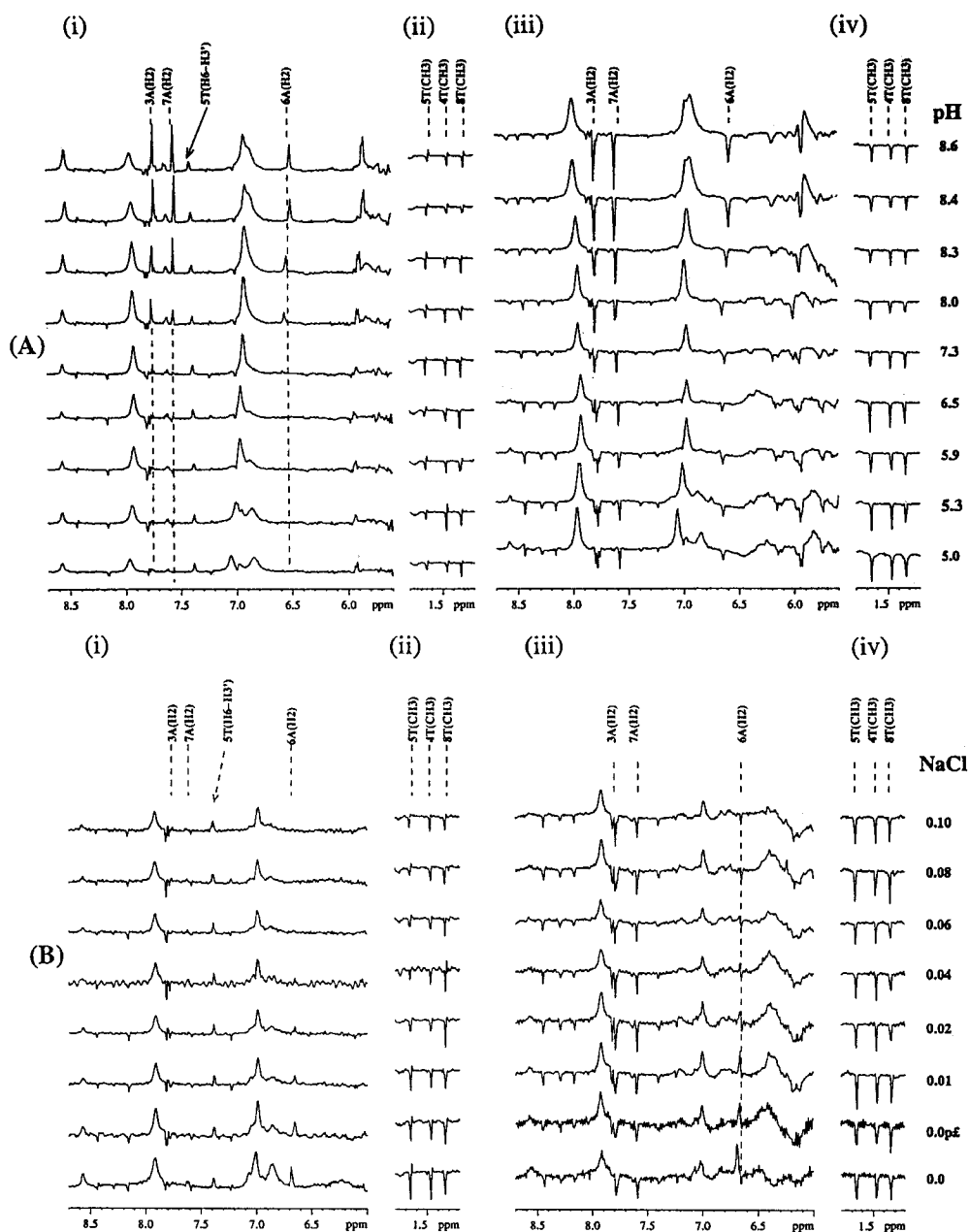
FIG. 2: Comparison of the cross-sections through the NOESY [panels: (i) and (ii)] and ROESY [panels: (iii) and (iv)] spectra taken along F2 at the F1 frequency of the water signal of d(CCATTAATGG)2 showing nOe/rOe projection of crosspeaks between protons of the DNA and water at 15°C (panel: A) at different pH and (panel: B) at different NaCl concentration (marked at the rightside of the panel).The NOESY and ROESY spectra were recorded at 120ms and 60ms mixing time, respectively. The low field region [panels: (i) and (iii)] between 8.7-5.75 ppm and higher field region [panels: (ii) and (iv)] between 1.8-1.2 ppm show the water-DNA nOe/rOe crosspeaks with H6, H2 or methyl protons, respectively. The assignments of the water-DNA crosspeaks are given at the top.

changed both in the NOESY [Fig. 2A(ii)] and in the ROESY [Fig. 2A(iv)] spectra (Table 2). *This allows us to conclude that in the major groove the presence of ammonia in the solution does not influence on the process of exchange between bound and bulk water.*

(II) Effect of pH on the hydration in the minor groove in d(CCATTAATGG)$_2$ duplex

(i) pH 5.0 - 8.0

Figs. 2A(i) and 2A(iii) show that the intensities of the crosspeaks between water and H2A do not change between pH 5.0 - 8.0. At pH < 8.0, the normalized intensities of these peaks in NOESY spectra are vanishing to the zero [Fig. 2A(i), Table 2], and they come to a stable value in the ROESY experiments [Fig. 2A(iii), Table 2]. The nOe crosspeaks of water-H2A which are present in ROESY spectra and absent in NOESY are interpreted[1h,2] as direct intermolecular nOe/rOe with short residence time of ~ 0.36ns for the bound-water [the R_{nOe} vanishes when $\omega_0\tau_c = \sqrt{5}/2$ (τ_c ~ 360ps at 500 MHz)[1c,h,2], which indicates significant mobility of the bound-water near H2A protons in the minor groove of the duplex d5'($^1C^2C^3A^4T^5T^6A^7A^8T^9G^{10}G)_2$3'. To confirm the conclusion that at pH < 8.0 the main contribution to the intensity of the water-H2A crosspeaks is direct dipole-dipole contribution, we have studied the dependence of the intensities of crosspeaks from mixing time and temperature. In Figs. 1A(i) and 1A(ii), the cross-sections through the ROESY spectra at different mixing times are presented for 8.7 - 6.0 ppm regions. Inspection of these figures shows that the absolute intensities increase with the increase of the mixing time only upto 60 ms, and then at longer mixing times they remain essentially unchanged, presumably because of complications caused by longitudinal relaxation and spin diffusion. Fig. 1A(iii) shows, on the other hand, that the normalization of the intensities of the crosspeaks to the corresponding diagonal peaks eliminates the effects of longitudinal relaxation and non-uniform excitation resulting from the spinlock used in the NOESY/ROESY type experiment. For the water-H2A crosspeak, the normalized intensity linearly increases upto 100 ms in ROESY experiment (in the same way as for water-MeT crosspeaks), which as expected from eq. 4 (experimentals), if the crosspeak arises from straight dipole-dipole relaxation between non-exchangeable protons of DNA and water molecule. This experiment enables us to define mixing time of 40 or 60 ms for ROESY and 80 or 120 ms for NOESY experiments, thereby giving crosspeaks that are free from spin diffusion. We have also examined the modulation of both the intensities of the water-H2A and water-imino protons crosspeaks depending from temperature[1d,7]. Fig. 1B shows the cross-sections through water line in the NOESY [Figs. 1BA(i) and 1B(ii)] and ROESY [Fig. 1B(iii) and 1B(iv)] spectra at 10°, 15° and 25°C (pH 7.0, 0.1 M NaCl) in the regions of

TABLE 2: The normalized intensities of the crosspeaks of the NOESY ($\tau_m = 120ms$) and ROESY (the values are in the brackets) ($\tau_m = 60ms$) spectra of B-DNA d(CCATTAATGG)$_2$ at different pH.

crosspeak	pH8.6*	pH8.5*	pH8.3	pH8.0	pH7.3	pH6.5	pH5.9	pH5.3	pH5.0
^{5}T(CH$_3$)-W	0.003 (0.013)	0.004 (0.015)	0.003 (0.013)	0.004 (0.012)	0.004 (0.012)	0.004 (0.014)	0.005 (0.014)	0.003 (0.017)	0.004 (0.017)
^{4}T(CH$_3$)-W	0.004 (0.010)	0.005 (0.011)	0.005 (0.010)	0.005 (0.009)	0.006 (0.010)	0.005 (0.010)	0.005 (0.016)	0.005 (0.014)	0.005 (0.017)
^{8}T(CH$_3$)-W	0.005 (0.012)	0.005 (0.010)	0.005 (0.010)	0.007 (0.009)	0.006 (0.009)	0.005 (0.011)	0.006 (0.012)	0.006 (0.013)	0.005 (0.017)
^{7}A(H2)-W	0.061 (0.058)	0.052 (0.055)	0.030 (0.039)	0.017 (0.024)	0.010 (0.015)	~0 (0.018)	~0 (0.014)	~0 (0.018)	~0 (0.016)
^{3}A(H2)-W	0.062 (0.057)	0.052 (0.053)	0.035 (0.045)	0.021 (0.027)	0.003 (0.021)	~0 (0.018)	~0 (0.017)	~0 (0.021)	~0 (0.017)
^{6}A(H2)-W	0.062 (0.079)	0.052 (0.067)	0.040 (0.052)	0.029 (0.037)	0.007 (0.027)	~0 (0.027)	~0 (0.027)	~0 (0.030)	~0 (0.027)
^{2}C(H6-H1')	0.026 (0.015)	0.023 (0.010)	0.016 (0.011)	0.014 (0.010)	0.017 (0.011)	0.013 (0.011)	0.013 (0.011)	0.012 (0.008)	0.014 (0.010)
^{3}A(H8-H1')	0.027 (0.015)	0.027 (0.015)	0.019 (0.012)	0.016 (0.014)	0.016 (0.012)	0.014 (0.012)	0.015 (0.012)	0.016 (0.013)	0.015 (0.014)
^{4}T(H6-H1')	0.039 (0.021)	0.035 (0.020)	0.023 (0.015)	0.021 (0.015)	0.018 (0.014)	0.016 (0.013)	0.017 (0.013)	0.017 (0.014)	0.021 (0.017)
^{5}T(H6-H1')	0.038 (0.020)	0.037 (0.021)	0.024 (0.013)	0.021 (0.012)	0.018 (0.011)	0.015 (0.010)	0.018 (0.010)	0.017 (0.014)	0.018 (0.012)
^{6}A(H8-H1')	0.030 (0.015)	0.026 (0.014)	0.019 (0.012)	0.017 (0.012)	0.015 (0.010)	0.014 (0.010)	0.015 (0.008)	0.015 (0.009)	0.017 (0.012)
^{7}A(H8-H1')	0.019 (0.011)	0.020 (0.011)	0.013 (0.009)	0.012 (0.008)	0.013 (0.009)	0.011 (0.010)	0.011 (0.010)	0.012 (0.011)	0.013 (0.009)
^{8}T(H6-H1')	0.033 (0.018)	0.034 (0.019)	0.021 (0.015)	0.020 (0.016)	0.018 (0.013)	0.018 (0.012)	0.014 (0.012)	0.013 (0.014)	0.015 (0.13)
^{5}T(H6-CH$_3$)	0.019 (0.016)	0.019 (0.017)	0.015 (0.014)	0.015 (0.016)	0.014 (0.014)	0.013 (0.016)	0.013 (0.014)	0.013 (0.014)	0.014 (0.015)
^{4}T(H6-CH$_3$)	0.017 (0.016)	0.017 (0.014)	0.014 (0.014)	0.013 (0.013)	0.013 (0.014)	0.012 (0.013)	0.012 (0.012)	0.011 (0.013)	0.012 (0.012)
^{8}T(H6-CH$_3$)	0.014 (0.012)	0.014 (0.012)	0.011 (0.012)	0.012 (0.011)	0.010 (0.008)	0.010 (0.010)	0.010 (0.010)	0.011 (0.010)	0.010 (0.011)
^{2}C(H6-H6)	0.179 (0.147)	0.178 (0.130)	0.134 (0.128)	0.131 (0.153)	0.126 (0.134)	0.127 (0.140)	0.129 (0.120)	0.124 (0.116)	0.129 (0.125)
^{3}T(H6-H3')	0.055 (-)	0.057 (-)	0.032 (-)	0.032 (-)	0.025 (-)	0.020 (-)	0.023 (-)	0.024 (-)	0.025 (-)
^{6}A(H2)-^{7}A(H2)	0.026 (-)	0.027 (-)	0.015 (-)	0.016 (-)	0.014 (-)	0.013 (-)	0.013 (-)	0.014 (-)	0.014 (-)
^{7}A(H2)-^{7}A(H1')	0.003 (0.005)	0.004 (0.006)	0.002 (0.007)	0.003 (0.004)	0.004 (0.007)	0.003 (0.007)	0.003 (0.005)	0.004 (0.006)	0.004 (0.006)
^{7}A(H2)-^{5}T(H1')	0.006 (0.008)	0.006 (0.008)	0.003 (0.007)	0.003 (0.004)	0.004 (0.004)	0.003 (0.005)	0.003 (0.004)	0.004 (0.004)	0.004 (0.005)
^{7}A(H2)-^{8}T(H1')	0.007 (0.011)	0.008 (0.011)	0.007 (0.011)	0.006 (0.008)	0.005 (0.007)	0.006 (0.011)	0.005 (0.011)	0.006 (0.013)	0.006 (0.010)

In the light-shadow area, the data of water-DNA proton crosspeaks are presented. In the dark-shadow area the reference crosspeaks CH$_3$-H6 are presented. *it should be pointed out that at higher pH 8.5 - 8.6 the concentration of salt has been raised till to the value ~ 0.5M, this leads to an increase of the effects of autorelaxation and spin diffusion.

aromatic and methyl protons. The normalized intensities of water-H2A crosspeaks are presented together with the intensities of the reference crosspeaks, T(H6-CH$_3$), in Table 3. It can be clearly seen from Fig. 1B that the absolute intensities of crosspeaks for water-H2A or water-MeT do not change noticeably upon the change of temperature. On the other hand, the normalized intensities (Table 3) of these crosspeaks show clearly a reduction with the increasing of temperature in the same way as found for the normalized intensities of the reference crosspeaks, which, as expected, are owing to the decrease of correlation time with the increasing of temperature[1d].

The above observations lead to the following conclusions: (a) The hydration pattern in the minor groove in the physiological pH, under our NMR measurement condition, is similar to the ones found in the X-ray structure[21,22] (b) The residence time of this boumd-water is in the range of 0.3 - 0.5 ns, which is closer to the residence time of the bound water found on the surface of protein[1a,c].

(ii) pH : 8.0 - 8.6

From pH 8.0 to 8.6, the water-H2A crosspeaks intensities gradually increase [Fig. 2A(i) and 2A(iii)], while water-MeT crosspeaks remain virtually unchanged [Figs. 2A(ii) and 2A(iv)], in both NOESY and ROESY experiments, which become clearly evident from their comparison with that of the unchanged absolute intensity of the reference H6-H3' dipole-dipole interaction crosspeak for the [5]T residue [Fig. 2A(i)]. Table 2 shows the normalized intensities as a function of pH, and because of the fact that the normalized intensty is free from longitudinal contribution (see eq. 2 in the experimental part), therefore this Table 2 shows   a semi-quantitative evaluation of the pH effect on the hydration. At the mixing times of 60 and 120 ms in ROESY and NOESY experiments, respectively, at pH 8.6, the intensities of the water-H2A crosspeaks are very closely similar, but with the reverse sign [Figs. 2A(i) and Fig. 2A(iii)]. These can be compared to the experiments performed at pH < 8.0, which show that the intensities of these peaks in NOESY spectra are vanishing to the zero [Fig. 2A(i)] whereas the crosspeak intensities in the ROESY experiments are reduced ca. 2 times and then come to a stable value [Fig. 2A(iii)].

In principle, the increase of intensities of the H2A-water nOe peaks could arise via any of the following mechanisms: (1) Through the relay effect (eq. 4) between H2A and bulk water through imino protons of T. (2) Through a direct dipole-dipole interaction[1c] (or/and relayed magnetization transfer) between H2A and ammonia catalyst which is in quick exchange with water and their resonances are coalesced with water signal. (3) The direct dipole-dipole interaction between bound water and H2A protons. Since, all of the above mechanisms would give rise to a negative nOe and positive rOe, we have performed the following studies: (a) The influence of pH on the

TABLE 3: The normalized intensities of the crosspeaks of the NOESY and ROESY specta of B-DNA d(CCATTAATGG)$_2$ at different temperature at pH = 7.0.

The type of crosspeaks	ROESY (τ_m = 40ms)			NOESY (τ_m = 80ms)		
	10°C	15°C	25°C	10°C	15°C	25°C
^{7}A(H2)-W	0.012	0.011	0.007	~0	~0	~0
^{3}A(H2)-W	0.011	0.007	0.004	~0	~0	~0
^{6}A(H2)-W	0.013	0.012	0.008	~0	~0	~0
^{5}T(CH$_3$)-W	0.009	0.012	0.005	0.007	0.003	0.005
^{4}T(CH$_3$)-W	0.011	0.008	0.004	0.008	0.005	0.004
^{8}T(CH$_3$) W	0.009	0.006	0.003	0.006	0.004	0.004
^{5}T(CH$_3$-H6)	0.023	0.022	0.015	0.020	0.019	0.014
^{4}T(CH$_3$-H6)	0.026	0.020	0.014	0.021	0.020	0.013
^{8}T(CH$_3$-H6)	0.023	0.016	0.015	0.021	0.018	0.012

overall rate of exchange of imino protons (k_{ex}) to consider the possible correlation between k_{ex} and the increase of intensity water-H2A crosspeak. (b) The influence of NaCl and NH$_4$Cl concentration on the intensity water-H2A crosspeak at neutral pH. The result of these studies are as follows.

(A) Evaluation of the significance of three site pathway H2A-imino-water in relay mechanism at pH. 8.0-8.6. From the eq. 4 and 6 presented in the experimental section, it is clear that the relay effect can be enhanced through the increase of the rate of exchange of the imino protons by the addition of a catalyst at a certain pH. In Fig. 3A, the logarithmic plots of the observable exchange time (τ_{ex}) of the imino protons of the AT and GC basepairs versus pH at 15°C are presented. Clearly, the τ_{ex} dependence on pH can be divided into two distinct regions: first, between pH 5.0 to 7.0 where the overall rate of exchange is pH independent, and, second, between 7.3 and 8.6 where the $\tau_{ex} = 1/k_{ex}$ is linear with increasing of pH, which are well described in the recent studies[13b-d]. In the plateau (pH 5.0 - 7.0), the value of k_{ex} slightly varies 0.45 ± 0.10s^{-1} for all three types of AT basepair. There are some apparent similarities[11] in the behaviour of the overall rate of exchange of the imino protons with the intensities of water-H2A rOe crosspeaks (compare Fig. 2A and Fig. 3A) in the pH range of 5.0 - 8.6. Although their origins have been explained[11] on the basis of the relayed magnetization transfer mechanism through imino protons of DNA to water, but some discrepancy clearly exists (vide infra).

If the relayed magnetization transfer mechanism through the imino proton is the reason for the above observation, we should be able to estimate the relay effect at pH >
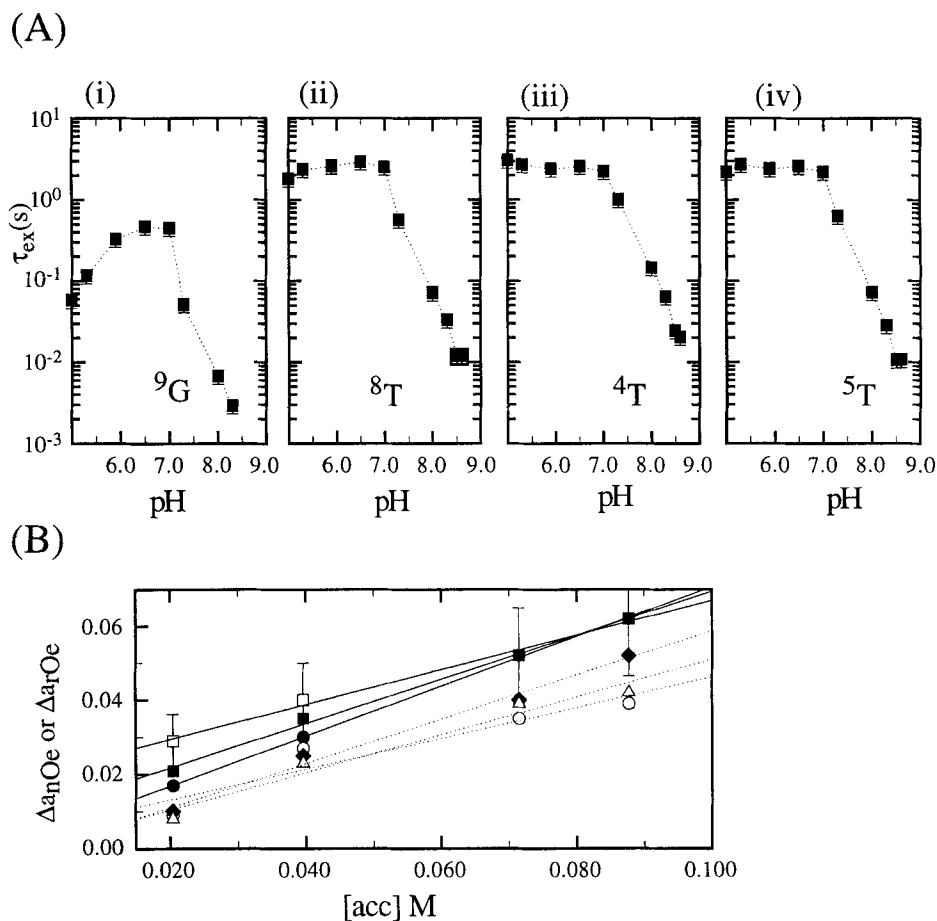
FIG. 3: Panel: A shows exchange time in the logarithm scale [τ_{ex} (s)] versus pH at 15°C for [9]G [panel: (i)], [8]T [panel: (ii)], [4]T [panel: (iii)] and [5]T imino [panel: (iv)] protons of $d5'(C^1C^2A^3T^4T^5A^6A^7T^8G^9G^{10})_2^3$. Panel: B shows normalized intensities of the 2HA-water crosspeaks, derived from equation given in Table 4, from the NOESY (Δa_{nOe}) (solid line) and ROESY (Δa_{rOe}) (dashed line) experiments versus the concentration of the base ([acc]M) for the [3]A (■, ○), [6]A (□, ◆) and [7]A (●, △) residues.

8 in the following manner: the normalized intensity becomes proportional to $\approx -R_{H2\text{-}W}\tau_m + \left(\frac{1}{2}\right)R_{H2\text{-}NH}k_{ex}\tau_m^2 +...$ (see eqn. 2 in the experimental section for the explanatory notes). For data obtained from the NOESY spectra, it follows that the first term is zero throughout the whole pH range, whereas the total crosspeak intensity at pH > 8 is represented by the second term, $\left(\frac{1}{2}\right)R_{H2\text{-}NH}^{nOe}k_{ex}\tau_m^2$. The cross-relaxation rate, $R_{H2\text{-}NH}^{nOe}$, is dependent upon the effective tumbling time of the whole molecule (which is ~4ns), and it is relayed with cross-relaxation rate, $R_{H2\text{-}NH}^{rOe}$, obtained in the ROESY experiment as $R_{H2\text{-}NH}^{nOe} = R_{H2\text{-}NH}^{rOe}/2$.

The subtraction of the water-H2A crosspeak intensities at pH 6.5 from pH >8.0 in ROESY experiments gives the estimated value of the "pure" relay effect $[\sim\left(\frac{1}{2}\right)R_{H2\text{-}NH}^{rOe}k_{ex}\tau_m^2]$ (eq. 9). This value should be approximately two times less than the corresponding value obtained from the NOESY crosspeaks intensities at $\tau_m^{nOe} = 2\tau_m^{rOe}$, and, *indeed, they are very close to this ratio* (Table 2).

Our experimental data show that the increases of water-H2A crosspeak volumes with pH are quite similar for all basepairs [Figs. 2A(i), 2A(iii) and Table. 2] in the duplex, but this is *not* correlated to the relative overall rate of exchange of the imino protons. Remarkably, the overall k_{ex} (Table 4) under our experimental condition is *two times different* for [7]A-[4]T compared to [3]A-[8]T and [6]A-[5]T basepairs, which suggest that the relay effect is not through the imino proton.

Moreover, in order to evaluate the significance of relay effect as one of the possible mechanisms, we have also estimated the crossrelaxation rate $R_{H2\text{-}NH}^{rOe}$ /or $R_{H2\text{-}NH}^{nOe}$ based on eqn. 9, $R_{H2\text{-}NH}^{rOe} = 2\left\{\dfrac{\left(a_{pH\geq8.0}^{rOe}\right) - \left(\overline{a_{pH<8.0}^{rOe}}\right)}{k_{ex}\tau_{m(rOe)}^2}\right\}$, using the difference of the normalized intensities obtained from the ROESY/NOESY spectra at pH>8 and avarage value of crosspeak volumes at pH<8 $\left(\overline{a_{pH<8.0}^{rOe}}\right)$. It is expected that the crossrelaxation rates $R_{H2\text{-}NH}^{rOe}$ /or $R_{H2\text{-}NH}^{nOe}$ between H2A and imino proton would not significantly vary through the pH range of 5.0 - 8.6, just in the same way as the reference aromatic-H1' nOe crosspeaks (Table 2) of this duplex. The estimated cross-relaxation rates, $R_{H2\text{-}NH}^{rOe/nOe}$, based on observable experimental values for k_{ex} are presented in Table 4 (see Methods for the notes on the abbreviations), showing that, despite the experimental error ($\pm$ 25%), the tendency of decreasing of $R_{H2\text{-}NH}^{rOe}$ /or $R_{H2\text{-}NH}^{nOe}$ with the increasing of pH is clearly detectable in Table 4. This again shows that the relayed magnetization transfer does not take place through the imino proton of DNA at pH>8.

These data unambiguously demonstrate that although there is an apparent similarity in the tendency of intensity increase of water-H2A crosspeak with the overall k_{ex}, the analysis performed above shows that *the mechanism of the relay pathway*

TABLE 4: The observable rate of exchange, normalized intensities in the NOESY and ROESY experiments and cross-relaxation rate.

pH	The rate of exchange (s^{-1})			NOESY (τ_m = 120ms)						ROESY (τ_m = 60ms)					
				Δa^f_{nOe}			$R^{\P}_{H2\text{-}NH}(s^{-1})$			Δa_{rOe}			$R_{H2\text{-}NH}(s^{-1})$		
	$^3T_{(NH)}$	$^5T_{(NH)}$	$^4T_{(NH)}$	(^3A)	(^6A)	(^7A)	$(^3A\text{-}^8T)$	$(^6A\text{-}^5T)$	$(^7A\text{-}^4T)$	(^3A)	(^6A)	(^7A)	$(^3A\text{-}^8T)$	$(^6A\text{-}^5T)$	$(^7A\text{-}^4T)$
8.0	14	14	7	0.021	0.029	0.017	0.208	0.287	0.337	0.009	0.010	0.008	0.357	0.396	0.634
8.3	31	36	16	0.035	0.040	0.030	0.165	0.154	0.260	0.027	0.025	0.023	0.484	0.386	0.798
8.5	85	95	41	0.052	0.052	0.052	0.084	0.076	0.176	0.035	0.040	0.039	0.229	0.234	0.528
8.6	84	94	50	0.062	0.062	0.061	0.103	0.091	0.169	0.039	0.052	0.042	0.257	0.310	0.476

f- Δa_{nOe} or Δa_{rOe} were calculated as $\Delta a_{nOe} = \left(\dfrac{a_{ij}}{a_{ii}}\right)^{nOe}_{pH \geq 8.0} - \left(\dfrac{a_{ij}}{a_{ii}}\right)^{nOe}_{pH < 8.0}$; $\P$ - $R_{H2\text{-}NH}$ was calculated

from equation $\Delta a_{nOe} = (1/2)R^{nOe}_{H2\text{-}NH}k_{ex}\tau_m^2$

through imino proton could not be considered as one of the main reasons for the observed increase of these intensities.

(B) Dipole-dipole interaction or/and relayed magnetization transfer between bound H2A and ammonia catalyst: Effect of concentration of NaCl and NH4Cl on Hydration of d(CCATTAATGG)2 at neutral pH.

While NaCl or NH4Cl at low to moderate salt concentration (0 - 100 mM) at pH 7 shows no change in the intensities of the crosspeaks between water and H2A proton or methyl protons in the NOESY and ROESY spectra (Fig. 2B), there is a clear linear dependence of Δa_{nOe} and Δa_{rOe} (see the footnote of Table 4 for explanation) of each AT basepair on the concentration of the ammonia base catalyst with almost identical slope, ~ 0.57 ± 0.10 (M^{-1}) (Fig. 3B). This shows that the concentration of base (ammonia in this case) perhaps plays a critical role in the building of water-H2A nOe at an basic pH, not the salt. This also means that the relayed magnetization transfer does not take place from H2A to water through imino proton, but through leakage of magnetization (direct dipole-dipole or a relayed process) through ammonia as a base.

(C) Direct dipole-dipole interaction between bound water-H2A protons.

It should be however noted that the straight dipole-dipole interaction between water and H2A can not completely ruled out by the data presented in this work. It can be argued that if the above effect is present at higher pH > 8.0, then one should expect an increase of the residence times of water from 0.36 ns to >1 ns[1h,7] In fact, the possibility of a dipole-dipole interaction between water and H2A becomes quite

feasible, if we consider the ratio of nOe to rOe of the water-H2A crosspeaks at pH 8.6, $\tau_m^{nOe} = 2\tau_m^{rOe}$, which is close to one. This is what should be expected in case of a straight dipole-dipole interaction, but the longer residence time associated with this straight dipole-dipole interaction is contradicted by the thermodynamic instability of DNA at the alkaline pH.

Experimental section

(A) NMR sample preparation

The oligomer $d5'(^1C^2C^3A^4T^5T^6A^7A^8T^9G^{10}G)_2 3'$ was synthesized by the phosphoamidite method using DMTrA[bz]-, DMTrG[ibu]-, DMTrC[bz]- and DMTrT-3'-β-cyanoethylphosphoamidite units on a Gene Assembler Plus DNA synthesizer (Pharmacia-Kabi). After completion of the synthesis, 5'-O-DMTr of oligomer was removed by acid treatment on support, then the support was suspended in 32% ammonia-water at ambient temperature for 7 days to cleave oligo-DNA from the support as well as to remove protecting groups. The duplex has been purified on a DEAE-Sephadex A-25 ion exchange column, and then it was passed through a Dowex 50W X8 column to give the sodium salt. The sample was dissolved in 0.4 ml of the buffer [0.1 M NaCl, 10 mM NaD_2PO_4, 10 μM EDTA in 10% D_2O: 90% H_2O. Sample concentration: 4.3 mM]. The pH of the solution was measured after addition of each aliquot (1 - 5 μL) of stock solution containing ammonia in ammonium chloride (4.0 M, pH 9.2) in the NMR tube before and after NMR experiment and the final concentration of the base [acc] at each buffer concentration was calculated using the following equation[13a]: $[acc]^{-1} = (1 + 10^{pKacc-pH})/[total buffer]$. (eq. 10)

(B) NMR experiment

^{1}H NMR spectra were recorded on a Bruker AMX- and DRX 500 NMR spectrometer (^{1}H at 500 MHz). Phase-sensitive NOESY experiments with water suppression is achieved by the use of two short spinlock pulses, $SL_{\varphi 4}$ and $SL_{\varphi 5}$ as described by Otting et al[1b] using the following parameters: mixing times (τ_m) were varied 0.01, 0.02, 0.03, 0.04 and 0.06s to measure the exchange rates and 0.120s to observe the spatial contact of the non-exchangeable protons with water; 2K/ or 4K complex data points in t_2, 128 /or 512 complex data points in t_1, the relaxation delay between pulse sequence is 2.0s, a sweep width of 10204.082 Hz is used in both dimensions, $SL_{\varphi 4}$ and $SL_{\varphi 5}$ are equal to 0.5 ms and 3 ms, respectively, the delay between spinlock pulses τ is equal to 167μs, the carrier was set at the water frequency, 32 scans/FID were used for quadrature detection in F_1 - dimension with the time

proportional phase incrementation (TPPI). 2D data sets for ROESY spectra with the water suppression are achieved with one short spinlock pulse, $SL_{\varphi 3}$, as described by Otting et al[1b]. During the mixing time sequence of $n(\pi/6)$ pulses with length 3.4 μs separated by delay, Δ, (34.5μs) provides the similar effect as spin-lock $SL_{\varphi 4}$ of the NOESY experiment so that the spectra were recorded with spinlock duration of 0.01, 0.02, 0.03, and 0.04s or 0.06s using 6.25 kHz rf field for all pulses and a recycle delay of 2s. Typically 2K /or 4K data points were collected for each t_1 128 /or 512 values during experiments using a sweep width of 10204.082 Hz. The correction of crosspeak volumes due to off-resonance effect has been performed using the literature procedure[14]. A 3 ms saturation pulse is applied after data acquisition. The spectral excitation profile in these experiment is proportional to $\sin(\Omega\tau)$ where Ω is the angular frequency relative to the carrier and $\tau = 167$ μs. The nonuniform spectral excitation in F2-dimension have been corrected by it multiplying on $1/\sin(\Omega\tau)$ function. NOE and ROE crosspeak volumes were measured using the program AURELIA[15] with segmentation level 0.1 and 1000 iterations. The k_{ex} have been calculated from the line-width of imino protons[8d] or from a combination of ROESY and NOESY experiments[8c].

(C)   The approximation of the relay mechanism pathway in nOe

The formalism describing the relay mechanism for three-site system where effects of both cross-relaxation and chemical exchange are treated simultaneously was first applied by Ernst et al[10,16], which was later used for theoretical evaluation of three-site model[11] as well as for the experimental evaluation of relay artifacts in the ROESY spectrum[9]. The matrix exponential of Bloch equations[10,16,17] can be presented in power series at approximation $\tau_m \to 0$[18]:

$$a_{ij}(\tau_m) \approx (\delta_{ij} - L_{ij}\tau_m + \frac{1}{2}\sum_k L_{ik}L_{kj}\tau_m^2)a_{jj}(0) \qquad (eqn.\ 1)$$

where a_{ij} and $a_{jj}(0)$ are the intensities of the crosspeaks correlating resonance i and j[10,18] at mixing time τ_m and the diagonal peak intensities at $\tau_m = 0$. L_{ij} is the element of the longitudinal relaxation matrix (**L**)[16,17] which is the superposition of exchange matrix (**K**) as well as cross-relaxation matrix (**R**), consisting of cross-relaxation (R_{ij}) and auto-relaxation (R_{ii}) elements which were completely described in [16,17]. We do not take in to account of the contribution of translation relaxation matrix, because it has been shown[11] that for the crosspeak intensity of nonexchangeable proton with water translation-diffusion is less important than chemically relayed magnetization transfer. Effect of longitudinal relaxation on intensities of crosspeak could be eliminated by dividing the crosspeak intensities by the intensities of diagonal peaks by using eqn. 1 at the same mixing time as was proposed by Macura[18-20]. In a three-site model of the chemical equilibrium, when a water molecule (A) and an exchangeable proton (B) are involved in

an exchange process with a constant rate (k_{ex}), but A and the nonexchangeable proton (C) as well as B and C are involved in the cross-relaxation process with crossrelaxation rates (R_{CA}) and (R_{BC}), the expressions for the normalized intensity of the crosspeak between A and C proton a_{ij}^* at short mixing times (*i.e.*, when $\tau_m \to 0$) and $k_{ex} \gg 0$ could be approximated as in eqn. 2:

$$a_{CA}^* \approx -R_{CA}\tau_m + \left(\tfrac{1}{2}\right)R_{CB}k_{ex}\tau_m^2 + \dots \qquad (\text{eqn. 2})$$

The cross-relaxation rates in the NOESY and ROESY experiments for macromolecules, where $\omega_0\tau_c \gg 1$, are as follows[18]:

$$R_{ij}^{nOe} = -q\,\tau_c r_{ij}^{-6}; \; R_{ij}^{rOe} = +2q\,\tau_c r_{ij}^{-6} \ , \qquad (\text{eqn. 3})$$

where $q = 0.1\left(\mu_0/4\pi\right)^2(\hbar)^2\gamma^4$ ($\hbar$ is Plank constant devided by 2π and γ is giromagnetic ratio of proton), r_{ij} is the distance between protons i and j.

Using eqns. 2 and 3, the expressions for the normalized intensities of the crosspeaks in the NOESY and ROESY spectra could be estimated as:

$$\begin{cases} a_{CA}^{*rOe} \cong -2q\tau_c r_{CA}^{-6}\tau_m - q\tau_c r_{CB}^{-6}k_{ex}\tau_m^2 \\ a_{CA}^{*nOe} \cong q\tau_c r_{CA}^{-6}\tau_m + \left(\tfrac{1}{2}\right)q\tau_c r_{CB}^{-6}k_{ex}\tau_m^2 \end{cases} \qquad (\text{eqn. 4})$$

The quadratic terms in τ_m signify a relay process due to indirect magnetization transfer pathway owing to the exchange and dipole-dipole cross-relaxation through the third nucleus[10]. Note that a_{CA}^{*noe} or $a_{CA}^{*roe} \neq 0$ if R_{CA}^{nOe} or $R_{CA}^{rOe} \approx 0$

(D) The formalism for the exchange process [13]

The theory of the exchange formalism is well described[13], and through out this work, we have used the literature terminology[13a].

Exchange of the imino proton of a basepair is a two-step process requiring the opening of the basepair controlled by the rate constant for opening of the basepair, (k_{op}), which is related to the basepair lifetime in the closed state: ($\tau_o = 1/k_{op}$). The process of chemical exchange then proceeds[13] from the basepair open-state with the rate constant $k_{ex,acc,open} = 1/\tau_{ex,acc,open}$, where $\tau_{ex,acc,open}$ is the exchange time from the open pair. The dissociation constant for formation of the open-state is defined by $K_{diss} = \dfrac{k_{op}}{k_{cl}}$, where k_{cl} is the rate constant for the closing of the basepair, and the life-time of the open-state of the basepair is defined by $\tau_{open} = 1/k_{cl}$. When $K_{diss} \ll 1$, the kinetics of imino protons is first order, and the exchange time ($\tau_{ex} = 1/k_{ex}$), with observable rate of exchange (k_{ex}), has been defined by Gueron[13a,d] as in eqn. 5 :

$$\tau_{ex} = \tau_o + \left(\tau_{ex,acc,open}/K_{diss}\right) \qquad (\text{eqn. 5})$$

It has been shown[13] that the transfer of the imino proton to acceptor (acc), which could

be either a catalyst or an intrinsic acceptor such as nucleic acid, proceeds from the open-state as in the case of the monomer. The exchange time for monomer is given by[13a,d]:

$$\tau_{ex,acc} = \left(1+10^{pK(nu)-pK(acc)}\right)/k_{coll}[acc] \quad (eqn.\ 6)$$

where [acc] is the concentration of the acceptor and k_{coll} is the rate constant of diffusion-controlled collision.

$$k_{ex,acc,open} = \alpha\, k_{ex,acc} \qquad\qquad (eqn.\ 8)$$

where $k_{ex,acc} = 1/\tau_{ex,acc}$. Factor α has been found to be not far from unity[13a].

Acknowledgments

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