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Direct estimation of base-pair exchange kinetics in oligo-DNA by a combination of NOESY and ROESY experiments

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ABSTRACT

A new method for the determination of the kinetics of exchange of the imino protons of DNA duplex is reported using a combination NOESY and ROESY experiments at short mixing times (≤20 ms). These results have been compared with the commonly used longitudinal relaxation approach through the T_1 measurement. To calculate k_{ex} and τ_{ex} by ROESY-NOESY experiment, the volume of the cross-peaks between imino protons and water in the NOESY and ROESY spectra have been measured separately from the magnetization term. This work shows that the present approach for the measurement of the kinetics of *slow exchanging* imino protons of DNA duplex is comparable to the saturation recovery experiment in which the exchange rate can be accelerated by the addition of a base catalyst. The present ROESY-NOESY approach has been found to be particularly useful and reasonably accurate for the measurement of exchange kinetics of both the *fast- and slow-exchanging* imino protons in DNA duplex both under non-physiological and physiological condition where the saturation recovery method can not be used.

INTRODUCTION

Oligo-DNA analogues act as antisense repressors at the transcriptional and translational level of gene expression by the specific base pairing which stabilizes the duplex formed between the oligonucleotide reagent and the target sequence. The strength and efficiency of the base pairing depends upon the rate of exchange of imino protons of DNA duplex with water which is believed to occur from an open state of the base pair[1–3]. In the following equilibrium process of the exchange reaction, [NH*···N⇄NH* + HOH⇄NH + HOH*⇄NH···N], it is only however possible to observe the chemical shifts of the hydrogen-bonded imino protons (NH···N) and one assumes that the formation of non-hydrogen bonded imino protons (NH* or NH) due to opening of the base-pairing (k_{op}) is very slow compared to the closing of the base-pair (k_{cl}) but the exchange rate (k_3) between NH* or NH and water is very fast ($k_3 >> k_{op}$) and is not observable in the NMR time scale. The observable rate of exchange (k_{ex}) of water is defined by the k_{op} which is the limiting rate of the exchange process if $k_{cl} >> k_3$. The exchange from the open state of the DNA duplex is catalyzed by either proton acceptors such as added catalyst[4,5] or the temperature[6–8]. When the exchange by added catalyst dominates, the overall exchange rate of the imino proton is: $k_{ex} = k_{op}k_b[B]/(k_{cl} + k_b[B])$ ... (eqn. 1). Taking into account, that $\tau_{ex} = 1/k_{ex}$, eqn. 1 could be rewritten as[4]: $\tau_{ex} = \tau_{op} + D[B]^{-1}$ ··· (eqn. 2a), where $\tau_{op} = 1/k_{op}$ is the life-time of the closed base pair and $D = k_{cl}/(k_{op}k_b)$. According to eqn. 1, the life-time of the base pair, τ_{op}, could be estimated as the limit of the τ_{ex} at infinite concentration of catalyst[9].

The kinetics of the exchange process affects the experimental longitudinal spin-lattice relaxation rate (T_1) of the imino protons of DNA duplex. This has mostly been monitored by selective measurements of T_1 4–8 where only the magnetization of the N-H protons is perturbed by inversion or saturation. The experimental T_1 is related to the exchange rate (k_{ex}) by $1/T_1 = k_{ex} + 1/T_{1*}°$... (eqn. 3)[4,5]. The most obvious limitations of this approach are two fold: (1) the rate of exchange (k_{ex}) can not be determined without substraction of the longitudinal relaxation time $T_{1*}°$, which contains both proton-proton dipolar interactions ($T_1°$) and the process of exchange without added catalyst. (2) In order to determine the exchange process reliably, it is necessary that the rate of opening of the base-pair (k_{op}) of DNA duplex should be relatively slow, which then can be accelerated by the catalysis[4,5] or by temperature change[6–8], assuming that the proton-proton dipolar interactions do not change with temperature[1] or the added base catalyst[4,5]. This means that k_{ex} and τ_{ex} determined by T_1 method reflects the non-physiological state of the exchange process of the imino proton of the base pair.

The alternative 2D exchange experiment[10–12] provided unique opportunity to estimate the exchange rates separately from the magnetization terms in a quantitative manner, using a two-state model, [NH*···N⇄HOH], at several mixing times under a physiological condition. The equations[10,11] describing the dependence of the intensities of the diagonal (a_{ii}) and the cross-

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peak (a_{ij}) on the mixing time relating the rate of exchange (k_{ex}) for this model are as follows:

$$a_{ii} = x_N \exp[-(R_{1N} + k_{ex})\tau_m]$$
$$a_{ij} = \frac{x_N(k_{ex})}{(R_{1N} + k_{ex} - R_{1W})} \{\exp(-R_{1W}\tau_m) - \exp[-(R_{1N} + k_{ex})\tau_m]\} \quad \text{(eqn. 4)}$$

where x_N, x_W are the mole fractions, and R_{1N} and R_{1W} are the longitudinal relaxation of hydrogen-bonded imino protons and water. This approach has been utilised to calculate the rate of exchange of the amide protons of viomycin in water using mixing times longer than 200 ms[11]. Clearly, the reliability of this NOESY approach[10,11] by two-proton approximations should be estimated using the ROESY experiment. Indeed, eqn. 4 can be rewritten for the ROESY experiment because the cross-relaxation process in the multi-spin systems can be described in the same manner both for NOESY and ROESY by the Bloch equations[13–15]. To define the rate of exchange through the relative intensity of ROESY experiment the formalism of Macura and Ernst[16] has been used in the present work. The expression of the normalised cross-peaks intensity $a^{roe}_{ij}/a^{roe}_{i0}$ and diagonal peak intensity $a^{roe}_{ii}/a^{roe}_{i0}$ are as follows:

$$\frac{a^{roe}_{ii}}{a^{roe}_{i0}} = \exp[-(R_{1N} + k_{ex})\tau_m]$$
$$\frac{a^{roe}_{ij}}{a^{roe}_{i0}} = \frac{(k_{ex})}{(R_{1N} + k_{ex} - R_{1W})} \{\exp(-R_{1W}\tau_m) - \exp[-(R_{1N} + k_{ex})\tau_m]\} \quad \text{(eqn. 5)}$$

The respective relaxation rates due to external sources[16] R_{1N} and R_{1W} has been shown[14] to be determined by $T_{1\varrho}^{-1} = T_2^{-1}$ for on-resonance conditions.

We have calculated the rate of exchange without catalyst using both NOESY and ROESY data according to eqns. 4 and 5 respectively, for the fast exchanging imino protons in duplex (I) for which reliable T_1 measurement is impossible because the addition of the base catalyst or increase of temperature would accelerate the exchange process to such an extent that considerable line broadening takes place giving an unrealistic error in the measurement.

$[^{5'}p[d(^1T^2G^3T^4T^5T^6G^7G^8C]^{3'}]^*$ $[^{5'}p[d(^9C^{10}C^{11}A^{12}A^{13}A^{14}C^{15}A]^{3'}]$ Duplex (I)

Table 1 shows that rate of exchange obtained from the NOESY experiments is found to be 2–3 times slower than the rate obtained from the ROESY data. Presumably, the main reason for this discrepancy is that no magnetic interactions with other protons have been taken into in the calculations using data of the NOESY (eqn. 4) or ROESY (eqn. 5) experiments *(vide infra)*. It should be noted from the trend of k_{ex} and τ_{ex} at different mixing times with either NOESY or ROESY approach using two-proton approximation (Table 1) that as the mixing time increases, even in the narrow range of shorter mixing times (*i.e.* between 20–60 ms) the difference in the values of k_{ex} and τ_{ex} are quite variable because of implicit errors in simple two-proton approximation. On the other hand, the calculations of k_{ex} and τ_{ex} using our NOESY/ROESY approach (eqn. 9) shown in Table 1 show, in sharp contrast, that the error is not more than 20% irrespective of which mixing time is chosen in the same narrow range of shorter mixing times (*i.e.* between 20–60 ms).

In this work, we exploit the fact that the NOE cross peaks from the NOESY and ROESY spectra can include not only cross-relaxation and chemical exchange but also two-step magnetization transfer (spin diffusion)[13]. For the initial buildup rate approximation (*i.e.*, when $\tau_m \rightarrow 0$), the matrix exponential of

Table 1. Rate constants (k_{ex}) and life-time (τ_{ex}) determined for the exchange process of the imino protons of the ^{3}T-^{13}A, ^{4}T-^{12}A, ^{5}T-^{11}A and ^{6}G-^{10}C base-pairs of the $[^{5'}p[d(T^1G^2T^3T^4T^5G^6G^7C^8]^{3'}]^*[^{5'}p[d(C^9C^{10}A^{11}A^{12}A^{13}C^{14}A^{15}]^{3'}$ duplex at 15°C by either NOESY or ROESY experiments or by a combination of ROESY and NOESY experiments.

Imino proton	$(R_{1N} + k_{ex})$¥	ROESY* *(Two-proton approximation)*												NOESY-ROESY† *(Present approach)*	
		The rate of exchange (k_{ex})(s^{-1}) Mixing time (ms)						The life-time (τ_{ex}) (ms) Mixing time (ms)						k_{ex} (s^{-1})	τ_{ex} (ms)
		20	40	60	112	200	250	20	40	60	112	200	250		
^{3}T-^{13}A	18.6±0.9	3.68	3.76	3.62	2.86	3.02	2.60	272	266	276	349	331	384	2.6±0.39	384±57
^{4}T-^{12}A	15.0±0.6	4.56	2.85	2.14	1.98	2.10	1.90	219	351	467	505	476	526	1.1±0.17	909±136
^{5}T-^{11}A	14.2±0.6	4.84	3.12	2.95	2.40	3.30	3.30	207	321	338	417	303	303	1.4±0.21	714±107
^{6}G-^{10}C	21.2±1.4	7.75	5.92	5.53	5.63	4.73	4.40	129	169	181	178	211	227	3.4±0.51	294±44

Imino proton	$(R_{1N} + k_{ex})$¥	NOESY# *(Two-proton approximation)*																	
		The rate of exchange (k_{ex})(s^{-1}) Mixing time (ms)									The life-time (τ_{ex}) (ms) Mixing time (ms)								
		20	40	60	112	200	250	350	500	650	20	40	60	112	200	250	350	500	650
^{3}T-^{13}A	5.6±0.2	3.06	2.07	1.64	1.59	1.40	1.23	1.16	1.18	1.25	327	483	609	629	714	813	862	847	800
^{4}T-^{12}A	4.2±0.2	1.38	1.51	1.70	0.94	0.66	0.57	0.61	0.65	0.73	725	662	588	1063	1515	1754	1639	1538	1369
^{5}T-^{11}A	4.7±0.2	1.10	2.18	1.97	1.18	0.91	0.82	0.90	0.84	0.92	909	459	508	847	1098	1219	1111	1087	1086
^{6}G-^{10}C	6.6±0.4	4.20	3.12	2.57	2.10	1.60	1.50	1.70	1.80	1.90	238	321	389	476	625	666	588	555	526

*The k_{ex} and τ_{ex} have been obtained using ROESY experiment at of τ_m = 20, 40, 60, 112, 200 and 250 ms using two-proton approximation (eqn. 5).

#The k_{ex} and τ_{ex} have been obtained by NOESY experiment at τ_m = 20, 40, 60, 112, 200, 250, 350, 500 and 650 ms and using eqn.4. Data shows that up to 2–3 times differences in values for k_{ex} and τ_{ex} are obtained between ROESY and NOESY experiment. It is noteworthy that the NOESY experiment at longer mixing times ($\tau_m \geq 200$ ms) do not exhibit any large changes in k_{ex} and τ_{ex} values compared to the data at shorter mixing times ($\tau_m \leq 200$ ms). Note that the values for k_{ex} from NOESY spectra[11] at $\tau_m \geq 200$ ms were not dependent on mixing time used which is also consistent with our finding that the contribution from the diffusion phenomenon remains steady at $\tau_m \geq 200$ ms.

¥.-$(R_{1N} + k_{ex}) = [\ln(a_{ii}/a_{io})]/\tau_m$, where a_{ii} is the intensity of diagonal peaks normalized to the intensity of the diagonal peak a_{io} at τ_m = 0 ms, R_{1N} is the longitudinal relaxation term for the imino protons (N).

†The k_{ex} and τ_{ex} have been calculated using eqn. 9 from a combination of NOESY and ROESY experiments. In the table the average data for mixing times 20, 40 and 60 ms have been presented with error limits.

Bloch equations $A(\tau_m) = S[\exp(-\mathbf{R}\tau_m)]$, [where $A(\tau_m)$ is a matrix of peak volumes, τ_m is mixing time, **R** is relaxation matrix with cross-relaxation (R_{ij}) and auto-relaxation (R_{ii}) constants, S is the magnetization defined as diagonal matrix of peak intensities at $\tau_m = 0$] can be written in power series[13] and for short mixing period, $R_{ij} << 1$,

$$\frac{a_{ij}}{a_{io}} = (k_{op} + \sigma)\tau_m[1 - \frac{(R_{NN} + R_{1W})\tau_m}{2}] + \frac{1}{2}\sum_{k \neq NW}\sigma_{Nk}\sigma_{kW}\tau_m^2 \qquad \text{(eqn. 6)}$$

where a_{ij} is the intensity of the cross-peaks normalized to the intensity of the diagonal peak, a_{io}, at $\tau_m = 0$ ms, R_{NN} is the longitudinal relaxation term for the imino protons (N) and R_{1W} is the longitudinal relaxation term for water (W), σ is cross-relaxation rate. A diffusion relaxation term[13] should be included, which represents multiple-step magnetization transfer between N and W protons and their common neighbour(s) (k): (N↔k↔W). The difference in the σ term between NOESY and ROESY spectra is $2\sigma^{noe} = -\sigma^{roe}$ ($\omega\tau_c > 1$)[15], in which the sign difference implies that the spin-diffusion contribution to cross-peak volumes is additive to both the exchange and magnetisation processes in NOESY spectra, but in ROESY spectra it has the same sign as the exchange process (in-phase with diagonal peaks) but opposite sign to the direct magnetization transfer. Moreover, at a given mixing time, ROESY cross peaks are approximately four times more sensitive to spin diffusion than NOESY cross peaks[13]. The equations for the NOESY and ROESY cross-peaks and diagonal peaks could be written:

$$\begin{cases} \frac{a_{NN}^{noe}}{a_{N0}^{noe}} \approx [1 - (R_{1N}^{noe} + k_{ex})\tau_m] \\ \frac{a_{NW}^{noe}}{a_{N0}^{noe}} \approx (k_{ex} + q_{NW}\tau_c)\tau_m[1 - \frac{(R_{NN}^{noe} + R_{WW}^{noe})\tau_m}{2}] + 0.5\sum_{k \neq N,W} q_{Nk}q_{kW}\tau_c^2\tau_m^2 \end{cases} \qquad \text{(eqn. 7)}$$

where $R_{NN}^{noe} = R_{1N}^{noe} + k_{ex}$; $R_{WW}^{noe} = R_{1W}^{noe}$

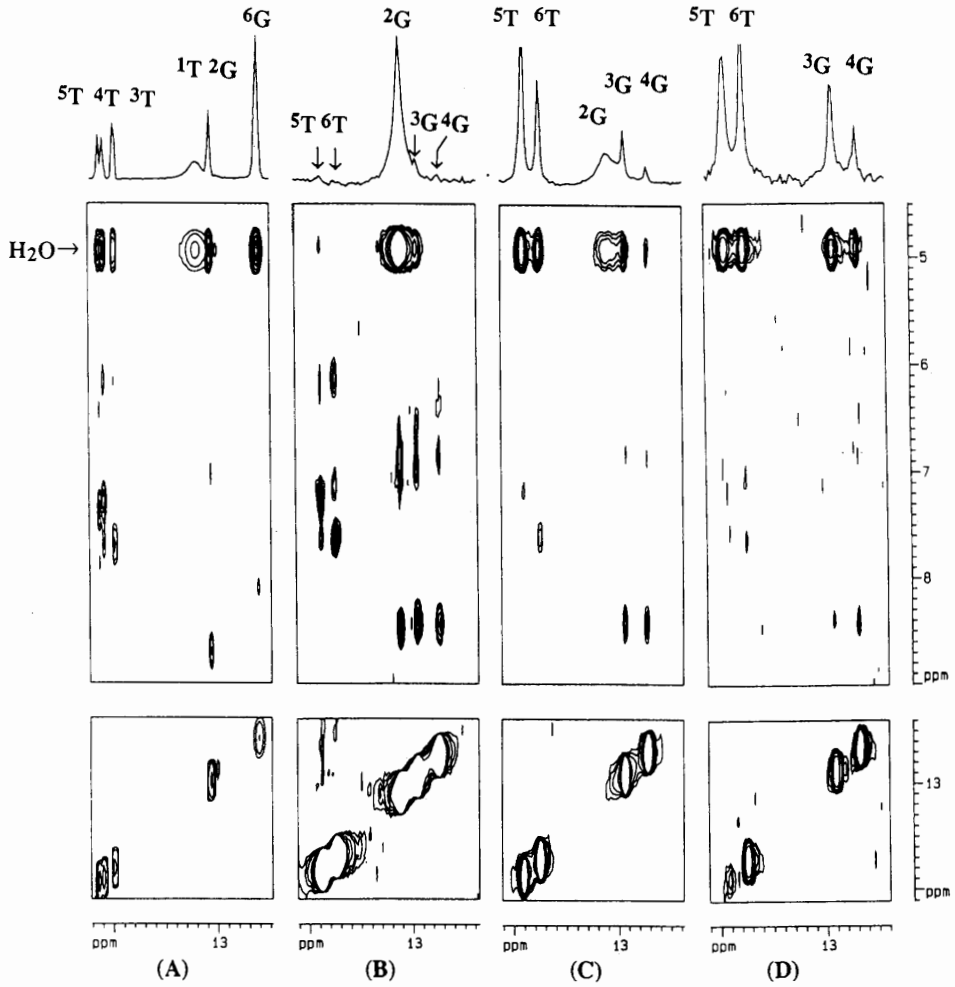

Figure 1. Plots of the spectral regions (f_1: δ14.1−12.5 ppm for imino protons; δ9.0−4.5 ppm for aromatic, amino and H1′ protons; f_2: δ14.1−12.5 ppm for imino protons) from the NOESY spectra (τ_m = 30 ms) at 15°C, recorded at 500 MHz (^{1}H) for duplex (I) (Panel **A**), and (II) (Panels **B**−**D**). The water chemical shift in panels **A**−**D** is indicated by the horizontal arrow. Horizontal cross section along f_2 at the f_1 frequency of the water line in the NOESY spectrum showing cross peaks of imino protons with water. Panel **A** shows strong cross peaks between all imino protons of duplex (I) and water at δH_2O at pH 7.4 without any base catalyst. These strong cross peaks at δH_2O indicate a fast exchange process of all imino protons in duplex (I) compared to the NOESY spectra of slow exchanging imino protons in duplex (II) in pH 8.8 in panel **B** in which no base catalyst has been added. Note that the strong cross peak at the δH_2O in panel **B** shows imino proton cross-peak of fraying ^{2}G with water while other imino protons, *i.e*, ^{5}T, ^{6}T, ^{3}G and ^{4}G, have negligible cross-peak with water. It is also noteworthy in panel **B** that there are considerably large cross-peaks with imino and aromatic protons due to magnetic relaxation process which start to be reduced as the concentration of the base catalyst is increased as evident by the comparison with the subspectra (**C**) with 0.029 M base added, and subspectra (**D**) with 0.160 M base added.

$$\begin{cases} \frac{a_{NN}^{roe}}{a_{N0}^{roe}} \approx [1-(R_{1N}^{roe}+k_{ex}+0.5\,q_{NW}\,\tau_c)\tau_m] \\ \frac{a_{NW}^{roe}}{a_{N0}^{roe}} \approx (k_{ex}-2\,q_{NW}\,\tau_c)\tau_m[1-\frac{(R_{NN}^{roe}+R_{WW}^{roe})\tau_m}{2}]+2\sum_{k\neq N,W} q_{Nk}\,q_{kW}\,\tau_c^2\,\tau_m^2 \end{cases} \quad \text{(eqn. 8)}$$

where $R_{NN}^{roe} = R_{1N}^{roe} + k_{ex} + 0.5\ q_{NW}\ \tau_c$; $R_{WW}^{roe} = R_{1W}^{roe}$, (q_{NW}) is a constant 0.1 $(\frac{\mu_0}{4\pi}\ h\gamma^2 r_{NW}^{-3})^2$; τ_c is the rotation correlation time; r_{NW} is the distance between imino protons N and water. We assumed that the direct magnetization term between water and imino protons could be neglected as it was evident from the extent of magnetic relaxation between imino and aromatic protons and almost complete absence of such relaxation with imino protons of ^{5}T, ^{6}T, ^{3}G and ^{4}G and water without catalyst (Panel B in Fig. 1) in duplex (II) in which the exchange process has been thoroughly investigated by several groups of workers using T_1 measurements[4–8].

d(^{1}C ^{2}G ^{3}C ^{4}G ^{5}A ^{6}A ^{6}T ^{5}T ^{4}C ^{3}G ^{2}C ^{1}G)
(G C G C T T A A G C G C)d Duplex (II)

It has also been found in this work that the volume of the cross-peaks due to magnetic relaxation considerably decrease as the concentration of the base catalyst increase (compare panel B with C and D in Fig. 1). The cross-relaxation rate is not dependent on the concentration of the base catalyst and the loss of the magnetization between imino and aromatic protons is due to the acceleration of the exchange rate of imino protons with water as the base concentration increases. Indeed, the cross peak between water and the slow exchangeable imino proton of G-C base pair is not observable before the addition of the base catalyst but under this neutral condition the magnetic relaxation cross peaks between imino and aromatic protons have been observed (Fig. 1). Only after the addition of the base catalyst did the cross peaks between water and imino protons appear. This means that we can safely omit the magnetization term, $q_{NW}\tau_c$, between imino proton and water in equations describing NOESY and ROESY spectra and substract eqn. 8 from 7 to give eqn. 9 which has the new function $(4\ a_{NW}^{noe}/a_{N0}^{noe} - a_{NW}^{roe}/a_{N0}^{roe})$ that does not include the diffusion term over the range of τ_m values where $\sigma\tau_m < 1$.

$$4\left(\frac{a_{NW}^{noe}}{a_{N0}^{noe}}\right)-\left(\frac{a_{NW}^{roe}}{a_{N0}^{roe}}\right) = k_{ex}\tau_m[3-(\frac{4(R_{NN}^{noe}+R_{WW}^{noe})\tau_m}{2}-\frac{(R_{NN}^{roe}+R_{WW}^{roe})\tau_m}{2})] \quad \text{(eqn. 9)}$$

where, $(R_{NN}^{noe}+R_{WW}^{noe}) = [\ln(\frac{a_{NN}^{noe}}{a_{N0}^{noe}})]/\tau_m$ and $(R_{NN}^{roe}+R_{WW}^{roe}) = [\ln(\frac{a_{NN}^{roe}}{a_{N0}^{roe}})]/\tau_m$

MATERIAL AND METHODS

^{1}H NMR spectra were recorded on a Bruker AMX-500 NMR spectrometer (^{1}H at 500 MHz). Phase-sensitive NOESY experiments with the water suppression is achieved by the use of two short spinlock pulses, $SL_{\psi 4}$ and $SL_{\psi 5}$ as described by Otting et al[17] at 288 K using the following parameters: mixing time (τ_m) was varied 0.020, 0.040, 0.060, 0.112, 0.200, 0.250, 0.350, 0.500 and 0.650 s for duplex (1) and 0.010, 0.015, 0.020 0.025 and 0.030 s for duplex (2); 90° pulse width of 10.25 μs; 2048 complex data points in t_2, 128 complex data points in t_1, the relaxation delay between pulse sequence is 2.0 s, a sweep width of 10204.082 Hz is used in both dimensions, $SL_{\psi 4}$ and $SL_{\psi 5}$ are equal to 0.5 ms and 3 ms, respectively, the delay between spinlock pulses τ is equal to 167 μs, the carrier was set at the water frequency, 128 or 64 scans/FID were used, for quadrature detection in F_1-dimension, time proportional phase incrementation (TPPI) is applied. The influence of the spinlock pulses in the NOESY experiment on the cross-peak and diagonal intensities has been controlled by blank NOESY experiment in which no spinlock pulses (i.e. reduction of water intensity by 45-τ-45 pulse sequence) are included. The difference between buildup intensities of the cross-peak and diagonal intensities in these two sets of experiments were well within the range of experimental error (±20%). Two-dimensional data sets for ROESY spectra with the water suppression is achieved using 1−1 echo sequence[18] and were collected in the phase-sensitive mode. The spectral excitation profile in this experiment is proportional to $\sin\omega\tau_1(1-\cos\omega\tau_2)$ where τ_1 and τ_2 are echo delays in case when $\tau_1 = \tau_2/2 = \tau = 150$ μs resulting in an excitation profile proportional to $\sin^3(\omega\tau)$. Typically 2048 data points were collected for each 128 t_1 values during experiments using a sweep width of 10204.082 Hz. The spectra was recorded with spinlock duration of 0.020, 0.040, 0.060, 0.112, 0.200, 0.250 s for duplex (1) and 0.010, 0.015, 0.020 0.025 and 0.030 s for duplex (2), using 6.25 kHz rf field for all pulses (36.5 μs 90° pulse with power level 14 dB) and a recycle delay of 2 s. The reduction of cross-peak volumes due to off-resonance effect, using a 6.25 kHz spinlock is not unduly large (maximum 20%) for imino protons. This is clearly evidenced through the comparison of the relative intensities of the cross-peaks of the imino protons with water in the ROESY (Fig. 2B) spectra, after the normalization of the cross-peaks by the corresponding diagonal peaks at $\tau_m = 0$, which corrects the offset of all the diagonal peaks with those relative intensities in the NOESY (Fig. 2A) spectra. This comparison shows that the NOE intensities in both NOESY and ROESY experiments are more or less comparable as a result of smaller off-resonance effect in the ROESY spectra. Our results of offset corrections and normalization shown in Figs. 2A and 2B may be compared with the results of the large off-resonance effects on the ROESY spectra for the same dodecamer duplex (2) by Wüthrich *et al.*[19] in which no offset corrections have been performed, and as a result NOESY cross-peaks are much larger than the ROESY cross-peaks between imino proton and water. The use of a weaker spinlock[20] would have a larger influence on the cross-peak volumes for imino protons (for example in a 4 kHz spinlock the imino protons cross-peak volumes would be decreased by 40%). A 4 ms saturation pulse is applied after data acquisition. The NOESY and ROESY data were collected with a nonspinning sample to avoid t_1 noise. The 1024×128 data points were resolution enhanced by a shifted squared sine-bell window function in the t_1 and lorentzian function in t_2 directions, then Fourier transformed and phase adjusted. All of the spectra were baseline-corrected in both dimensions using polynomials. NOE and ROE cross peak volumes were measured using the program AURELIA[21] with segmentation level 0.5 and 1000 iterations. The longitudinal relaxation times (T_1) were measured applying the selective saturation recovery experiment with solvent elimination using the 1331 pulse sequence[4,5]. The carrier frequency was placed at the water frequency, and the interval between the hard pulses was adjusted. The imino proton resonances were saturated individually and 26 values for the recovery delay were used for each T_1 measurement with a delay of 10s between scans. The T_1 values were obtained after baseline-correction using polynomials and from exponential nonlinear least-square fits of intensities and areas as a function of recovery delay. The errors in the determination of the T_1 value have been estimated by the repetition of the experiment three times, and they were baseline-corrected in both dimensions using polynomials. The error was found to be ± 15%

Table 2. Comparison of the base-pair life time (τ_{ex}) and the rate of exchange (k_{ex}) of residues ^{5}T, ^{6}T, ^{3}G and ^{4}G of d(CGCGAATTCGCG)$_2$ at 15°C in ammonia-ammonium chloride buffer containing 150 mM NaCl, and 0.5 mM NaEDTA (90%H_2O, 10%D_2O) at pH 8.8 as a function of inverse base catalyst concentration obtained (i) by saturation recovery experiment, and (ii) using a combination of NOESY and ROESY experiments.

[base]$^{-1}$ (M^{-1})	^{5}T					^{6}T				
	Saturation recovery expt. T_{1*}° = 270±15 (ms)#			NOESY-ROESY combination†		Saturation recovery expt. T_{1*}° = 325±25 (ms)#			NOESY-ROESY combination†	
	T_{1exp}¥	k_{ex} (s^{-1})	τ_{ex} (ms)	k_{ex} (s^{-1})	τ_{ex} (ms)	T_{1exp}¥	k_{ex} (s^{-1})	τ_{ex} (ms)	k_{ex} (s^{-1})	τ_{ex} (ms)
34.7	41±5	21±3	48±8	21±4	48±8	78±5	9.7±2	103±13	8.9±2	112±16
19.1	24±3	38±5	26±4	30±5	33±4	58±2	14.2±3	71±10	13.8±4	72±11
16.0	22±4	42±6	24±4	40±5	25±4	59±5	13.9±3	72±10	15.2±3	66±10
13.2	21±2	44±6	23±4	42±5	24±4	52±4	16.2±3	62±8	17.2±3	58±8
11.6	17±4	55±8	18±4	59±7	17±3	43±3	20.2±4	50±7	17.9±3	56±8
9.0	14±3	68±10	15±4	67±10	15±3	36±3	24.7±5	40±7	21.3±4	47±7
8.3	13±3	73±11	13±4	71±11	14±3	38±4	23.2±5	43±7	25.0±4	40±6
7.6	13±3	70±11	14±4	77±11	13±3	41±5	21.3±5	47±7	31.3±5	32±6
6.2	12±3	80±12	13±4	100±15	10±3	30±5	28.6±6	35±6	31.3±5	32±6
[base]$^{-1}$ (M^{-1})	^{3}G					^{4}G				
	Saturation recovery expt. T_{1*}° = 200±15 (ms)#			NOESY-ROESY combination†		Saturation recovery expt. T_{1*}° = 218±17 (ms)#			NOESY-ROESY combination†	
	T_{1exp}¥	k_{ex} (s^{-1})	τ_{ex} (ms)	k_{ex} (s^{-1})	τ_{ex} (ms)	T_{1exp}¥	k_{ex} (s^{-1})	τ_{ex} (ms)	k_{ex} (s^{-1})	τ_{ex} (ms)
34.7	117±7	3.5±0.6	282±27	3.4±0.6	294±29	**	**	**	**	**
19.1	95±7	5.5±0.9	181±16	5.7±0.9	175±17	160±15	**	**	**	**
16.0	91±6	6.0±1.1	167±14	6.6±1.0	152±15	161±12	**	**	**	**
13.2	75±7	8.3±1.2	120±12	7.7±1.1	130±13	150±10	**	**	**	**
11.6	70±9	9.3±2.0	108±10	9.7±1.4	103±11	132±8	3.0±0.5	335±33	3.3±0.5	303±30
9.0	53±4	13.9±2	72±8	14.1±3	71±8	121±8	3.7±0.5	272±27	4.2±0.6	238±24
8.3	51±4	14.6±3	68±8	12.5±3	80±9	116±6	4.0±0.6	248±25	4.3±0.6	233±23
7.6	50±4	15.0±3	67±8	14.3±3	70±8	114±6	4.2±0.6	239±23	4.8±0.6	208±21
6.2	49±3	15.4±3	65±7	16.7±3	60±7	106±7	4.8±0.7	206±19	5.0±0.6	200±20

$T_1^{*\circ}$ the longitudinal relaxation time in the absence of added catalyst.
¥ T_{1exp} the experimental longitudinal relaxation time with added catalyst. The values of τ_{ex} and k_{ex} have been calculated according to formula (3).
† The k_{ex} and τ_{ex} have been calculated using eqn. 9 from a combination of NOESY and ROESY experiments
** can not be calculated due to slow exchange.

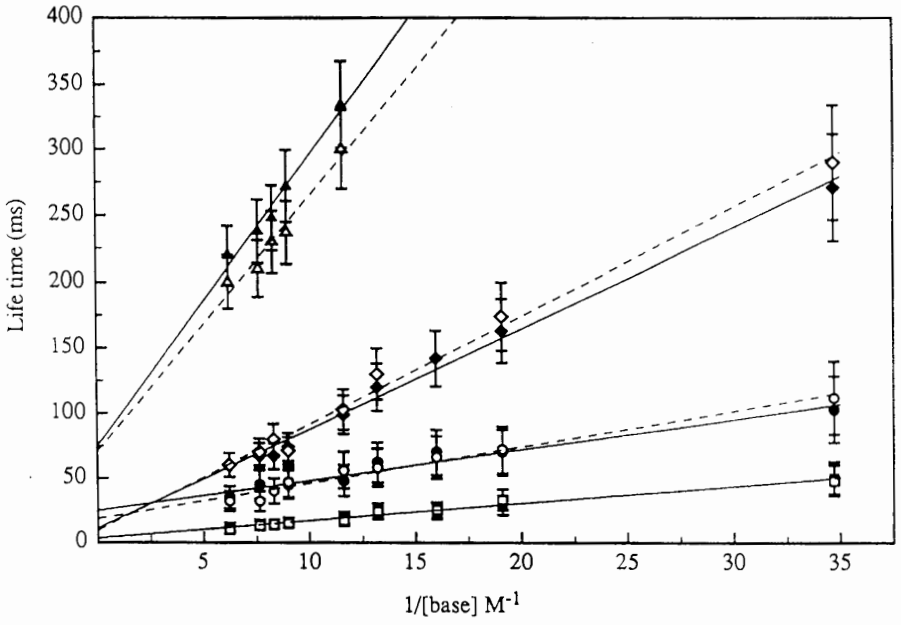

Figure 3. The life-time of exchange process in the duplex d(CGCGAATTCGCG)$_2$ at 15°C as a function of inverse base catalyst [NH_3 buffer at pH 8.8] concentration (see Table 2) obtained by the ^{1}H NMR selective saturation recovery T_1 experiment (filled symbols) and by a combination of NOESY-ROESY method (unfilled symbols) for the imino protons: ^{5}T (■ · and □), ^{6}T (● and ○), ^{3}G (◆ and ◇) and ^{4}G (▲ and △). To obtain the base-pair life-times ($\tau_{op} = 1/k_{op}$) according to eqn. 2 as described in the text, straight line extrapolations through linear regression analyses have been used for the T_1 measurement experiment (solid line) and NOESY-ROESY method (dashed line).

be less then ±15%. To calculate k_{ex} and τ_{ex} by saturation recovery experiment the longitudinal relaxation T_{1exp} with added catalyst and T_{1*}° without catalyst have been measured using eqn. 3[1–4]. To calculate k_{ex} and τ_{ex} by ROESY-NOESY experiment, the volume of the cross-peaks between imino protons and water in the NOESY and ROESY spectra have been measured separately from the magnetization term using eqn. 9. In Fig. 3, the relative intensity of the cross-peaks and semilog $[\ln(a_{ii}/a_{io})]$ of diagonal peaks normalised to the value of the diagonal peaks at zero mixing time of NOE build-up curves at different mixing time in the NOESY and ROESY spectra have been presented as a function of concentration of ammonium catalyst for the imino proton of ^{6}T as an example. Fig. 3 shows that as the concentration of the catalyst is increased both the intensity of cross-peaks and the values for $(R_{1N} + k_{ex}) = -[\ln(\frac{a_{ii}}{a_{io}})]/\tau_m$ obtained from NOESY and ROESY are increased. Moreover, it may be noted that in the absence of the catalyst (Fig. 3) or at a slow exchange condition [for example for ^{4}G imino proton of the duplex (II) or ^{3}T, ^{4}T, ^{5}T residues of the duplex (I), data not shown] the relative intensity of the cross-peaks at an identical mixing time (< 20 ms) in the ROESY experiments are larger than the corresponding cross-peaks in the NOESY experiment. Only in the fast exchanging conditions [for example, under the influence of a catalyst (Fig. 3)], the behaviour of the relative intensity of the cross-peaks in NOESY and ROESY spectra is qualitatively similar. Concurrently, the intensity of the cross-peaks between imino and aromatic protons, owing to dipole-dipole relaxation process (Fig. 1) are strongly reduced. All of these data are consistent with our present finding that the contribution from the diffusion phenomenon play an important role in the magnetic transition process between exchangeable imino protons and water even at a shorter mixing times ($\tau_m \leq 200$ ms) and remains steady at a longer mixing times ($\tau_m \geq 200$ ms).

The values for $(R_{1N} + k_{ex}) = -[\ln(\frac{a_{ii}}{a_{io}})]/\tau_m$ from the diagonal peaks in the NOESY spectra for any imino protons are 2–3 times smaller than the corresponding proton in the ROESY spectra [Table 1 for the duplex (I) and Fig. 3 for the duplex (II)]. It is noteworthy that the respective relaxation rates[14] R_{1N} is however determined by $T_{1\varrho}^{-1} = T_2^{-1}$ for ROESY and by T_1^{-1} in NOESY experiments. This means that the diagonal peaks in the NOESY spectra could be observed at a much longer mixing time compared to ROESY spectra.

Fig. 2 shows that the curves fitted by linear regression analysis using eqn. 2 for both methods appear fairly linear. There is a good agreement in the estimation of the life-time by both methods at different concentration of catalyst and also for the base-pair life-time (τ_{op}) obtained by extrapolation to infinite base concentration. These data are consistent with the results of other investigations by saturation recovery experiment on the same duplex (II)[4,5]. These data strongly shows that both approaches (saturation recovery and ROESY–NOESY method) produce the same sort of information in the condition of added catalyst experiment. The saturation recovery method is however not a valid approach when the catalyst can not be used because of fast exchange process of DNA base-pairs, or if the temperature should be kept constant in order to compare the melting points (T_m) of a duplex with those of the exchange properties under an uniform condition. In these situations, only the ROESY-NOESY approach can be used with a reasonable accuracy.

A comparison of the rate of exchange of the imino proton cross peaks at the chemical shift of water for the duplex (I) in neutral condition (pH 7.4) at 15°C [panel A in Fig. 1] with that of the duplex (II) at pH 8.8, without additional base catalyst, at 15°C [panel B in Fig. 1] clearly show that the imino protons of duplex (I) are indeed very fast exchanging at the neutral pH compared to the undetectable slow exchange process in the duplex (II) even at pH 8.8. We have successfully used our approach of ROESY-NOESY combination for the determination of k_{ex} of these fast-exchanging imino protons of duplex (I) which have been found to be between 1–3 s^{-1} (Table 1). In the fast exchanging duplex, any addition of the base catalyst or an increase of temperature would increase their k_{ex} and give considerable error in the T_1 measurement. Moreover, these measurements would only reflect the k_{ex} and τ_{ex} under the condition of external influence. Clearly, the present approach is preferred to the T_1 measurement when the k_{ex} and τ_{ex} should be measured in a duplex in a native physiological condition for both slow and fast exchanging imino protons of the base pair of DNA duplex. In our NOESY-ROESY approach, we can extract exchange rates of imino protons of DNA duplex in either pure aqueous solution or in any other buffered media at any pH of choice. This means that the combination of NOESY-ROESY experiments should allow us to examine the effect of any extraneous agent of biological importance (such as drug, metal ion, protein, carbohydrate etc.) on the DNA duplex stability through the measurement of the exchange rates of the imino protons of the duplex.

The main limitation of the present ROESY-NOESY approach is that it is time consuming. But the use of field gradients could potentially reduce the experimental time due to the shorter phase-cycling in these type of experiments[23].

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