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Poor Hydration Enhances The Activation Energy of the Exchange Rate of the Base-paired Imino Protons with Water at the Core Part of the DNA Duplex

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Abstract: Here we report the exchange rates (k_{ex}) of imino protons of $d[{}^5p(T^1G^2T^3T^4T^5G^6G^7C^8)^3]$; $d[{}^3(A^{15}C^{14}A^{13}A^{12}A^{11}C^{10}C^9)p^5]$ (duplex I) with water at different pH and temperature to give the life-times (τ_o) of the closed state of the base-pairs. The τ_o of the closed state of the base-pairs is uniform ($E_a \approx 25 \pm 5$ kcal/mol) in the duplex I, and varies between 0.2 - 4 ms. A plot of the natural log of various exchange rates of the imino protons of the base-pair of the duplex I within the pH range of 6.1 to 8.6 as a function of the inverse of temperature gave the activation energy (E_a) of the exchange process of imino protons with the bound water (hydration). It has been found that although τ_o are in the same range but the E_a of the exchange processes of the open state of imino protons with the bound water are very different, and they are strongly dependent upon the location of the nucleotide residues along the DNA duplex: 22.3±3.3 kcal/mol for the core base-pair T⁴-A¹², 16.2 ± 2.4 kcal/mol for the base-pair T⁵-A¹¹, 10.5 ± 1.6 kcal/mol for the base-pair T³-A¹³, 12.3 ± 1.8 kcal/mol for the base-pair G⁶-C¹⁰ and 2.4 ± 0.4 kcal/mol for the base-pair G²-C¹⁴. The comparison of the activation energies of the exchange process of imino protons and water with that of the water abundance in the first spine of hydration between fully-matched duplex I and the analogous G⁷-A⁹ mismatched duplex II, ($d[{}^5p(T^1G^2T^3T^4T^5G^6G^7C^8)^3]$; $d[{}^3(A^{15}C^{14}A^{13}A^{12}A^{11}C^{10}A^9)p^5]$), determined by a combination of NOESY and ROESY experiments, suggests for the first time that the relative exchange of imino protons of the base-pairs in the DNA duplex is more rapid when there is an abundance of water at the first spine of hydration. This result also showed unambiguously that the core of the DNA is by and large devoid of water and the energy penalty of water entering the core is very high. This is consistent with our earlier work which showed that as the water activity in the minor and major groove of DNA increases, the T_m decreases (ref. 1), suggesting the water poisoning as the principal factor for base-pair mismatch, frame-shift and mutation in our DNA replication machinery.

The affinity of nucleic acids for water (hydration) in aqueous medium is of considerable interest to understand the dynamic characters of the conformational transitions of DNA or RNA duplex, DNA-RNA hybrid duplex, RNA pseudoknots as well as of RNA catalysis in hammerheads and hairpins. The critically controlled hydration in the active site of DNA polymerase-DNA complex seems to dictate the high fidelity of the DNA synthesis (one error in $\sim 10^9$) in our replication machinery. Water that sustains our life process could most probably interfere with the fidelity of our DNA replication by competitive hydrogen-bonding with heteroatoms involved in Watson-Crick base-pairing (water poisoning) under certain circumstances, promoting base-pair mismatch, frame shift and mutation. We reported earlier¹ through comparison of the relative NOE intensities of aromatic, anomeric and methyl protons with water within a set of four analogous DNA duplexes with varying T_m that it is only the least stable mismatched DNA duplex (as evident by their respective T_m) which has a continuous spine of hydration and ribbon structures throughout both the core part and the terminals. Our NMR work qualitatively showed¹ that as the stability (T_m) of the DNA duplex increased, both the exchange rates of the imino protons as well as the water activity in the minor and major grooves decreased considerably.

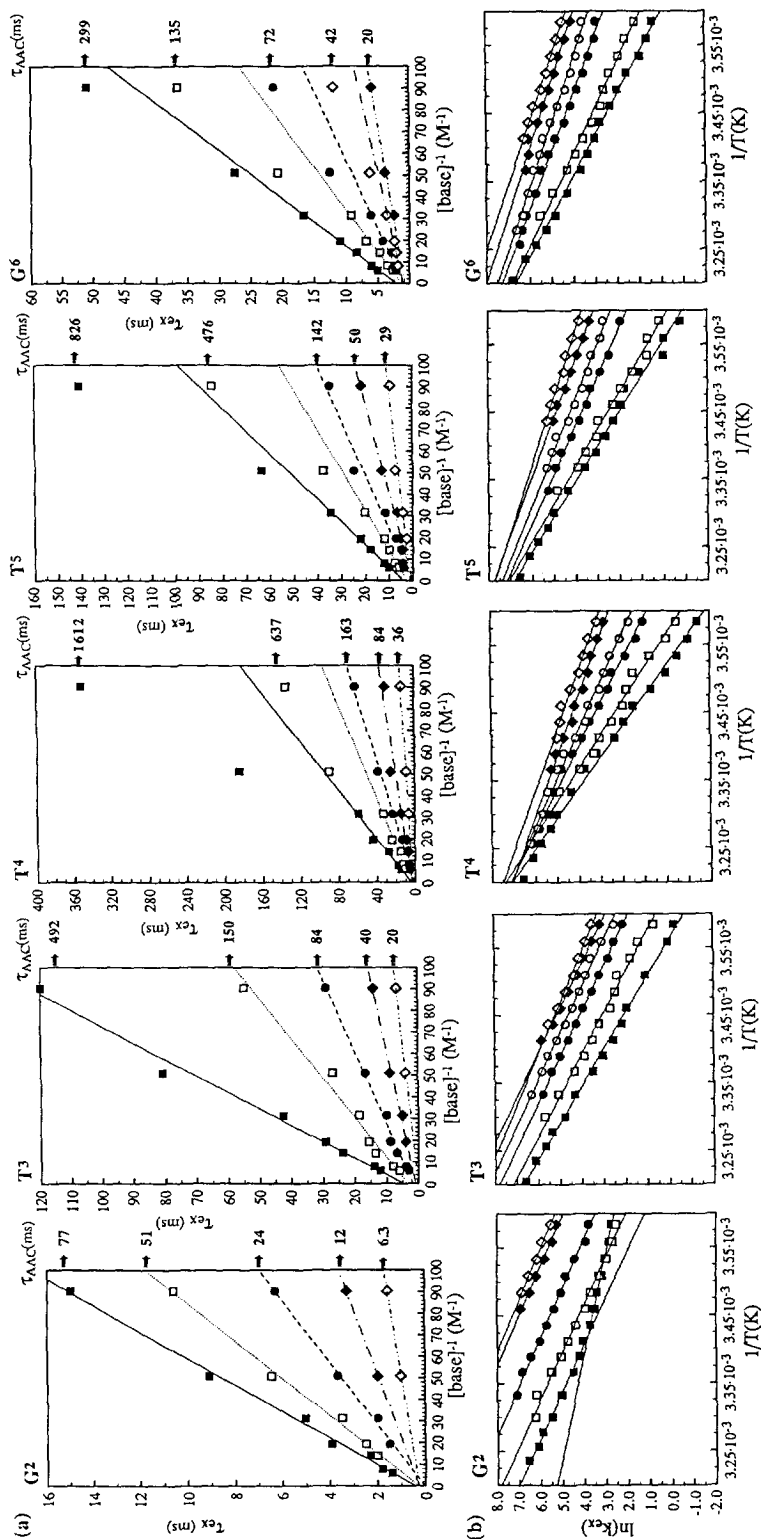


Fig. 1: (a) A plot of observable proton exchange life-time (τ_{ex} in ms) versus inverse of added base catalyst concentration ($[base]^{-1} M^{-1}$) at different temperatures: (■) 6 °C, (□) 10 °C, (●) 14 °C, (◇) 22 °C for the imino protons of the base-pairs of the B-DNA duplex $d[5'p(T^1G^2T^3T^4T^5G^6G^7C^8)^3]_1 d[3'(A^{15}C^{14}A^{13}A^{12}A^{11}C^{10}C^9)p^5]$. See the experimental section for exact protocol for the additions of the base catalyst at pH range of 8.1 - 8.8. The closed state life-times (τ_o) of the base-pairs have been obtained by the extrapolation to the infinite concentration of the catalyst at different temperatures, which are presented at the top left corner of the graph. The life-times of the base-pairs at the above temperatures in the absence of the base catalyst (τ_{AAC}) (ms) are presented along the right axis of the graph by arrow for comparison. (b) The plot of logarithm of the observable rate of exchange (k_{ex} in s^{-1}) of imino protons versus inverse of temperatures $[1/T$ (K)] at different pH [(■) 6.1, (□) 7.3, (●) 7.9, (◇) 8.1, (◆) 8.3, (◇) 8.6] showing a linear relationship. The slope of the linear graph gives the activation energy (E_a) at different pH.

We report here for the first time three important experimental findings: (i) that the life-times (τ_0) of the closed state of the base-pairs are uniform ($E_a \approx 25 \pm 5$ kcal/mol) in our duplex and vary between 0.2 - 4 ms, (ii) although τ_0 are in the same range but the activation energies (E_a) of the exchange processes of imino protons with the bound water are different, and they strongly depend upon the location of the nucleotide residues along the DNA duplex, and (iii) as the water activity in the first spine of hydration decreases, the activation energy of the exchange process of imino protons with the bound water increases (~ 22 kcal/mol at the core base-pair T⁴-A¹² to ~ 2 kcal/mol at the termini).

A perusal of the pH dependence of the exchange life-time ($\tau_{ex} = 1/k_{ex}$) of the imino protons with water within the pH range of 6.1 - 8.8 at different temperatures (6° - 22°C at 2° intervals) in the heptameric B-DNA duplex (I), d[⁵p(T¹G²T³T⁴T⁵G⁶G⁷C⁸)³]: d[³(A¹⁵C¹⁴A¹³A¹²A¹¹C¹⁰C⁹)p⁵] (Fig. 1a), shows that the imino proton T⁴ nucleotide of the core part of the duplex exchanges much more slowly than the imino protons of the flanking base-pairs (*i.e.* T³/G² at the 5'-end and T⁵/G⁶ at the 3'-end). The life-times (τ_0) of the closed state of the base-pairs in this heptameric duplex were obtained by extrapolation of the imino proton exchange time to infinite concentration of the base catalyst at different temperatures (Fig. 1a). This shows that the life-times τ_0 of the closed state of the base-pairs in this heptameric DNA duplex are indeed very small (*i.e.* between 0.2 - 4 ms). Most importantly, the life-times τ_0 of all the base-pairs are almost the same within the experimental error and they are both temperature and sequence-independent. We have observed such a trend of small τ_0 for other hexameric and heptameric DNA^{16b} and DNA-RNA duplexes^{16b} in contrast to the larger 12-mer^{16a} or 20-mer^{16b} DNA duplex (3 - 20 ms), which show sequence-dependent exchange rates. Our results of life-times of the base-pair closing in the heptameric DNA duplex with three A-T base-pairs are in contrast with those found in 11-mer with four A-T base-pairs involving 5'-AT step ($\tau_0 \approx 80$ - 100 ms) whereas oligo-DNAs with three A-T base-pairs in a nonamer or four A-T base-pairs in decamer had $\tau_0 \approx 10$ - 12 ms¹². These observations however show that τ_0 is directly dependent on the length of DNA duplex as well as the types of the base-pair¹².

We have subsequently shown here (*vide infra*) that although the life-times of the closed state of all imino protons are in the same range in our duplex I, but the activation energies E_a of their exchange process with the bound water are indeed different, and this E_a can be directly correlated with the water availability in the first spine of hydration¹⁻⁴ as evident by a combination of NOESY and ROESY experiments^{1a}.

A plot of the natural log of various exchange rates of the imino protons of the base-pair with water of the heptamer within the pH range of 6.1 to 8.6 as a function of the inverse of temperature shows linear dependency (Fig. 1b). The slope of the vant' Hoff plot thus gave the activation energy (E_a) of the exchange process of imino protons with the bound water (Fig. 1b) at pH range of 6.1 - 8.8. Finally, a plot of the change of E_a of the imino proton of the core base-pair (*i.e.* T⁴) and its 3'- and 5'-flanking base-pairs as a function of different pH clearly shows that the E_a of the exchange of imino protons with water is indeed pH dependent (Fig. 2). At the basic pH (pH > 8.1), the exchange rates of all imino base-pairs with water are more or less similar within the experimental error ($\pm 15\%$) but within the pH range of 6.1 - 7.6, the individual exchange rates of each imino protons with water are highly dependent on the exact location of the base-pair in the DNA duplex (Fig. 2).

The high E_a of the exchange rates of all imino protons with water of the heptameric matched duplex I (28 - 44 kcal/mol) compared to Dickerson's 12-mer DNA duplex (14-15 kcal/mol) suggests⁵⁻⁸ that the large

activation energy in the former is due to contributions from both base-pair opening and helix opening as Tinoco et al originally reported for G-T mismatched 12-mer⁷ [d(C¹G²T³G⁴A⁵A⁶T⁶T⁵C⁴G³C²G¹)₂] and 13-mer [d(C¹G²C³AG⁴A⁵A⁶T⁶T⁵C⁴G³C²G¹)₂] containing extra adenine⁷. In their G-T mismatched 12-mer⁷, they observed that the E_a for base-pairs 4, 5, and 6 are 30 ± 5 , 37 ± 8 and 48 ± 9 kcal/mol at pH 6 (a similar trend was also observed for 13-mer⁷ at pH 8), which is consistent with our present study at pH 6.1. It should be noted that our present estimation of the total E_a representing both the exchange of the imino protons with water at the open state with water and the E_a of the barrier of the base-pair and helix opening-closing rates, has been performed at seven different pHs in the pH range of 6.1 - 8.8. This detailed pH dependent estimation of E_a has enabled us to distinguish the E_a of the exchange of the imino protons at the open state with water from the E_a of the barrier of the base-pair and helix opening-closing rates.

It can be seen from Fig. 2 that E_a of all imino protons of the base-pairs of G², T³, T⁴, T⁵ and G⁶ residues have two plateaus: one representing the E_a of the barrier of the opening-closing of the base-pair and the helix at pH >7.7 ($\sim 25 \pm 5$ kcal/mol), which is the same for all imino protons within the experimental error; the second plateau at pH < 7.5 represents the E_a of the exchange of imino protons at the open state with water plus the E_a of the barrier of the base-pair and helix opening-closing rates. The difference between the values of these two plateaus (ΔE_a) represents the E_a of the barrier of the exchange process of imino protons with the bound water from the base-pair in the open state. It can be seen from ΔE_a that the exchange rate with water is strongly dependent upon where a particular base-pair is located along the DNA duplex: 22.3 ± 3.3 kcal/mol for the core base-pair T⁴-A¹², 16.2 ± 2.4 kcal/mol for the base-pair T⁵-A¹¹, 10.5 ± 1.6 kcal/mol for the base-pair T³-A¹³, 12.3 ± 1.8 kcal/mol for the base-pair G⁶-C¹⁰ and 2.4 ± 0.4 kcal/mol for the base-pair G²-C¹⁴.

The above non-uniform E_a of the exchange rates of the imino protons with water of the base-pair of the heptameric matched duplex I with water have been finally compared with the water activity inside the major and the minor grooves at pH 6.4 using a combination of our NOESY and ROESY experiments^{1a} (Fig. 3). The signs of the cross-peaks for the non-exchangeable protons with water in the ROESY spectra compared with the NOESY spectra show the relative rates of the exchange of water molecules in the first spine of hydration with the bulk water on the molecular tumbling correlation scale^{3,4}. The relative abundance of the negative cross-peaks in the ROESY spectra at 15 °C (Fig. 3) of the heptameric duplex I shows that it is H2A of A¹³-T³ and A¹⁵-T¹ that have the bound water. A similar comparison of the abundance of hydration of the fully matched heptamer I ($T_m = 30$ °C)¹ with its analogous single mismatched heptamer, d[⁵p(T¹G²T³T⁴T⁵G⁶G⁷C⁸)₃]: d[³(A¹⁵C¹⁴A¹³A¹²A¹¹C¹⁰A⁹)p⁵] duplex (II) ($T_m = 25$ °C)¹ shows in contrast that H2 protons of all adenine moieties in the latter have NOE cross peaks with water. Additionally, it has been found¹ that all imino protons of the base-pair in G⁷-A¹⁰ mismatched duplex II have very similar exchange rates with water at pH 6.4, which are 10-15 fold faster at the core than the matched duplex I at an identical pH and temperature.

The comparison of the activation energies of the exchange process of imino protons and water with the water abundance in the first spine of hydration (*i.e.* bound water) suggests that the relative exchange of imino protons of the base-pairs in the DNA duplex is more rapid when there is an abundance of water at the first spine of hydration¹ as in the G⁷-A¹⁰ mismatched duplex II compared to matched duplex I. Since the NOESY and ROESY experiments to estimate the relative level of first spine of hydration in the duplexes I and II have been performed at a temperature of 15 °C, which is well below their T_m [UV measured (25 μ M) T_m of the

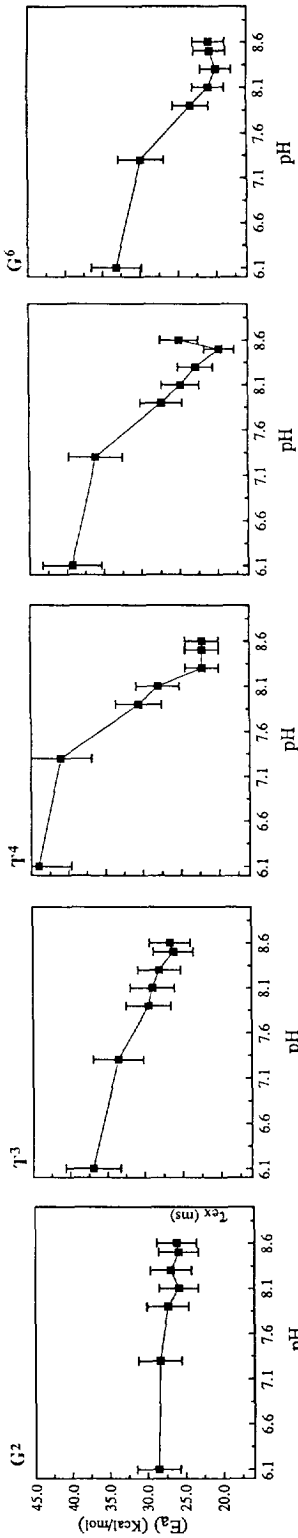


Fig. 2: The plot of total E_a as a function of pH for different nucleotide residues in duplex I. The calculated E_a represents the sum of opening-closing of the base-pair and the helix as well as the exchange of imino protons with water.

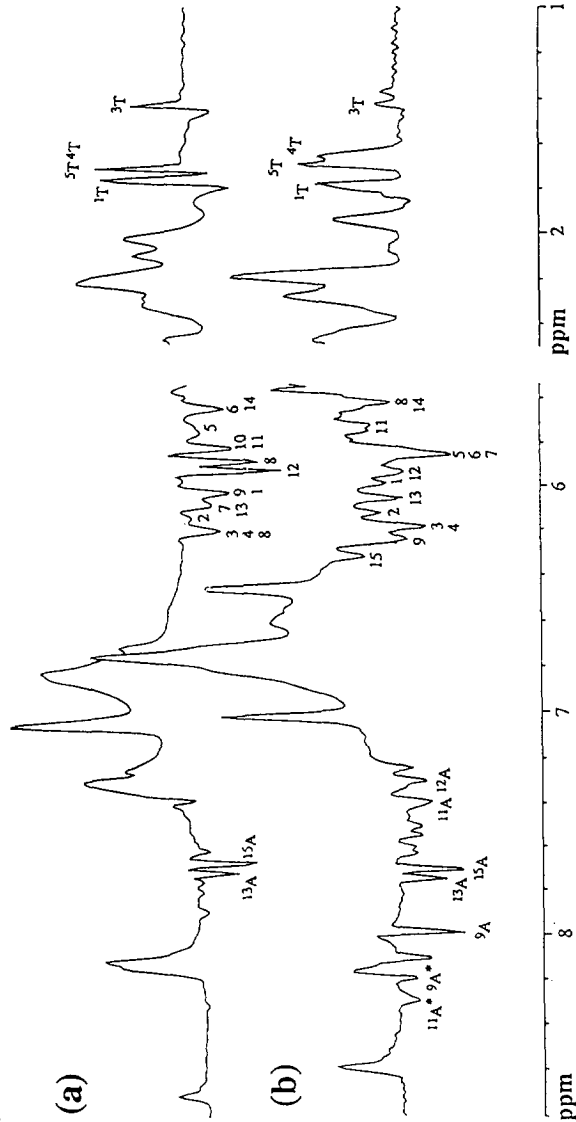


Fig. 3. $^1\text{H-NMR}$ spectra at 500 MHz showing cross-sections of the ROESY spectra through the cross section of the ROESY spectrum along the f_2 (δ 9.0-5.5 ppm and δ 2.5-1.0 ppm) at the f_1 frequency of the water line with the spinlock duration of 0.06 s for (a) duplex (I) and (b) duplex (II) using a literature experimental condition (ref. 1). The upfield region from δ 2.5-1.0 ppm was inverted for improved readability. Resonance assignments for the H^1 protons in δ 6.5-5.5 ppm region are indicated by the Arabic number which are identical with the nucleotide number in the duplexes (I) and (II). Resonance assignments (ref. 1) for the aromatic H2A protons (in δ 9.0-7.0 ppm region) and methyl thymine protons (in δ 2.5-1.0 ppm region) are indicated by the nucleotide number and nucleobase whereas the aromatic H8A are labelled by *.

duplex I = 30 °C and T_m of the duplex II = 25 °C^{1a,b}, we therefore are here measuring the hydration of both duplexes in their stable double stranded form. This is clearly evident from our earlier study on the dependence of chemical shift versus temperature on duplexes I and II, where we showed that the chemical shifts of aromatic and anomeric proton resonances remained unchanged upto 40 °C for duplex I and 25 °C for duplex II at 10 mM NMR concentration (see Fig. 11 in ref 1b). This also means that our above experimentally observed energy of activation of the exchange rate of imino protons with the bound water actually reflects the energy of activation of the hydration in the bound state (*i.e.* first spine of hydration).

This result shows for the first time that the core of the matched DNA is by and large devoid of water, or at least the energy penalty of water entering the core is very high, specially under condition when the DNA duplex stability is high. This leads us to speculate that the water availability in the DNA replication machinery¹⁸ must be quite negligible in order to maintain the high fidelity of the correct base-pairing with as little mismatch or frame shift as possible.

Experimental

¹H NMR spectra were recorded on a Bruker AMX-500 NMR spectrometer (¹H at 500 MHz). Phase-sensitive NOESY experiments with the water suppression is achieved by the use of two short spinlock pulses, SL_{φ4} and SL_{φ5} as described by Otting et al^{3,20} using the following parameters: mixing times (τ_m) were varied 0.01, 0.02, 0.03, and 0.04s to measure the exchange rates and 0.120s to observe the contact of the non-exchangeable protons with water; 90° pulse width of 10.25 μ s; 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 2.0 s, a sweep width of 10204.082 Hz is used in both dimensions, SL_{φ4} and SL_{φ5} are equal to 0.5 ms and 3 ms, respectively, the delay between spinlock pulses τ is equal to 167 μ s, the carrier was set at the water frequency, 32 scans/FID were used for quadrature detection in F₁-dimension with the time proportional phase incrementation (TPPI) applied. The influence of the spinlock pulses on the relaxation properties of water and its magnetization transfer to the exchangeable protons and on the relative intensity of the cross- and diagonal peaks, obtained after their normalizations by the diagonal peaks at $\tau_m = 0$ in the NOESY/ROESY experiments^{16a}, has been assessed in the following manner: firstly, a blank NOESY experiment has been performed in which no spinlock pulses (*i.e.* reduction of water intensity by 45- τ -45 pulse sequence)^{16a} are included, secondly, by NOESY experiments with spinlock pulses with a recycle delay of 5 or 10s have been performed in order to examine how the water relaxation is influenced by the delay. These control studies have shown that the difference between the relative build-up intensities of the crosspeak and diagonal intensities in the above two sets of experiments were well within the range of the experimental error ($\pm 20\%$). Two-dimensional data sets for ROESY spectra with the water suppression is achieved with one short spinlock pulse, SL_{φ3}, as described by Otting et al^{3,20}. During the mixing time sequence of $n(\pi/6)$ pulses with length 3.4 μ s separated by delay, Δ , (9.9 μ s) provides the similar effect as spin-lock SL_{φ4} of the NOESY experiment^{3,20} so that the spectra were recorded with spinlock duration of 0.010 0.02, 0.03, and 0.04s or 0.120s using 6.25 kHz rf field for all pulses (36.5 μ s 90° pulse with power level 14 dB) and a recycle delay of 2 s. 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

procedure¹⁹. A 3 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 x 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 AURELIA²¹ with segmentation level 0.5 and 1000 iterations. The spectral excitation profile in these experiment is proportional to $\sin(\Omega\tau)$ where Ω is the angular frequency relative to the carrier and $\tau = 167 \mu\text{s}$. In the Fig.3 the spectra have been multiplied by this function, correspondingly.

The assignment of imino protons are as reported in the literature^{1a,b}. The samples were 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: 10 mM]. The pH of the solution was measured after addition of each aliquot (5 μL) of stock solution containing ammonia in ammonium chloride (4.26 M, pH 9.2) in the NMR tube before and after NMR experiment and the final concentration of the base at each buffer concentration was calculated in the pH range of 8.1 to 8.8 using the following equation⁹⁻¹⁶: $[\text{base}]^{-1} = (1 + 10^{\text{pK}_a - \text{pH}}) \times [\text{total buffer}]^{-1}$. These solutions were then subjected to a combination of NOESY and ROESY experiments¹⁶ for the measurement of exchange rates.

The k_{ex} have been calculated from the line-width of imino protons in the following manner: A combination of ROESY and NOESY experiments¹⁶ were used to calculate the pure rate of exchange (k_{ex}) at 6 °C, 8 °C and 10 °C independently for each imino proton at a particular pH. These exchange rates were then subtracted from the value $\pi\Delta$, where Δ is line-widths (which have both the magnetization and k_{ex}) at the corresponding temperature and pH to calculate the pure dipole-dipole relaxation term which has been found to be constant upto 25 °C¹⁷. This constant dipole-dipole relaxation term was then subtracted from all other value $\pi\Delta$ of imino protons at higher temperatures (>10 ° to 22 °C) at the same pH to calculate the pure rate of exchange.

The activation parameters for the exchange process were obtained from the dependence of the exchange rate (k_{ex}) constants on temperature using the Arrhenius equation: $\ln(k_{\text{ex}}) = \ln(A) - E_a/RT$, where E_a is the activation energy and A is the frequency factor. The errors for these parameters are the standard deviation of the linear regression analysis which have been presented with average value of the E_a .

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