Alteration of A•T Base-Pair Opening Kinetics by the Ammonium Cation in DNA A-Tracts

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ABSTRACT: We have investigated by NMR the effects of NH_4^+ on the chemical shifts, on the structure, and on the imino proton exchange kinetics of two duplexes containing an A-tract, $[d(CGCGAATTCGCG)]_2$ and $[d(GCA_4T_4GC)]_2$, and of a B-DNA duplex,$[d(CGCGATCGCG)]_2$. Upon NH_4^+ addition to [d(CGCGAATTCGCG)]$_2$, the adenosine H2 protons, the thymidine imino protons, and the guanosine imino proton of the adjacent G•C pair show unambiguous chemical shifts. Similar shifts are observed in the A-tract of $[d(GCA_4T_4GC)]_2$ and for the A5(H2) proton of the B DNA duplex $[d(CGCGATCGCG)]_2$. The localization of the shifted protons suggests an effect related to NH_4^+ binding in the minor groove. The cross-peak intensities of the NOESY spectra collected at low and high NH_4^+ concentrations are comparable, and the COSY spectra do not show any change of the sugar pucker. This indicates a modest effect of ammonium binding on the duplex structures. Nevertheless, the imino proton exchange catalysis by ammonia provides evidence for a substantial effect of NH_4^+ binding on the A•T base-pair kinetics in the A-tracts. Proton exchange experiments performed at high and low NH_4^+ concentrations show the occurrence of two native conformations in proportions depending on the NH_4^+ concentration. The base-pair lifetimes and the open-state lifetimes of each conformation are distinct. Exchange from each conformation proceeds via a single open state. But if, and only if, the NH_4^+ concentration is kept larger than 1 M, the A•T imino proton exchange times of A-tract sequences exhibit a linear dependence versus the inverse of the NH_3 proton acceptor concentration. This had been interpreted as an indication for two distinct base-pair opening modes (Wärmländer, S., Sen, A., and Leijon, M. (2000) *Biochemistry 39*, 607−615).

Almost all our knowledge on the base-pair opening kinetics of nucleic acids relies on the analysis of the exchange times of the H-bonded imino protons with water. It is well established that the imino protons are sequestered within the closed base pairs. The formalism of catalyzed imino proton exchange from a base pair has been extensively described (see ref *1* for a recent review). It depicts a process requiring base-pair opening followed by proton transfer to a proton acceptor acting as an exchange catalyst.

During the past 20 years, proton exchange studies in B (*2*), B′ (*3*), and Z (*4*) DNA duplexes, in drug−DNA complexes (*5*), in tRNA (*6*), in RNA duplexes (*7*), and in triple helixes (*8*) have validated the two-state (open/closed) model of the base pairs. In this model the plot of the proton acceptor contribution to the imino proton exchange time versus the inverse of the acceptor concentration is a straight line, whose extrapolation at infinite catalyst concentration yields the base-pair lifetime. The possible occurrence of multiple opening modes has been evoked (*9*). Base-pair opening modes occurring at a rate slower than the imino proton exchange rate would escape proton exchange investigations. On the contrary, multiple opening modes operating at a rate faster than imino proton exchange would be detected by the nonlinearity of the plot of the exchange times versus the inverse of the acceptor concentration. The systematic observation of a linear relation between the exchange contribution of added catalysts and the inverse of the catalyst concentration argued until now for a single opening mode of the pairs.

Evidence which could favor the existence of a second opening mode has recently emerged from an imino proton exchange study of $[d(CGCGAATTCGCG)]_2$ and $[d(CGCA_8CGC)/d(GCGT_8GCG)]$ (*10*). The imino proton exchange catalysis was investigated in a wider range of NH_3 concentrations than in earlier studies. This allowed the observation of a feature that was previously unnoticed: the plot of the exchange times of most the A•T imino protons is nonlinear versus the inverse of the NH_3 concentration. This effect could indicate the occurrence of two base-pair opening modes. But it could also be due to a structural change induced by NH_3 or NH_4^+. This possibility was examined in ref *10* and rejected because the NOESY[1] spectra give no indication of such change. The authors therefore concluded in favor of two concurrent opening modes. In view of the importance of this result with regard to the description of the base-pair motion in nucleic acids, we have examined the effect of NH_4^+ on

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[1] Abbreviations: NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy; COSY, correlation spectroscopy.

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the NMR spectrum of $[d(CGCGAATTCGCG)]_2$ and reinvestigated the proton exchange catalysis by NH_3 using a complementary approach.

It is well-known that the large ammonia concentration required in proton exchange studies (up to 6 M !) induces intermolecular interactions between the duplexes (*11*, *12*). When the proton exchange times are determined from inversion−recovery measurements, as in ref *10*, they may be affected by the variation of the dipole−dipole relaxation rate (*11*, *13*). In the present work, we therefore measured the imino proton exchange times using magnetization transfer experiments, which are not subject to this artifact. An other problem is the possible conformational change mentioned above. Examination of the proton spectra of $[d(CGCGAATTCGCG)]_2$ revealed that NH_4^+, but not Na^+, induces a shift of the A(H2) and imino protons. This suggested a conformational change induced by NH_4^+. At the very least, it showed that some change is occurring in NH_4^+ solution even through the intensity of the NOESY cross-peaks remains nearly unchanged. Indeed, when the exchange experiments were carried out at NH_4^+ concentration larger than 1M, the linearity of the imino proton exchange time versus $1/[NH_3]$ was restored. We could thus demonstrate that the nonlinearity observed in ref *10* is not due not to one native structure associated with multiple opening modes but to the existence of two native structures, in proportions depending on the NH_4^+ concentration, each associated with its own single opening mode. The structural change induced by NH_4^+ was explored by NOESY and COSY measurements. Similar effects of NH_4^+ were also observed on the exchange kinetics of the A•T imino protons and on the chemical shifts of $[d(GCA_4T_4GC)]_2$, suggesting a feature characteristic of the A-tracts DNA duplexes. The localization of the shifted protons points toward an effect related to NH_4^+ binding in the narrow minor groove of the A-tracts.

MATERIALS AND METHODS

Oligonucleotide Synthesis. The DNA sequences d(CGCGAATTCGCG), $d(GCA_4T_4GC)$ and d(CGCGATCGCG) were synthesized using β-cyanoethyl phosphoramidites on a 2 or 10 μM scale and purified on a DEAE column.

Proton Exchange Experiments. All the proton exchange experiments were performed at 20 °C in the presence of 0.1 M NaCl. To distinguish the effect of NH_3 on the imino proton exchange kinetics from those induced by NH_4^+ on the duplex structure, we measured the imino proton exchange times versus the NH_3 concentration according to two different procedures. A first series of proton exchange measurements was performed at constant pH (8.8) with a constant 0.32 NH_3/NH_4^+ ratio. In these experiments, the NH_3 concentration was increased from 2 mM to 1.4 M by successive additions of small alliquots of a 6.5 M ($[NH_3] + [NH_4^+]$) stock solution. Hence, the NH_4^+ concentration increased in proportion from 6.3 mM up to 4.4 M. Such experiments will be henceforward designed as "experiments performed at variable ($[NH_3] + [NH_4^+]$) concentration". A second proton exchange study was performed at a constant 2 M ($[NH_3] + [NH_4^+]$) concentration. The NH_3 concentration was in that case controlled by changing the pH sample from 7.0 to 9.1, a range such that the exchange catalysis due to hydroxyl and to base deprotonation could be neglected. The NH_4^+ concentration remained within the narrow concentration range of 1.22−1.99 M. These experiments will be designed as "experiments performed in 2 M ($[NH_3] + [NH_4^+]$) ". The imino proton hydroxyl catalysis and the effect of the pH change on the duplex structure were measured in separate experiments.

Sample preparation. The duplex concentration was determined from the UV absorbance using the A^{260} values computed according to a nearest neighbor model (14). The strand concentrations ranged from 1 to 6 mM. The NMR samples contained 1 mM ethylenediamine tetraacetic acid and 0.2 mM 2,2-dimethyl-2-silapentane-5-sulfonate, whose methyl peak was set to 0 ppm for chemical shift reference. The sample pH was measured at room temperature before and after each experiment. In the experiments performed at variable ($[NH_3] + [NH_4^+]$) concentration, it was adjusted using 0.1 to 1 M HCl and NaOH solutions. In the experiments performed in 2 M ($[NH_3] + [NH_4^+]$), it was adjusted by mixing two DNA samples prepared in 2 M ($[NH_3] + [NH_4^+]$), respectively, at pH 6.9 and 9.3. Nonbuffered samples were kept under nitrogen atmosphere in the NMR tube. The effects pH, NH_4^+, and Na^+ on the proton chemical shifts were measured at pH 8.8 in 90% H_2O, 10% D_2O solution, and in 99.9% D_2O. NH_4Cl or ND_4Cl and NaCl were added from 5 M stock solutions in H_2O or in D_2O.

NMR Methods. Unless otherwise stated, all the NMR experiments were performed at 20 °C on a 500 MHz Varian Unity INOVA spectrometer using a penta probe. Two-dimensional spectra devised to investigate the effect of NH_4^+ on the oligonucleotide structure were acquired in the hypercomplex mode (*15*). The NOESY spectra were collected with mixing times of 40, 60, 80, 100, and 120 ms using a relaxation delay of 2 s in H_2O solution and 6.2 s in D_2O in order to avoid saturation of the H2 protons. The COSY spectra were acquired at 20 °C and at 45 °C to improve the spectral resolution. The cross-strand H2−H1′ distances were measured from the built-up of their NOE cross-peaks calibrated to the intra-residue H6−H5 cross-peaks of C3 and C11 (interproton distance 2.45 Å). The imino protons of $[d(GCA_4T_4GC)]_2$ were assigned from NOESY spectra collected with mixing times of 50 and 250 ms by their cross-peaks with the A(H2) protons identified in a previous publication (*16*). Except for magnetization transfer experiments, the H_2O signal was suppressed by a jump-and-return (JR) pulse sequence (*17*).

Exchange Time Measurements. Most of the exchange times were determined by magnetization transfer from water (*7*). After selective inversion of the water magnetization, the time dependence of the imino proton magnetization is given by

$$M_{z(t)} = M_0\left[1 - \frac{1 - (M_{w(0)}/M_w)}{\tau_{ex}(R_i - R_w)}\right] \times [\exp(-tR_w) - \exp(-tR_i)] \quad (1)$$

where τ_{ex} is the imino proton exchange time, M_0 and M_w are the imino proton and water equilibrium magnetizations, $M_{w(0)}$ is the water magnetization right after the selective inversion pulse, and R_i and R_w are the imino proton and the water longitudinal relaxation rates. The water magnetization was inverted by a DANTE sequence (*18*) of 30 hard 6° pulses separated by 50 μs intervals. The small water residual transverse component due to pulse imperfections was sup-

pressed by a 23 G/cm Z-gradient applied after the DANTE sequence during 0.5 ms and by a 0.01 G/cm Z-gradient applied during the magnetization transfer delay. The signal was detected using an echo water suppression subsequence. The recovery rate was set to 15 s to allow full relaxation of the water magnetization. Unwanted cross-relaxation effects were limited by using magnetization transfer delays shorter than 160 ms. The ratio $M_{w(0)}/M_w$ and the water and imino proton relaxation rates were measured in separate inversion−recovery experiments using 12 time increments. Thirty time increments were used to measure the imino proton relaxation rates. The exchange times were computed from the best fit of expression [1] using 16−24 time increments. The exchange contribution of NH_3, τ_{ex,NH_3} was determined from the exchange times measured in the presence (τ_{ex}) and in the absence (τ_{ex0}) of ammonia by

$$\tau_{ex,NH_3} = (1/\tau_{ex} - 1/\tau_{ex0})^{-1}$$

In some cases, the exchange time contribution of the proton acceptor was obtained from the difference of the imino proton relaxation rates, R_0 and R_{ex}, measured by inversion−recovery in the absence and presence of proton acceptor according to

$$\tau_{ex,NH_3} = (R_{ex} - R_0)^{-1}$$

To avoid spurious effects due to the salt-induced change of the relaxation rates (*13*), this method was only applied when the ionic strength change due to the added proton acceptor is negligible. This was the case, for example, for the exchange time measurements versus pH.

Imino Proton Exchange Theory. The formalism of catalyzed proton exchange has been extensively described (*1*). The salient features relevant to this study are summarized below. In an isolated nucleoside, the imino proton exchange rate induced by a proton acceptor such as NH_3 is

$$k_{ex,acc} = k_{coll}[acc]/(1 + 10^{\Delta pK})$$

where k_{coll} is the collision rate, [acc] the proton acceptor concentration, and ΔpK the pK difference between the imino proton ($pK_G = 9.3$, $pK_T = 10.3$) and the proton acceptor ($pKNH_3 = 9.3$).

Imino proton exchange from a base pair is a two-step process requiring base-pair opening, followed by transfer to a proton acceptor. The proton acceptor contribution to the exchange time is given by

$$\tau_{ex,cat} = \tau_0 + 1/(k_{ex,acc}\alpha K_{diss})$$

where τ_0 is the base-pair lifetime, K_{diss} the base-pair dissociation constant, and $k_{ex,acc}$ the proton-transfer rate from the open pair. α is an accessibility factor, which should be equal to one for an imino proton fully accessible in the open pair. According to this model, the plot of $\tau_{ex,cat}$ versus the inverse of catalyst concentration is a straight line whose extrapolation to infinite catalyst concentration yields the base-pair lifetime τ_0. The apparent dissociation constant, αK_{diss}, is obtained according to expression 2 from the ratio of the rates of exchange catalysis measured in the duplex and for the isolated nucleoside (*9*). The apparent open-pair lifetime, $\alpha\tau_{open}$, is equal to the product $\tau_0\alpha K_{diss}$.

RESULTS AND DISCUSSION

Effect of NH_4^+ on the Base-Pair Kinetics and Chemical Shifts of [d(CGCGAATTCGCG)]$_2$. Ammonia-Catalyzed Imino Proton Exchange. At 20 °C, pH 8.8, in the absence of ammonia, the exchange time of the imino proton of pair A5•T is about 1.5 s. For A6•T, C3•G, and G4•C, it is longer than 2 s, the upper limit of the values accessible to magnetization transfer, and shorter than 30 s, the lower limit of the values accessible to H/D real time exchange experiments. The NH_3 contribution, τ_{ex,NH_3}, to imino proton exchange from pairs C3•G, G4•C, A5•T, and A6•T measured at variable ($[NH_3] + [NH_4^+]$) concentration and in 2 M ($[NH_3] + [NH_4^+]$) is plotted versus $1/[NH_3]$ in Figure 1. The τ_{ex,NH_3} values measured at variable ($[NH_3] + [NH_4^+]$) concentration are in agreement with those, measured in the same condition, by Wärmländer et al. For A6•T imino proton in particular, the plot of τ_{ex,NH_3} versus $1/[NH_3]$ presents a "curvature" around $1/[NH_3] \sim 30\ M^{-1}$ and $\tau_{ex,NH_3} \sim 0.1$ s, as reported in re *10*. The corresponding NH_4^+ concentration is about 0.1 M. Similarly, we find that the τ_{ex,NH_3} values measured for A5•T imino proton in the higher and lower NH_3 concentration ranges are not aligned on the same straight line (Figure 1). The good agreement of the τ_{ex,NH_3} values obtained by magnetization transfer (this work) and from the variation of the inversion−recovery rate upon ammonia addition (*10*) confirms the nonlinearity of the NH_3 contribution to A6•T and A5•T imino proton exchange in such condition. Furthermore, the exchange times are nearly unchanged in the presence of 2 M NaCl or KCl (Figure 1). This indicates that the nonlinearity of τ_{ex,NH_3} versus $1/[NH_3]$ is not due to the variation of the ionic strength caused by the increasing NH_4^+ concentration.

In the range of the NH_3 concentrations explored, the plots of τ_{ex,NH_3} versus $1/[NH_3]$ are linear for the G•C imino protons (Figure 1). Their slopes reflect the high stability of the G•C pairs. τ_{ex,NH_3} becomes inaccessible to NMR measurements when $1/[NH_3] > 80\ M^{-1}$ in the case of C3•G and $1/[NH_3] > 1/20\ M^{-1}$ in the case of G4•C. It should be noted that a nonlinearity of the plot the τ_{ex,NH_3} versus $1/[NH_3]$ could be missed since the measurements were restricted to a rather narrow concentration range.

In 2 M ($[NH_3] + [NH_4^+]$), 20 °C, pH 6.1, the exchange times of the imino protons of the four inner pairs fall in a range of value ($2\ s < \tau_{ex} < 30\ s$) inaccessible to NMR measurements.

The two magnetization transfer experiments from water displayed in Figure 2 show the effect of NH_4^+ on the imino proton exchange catalysis of A6•T. Both experiments were performed with the same NH_3 concentration, $90\ M^{-1}$, either in the presence of 35 mM $[NH_4^+]$, pH 8.8, or in 1.99 M $[NH_4^+]$, pH 7.04. The exchange times derived according to eq 1 from the evolution of the imino proton magnetization after water inversion are respectively 0.14 and 1 s. The insensitivity of A6•T imino proton to hydroxyl catalysis in this pH range (Figure 3), together with the identity of the chemical shifts measured in this pH range, establishes that the difference is related to the NH_4^+ concentration.

The plots of τ_{ex,NH_3} versus $1/[NH_3]$ in 2 M ($[NH_3] + [NH_4^+]$), pH 7.1−9.1, are displayed in Figure 1. The absence

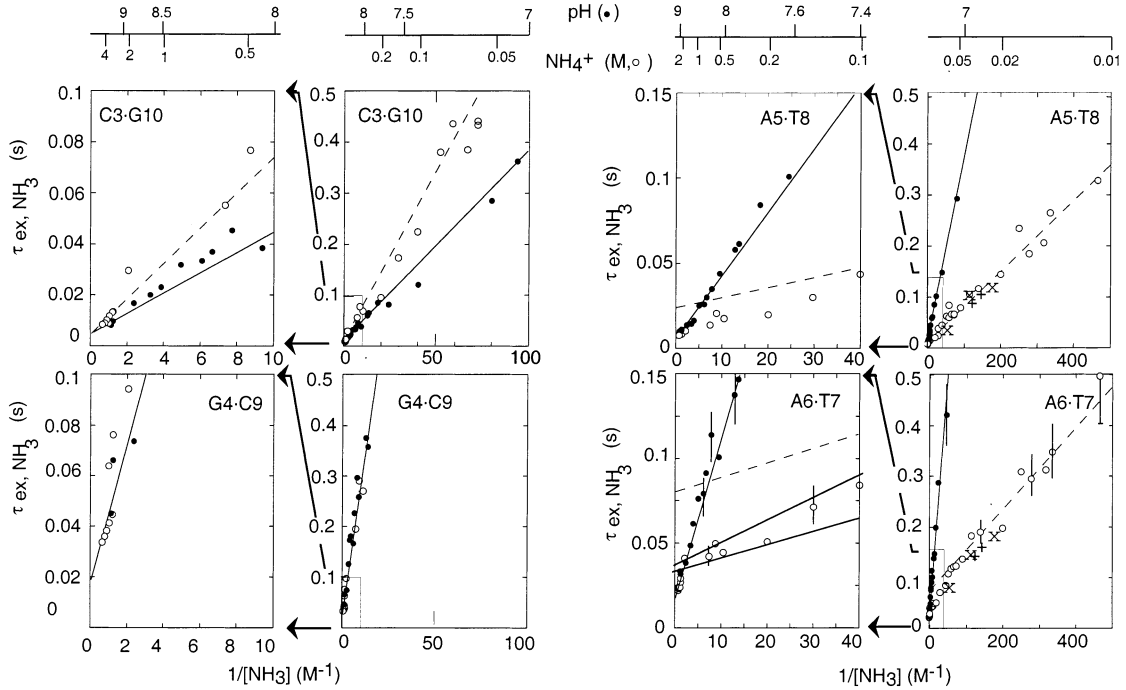

FIGURE 1: Ammonia contribution, τ_{ex,NH_3}, to imino proton exchange in [d(CGCGAATTCGCG)]$_2$ vs 1/[NH_3]. For each base pair, the lower left corner of the right panel is enlarged on the left side. (O) τ_{ex,NH_3} was measured at variable ([NH_3] + [NH_4^+]) concentrations, pH 8.8. Hence, [NH_3] and [NH_4^+] were increased in the same proportion, respectively, from 2 mM to 1.4 M and 6.3 mM up to 4.4 M. The exchange times are unchanged in 2 M NaCl (+) or KCl (X) solutions. (l) τ_{ex,NH_3} was measured in 2 M ([NH_3] + [NH_4^+]). [NH_3] was controlled by the solution pH (pH 7.1−9.1). [NH_4^+] was therefore maintained between 1.22 and 1.99 M. The upper horizontal scale indicates the NH_4^+ concentration in the experiment performed at variable ([NH_3] + [NH_4^+]) concentration, pH 8.8, and the pH of the measurements in 2 M ([NH_3] + [NH_4^+]). The ammonia contribution to imino proton exchange vs 1/[NH_3] is a straight line when the NH_4^+ concentration is lower than 30 mM (dashed lines) or higher than 1 M (full lines). The full and the dashed lines of the right and left panels have, respectively, the same slopes and the same extrapolations at 1/[NH_3] = 0. The nonlinearity of the plot of the τ_{ex,NH_3} values measured at variable ([NH_3] + [NH_4^+]) concentration for the imino protons of A6•T and A5•T is related to a conformational change induced by NH_4^+. T = 20 °C, [NaCl] = 0.1 M.

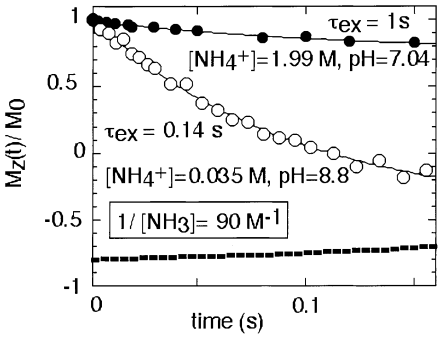

FIGURE 2: Evolution of the normalized magnetization of A6•T imino proton after selective inversion of the water magnetization. (l) In 2 M ([NH_3] + [NH_4^+]), pH 7.04, and (O) in 46 mM ([NH_3] + [NH_4^+]), pH 8.8. The NH_3 concentration is the same in both experiments: 1/[NH_3] = 90 M^{-1}. The solid lines are the best fits computed according to expression 1, and the corresponding exchange times values are indicated. The heavy dashed line shows the evolution of the water magnetization. T = 20 °C, [NaCl] = 0.1 M.

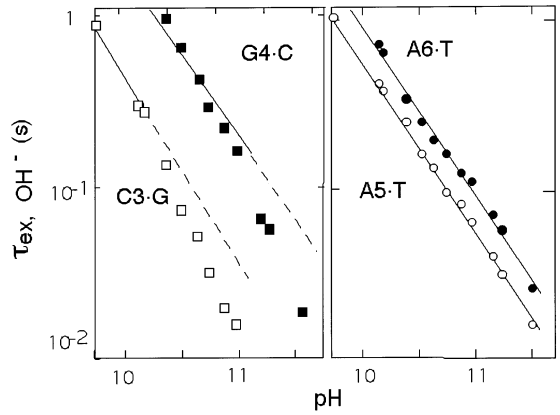

FIGURE 3: Imino proton hydroxyl catalysis, τ_{ex,OH^-} in [d(CGCGAATTCGCG)]$_2$. (**o**) C3•G, (n) G4•C, (O) A5•T, and (l) A6•T. The full line with a −1 slope is characteristic of catalysis by OH^-. Deviation from linearity above pH 10.4 and 11.4 for, respectively, C3•G and G4•C reflects the deprotonation of the outermost pairs. T = 20 °C, [NaCl] = 0.1 M.

of effect of pH on the imino, aromatic, and methyl proton chemical shifts establishes that the duplex structure is not modified in this pH range (Figure S1). For A6•T and A5•T, τ_{ex,NH_3} versus 1/[NH_3] does not show the nonlinearity observed when both [NH_3] and [NH_4^+] are increased together. Instead, the plot of τ_{ex,NH_3} is a straight line, as expected in a two-state model of the pairs. By comparison with the measures at low NH_4^+ concentration, the slopes of the plots indicate an increase of the base-pairs stabilities, but the base-pair lifetimes and dissociation constants are consistent with those derived from the measurements performed in the higher concentration range of the experiments at variable ([NH_3] + [NH_4^+]) concentration. The comparison of the experiments performed in 2 M and variable ([NH_3] + [NH_4^+]) concentrations shows that τ_{ex,NH_3} versus 1/[NH_3] is a straight line when [NH_4^+] < 30 mM and when [NH_4^+] > 1M.

These observations are inconsistent with the existence of two distinct open states. This suggests that the nonlinearity of the plots of τ_{ex,NH_3} measured at variable ([NH_3] + [NH_4^+])

Table 1: Lifetimes τ_0, Apparent Dissociation Constants αK_{diss}, and Apparent Open State Lifetimes $\alpha\tau_{Op}$ at 20 °C of the Four Inner Base Pairs of $[d(CGCGAATTCGCG)]_2$ and $[d(GCA_4T_4GC)]_2$[a]

		$[d(CGCGAATTCGCG)]_2$				$[d(GCA_4T_4GC)]_2$			
	[NH_4^+]	C3•G	G4•C	A5•T	A6•T	A3•T	A4•T	A5•T	A6•T
τ_0 (ms)	<30 mM	6.3 ± 1	*b*	23 ± 3	81 ± 5	2 ± 1	71 ± 5	145 ± 10	135 ± 10
	>1 M	6.3 ± 1	21 ± 3	5 ± 1	20 ± 3	*c*	*c*	85 ± 7	40 ± 4
αK_{diss} **(× 10^7)**	<30 mM	9 ± 1	*b*	79 ± 5	66 ± 7	160 ± 10	2 ± 0.3	6 ± 1	4 ± 0.5
	>1 M	5 ± 1	1 ± 0.07	17 ± 2	5 ± 0.7	*c*	*c*	0.6 ± 0.1	0.5 ± 0.1
$\alpha\tau_{op}$ (ns)	<30 mM	5.6 ± 1.5	*b*	180 ± 35	535 ± 86	32 ± 18	14 ± 3	87 ± 20	54 ± 11
	>1 M	3.1 ± 1.1	2.1 ± 0.4	8.5 ± 2.7	10 ± 3	*c*	*c*	5 ± 1.2	2 ± 0.6

[a] The kinetic parameters describing the base-pair opening kinetics of each species are derived from imino proton catalysis by NH_3 measured at low ([NH_4^+] < 30 nM) and high (1 < [NH_4^+] < 4.4 M) ammonium concentrations (see Figures 1 and 3. [b] Exchange times inaccessible to NMR measurements. [c] Not measured due to spectral overlap.

concentration is due to the simultaneous presence of two exchanging conformations of the duplex, in proportions depending on the NH_4^+ concentration. The plot of τ_{ex,NH_3} versus 1/[NH_3] is nonlinear when the increase of the ammonia concentration change the native conformation from the low NH_4^+ to the high NH_4^+ conformation.

The A•T base-pair lifetimes and open-state lifetimes of each conformation are distinct, but for each conformation, the imino proton exchanges by way of a single base-pair opening mode. For A6•T, the lifetimes are respectively 81 ms and 535 ns in low NH_4^+ and 20 ms and 10 ns in high NH_4^+. The kinetics parameters derived from the fits of the exchange experiments (solid and dashed lines of Figure 1) at low ([NH_4^+] < 30 mM) and high ([NH_4^+] > 1M) ammonium concentrations are displayed in Table 1. The lifetimes of the A•T pairs of the species that dominates at high [NH_4^+] concentration are consistent with the published values measured at high ammonia concentration (1). At low [NH_4^+] concentration, the A•T base-pair lifetimes are about 4 times longer. The open-state lifetimes of the A•T pairs of each species are in the upper and lower ranges of the values measured in B DNA duplexes (19). NH_4^+ has little effect (C3•G) or no effect (G4•C) on the kinetics of the G•C pairs.

Hydroxyl Catalysis. The hydroxyl contribution, τ_{ex,OH^-}, to imino proton exchange is plotted versus pH in Figure 3 for the four inner pairs. The apparent base-pair dissociation constants may be determined from the hydroxyl catalysis when deprotonation of the bases is negligible. The half-dissociation pH of a base pair, $pH_{1/2}$, is related to its dissociation constant and to the imino proton pK_i by $pH_{1/2} = pK_i - \log(K_{diss})$ (*20*). The OH^--induced base-pair dissociation first affects C1•G whose dissociation constant at 20 °C is expected to be in the range of 0.1 (20).

The plots of log τ_{ex,OH^-} versus pH have a slope of −1 up to about pH 10.3 (Figure 3). The apparent dissociation constants are respectively 3.5×10^{-4} for G2.C (data not shown), 0.25×10^{-6} for C3•G, 0.05×10^{-6} for G4•C, 0.2×10^{-6} for A5•T, and 0.12×10^{-6} for A6•T. It is noteworthy that the values for the A•T pairs are much smaller (about 50 times) than those derived from the NH_3 catalysis measured at low NH_4^+ concentration (Table 1). Lower apparent dissociation constants are commonly found for negatively charged proton acceptors (*9*). This effect is due to their low local concentration near the negatively charged duplex. Nevertheless, the large difference between the apparent dissociation constants derived from exchange catalysis by NH_3 and OH^- for the A•T pairs suggests a restricted access of OH^- in the A-tract.

The pH-dependent disruption of the outermost pairs is expected to occur around pH 10.3 (see above). The deviations from linearity observed around pH 10.3 and pH 11 for, respectively, C3•G and G4•C indicate a destabilization of the pairs due to end-fraying propagation. This may also account for the observation of shorter lifetimes at pH 9.5 than at pH 8.8 (*10*). The deprotonation of the terminal pair probably modifies the kinetics of the A•T pairs. For this reason, the hydroxyl catalysis of A6•T and A5•T imino protons cannot provide argument for or against the occurrence of multiple opening modes.

NH_4^+-Induced Chemical Shifts in $[d(CGCGAATTCGCG)]_2$. Due to the negative charge of the phosphate groups, the DNA structure may depend on the cation concentration. A well documented example is that of the B to Z transition (*21* and references therein). The high NH_3/NH_4^+ concentration required to determine the base-pair lifetimes may affect the duplex structure. This possibility had been already examined in particular in B DNA duplexes (*12*, *14*). It is generally observed that the effects of NH_4^+ and Na^+ on the NMR spectra are roughly comparable. The line broadening observed at high ionic strength is attributed to the increase of the correlation time induced by intermolecular interactions (*11*, *13*). At high duplex concentration, the relatively large chemical shifts of the aromatic protons of the terminal pairs are indicative of end-to−end stacking interactions.

The effects of Na^+ and NH_4^+ on the aromatic and imino proton regions of the $[d(CGCGAATTCGCG)]_2$ spectrum are displayed in Figure 4. The comparison of the spectra obtained in 15 mM and 1.92 M NaCl indicates that Na^+ has little effect on the duplex structure. On the contrary, NH_4^+ induces chemical shift variations, especially for A5(H2) and A6(H2), which are upfield shifted by, respectively, 0.3 and 0.18 ppm in 3.8 M NH_4^+. The NH_4^+-induced shifts of T7(H3), T8-(H3), G4(H1), and, to a lesser extend, A6(H8) are also significantly larger that those induced by Na^+. The small chemical shifts induced by NH_4^+ and Na^+ on the methyl and sugar protons are comparable (not shown). The gradual shifts of the A(H2) protons versus [NH_4^+] indicate a fast exchange on the NMR time scale between different conformations. The localization of the protons shifted by NH_4^+ in the AATT region correlates with the effect of NH_4^+ on the A•T base-pair kinetics. The observation for the T imino protons of downfield shifts for [NH_4^+] < 1 M and of upfield shifts at higher NH_4^+ concentration suggests that the conformational exchange could involve more than two species. At 0 °C, the 800 MHz spectrum does not show line splitting

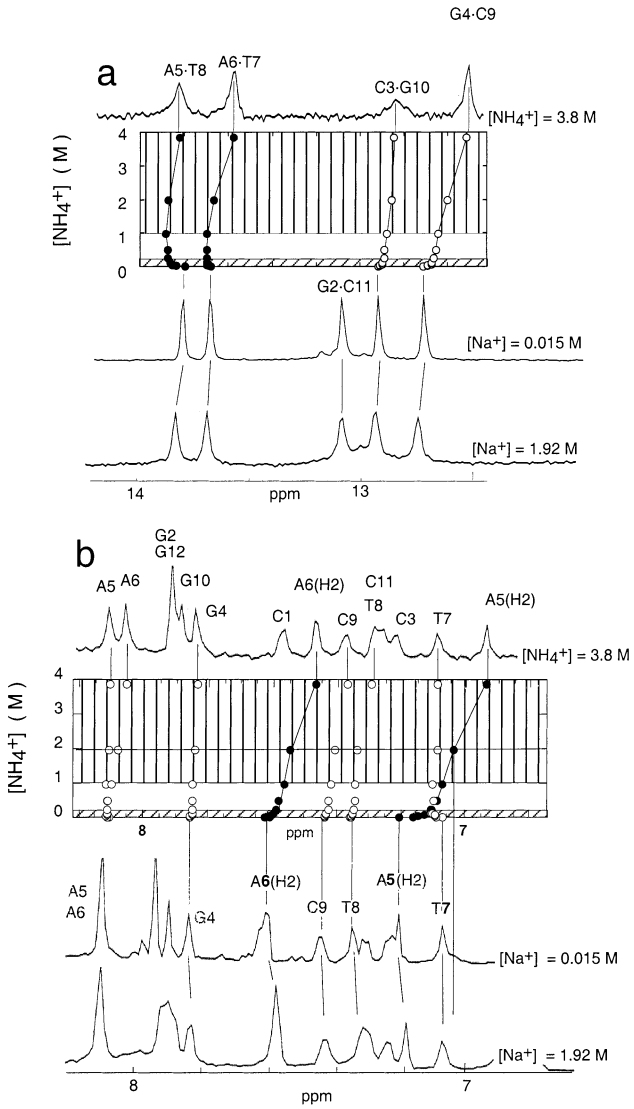

FIGURE 4: Imino (a) and aromatic (b) proton chemical shifts vs $[NH_4^+]$ and $[Na^+]$ in $[d(CGCGAATTCGCG)]_2$. Na^+ addition has little effect on the chemical shifts. By contrast, specific shifts are induced by NH_4^+, in particular, for A5(H2) and A6(H2) (black circles) and for the G4 and T imino protons. The shifts indicate an NH_4^+-dependent equilibrium between distinct species. The diagonally and vertically stippled areas show, respectively, the NH_4^+ concentration ranges that allowed the characterization of the base-pair kinetics of the species that predominate at low and high ammonium concentrations. $T = 20$ °C

or line broadening indicating that the conformational exchange approaches the slow exchange condition.

Other Duplexes. With a tract of four consecutive A•T pairs lacking a 5′ T−A 3′ step, $[d(CGCGAATTCGCG)]_2$ belongs to the B′ DNA family. The A-tracts of B′ DNA duplexes have a number of structural and kinetics properties. The lifetimes of the A•T pairs are markedly longer than in B DNA duplexes (*3*). Their narrow minor groove is occupied by a network of H-bonded water molecules (the hydration spine, *22*), whose residence times are slightly longer than those of the hydration water molecules of B DNA duplexes (*23*). A recent investigation on NH_4^+ binding indicates a specific localization of NH_4^+ in the narrowest section of the minor groove of $[d(GCA_4T_4GC)]_2$, close to the A5•T and A6•T pairs (*16*). This prompted us to select this duplex for an imino proton exchange study.

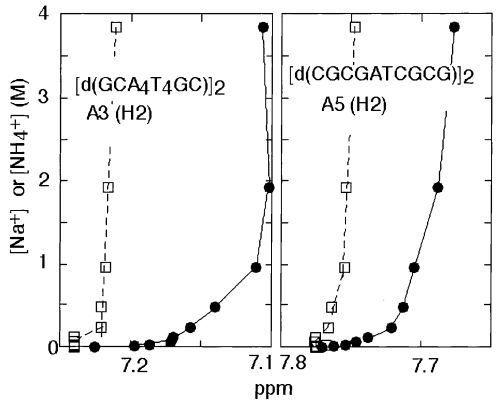

FIGURE 5: Chemical shifts of A5(H2) in $[d(CGCGATCGCG)]_2$ (right panel) and of A3(H2) in $[d(GCA_4T_4GC)]_2$ (left panel) vs the NaCl (□), and the NH_4Cl (●) concentrations. pH 8.8, $T = 20$ °C.

We also examined the effect of NH_4^+ on the imino proton exchange rates of $[d(CGCGATCGCG)]_2$, a duplex which we consider a benchmark for imino proton exchange studies on B DNA.

$[d(GCA_4T_4GC)]_2$. As with $[d(CGCGAATTCGCG)]_2$, the plots of τ_{ex,NH_3} versus $1/[NH_3]$ are linear for A6•T and A5•T imino protons in the experiments performed in 2 M ($[NH_3] + [NH_4^+]$), whereas they present a curvature around $NH_4^+ \sim 0.1$ M in the experiment at variable ($[NH_3] + [NH_4^+]$) concentration (Figure S2). The kinetics parameters derived from the exchanges times measured at low ($[NH_4^+] < 0.03$ M) and high NH_4^+ ($NH_4^+ > 1$ M) concentrations are displayed in Table 1. The parameters could not be determined at high NH_4^+ concentration for pairs A4.•T and A3•T. due to overlap of their imino proton peaks. Once again, the chemical shifts induced by NH_4^+ on the A(H2) protons are much larger than those induced by Na^+ and as for $[d(CGCGAATTCGCG)]_2$, the largest NH_4^+-induced shift is that of the first adenine of the A-tract (Figure 5).

$[d(CGCGATCGCG)]_2$. Imino proton exchange at variable ($[NH_3] + [NH_4^+]$) concentration has been extensively investigated in this duplex (*2*, *13*). For comparison, we measured the imino proton exchange catalysis in 2 M ($[NH_3] + [NH_4^+]$). The rates of exchange catalysis of the G•C imino protons are identical to those previously measured at variable ($[NH_3] + [NH_4^+]$) concentration. For A5•T imino proton, τ_{ex,NH_3} versus $_{1/[}NH_3]$ extrapolates to the same lifetime, ca. 3 ms, as that in the experiments performed at variable ($[NH_3] + [NH_4^+]$) concentration, but the slope is 2.5 times larger. This indicates either a restricted access for NH_3 or a shorter open-state lifetime in 2 M ($[NH_3] + [NH_4^+]$). In the experiments performed at variable ($[NH_3] + [NH_4^+]$), the slope variation should induce a curvature of the plot of τ_{ex,NH_3}. However, considering the limited accuracy of the exchange time measurements in the millisecond range and the weakness of the effect, the corresponding curvature is beyond detection. Although NH_4^+ has little effect on the exchange catalysis of A5•T imino proton, we observe a specific NH_4^+-induced shift of A5(H2) with amplitude comparable to those measured for the A(H2) protons of A-tracts (Figure 5).

Structural Change Induced by NH_4^+. The observation of different values of the base-pair lifetimes at low and high NH_4^+ concentrations in $[d(CGCGAATTCGCG)]_2$ and $[d(GCA_4T_4GC)]_2$, shows that the anomalous imino proton exchange kinetics of their A•T pairs is related to the NH_4^+

concentration and that it does not reflect the existence of two distinct opening mode of the A•T pairs. NH_4^+ binding in the A-tract narrow groove may explain the alteration of the base-pair kinetics, but the amplitude of the NH_4^+-induced shift of the A(H2) and imino protons suggests a structural effect of this cation. Nevertheless, the nature of the structural change remains unclear. The coupling constants measured on the COSY spectra of $[d(CGCGAATTCGCG)]_2$ in 0.1 M NaCl and in 0.5 M NaCl or NH_4Cl do not show any indication for an effect of NH_4^+ on the sugar puckers. The cross-peaks intensities of NOESY spectra collected at different Na^+ and NH_4^+ concentrations are comparable within experimental error (Figure S3). The cross-strand distance between A(H2) and the H1′ proton of the residue adjacent to the paired thymidine in the 3′ direction is correlated with the minor groove width (*24*). The values measured in 0.1 M NaCl are in good agreement with those previously reported (*24*, *25*). For $[d(CGCGAATTCGCG)]_2$ we measure 3.8 ± 0.2 Å for A6(H2)−T8(H1′) and 4.1 ± 0.2 Å for A5(H2)−C9(H1′). For $[d(GCA_4T_4GC)]_2$ they are in the range of 3.7 ± 0.2 Å for A6(H2)−T8(H1′) to 4.6 ± 0.3 Å for A3(H2)−G11(H1′), and for $[d(CGCGATCGCG)]_2$ we find 4.1 ± 0.2 Å. The distances measured on the NOESY spectra collected in 1 M NH_4Cl are not significantly different. This shows that the groove width is not strongly affected by NH_4^+ binding (Figure S3). We note that this result is consistent with a molecular dynamic simulation predicting that monovalent cation binding in the minor groove of the AATT segment should not affect the groove width (*26*).

The base-pair kinetics is quite sensitive to the nucleic acid structure. The G•C lifetimes, for example, are increased by 2 orders of magnitude in Z DNA (*27*), and the A•T lifetimes of A-tracts are typically 10−100 times longer than in B DNA duplexes (*3*). In this context, it is conceivable that the modest differences (a factor of 2 to 4) of the A•T lifetimes measured at low and high ammonia concentrations could result from a minor structural rearrangement which would only slightly affect the NMR spectra.

Recent investigations reporting the presence of mono (*28*) and divalent cations (*25*) in the minor groove of A-tracts infer that cation binding may contribute to DNA bending. Ammonium, in particular, is suggested to have a crucial role in the A-tract curvature (*29*). But a macroscopic curvature resulting from the accumulation of small local distortions of the DNA helix might not be detected by COSY and NOESY experiments which are sensitive to local structure, i.e., to dihedral angles and to distances shorter than 5 Å.

CONCLUSION

The imino proton exchange catalysis of $[d(CGCGAATTCGCG)]_2$ and $[d(CGA_4T_4CG)]_2$ reveals an alteration by NH_4^+ of the A•T opening-closing kinetics in DNA A-tracts. This, together with the observation of chemical shifts specific of NH_4^+, argues for the occurrence of two fast exchanging structures of the A-tract. The lifetimes of the closed and open states of each structure are distinct, but from each structure, imino proton exchange occurs via a single opening mode. When the ($[NH_3] + [NH_4^+]$) concentration is increased, the combination of the effects of NH_3 on the exchange catalysis and of NH_4^+ on the A-tract structure results in a nonlinear dependence of the exchange contribution of the added catalyst versus $1/[NH_3]$. The present study shows that this may disturb the evaluation of the base-pair kinetic parameters. Nevertheless, due to its small size, high p*K*, good solubility, neutral character, and lack of detectable proton on the NMR spectrum at alkaline pH, NH_3 remains certainly the best choice for imino proton exchange studies. The nonlinearity of the trimethylamine exchange contribution (*10*) suggests that the trimethylamine cation and NH_4^+ might have a comparable effect on the structure and base-pair kinetics of $[d(CGCGAATTCGCG)]_2$.

The similarity of the chemical shifts induced on the H2 protons of $[d(CGCGATCGCG)]_2$ (0.13 ppm) and of the A-tracts (0.14−0.27 ppm) suggests that NH_4^+ binds with comparable affinities on both kind of sequences and has comparable structural effect. Nevertheless, NH_4^+ has only a modest effect on the closing rate of pair of the isolated A.T.

Once again, the base-pair kinetics proves to be a very sensitive probe of nucleic acid structures. The scantiness of the spectroscopic indications on the structural change induced by NH_4^+ contrasts with the conspicuous effects on the imino proton exchange. The structural changes induced by ammonium on the A-tract remain nonelucidated. The residual dipolar coupling methodology which extends the range of the distances accessible to NMR is a promising tool for further structural investigations.

Earlier proton exchange experiments in A-tracts focused on the determination of the base-pair lifetimes. For this reason, they were performed in the presence of high ($[NH_3] + [NH_4^+]$) concentrations (*1*, *3*, *30*). Hence, they describe the base-pair opening kinetics of the conformation stabilized by NH_4^+. It is likely that, as shown for $[d(CGCGAATTCGCG)]_2$, $[d(CGCA_8CGC)/d(GCGT_8GCG)]$ (*10*), and $[d(GCA_4T_4GC)]_2$, the A•T lifetimes in A-tract at low NH_4^+ concentration are appreciably longer than reported earlier.

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SUPPORTING INFORMATION AVAILABLE

Figure S1 shows that the chemical shifts of the imino, aromatic, and methyl proton of $[d(CGCGAATTCGCG)]_2$ are unchanged between pH 7.1 and 9.1. This establishes that the duplex structure is not altered by the pH variation in the exchange experiment performed in 2 M ($[NH_3] + [NH_4^+]$), pH 7.1−9.1. Solution conditions: $T = 20$ °C, [NaCl] = 0.1 M, $NH_4^+ = 2$ M. Figure S2 shows the effect of NH_4^+ on the exchange catalysis by NH_3 of the imino proton of A6•T in $[d(GCA_4T_4GC)]_2$. Solution conditions: $T = 20$ °C, [NaCl] = 0.1 M. Figure S3: The aromatic-H1′ region of the NOESY spectra of $[d(CGCGAATTCGCG)]_2$ in 0.5 M NaCl and in 0.5 M NH_4Cl. This material is available free of charge via the Internet at http://pubs.acs.org.

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