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"Asymmetric" Opening Reaction Mechanism of Z-DNA Base Pairs: A Hydrogen Exchange Study†

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ABSTRACT: With the tritium–Sephadex method, the hydrogen-exchange kinetics of the five NH protons of guanine and cytosine residues in Z-form poly(dG-dC)·poly(dG-dC) were measured as a function of temperature and catalyst concentration. Over the measured temperature range from 0 to 34 °C, two classes of protons with constant amplitudes are found. The three protons of the fast class, which were assigned to the guanine amino and imino protons, have an exchange half-time in the minute time range (at 20 °C the half-time is 2.5 min) and an activation energy of 18 kcal mol^{-1}. Since these two types of protons exchange at the same rate in spite of their grossly different p*K* values, the exchange of these protons must be limited by the same nucleic acid conformational change. The two cytosine amino protons of the slow class are especially slow with exchange half-times in the hour time range (at 20 °C the exchange half-time is 1 h) and the activation energy is 20 kcal mol^{-1}. The exchange of these two protons is not limited by some nucleic acid conformational change as shown by the marked exchange acceleration of these protons upon addition of 0.2 M imidazole. In addition, we have also reexamined the hydrogen–deuterium exchange kinetics of the amino protons of guanosine cyclic 2′,3′-monophosphate by a spectral difference method using a stopped-flow spectrophotometer. The measured kinetic process is monophasic with a rate constant of 3 s^{-1} at 20 °C, which is in the same range as the predicted rate constant of the guanine amino protons. From the measurement of this exchange rate as a function of temperature between 10 and 35 °C, an apparent activation energy of 16 kcal mol^{-1} is found. The very peculiar dependence of the rate constant upon pH confirms the proposed general exchange mechanism that requires a preprotonation on the N_7 position of guanine. A quantitative analysis of the Z-form poly(dG-dC)·poly(dG-dC) hydrogen-exchange data with the classical two-step exchange mechanism (closed $\rightleftarrows$ open $\rightarrow$ exchanged) leads to the prediction of a non-opening-limited exchange of the guanine amino protons, thus conflicting with the experimental data. We discuss two extreme exchange mechanisms that can account for our set of data. The common feature of both mechanisms is the existence of an "asymmetric" open state where the guanine protons are more likely to be in contact with the solvent than the cytosine protons.

There is now ample experimental evidence showing that the conformation of a macromolecule in solution is subjected to thermally driven fluctuations [for recent reviews, see Karplus & McCammon (1981) and Sarma (1981)]. In double-stranded nucleic acids a major fluctuation mode is characterized by the opening and closing of the base pairs, the so-called "breathing" reaction (Englander & Kallenbach, 1983). As the nucleic acid hydrogen exchange kinetic is modulated by this base-pair opening reaction, the hydrogen-exchange measurements are a valuable approach to the study of the mechanism of the opening and closing reaction in duplexes.

In a previous study, using the tritium–Sephadex method worked out by Englander and colleagues, we have shown that the Z conformation of poly(dG-dC)·poly(dG-dC) is characterized by a surprisingly overall slow exchange rate of the five protons involved in hydrogen bonds between guanine and cytosine residues (Ramstein & Leng, 1980). In particular, the exchange of two protons is remarkably slow, their exchange half-time being 7 h at 0 °C. These results were confirmed by infrared spectroscopy (Pilet & Leng, 1982) and more recently by NMR (Mirau & Kearns, 1984).

The protons of poly (dI-br^5dC)·poly(dI-br^5dC) in Z form presented a similar overall slow exchange kinetic with a slow class of protons having an exchange half-time of 14 h at 0 °C (Hartmann et al., 1982). A comparison of these results with those previously obtained with poly(dG-dC)·poly(dG-dC) in the Z form allowed us to identify the two slow-exchanging protons with exchange half-times in the hour time range as being the cytosine amino protons. Having identified the exchanging protons and postulating for the Z-form DNA the same exchange mechanism as for the B-form DNA, we were able to give a first quantitative description of the Z-form dynamic structure. The main conclusion was that the open state is approximately 50 times less likely in the Z-form DNA as compared to the B-form DNA.

In order to obtain a more detailed picture of the opening and closing reaction of the base pairs in the Z-form DNA, we have measured the proton-exchange kinetics as a function of temperature and of proton-exchange catalyst concentration. In addition, we have studied the exchange kinetics of the guanosine cyclic 2′,3′-monophosphate amino protons as a function of temperature and pH. Altogether, these results lead us to the conclusion that the exchange process operative so far in B DNA and A RNA, in which open states both the guanine and cytosine protons are exchanging freely and simultaneously with solvent, cannot account for the proton-exchange kinetics in Z DNA. In contrast, these results suggest that the guanine residue is much more likely to be in contact with the solvent than the cytosine residue. We discuss two extreme mechanisms that meet this requirement.

Experimental Procedures

Materials

Poly(dG-dC)·poly(dG-dC) was obtained from P-L Biochemicals and guanosine cyclic 2′,3′-monophosphate was from

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Boehringer. For concentration determinations the following extinction coefficients were used: 6700 mol^{-1} cm^{-1} and 7000 mol^{-1} cm^{-1} at 260 nm for poly(dG-dC)·poly(dG-dC) in Z form and B form, respectively, and 13 700 mol^{-1} cm^{-1} at 252 nm for guanosine cyclic 2′,3′-monophosphate.

Sodium perchlorate (Merck), sodium cacodylate (Prolabo), Tris (Boehringer), sodium citrate (Merck), and imidazole (Janssen chimica) were used in the highest purity available. Deuterium oxide (99.5%) and tritiated water (1 Ci/mL) were purchased from C.E.A. (Saclay, France). A Tacussel Minisis pH meter was used to measure the pH. The pD values of the deuterium oxide solutions were determined by the approximation of Glasoe & Long (1960); $pD = pH_{meter} + 0.4$. All pH values were measured at 20 °C.

The chloro(diethylenetriamine)platinum(II) chloride, which we shall abbreviate as dien-Pt was a gift of Dr. J. P. Macquet (Toulouse). The stochiometric reaction between dien-Pt and poly(dG-dC)·poly(dG-dC) on the N_7 ring position of guanine was performed as follows. To a standard solution of B-form poly(dG-dC)-poly(dG-dC) with a phosphate concentration [P] = 5.7×10^{-4} M in 10 mM $NaClO_4$ the computed volume of a solution of dien-Pt (0.24 mg/mL), so as to modify one-fifth of the guanine, is added. The reaction was run at 37 °C for 24 h in the dark.

Methods

Tritium–Sephadex Method. To measure the hydrogen-exchange kinetics in poly(dG-dC)·poly(dG-dC) and platinated poly(dG-dC)·poly(dG-dC), we have used the tritium–Sephadex method of Englander & Englander (1972). Briefly, the macromolecule was first incubated in the presence of tritiated water (10 mCi) for 24 h at 0 °C. The free tritiated water and the labeled nucleic acid were separated by Sephadex filtration (G-25 fine grade). After being passed through the Sephadex column, the fractions containing the nucleic acid were examined for radioactivity in an Intertechnique SL 30 liquid scintillation counter and for nucleic acid concentration in a Cary 210 spectrophotometer. All the handling of the Sephadex columns and exchanging solutions were performed in a thermostated room.

Stopped-Flow Kinetics. To measure the rate of hydrogen exchange in guanosine cyclic 2′,3′-monophosphate, we followed the deuterium labeling rate with a Durrum D110 (Dionex) stopped-flow instrument interfaced with a Hewlett-Packard 9826 computer. The progress curves were obtained as time-dependent change in transmittance at 290 nm. As the change in transmittance is small (<10%), this latter is proportional to the optical density. One hundred points per kinetic measurement were stored under computer control. About five individual rate measurements were averaged to improve the signal to noise ratio. These data were then represented in a semilogarithmic plot and analyzed for amplitude and rate constant with a linear least-squares routine.

The mixing dead time of the instrument was found to be approximately 3 ms, and the slide width was 0.5 mm. The ultraviolet light source was a 75-W xenon arc lamp. The optical path length in the observation cell was 2.0 cm.

All experiments were performed with drive syringes with a 1 to 1 mixing ratio. The final concentration of the mononucleotide after being mixed with the D_2O buffer in the stopped-flow was always 5.8×10^{-5} M (0.8 optical density at 290 nm). The temperature of the flow system (drive syringes, mixing and observation chambers) was controlled and maintained within ±0.2 °C of the desired temperature.

Circular Dichroism Spectra. Before all hydrogen-exchange measurements on native poly(dG-dC)·poly(dG-dC) and platinated poly(dG-dC)·poly(dG-dC), in order to check the conformation of the polymer, we recorded routinely a dichroism spectra with a Jobin Yvon Mark IV dichrograph.

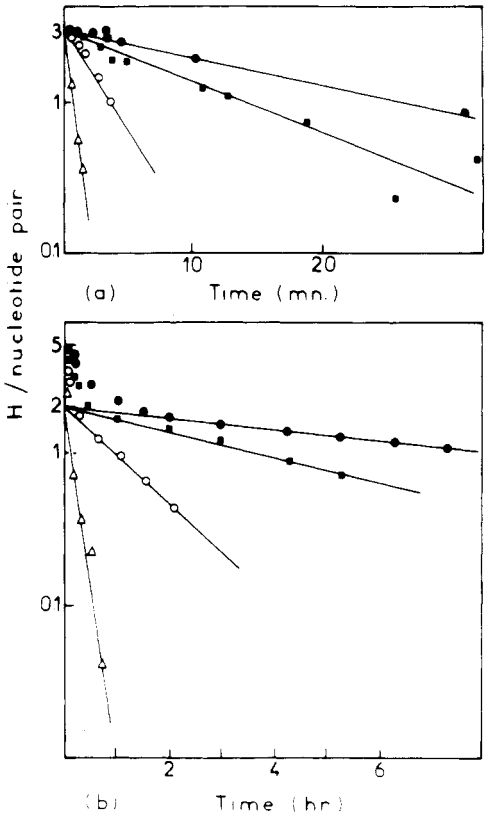


FIGURE 1: Hydrogen-exchange curves of Z-form poly(dG-dC)·poly(dG-dC) as a function of temperature. Buffer was 3 M sodium perchlorate and 10^{-2} M sodium phosphate, pH 7.5. (●) 0, (□) 10, (○) 20, and (△) 34 °C. (a) Semilogarithmic plot of the total hydrogen-exchange curves. The linear part corresponds to the exchange of the two protons of the slow class. (b) Semilogarithmic plot of the exchange of the fast class (three protons) obtained by subtracting the slow phase from the total exchange curve.

RESULTS

Z-Form DNA Hydrogen-Exchange Kinetics. (A) Effect of Temperature. We have measured the hydrogen-exchange kinetics of the five protons involved in hydrogen bonds between guanine and cytosine residues in Z-form poly(dG-dC)·poly(dG-dC) over a temperature range between 0 and 34 °C (Figure 1). At all measured temperatures, the exchange kinetic curves present two phases whose constant amplitudes are equal to three and two protons, respectively, for the fast and slow processes. The result that the three protons of the fast process exchange at the same rate at each temperature is very unexpected as will be discussed later (see Discussion). Activation energies determined from the slopes of the Arrhenius plots (Figure 2) are 18 kcal mol^{-1} and 20 kcal mol^{-1} for the fast and slow processes, respectively.

(B) Effect of Catalyst Concentration. To test the effect of catalyst concentration upon the proton-exchange kinetics in Z-form DNA, we have used poly(dG-dC)·poly(dG-dC) chemically modified by the dien-Pt on the N_7 of one-fifth of the guanine residues. This chemical modification stabilizes greatly the Z form, which is stable at lower ionic strength 0.2 M NaCl and 10 mM MgCl2, as compared to 3 M $NaClO_4$ for native poly(dG-dC)·poly(dG-dC) (Malfoy et al., 1981; Ushay et al., 1982). At this ionic strength, any possible interference between the salt (NaCl) and the catalyst (imidazole) should be negligible.

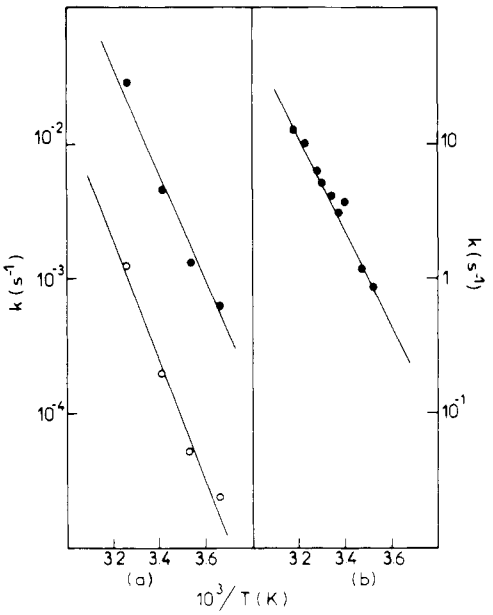


FIGURE 2: Arrhenius plots for hydrogen-exchange rate constants: (a) Z-form poly(dG-dC)·poly(dG-dC) fast phase (●) and slow phase (○). Buffer was 3 M sodium perchlorate and 10^{-2} M sodium phosphate, pH 7.5. (b) Guanosine cyclic 2′,3′-monophosphate. Buffer was 10^{-1} M sodium chloride and 10^{-3} M sodium cacodylate, pH 6.5.

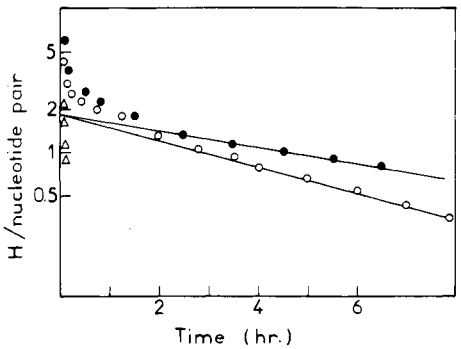


FIGURE 3: Hydrogen-exchange curve of platinated poly(dG-dC)·poly(dG-dC) (20% of the guanine residues are modified by the dien-Pt) at 0 °C as a function of catalyst concentration. (Z form) (●) 10^{-2} M magnesium chloride, 10^{-2} M sodium cacodylate, and 2×10^{-1} M sodium chloride, pH 6.5; (○) 10^{-2} M magnesium chloride, 10^{-1} M sodium cacodylate, and 2×10^{-1} M imidazole, pH 6.5. (B form) (△) 10^{-2} M sodium cacodylate, 2×10^{-2} sodium chloride, and 10^{-3} M EDTA, pH 6.5.

The hydrogen-exchange data in the absence and in the presence of 0.2 M imidazole (pH 6.5, 0 °C) of Z-form platinated poly(dG-dC)·poly(dG-dC) are presented in Figure 3, together with those corresponding to the B-form platinated poly(dG-dC)·poly(dG-dC). The hydrogen-exchange curves of the Z-form platinated poly(dG-dC)·poly(dG-dC) exhibit all the characteristic features of the Z-form native poly(dG-dC)·poly(dG-dC). The overall exchange is much slower than that for the B form, and the exchange curves reveal the presence of two exchange processes with exchange half-times in the minute and hour time ranges. The slow phase has an amplitude of nearly two protons with an exchange half-time of 5.5 h. Upon addition of catalyst there is a nearly 2-fold acceleration of the exchange rate whereas the amplitude stays constant. This result shows clearly that these slow protons are not exchanging according to an opening-limited mechanism (see Discussion). Incidentally, the equal acceleration of both protons implies also that these protons belong to the same chemical group.

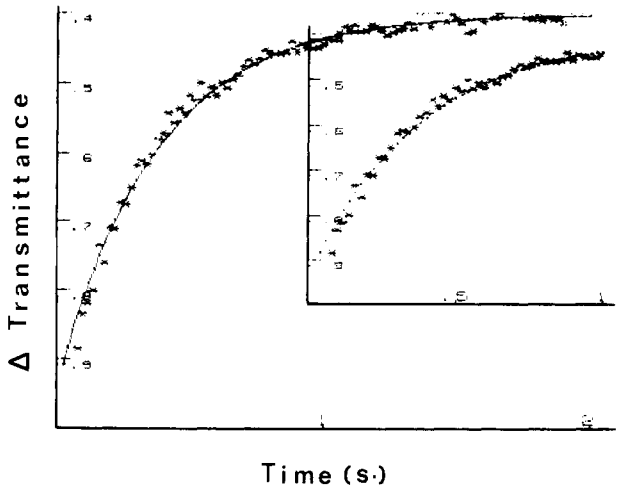


FIGURE 4: Typical kinetic trace for the hydrogen–deuterium exchange of guanosine cyclic 2′,3′-monophosphate at 20 °C observed by transmittance detection at 290 nm after stopped-flow mixing. Buffer was 10^{-1} M sodium chloride and 10^{-3} sodium cacodylate, pH 6.5.

The fast phase presents some heterogeneity in the exchange rates (as judged from the slight curvature of the semilogarithmic plot, results not shown) probably reflecting the presence of exchanging protons that belong to platinum group on the N_7 of the modified guanine residues. This could also explain that in the absence of imidazole the fast class has an amplitude of four protons instead of three for native poly(dG-dC)·poly(dG-dC). Therefore, we shall not try to make any quantitative comparison of the fast-class exchange rate in the presence and absence of catalyst.

Some results suggest that at high imidazole concentration this buffer interacts physically with the duplex (Mandal et al., 1979). In our experimental conditions the dichroism spectra of the Z-form poly(dG-dC)·poly(dG-dC) with and without imidazole are identical (results not shown). This result indicates that there is no strong interaction between the buffer and the double helix.

Exchange Rate of the Amino Hydrogens of Guanine: Effect of Temperature and pH. To interpret the hydrogen-exchange data of Z-form poly(dG-dC)·poly(dG-dC) in terms of dynamic structure, we have reexamined the chemistry of the guanine amino proton exchange by using the stopped-flow method with deuterium labeling.

A typical stopped-flow kinetic trace corresponding to the proton exchange in guanosine cyclic 2′,3′-monophosphate at 20 °C and recorded at 290 nm as transmittance is presented in Figure 4. The exchange curve is monophasic with an exchange rate of 3 s^{-1}. Evidently, this signal corresponds to the exchange of the amino protons, because owing to the much more favorable pK the exchange rate of the amino protons is expected to be 3 orders of magnitude greater (see Discussion). Under the same conditions of pH and temperature, the guanine amino protons in Z-form poly(dG-dC)·poly(dG-dC) have an exchange rate of 4.6×10^{-3} s^{-1}, which is 3 orders of magnitude smaller than that for the free guanosine.

We have measured the exchange rate over a temperature range from 10 to 35 °C. An Arrhenius plot of these results is given in Figure 2. The activation energy is 16 ± 1 kcal mol^{-1}. Cross et al. (1975) found for the amino protons of AMP an activation energy of 11.5 ± 0.5 kcal mol^{-1}.

The dependence of the guanine amino proton exchange rate on pH is shown in Figure 5. Three regions can be distinguished. Between pH 5 and pH 7.5, the exchange rate is essentially pH independent. Above pH 7.5, the exchange rate increases sharply with pH whereas below pH 5 there is a

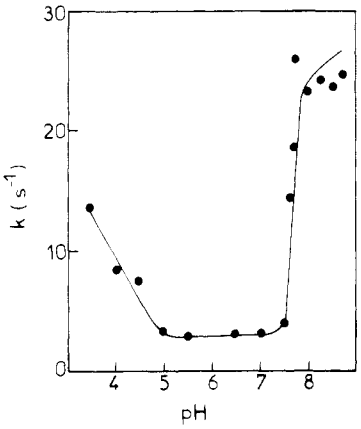


FIGURE 5: Hydrogen-exchange rate constant of guanosine cyclic 2′,3′-monophosphate as a function of pH at 20 °C. All buffers contained 10^{-1} M sodium chloride and 10^{-3} M sodium citrate for pH 3.5–5.5, 10^{-3} M sodium cacodylate for pH 6–7, and 10^{-1} M Tris-HCl for pH 7.5–8.75.

decrease of the exchange rate with pH. Deducing the proton-exchange rate from NMR line-width broadening, McConnell et al. (1983) obtained for the exchange rate of these protons the same peculiar pH dependence.

Discussion

Hydrogen Exchange in Double-Stranded G-C-Containing Nucleic Acid (A Form and B Form): A Survey. The experimentally measured hydrogen-exchange is modulated by two main factors: the chemistry of the proton-exchange process and the opening and closing of the base pairs. We shall briefly summarize these two aspects of the hydrogen-exchange process.

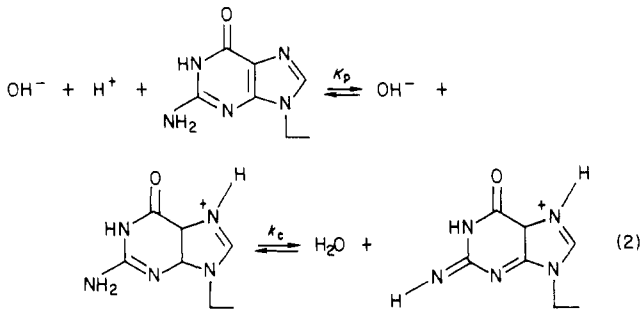
(A) Chemistry of Proton Exchange in Cytosine and Guanine. The chemistry of the exchange of the cytosine and guanine NH protons with water protons is now rather well understood. Quite generally, the overall chemical exchange rate is equal to the rate constant of a rate-limiting proton-transfer reaction between an acid and a base (Eigen, 1964). If the transfer is from a strong to a weak acid, the reaction will proceed at the encounter-limited rate; otherwise, the chemical-exchange rate will be a function of the difference in pK between the proton acceptor and donor according to the equation

$$k_{ch} = k_D \frac{10^{\Delta pK}}{1 + 10^{\Delta pK}}[B] \quad (1)$$

In this equation, [B] is the concentration in exchange catalyst, which in our case is either OH^- or the unprotonated imidazole molecule, and k_D is the diffusion-limited encounter rate constant, normally about 3×10^{10} M^{-1} s^{-1} (Teitelbaum & Englander, 1975).

Owing to the favorable pK (pK = 10), the cyclic guanine imino proton can be transfered directly to OH^- or to the imidazole molecule, and the value of the rate constant can be estimated directly from (1). Taking pK values of 10 and 16.7 for the imino proton and the OH^- ion, respectively, the predicted exchange rate at pH 7.5 and 0 °C is 3×10^3 s^{-1}.

In the case of the amino protons of guanine, direct removal of the proton by OH^- cannot take place because of the extreme pK value of these protons (Teitelbaum & Englander, 1975; McConnell, 1978). Furthermore, such a simple proton-transfer reaction cannot account for the pH-independent region of the curve shown in Figure 6. The mechanism proposed by Teitelbaum & Englander (1975b) and McConnell (1978) involves two proton-transfer steps:



The first step is a preequilibrium protonation of guanine at the N_7 position with an equilibrium constant K_p of about $10^{2.7}$ M^{-1}, which changes the pK from an extremely unfavorable pK value (pK = 30) to a pK value where the amino protons are withdrawn by OH^- at a diffusion-limited rate. The chemical-exchange rate constant for such a reaction is given by

$$k_{ch} = [H^+]K_p k_D \frac{10^{\Delta pK}}{1 + 10^{\Delta pK}}[B] \quad (3)$$

When [B] in (3) refers to the OH^- ion, the expression of the rate involves the product $[H^+][OH^-]$, which equals K_w. This accounts for the pH-independent region in Figure 6. Thus, our stopped-flow deuterium exchange brings further experimental support for the reaction mechanism 2. From (3), the predicted pH-independent rate at 0 °C is 5×10^{-2} s^{-1} [$K_w \sim 10^{-14.5}$, $k_D \sim 3 \times 10^{10}$ M^{-1} s^{-1}, and $K_p \sim 10^{2.7}$ M^{-1} values taken from Teitelbaum & Englander (1975)], which is reasonably close to the 30×10^{-2} s^{-1} value obtained from the Arrhenius plot of Figure 2.

A similar reaction scheme has been proposed for the cytosine amino protons, involving a preprotonation step at the ring N_3 position (Teitelbaum & Englander 1975a,b; McConnell, 1978) and for the adenine amino protons involving a preprotonation at the ring N_1 position (Cross et al.; Teitelbaum & Englander, 1975a). Because of the pK difference of the protonation reaction at position N_3 in cytosine (p$K \sim 4.7$) and at position N_7 in guanine (p$K \sim 2.7$), the exchange of the amino cytosine protons is expected to be around 100 times faster than the guanine amino protons. At 20 °C, the measured pH-independent rate is equal to 3 s^{-1} for the guanine amino protons whereas for the cytosine amino protons Nakanishi & Tsuboi (1978) found a value of around 30 s^{-1}.

As for general catalysis, McConnell et al. (1983) have shown that the exchange rate of the amino proton in guanine is completely unaffected by the addition of all the commonly used buffers (except phosphate), although these buffers catalyze as expected the exchange of the cytosine amino protons. Apparently, the increase in amino acidity that results from the N_7 protonation is too small for a direct removal of the guanine amino protons by the buffer. Especially, the presence of 1 M sodium perchlorate did not accelerate the exchange rate of the guanine amino protons. This result is important in connection with our work as we had to use a buffer containing 3 M sodium perchlorate to stabilize the Z form of the native poly(dG-dC)·poly(dG-dC). As a matter of fact, from this it can be confidently concluded that the measured guanine amino proton pH-independent exchange rate (3 s^{-1} at 20 °C) is an upper limit for the exchange rate of these protons in the Z-form poly(dG-dC)·poly(dG-dC). At 34 °C, the rate of the guanine amino protons corresponding to pH 7.5 might be slightly higher than the minimum pH-independent rate by a factor of around 3, as judged from the results at 27 °C of McConnell et al. (1983).

This brief summary shows that at the nucleotide level the hydrogen-exchange reactions in guanine and cytosine are well understood. In particular, our experimental stopped-flow results bring further experimental evidence that the exchange of the guanine amino protons does not present some unusual features and can be described by reaction 2.

(*B*) *Dependence on Structural Opening.* We turn now to the exchange rate of the five guanine and cytosine NH protons in polynucleotide duplex where cytosine and guanine are base paired. The most extensively studied double helix is the poly(rG)·poly(rC) by Teitelbaum & Englander (1975b). The rates of all the five protons are considerably slower than those in the case of the mononucleotides where the protons are freely exposed to the solvent. Three classes of protons with different exchange rates can be distinguished. The fast class has an amplitude of one proton whereas the two other classes have each an amplitude of two protons. On the basis of their response to general and specific base catalysis, the fast-class proton was assigned to the guanine imino ring proton, and the protons of the intermediate and slow class were assigned to the cytosine and guanine amino protons, respectively.

It is clear that the guanine imino proton involved in a hydrogen bond and furthermore deeply buried inside the double helix cannot exchange from the native state. Similarly, the position N_3 on the cytosine, which has to be protonated to allow the exchange of the cytosine amino proton, is also blocked. Thus, for exchange, these protons have to wait for a prior opening of the base pairs. Such a situation can be most simply described according to the two-step reaction proposed by Teiltelbaum & Englander (1975a,b):

$$\text{closed} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \text{open} \xrightarrow{k_{ch}} \text{exchanged} \qquad (4a)$$

$$K_{eq} = k_{op}/k_{cl} \qquad (4b)$$

where k_{op} and k_{cl} represent the opening and closing rates of the base pairs and K_{eq} is the opening equilibrium constant. k_{ch} is the chemical rate constant characterizing the transfer of an NH proton from a freely solvent-exposed nucleotide to the solvent. Its value can be estimated from (1) or (3). According to this reaction scheme, the flux of exchanging protons per time unit is given by

$$k_{EX} = k_{op}k_{ch}/(k_{cl} + k_{ch}) \qquad k_{cl} \gtrsim k_{op} \qquad (5a)$$

Depending on the relative values of k_{cl} and k_{ch}, two limiting cases for k_{EX} can be considered:

opening limited

$$k_{ch} > k_{cl} \qquad k_{EX} = k_{op} \qquad (5b)$$

preequilibrium

$$k_{cl} > k_{ch} \qquad k_{EX} = k_{ch}K_{eq} \qquad (5c)$$

In the first limiting case when $k_{ch} > k_{cl}$, every time the structure opens exchange takes place before reclosing. Thus, the exchange is opening limited. In the second limiting case, the so-called preequilibrium regime, as $k_{cl} > k_{ch}$ opening and closing reactions equilibrates before exchange takes place. Experimentally, the two limiting cases described in (5b) and (5c) can be easily distinguished. In the preequilibrium regime, the exchange rate is a function of k_{ch} and thus will be dependent on pH and general buffer catalyst, while in the opening-limited regime the exchange rate does not respond to such factors.

In the poly(rG)·poly(rC) duplex polymer the guanine imino proton has been found to be insensitive to pH and general buffer catalysis, implying that this proton exchanges at the opening-limited rate. In contrast, the NH_2 cytosine protons of the intermediate class exchange as a function of pH and general buffer catalysis and are thus exchanging according to a preequilibrium regime. This is quite reasonable because as we have seen previously the chemical exchange of the guanine imino proton is much faster than the exchange rate of the cytosine amino protons. Concerning the guanine amino protons, although they are the slowest exchanging protons, the comparison of the chemical exchange of cytosine and guanine amino protons on one hand and of the exchange rate of these protons in the double-stranded helix on the other have lead Teitelbaum & Englander (1975b) to the conclusion that these guanine protons can exchange from the closed state.

Although there is no extensive study available concerning the double-helix poly(dG-dC)·poly(dG-dC) in the B form, we have found that under the same experimental conditions (0.2 M NaCl, pH 7.5, 0 °C) the exchange curves for B-form poly(dG-dC)·poly(dG-dC) and A-form poly(rG)·poly(rC) are very similar. Thus, it can be confidently expected that all the major characteristics of the proton exchange in the A-form poly(rG)·poly(rC) can be extrapolated to the B-form poly(dG-dC)·poly(dG-dC).

Hydrogen-Exchange in Z DNA: The Opening Reaction Mechanism. (*A*) *Hydrogen Exchange in Z DNA: General Characteristics.* The exchange pattern of Z-form DNA is very characteristic. Besides the overall dramatic retardation of the exchange rate, there are only two kinetic classes instead of three for poly(rG)·poly(rC). The fast class has an amplitude of three protons that we were able to identify as the imino and amino guanine protons whereas the slow class, whose exchange rate is in the hour time range, has an amplitude of two protons. These were identified as the cytosine amino protons (Hartmann et al., 1982).

How does the hydrogen exchange depend upon the dynamic structure of Z DNA? The exchange curves as a function of temperature show that over the temperature range from 0 to 34 °C the imino and amino protons of guanine are exchanging at the same rate. This result is very unexpected because the chemical exchange rate of the guanine imino and amino protons are very different. At pH 6.5 and at 3 °C, the exchange rate of the guanine amino protons deduced from the Arrhenius plot of Figure 2 is 0.35 s^{-1} whereas the measured exchange rate of the guanine imino proton obtained by McConnel (1978) is 600 s^{-1}. Irrespective of the detailed exchange mechanism the only possible way for these protons to exchange at the same rate is to exchange according to the same opening-limited regime, and thus, the measured exchange rate of these protons reflects the rate of the conformational change that brings the exchanging protons in contact with the solvent. This rate constant is 2 orders of magnitude smaller than the corresponding opening rate constant for poly(rG)·poly(rC) (Teitelbaum & Englander, 1975b). Although we cannot at this point give any exact value concerning the rate constant of the reverse conformational change (exchanging state → native state), we can set an upper limit to this rate constant from these data. As for each conformational change that brings the guanine in contact with the solvent, the amino protons exchange before the reverse reaction takes place; the rate of the reverse reaction must be at least 5 times smaller than the chemical exchange of the guanine amino protons (for example, at 20 °C we must have $k_{cl} < 6$ s^{-1}).

In contrast to these guanine protons, the imidazole concentration dependence of the exchange rate of the cytosine amino protons in Z-form platinated poly(dG-dC)·poly(dG-dC)

Table I: Opening Parameters at 20 °C for Different Opening Reaction Mechanisms

	closed $\underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}}$ open $\xrightarrow{k_{ch}}$ exchange			
	k_{ch} (s^{-1})	k_{cl} (s^{-1})	k_{op} (s^{-1})	K_{eq}
2′,3′-cGMP[a]	3			
CMP[b]	26.4			
poly(dG-dC)·poly(dG-dC) (Z form)[c]		630	4.6×10^{-3}	7×10^{-6}

	closed $\underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}}$ {out (guanine) $\xrightarrow{k^G_{ch}}$ exchanged; in (cytosine) $\underset{k^C_{ch}/R}{\rightleftharpoons}$ exchanged}		
	k_{cl} (s^{-1})	k_{op} (s^{-1})	K_{eq}
poly(dG-dC)·poly(dG-dC) (Z form)[c]	$<6 \times 10^{-1}$	4.6×10^{-3}	$>7.7 \times 10^{-3}$

	exchanged $\xleftarrow{k^G_{ch}}$ out (guanine) $\underset{k^G_{cl}}{\overset{k^G_{op}}{\rightleftharpoons}}$ closed $\underset{k^C_{cl}}{\overset{k^C_{op}}{\rightleftharpoons}}$ out (cytosine) $\xrightarrow{k^C_{ch}}$ exchanged					
	k_{op}^G (s^{-1})	k_{cl}^G (s^{-1})	K_{eq}^G	k_{op}^C (s^{-1})	k_{cl}^C (s^{-1})	K_{eq}^C
poly(dG-dC)·poly(dG-dC) (Z form)[c]	4.6×10^{-3}	$<6 \times 10^{-1}$	$>7.7 \times 10^{-3}$	$>9.6 \times 10^{-4}$	$>1.3 \times 10^{2}$	7×10^{-6}

[a] Solvent was 0.1 M NaCl and 1 mM sodium cacodylate, pH 6.5. [b] Taken from Nakanishi & Tsuboi (1978). [c] Solvent was 3 M sodium perchlorate and 10 mM sodium phosphate, pH 7.5. The upper-case superscripts C and G refer to cytosine and guanine residues, respectively.

shows that the exchange of these protons is not limited by some conformational change. Instead, their exchange can be accounted for by preequilibrium mechanism 5c. The acceleration factor of the rate in presence of 0.2 M imidazole is around 2, which is in the same order of magnitude as the acceleration factor for the cytosine amino protons, found to be 7 (Teitelbaum & Englander, 1975b).

(*B*) *Analyzing the Data with the Two-Step Model* (*4a*). Postulating the classical two-step exchange mechanism (4a), from these data it is possible to evaluate the opening parameters, as was done for poly(rG)·poly(rC) (Teilelbaum & Englander, 1975b). As already discussed, the exchange rate of the fast class is equal to the opening rate k_{op} whereas the opening equilibrium constant K_{eq} can be deduced from the rate constant of the slow-class protons with (5c). From the values of K_{eq} and k_{op} we can calculate k_{cl}. The values of all these parameters are listed in Table I together with the measured chemical-exchange rates of guanine and cytosine amino protons. A close inspection of this table shows that k_{cl} is 2 orders of magnitude greater than the chemical exchange rate of the guanine amino protons, implying that these protons should exchange via a preequilibrium as in the case of the cytosine amino protons. The exchange rate temperature dependence of the three fast protons indicates clearly that this is not the case but that instead the exchange of the guanine amino protons is opening limited. We must then conclude that analyzing the Z-DNA exchange data with the classical two-step reaction 4a leads to some inconsistency, and thus this model as such is not operative in the case of Z DNA.

(*C*) *Two Possible Exchange Mechanisms for Z DNA.* The value of the closing rate k_{cl} obtained when analyzing the data with the opening reaction mechanism 4a is too high. Examination of the relations 4b and 5c shows that the value of k_{cl} relies ultimately upon the estimated value of the cytosine amino protons chemical-exchange rate and too high a value of k_{cl} could mean simply that we have overestimated this chemical-exchange rate. We can compute a lower limit for the factor by which the chemical exchange of the cytosine amino protons in the open state must be decreased to obtain a closing rate k_{cl} 5 times smaller than the chemical-exchange rate of the guanine amino protons, which would then be opening limited in agreement with the experimental data. The value of this estimated lower limit of the retardation factor is 10^3. To explain such an important slow down of the cytosine amino protons, we have to imagine an open state where, in contrast to the guanine, the cytosine does not swing out into the solvent but instead remains stacked in the inside of the double helix. This situation can most simply be described through the kinetic mechanism

$$\text{closed} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \left\{ \begin{array}{l} \text{out (guanine)} \xrightarrow{k^G_{ch}} \text{exchange} \\ \text{in (cytosine)} \underset{k^C_{ch}/R}{\longleftarrow} \text{exchange} \end{array} \right. \quad (6)$$

The upper case superscripts G and C refer respectively to the guanine and cytosine residues. Formally, (6) is identical with (4a). However, we have introduced the additional feature that the open state is very "asymmetric" in the sense that the guanine residue is in good contact with the solvent, contrary to the cytosine residue. This asymmetry is reflected quantitatively in the model by the presence of the retardation factor R.

Although we cannot rule out such a model with the data at hand, it seems to us not very realistic to assume such an open state where only the guanine protons are exchanging freely with the solvent, whereas the cytosine amino protons remain buried inside the double helix. Therefore, we favor the following extreme explantation: the closing rate k_{cl} determined with the opening equilibrium constant K_{eq} determined from the exchange rate of the cytosine protons is irrevelant for the guanine residue simply because the opening reactions of cytosine and guanine are two independent reactions. Therefore, the opening equilibrium constant K_{eq} would refer only to the opening reaction of the cytosine residue. In this case, the simplest kinetic mechanism for the hydrogen exchange in Z DNA is

$$\begin{array}{l} \text{exchanged} \xleftarrow{k_{ch}^G} \text{out (guanine)} \underset{k_{cl}^G}{\overset{k_{op}^G}{\rightleftharpoons}} \text{closed} \underset{k_{cl}^C}{\overset{k_{op}^C}{\rightleftharpoons}} \\ \qquad\qquad\qquad\qquad \text{out (cytosine)} \xrightarrow{k_{ch}^C} \text{exchanged} \end{array} \quad (7)$$

The upper-case superscripts G and C refer respectively as before to the guanine and cytosine residues, k_{op} and k_{cl} represent the opening and closing rates, respectively, and k_{ch} is the chemical-exchange rate constant. In the closed state, guanine and cytosine are hydrogen bonded and cannot exchange whereas in the out (cytosine) and out (guanine) states, cytosine and guanine protons respectively are exposed to solvent and can exchange freely. The corresponding opening

Table II: Equilibrium Thermodynamic Functions of the Cytosine Opening Reaction in the Case of Reaction 3 at 20 °C

$\Delta G°$ (kcal)[a]	$\Delta H°$ (kcal)[b]	$\Delta S°$ (eu)[c]
7	5.6	−4.8

[a] Calculated from $\Delta G° = RT \log K_{eq}^{C}$. [b] From the relation 5c, $k_{EX} = K_{eq}^{C} k_{ch}^{C}$, where k_{EX} is the exchange rate constant of the slow phase, by taking the natural logarithm of both sides and differentiating with respect to $1/T$, we obtain $E_{app} = E_{ch}^{C} + \Delta H°$, where E_{app} and E_{ch}^{C} refers to the activation energies of the slow phase (20 kcal mol^{-1}) and of the chemical-exchange rate of the cytosine amino protons (14.4 kcal mol^{-1}), respectively. [c] Calculated from $\Delta G° = \Delta H° - T\Delta S°$.

Table III: Activation Energies

	CMP[a]	2′,3′-cGMP	poly(dG-dC) (slow phase)	poly(dG-dC) (fast phase)
E_a (kcal mol^{-1})	14.4 ± 1	16 ± 1	20 ± 1	18 ± 1

[a] Taken from Nakanishi & Tsuboi (1978).

parameters for this model are listed in Table I. For the closing rate constant of guanine, k_{cl}, we can only estimate an upper limit using the opening-limited conditions (5b). In the same way, using the preequilibrium condition (5c), we can estimate a lower limit for the closing (k_{cl}) and opening (k_{op}) rate constants of the cytosine. It is interesting to notice on this table that, besides the inherent slowness of the opening and closing rates of the guanine residue, the opening equilibrium constant for the cytosine residue is at least 3 orders of magnitude smaller than the corresponding equilibrium constant for the guanine residue. This means that for each guanine residue the time spent in contact with the solvent is 10^3-fold greater than the corresponding time for the cytosine residue. The thermodynamic parameters of the cytosine opening reaction are listed in Table II. One notices the very unfavorable entropic contribution whereas the enthalpic part is not very different from the one found in poly(rA)·poly(rU) (Mandal et al., 1979).

Even though reactions 6 and 7 are essentially different, examination of the opening parameters listed in Table I shows that both models lead to similar qualitative conclusions. Namely, in the open state the base pair is asymmetric; the probability to be in contact with the solvent is higher for the guanine then for the cytosine residue.

Also, irrespective of the exchange mechanism, the opening and closing rates of the guanine residue are dramatically slow as compared to those obtained for all the other double helices (Teitelbaum & Englander, 1975a,b; Mandal et al., 1979; Early et al., 1981a,b; Munt et al., 1984; Mirau & Kearns, 1984; Pardi et al., 1982). One may wonder why the opening and closing rates in Z DNA are so slow. There is presently no clear answer to this question. It may be worthwhile at this point to discuss the value of the measured opening activation energy, which is 18 kcal/mol (Table III). This activation energy falls into the same range as the activation energies determined for more rapidly exchanging B-form and A-form duplexes (Mandal et al., 1979; Mirau & Kearns 1984), indicating that the very slow opening rate in Z DNA results from a smaller preexponential factor, that is to say, from a smaller activation entropy. The difficulty in finding among many possibilities the correct pathway leading to the swinging out of the base might then be the origin of the unusually slow opening rate. A similar explanation might hold for the closing rate.

Conclusions

Our study points to be conclusion that the open state in Z DNA presents some asymmetry; the guanine residue is much more likely than the cytosine residue to swing out making contact with the solvent. It is tempting to ascribe this characteristic feature to the difference in the conformation between the cytosine and guanine residues, which adopt in Z DNA the anti and syn conformations, respectively. Finally, we would like to point out that these unusual characteristics of the Z-DNA dynamic structure might play an important role in biological processes that require molecular recognition.

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Registry No. 2′,3′-cGMP, 634-02-6; poly(dG-dC), 36786-90-0; guanine, 73-40-5; imidazole, 288-32-4; hydrogen, 1333-74-0.

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