Volume 13 Number 10 1985 Nucleic Acids Research

Effect of B–Z transition and nucleic acid structure on the conformational dynamics of bound ethidium dimer measured by hydrogen deuterium exchange kinetics

Judith Markovits, Jean Ramstein[1], Bernard P.Roques[2] and Jean-Bernard Le Pecq

Laboratoire de Physico-chimie macromoléculaire, LA 147 CNRS, U 140 INSERM, Institut Gustave Roussy, 94805 Villejuif Cedex, [1]Centre de Biophysique Moléculaire du CNRS, 45000 Orléans, and [2]Departement de Chimie Organique, ERA 613 CNRS, U 266 INSERM, UER des Sciences Pharmaceutiques et Biologiques, Université Paris V, 75006 France

Received 6 August 1984; Revised 16 April 1985; Accepted 30 April 1985

ABSTRACT :

Ethidium dimer is shown to bind by intercalation, almost equally well, to the B and Z form of poly[(dG-m^{5}dC)].poly[(dG-m^{5}dC)], whereas the ethidium monomer shows a strong preference for the B form. The hydrogen-deuterium (H-D) exchange kinetics of the ethidium dimer bound to the B and Z form of poly[(dG-m^{5}dC)].poly[(dG-m^{5}dC)] could then be compared. The kinetics of the H-D exchange were strikingly slower when the dye was bound to Z DNA as compared to B DNA. The exchange kinetics were also modified when ethidium dimer was bound to tRNA and to a triple stranded structure. It is proposed that a dynamic fluctuation at the level of the nucleic acid could modulate the dynamic fluctuation at the level of the bound ligand.

INTRODUCTION :

The hydrogen exchange kinetic studies of DNA have recently been reviewed (1). They have permitted the characterization of the double helix dynamic structural fluctuation referred to as the breathing reaction. More recently, the hydrogen-deuterium (H-D) exchange kinetics of DNA bound ethidium dimer has been studied by fluorometry (2). The exchange kinetics of the DNA bound ethidium dimer (EthDi) resembled that of DNA itself. The time range of the exchange process, the effect of catalysts, and the activation energies were comparable in the two cases. Moreover, the exchange of EthDi bound to DNA was found to be independent of the dye-DNA binding affinity although this parameter was changed with ionic strength by several orders of magnitude (2). This raised the question whether the structural fluctuations governing the proton exchange kinetics in both cases, are correlated. It is known that intercalated ethidium is a probe of the torsional dynamics of the double helix (3-5). Therefore, it could also be hypothesized that the base pair opening process could induce a displacement of ethidium dimer in its binding site leading to its amino proton exchange. Because it was shown by NMR studies that several DNA ligands did not appreciably perturb the imino proton exchange in oligo-

nucleotides (7,8), a double helix fluctuation and the nucleic acid bound ethidium dimer H-D exchange might be correlated. If this were really the case, ethidium dimer could be used as a fluorescent probe of the dynamic structure of various nucleic acids. In order to investigate this possibility, the characteristics of the H-D exchange of ethidium dimer bound to nucleic acids of different structures has been investigated. The rate of proton exchange of Z DNA has been found to be much slower than that of B DNA (9). Therefore the comparison of the exchange kinetics of the same ligand bound to B or Z DNA would be of special interest. Indeed this comparison can be performed with ethidium dimer, because this dye binds almost equally well to B or Z DNA, as is reported in this paper. In addition, the H-D exchange of ethidium dimer bound to t-RNA and the triple stranded structure poly(A)-2 poly(U) was studied. The hydrogen exchange of several protons of tRNA is remarkably slower than those of DNA (10-11). The opening rate for the Hoogsteen base pair of poly(A)-2 poly(U) is much faster than that of the Watson-Crick base pair of poly(A) - poly(U) (12).

MATERIALS AND METHODS :

Ethidium dimer (EthDi)(4,7-Diazadecyl-5, 5'-bis) (3, 8 diamino-6 phenyl phenanthridinium dichloride dihydrochloride) was synthezised and purified as already described (13). Ethidium bromide (EthBr) was a product of Sigma. Poly[d(A-T)].poly[d(A-T)], poly [d(G-C)].poly[d(G-C)], poly[(dG-m^5dc)].poly[(dG-m^5dc)] were purchased from Boehringer. Prior to use, these products were extensively dialyzed against a buffer containing 50 mM sodium chloride and 10^{-3}M EDTA. For concentration determination the following extinction coefficients were used : 6800 $M^{-1}cm^{-1}$ for poly[d(A-T)].poly[d(A-T)] at 260 nm, 8400 $M^{-1}cm^{-1}$ for poly[(dG-dc)].poly[(dG-dC)] at 254 nm (14), 6900 $M^{-1}cm^{-1}$ and 6400 $M^{-1}cm^{-1}$ at 260 nm respectively for poly[(dG-m^5dC)].poly[(dG-m^5dC)] in B and Z conformation (15).

Poly(A) and poly(U) were purchased from Miles. Solutions containing mixtures of poly(A) and poly(U) were prepared according to the continuous variations method. The total nucleotide concentration was kept constant as the proportions of the poly(A) and poly(U) were varied. E. coli $tRNA^{fMet}$ and yeast $tRNA^{Phe}$ were extensively dialyzed prior to use. Concentrations of tRNA samples were determined by absorbance at 260 nm using an extinction coefficient of 5.64×10^5 $M^{-1}cm^{-1}$. Deuterium oxide (99.8%) was purchased from the Commissariat à l'Energie Atomique (Saclay, France). The hydrogen-deuterium exchange of ethidium dimer was measured at 25°C with a

Durrum Gibson D-110 stopped-flow instrument equipped with fluorescence detection. Data stored in a Minc 11/23 computer were analyzed by nonlinear regression using the Marquardt algorithm for a single or double exponential process (16) as already described (2). Circular dichroism spectra were recorded on a Roussel-Jouan dichrograph Mark IV.

Binding parameters were deduced from fluorescence titration as already described (17). The Marquardt algorithm (16) was used to fit the data to the McGhee and von Hippel equations (18).

Equilibrium dialysis experiments were performed in a three compartment cell as already described (19, 20). The two outer chambers were filled with 0.8 ml of poly[(dG-dC)] and poly[(dG-m^5dC)] respectively ($2x10^{-4}$M base pair). The inner chamber contained 0.8 ml of dimer solution at 10^{-6}M. The concentration of free dimer after equilibrium being negligible, the ratio of the total dimer concentration measured in each outer chamber equals the ratio of the corresponding poly(dG-m^5dC):poly (dG-dC) dimer binding constant. The dye concentration was determined by fluorometry after dilution (1:1) in 0.5% acetic acid-dimethylsulfoxide (21). "B buffer" was 0.05 M NaCl, 0.01 M Tris.HCl, pH 7.4, "Z buffer" was 0.05 M NaCl, 0.01 M Tris.HCl, 10 mM $MgCl_2$. All measurements were done at 25°C.

In order to determine the DNA to dye energy transfer, fluorescence excitation spectra were recorded and corrected as described (17, 22) using a SLM 800 spectrofluorometer controlled by a Minc 11/23 Digital computer.

RESULTS

I) Ethidium dimer intercalation in B and Z DNA.

It is well known that EthBr favors the Z→B transition as do other intercalating drugs (23,24). In addition, it was reported that the bis(methidium) spermine dimer could bind to the Z form of poly[(dG-dC)].poly[(dG-dC)] at high ionic strength (25). The Z to B transition of the poly[(dG-m^5dC)].poly[(dG-m^5dC)] occurs at a relatively low ionic strength. Because low salt concentration is required for the study of the EthDi H-D exchange, as will be discussed later, this polymer was selected.

In figure 1, the effects of EthDi and EthBr on the CD spectrum of poly[(dG-m^5dC)].poly[(dG-m^5dC)] in B or Z form are compared. EthBr (fig. 1A) clearly induces spectral modifications characteristic of the Z to B transition when it is added to the Z form of the polymer. By contrast, the

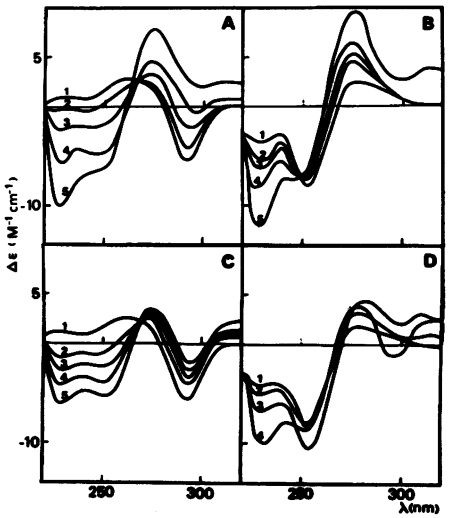

Figure 1 : Effect of EthDi and EthBr upon the circular dichroism spectra of poly[(dG-m^{5}dC)] in Z or B form. Temperature was 25°C.
A) EthBr + poly[(dG-m^{5}dC)] in Z buffer. The dye/base-pair ratios are 0, 0.10, 0.16, 0.26 and 0.56 for curve n° 1, 2, 3, 4 and 5 respectively.
B) EthBr + poly[(dG-m^{5}dC)] in B buffer. The dye/base-pair ratios are 0, 0.10, 0.16, 0.26 and 0.56 for curve n° 1, 2, 3, 4 and 5 respectively.
C) EthDi + poly[(dG-m^{5}dC)] in Z buffer. The dye/base-pair ratios are 0, 0.04, 0.06, 0.08 and 0.16 for curves n° 1, 2, 3, 4 and 5 respectively.
D) EthDi + poly[(dG-m^{5}dC)] in B buffer. The dye/base-pair ratios are 0, 0.5, 0.12 and 0.24 for curve n° 1, 2, 3, and 4 respectively. In all experiments nucleotide concentration is 10^{-4}M.

CD spectra of the polymer remains characteristic of the Z form when EthDi is added (fig. 1C). In order to interpret quantitatively these CD spectra, the binding of EthBr and EthDi to poly[(dG-m^{5}dC)] in Z and B buffer, where the polymer exists in the Z and B form respectively (15), was studied and compared to the results obtained with poly [(dG-dC)], which remains in the B form in these two buffers. Figure 2 shows the Scatchard representation of the binding of EthBr to poly [(dG-dC)] and to poly[(dG-m^{5}dC)] deduced from fluorescence titration. In B buffer (fig. 2A), the binding data are well fitted (continuous line) according to the McGhee-von Hippel equation (18). This demonstrates a preference of the dye for the methylated polymer. In Z buffer (fig. 2B), the binding to poly[(dG-dC)] is also well fitted according to the McGhee-Von Hippel equation, whereas the binding to

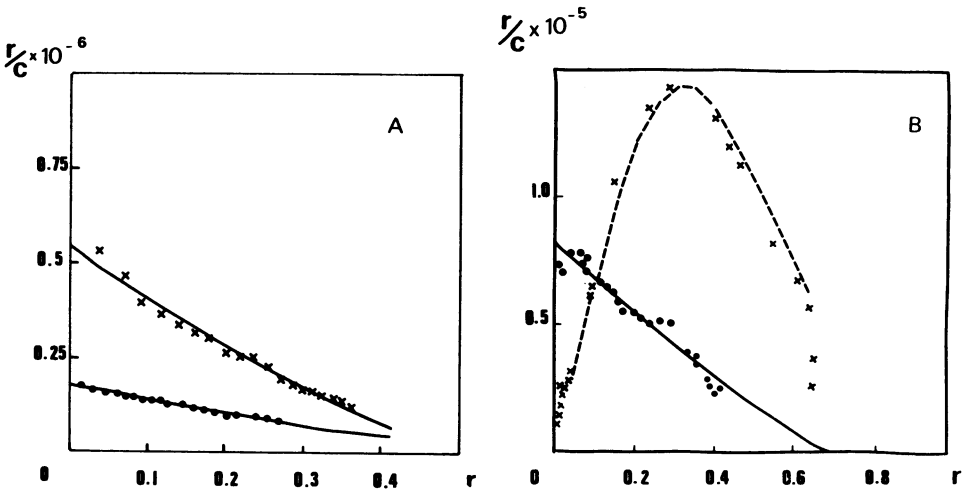

Figure 2 : Comparison of the binding of ethidium bromide to poly[(dG-dC)] and poly[(dG-m^{5}dC)] in B and Z buffer. Temperature was 25°C. The data has been deduced from fluorescence titration and plotted according to the Scatchard representation with r the number of ethidium molecule bound per base-pair and c the free dye concentration. The continuous lines are the best fits computed from the Mc Ghee-von Hippel equation (18) for non cooperative binding. The discontinuous line in B has been drawn to help follow the data.
(A), left, is in B buffer, (B), right, is in Z buffer :
×——× poly[(dG-m^{5}dC)] ●——● poly[(dG-dC)].

the methylated polymer is highly cooperative. The McGhee-von Hippel equation for cooperative processes (18) leads to a poor fit of these data in the latter case. In figure 1B, a discontinous line was simply drawn to help follow the data. Nevertheless, the extrapolated values of r/c for r equals 0 leads to the binding constant of EthBr for the Z form of the polymer.

The fluorescence titrations of poly[(dG-m^{5}dC)] or poly[(dG-dC)] by EthDi in the presence or absence of magnesium (Z or B buffer respectively) could not be analyzed in terms of binding parameters. Under the conditions which could be used (DNA base-pair concentrations greater than 10^{-6}M), the EthDi binding is always rigorously stoichiometric, implying that the binding association constant is at least greater than 10^7 M^{-1} in all cases.

Interestingly, the fluorescence characteristics of EthBr and EthDi, including fluorescence lifetimes, are very similar whatever the polymer

Table 1 : Binding parameters of EthDi and EthBr to poly(dG-m^5dC) in Z and B buffers.

Dialysis equilibrium		EthBr fluorescence titration		
EthBr	EthDi	polymer	Kap (M^{-1})	n
		poly (dG-m^5dC)		(a)
α_1 0.5	1.8	Z buffer	1.0×10^4	-
		poly (dG-dC)		
		Z buffer	8.5×10^4	1.5
α_2 3.0	1.2			
		poly (dG-m^5dC)		
		B buffer	5.4×10^5	1.8
α_1/α_2 6.0	1.5			
		poly (dG-dC)		
		B buffer	1.7×10^5	1.5

Dialysis equilibrium measurements were performed in a three compartment cell as described in the methods section : α_1 is the ratio on the concentration of bound dye in the poly(dG-m^5dC) compartment to the concentration of bound dye in the poly(dG-dC) compartment, in the Z buffer. α_2 is the same but in B buffer. α_1/α_2 represents the ratio of the binding constants for the Z to the B form of poly(dG-m^5dC).

Fluorescence titration : Kap binding constant, n number of base pairs covered, deduced from the best fit to the McGhee-von Hippel equation (18) of the results of Figure 2.
(a) deduced from extrapolation of r/c at r=0 value (Figure 2).

and the salt concentration (results not shown). The same was observed for EthBr and poly(dG-dC) at high salt concentration (25).

In addition, the ratios of the apparent binding constants of EthBr and EthDi for poly[(dG-m^5C)] and poly[(dG-dC)] in Z and B buffer could be determined by equilibrium dialysis using a three compartment cell (19, 20). The results are reported with those of the fluorescence titration in table 1.

In order to verify that EthDi binding to the Z form of poly[(dG-m^5dC)] occured according to the intercalation process, the DNA to dye energy transfer was measured. It was shown before that such transfer can occur only when the aromatic ring of the dye is directly in contact with a base pair and therefore is intercalated (17, 26). If normalized absorption and fluorescence excitation spectra are compared, they must superpose in the absence of energy transfer. If energy transfer from the

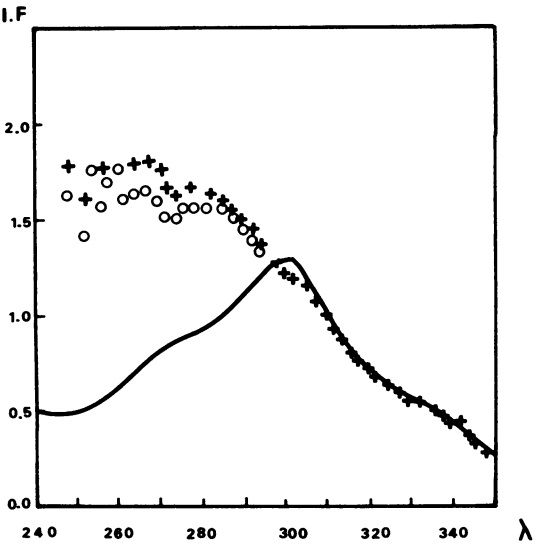

Figure 3 : Comparison of the corrected and normalized fluorescence excitation spectra of ethidium dimer bound to poly[(dG-m^{5}dC)] in Z and B buffer with the normalized absorption spectrum of ethidium dimer bound to poly[(dG-m^{5}dC)]. To record the absorption spectrum, the EthDi DNA complex solution (EthDi 0.5 x 10^{-5} M, poly[(dG-m^{5}dC)] 1.6×10^{-4} M in nucleotide) was read against a solution containing the same concentration of poly[(dG-m^{5}dC)] in B or Z buffer. All absorbances were then divided by the absorbance measured at λ = 310 nm.
The excitation spectra were recorded on solutions containing 0.5 10^{-6} M EthDi and 3×10^{-5} M of the corresponding polymer. After correction, all data were divided by the value measured at 310 nm. Emission was at 610 nm.
——— absorption spectra +———+ and o———o fluorescence excitation spectra in B and Z buffer respectively.

base pair to the dye occurs, the apparent quantum yield of fluorescence of the bound dye increases in the DNA absorption region (17, 26). In figure 3, it can be seen that the normalized and corrected fluorescence excitation spectra of EthDi bound to poly[(dG-m^{5}dC)] in Z and B buffer cannot be distinguished from the corresponding normalized absorption spectra at wavelength greater than 300 nm. Contrastingly, below 300 nm, in the DNA absorption region, the apparent quantum yield of the dye is increased similarly whether the dye is bound to the Z or B form of the polymer. The two corresponding fluorescence excitation spectra are very similar. At 260 nm, the fluorescence quantum yield of the bound dye is increased by a factor of 3 as compared to the visible region. This

factor is close to the value reported for EthDi bound to DNA or t-RNA (26). It can then be concluded that the dye intercalates in the Z or B form of the methylated polymer in a similar way.

The CD results, together with the binding data of the dyes to poly [(dG-dC)] and poly[(dG-m^5dC)] in the presence or absence of Mg^{++} and the energy transfer measurements allow the following conclusions to be drawn. EthBr can apparently bind to Z DNA, but the association constant is much smaller than the corresponding constant for B DNA. Interestingly, EthBr was found to bind to the Z form of poly[(dG-dC)] in $MgCl_2$ ethanol buffer with a binding constant also much smaller than the corresponding value for the B form (24). The Z - B transition is induced when a relatively low number of EthBr molecules are bound. EthDi can bind almost as strongly to poly[(dG-m^5dC)] in either B or Z form. The Z → B transition does not occur until the dye begins to bind according to the monointercalating mode when the polymer is almost saturated (27, 28). The same conclusion was also reached when the effect of EthBr and EthDi on the precipitation of poly[(dG-m^5dC)] by Z specific antibodies was studied (29). It was observed that, whereas EthBr suppresses the immuno-precipitation of the polymer in Z form at low binding density, EthDi was without effect, even at high binding density. Shafer et al. (25) also reported that the bis (methidium) spermine dimer was able to bind and intercalate to the Z form of poly(dG-dC) at high ionic strength.

II) H-D exchange kinetics of ethidium dimer bound to poly (dG- m^5dC) in B and Z form.

After mixing with deuterated water, the rate of exchange of the ligand protons, k_{ex}, is given by the following equation (2,30) :

$$k_{ex} = \frac{k_{-1}\, k_f + k_b\,(k_1[DNA] + k_f)}{k_{-1} + k_1\,[DNA] + k_f} \qquad [1]$$

with k_1, k_{-1} being the on-rate and off-rate constants relative to the DNA ligand equilibrium respectively, k_b and k_f being the exchange rate of the ligand from the bound and free state, respectively, and [DNA] the free DNA concentration.

When the ionic strength is low enough to keep the binding affinity high and DNA is in excess

$$k_1\,[DNA] \gg k_{-1}$$

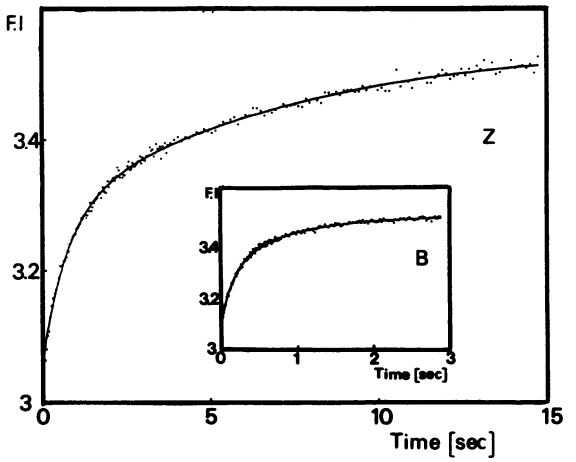

Figure 4 : Typical fluorescence signal for the H-D exchange reaction of EthDi bound to poly[(dG-m^5dC)] in Z buffer and B buffer (inset). The dots represent the experimental determinations. The continuous line is the best fit for a two exponential process. (EthDi 1.8×10^{-6}M, poly[(dG-m^5dC)] 1.8×10^{-4}M in nucleotide).

Equation [1] then reduces to :

$$k_{ex} = k_b + \frac{k_{-1}\, k_f}{k_f + k_1\,[DNA]} \qquad [2]$$

The second term of equation [2] accounts for the exchange process via the free dye. It was found negligible relative to k_b for DNA-EthDi complexes in buffer containing less than 1.0 M NaCl (2), where the EthDi binding constant remains high enough. In this case :

$$k_{ex} \simeq k_b \qquad [3]$$

The rate of H-D exchange of EthDi bound to the Z or B form of poly [(dG-m^5dC)] has been measured by stopped flow using fluorescence detection. Typical records are shown in figure 4. It is clear that the EthDi exchange which can be analysed according to a two exponential process is much slower for the Z form than for the B form of poly[(dG-m^5dC)]. As a control, the exchange rates were measured for poly[(dG-dC)] in the presence or absence of Mg^{++} in the same buffers. As shown in table 2, the presence of Mg^{++} has no effect on the exchange process in this case.

III) H-D exchange kinetics of ethidium dimer bound to tRNA.

The exchange behavior of EthDi bound to yeast $tRNA^{Phe}$ and E.coli

Table 2 : Relaxation times (s.) for the H-D exchange of EthDi bound to nucleic acids of different structures.

Polymer	$\tau_1$ex	$\tau_2$ex	$\bar{\tau}$ ex
Poly(dG-m^{5}dC) Z buffer	0.73	6.9	1.5
Poly(dG-m^{5}dC) B buffer	0.17	0.83	0.27
Poly(dG-dC) Z buffer	0.05	0.60	0.13
Poly(dG-dC) B buffer	0.05	0.55	0.14
C.T. DNA	0.13	1.08	0.28
Poly(dA-dT)	0.17	0.86	0.32
Poly(dA).poly(dT)	0.36	1.3	0.63
Poly(A).poly(U)	0.72	7.4	2.1
Yeast tRNA^{Phe}	0.16	1.65	0.50

$\tau_1$ex, $\tau_2$ex are the relaxation times corresponding to the fast and slow phase of the exchange respectively. $\bar{\tau}$ ex is the mean relaxation time computed according to Schwarz (36). Calf thymus (C.T.) DNA, poly(dA-dT), poly(dA). poly(dT), poly(A).poly(U) were in B buffer. tRNA was in the same buffer as in figure 3.

tRNA^{fMet} was investigated. As for DNA, the exchange is well accounted for by a two exponential process. However the exchange rates are significantly slower (table 2). The activation energies deduced from the variation of the exchange rates with temperatures between 10°C and 40°C are given in table 3.

The effect of catalyst concentration (imidazole) on the exchange rate is shown on figure 5. The slow and fast exchange phases are much less dependent on catalyst concentration than in the case of the DNA complex

Table 3 : Activation energies (Kcal/mol) of the H-D exchange reaction of EthDi bound to calf thymus (C.T.) DNA and $tRNA^{Phe}$

	Fast process	Slow process
C.T. DNA	4.5	13
tRNA(Phe)	4.0	15

The value of C.T. DNA hydrogen exchange was found to be 15.7 Kcal/mol (33). DNA was in B buffer and tRNA buffer was the same as in Figure 3.

(2). The catalyst effect on the exchange kinetics of DNA bound EthDi has been interpreted according to the well known scheme proposed to account for exchange kinetics of DNA protons (1).

$$\text{Closed} \underset{k_{Cl}}{\overset{k_{op}}{\rightleftharpoons}} \text{Open} \xrightarrow{k_{CH}[C]} \text{exchanged}$$

The exchange can take place only in the open state when EthDi amino groups are accessible to solvent. In the open state, the exchange rate constant is $k_{CH}[C]$, $[C]$ being the catalyst concentration. k_{op} and k_{cl} is the opening and closing rate respectively. According to this scheme, the

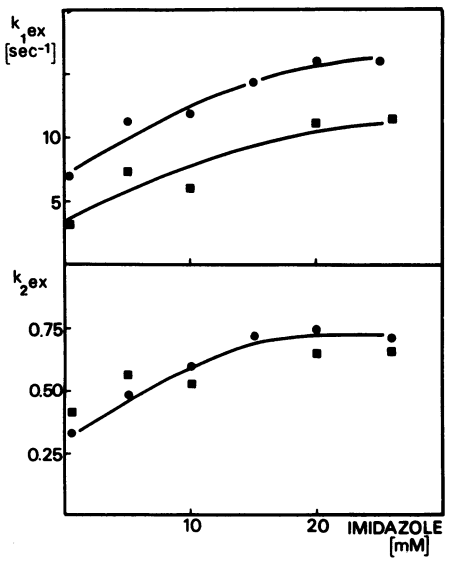

Figure 5 : Variations of the EthDi tRNA bound rates of exchange with catalyst (imidazole) concentration for the fast $k_1$ex (upper) and slow $k_2$ex rate (lower). ■—■ $tRNA^{Phe}$, ●—● $tRNA^{fMet}$ Buffer was 5 mM Na phosphate, pH 7.0, 2 mM $MgCl_2$. NaCl was added to keep ionic strength equal to 0.125 M as imidazole concentration was varied. tRNA is 3.7×10^{-6} M and EthDi is 1.5×10^{-6} M.

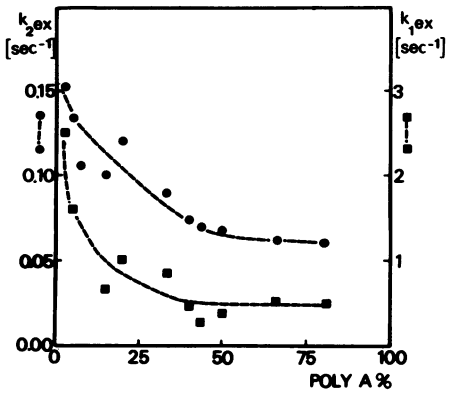

Figure 6 : Exchange rate of EthDi in the presence of a mixture of poly (A) and poly(U) as a function of the percentage of poly(A). $k_1$ex and $k_2$ex are the exchange rates corresponding to the fast and slow phase respectively. Total nucleotide concentration is kept constant (10^{-4} M). EthDi is 10^{-6} M. Buffer is 0.35 M NaCl, 0.05 M Tris HCl, pH 7.4.

exchange rate constant is calculated (1) :

$$k_{ex} = \frac{k_{op} \times k_{CH}[C]}{k_{Cl} + k_{CH}[C]} \qquad [4]$$

At high catalyst concentration $k_{ex} \sim k_{op}$.
Therefore, in the case of EthDi tRNA complexes, the k_{op} values for the slow and fast exchange phases, 0.6 and 13 sec respectively, can be compared to the corresponding values measured for EthDi complexed to DNA ; 4 and 33 sec. Equation [4] shows that a decreased dependence of k_{ex} with catalyst concentration corresponds to a smaller value of k_{Cl}. Therefore these results suggest that both k_{op} and k_{Cl} are significantly smaller for EthDi bound to tRNA than for EthDi bound to DNA.

IV) H-D exchange kinetics of EthDi in the presence of poly (A). poly (U) and poly (A). 2 poly (U).

Mixtures of poly(A) and poly(U) were prepared so that the total nucleotide concentration remained constant, but that the proportions of poly(A) to poly(U) were varied. EthDi was added at such concentration that there was less than 1 EthDi molecule per 100 nucleotides and the H-D exchange of EthDi was followed. The results are shown in figure 6.

It has been previously shown that EthDi did not bind to single stranded polynucleotides such as poly(A) and poly(U) (31). The presence of single stranded polymer in the mixture cannot interfere therefore with the H-D exchange measurement. It was also shown that EthBr does not bind to poly(A).2 poly(U) (32). With EthDi, binding measurements (results not shown) suggest that this dye displaces the poly(U) strand

from the triple stranded structure to bind to the double stranded poly(A).poly (U). At the low dye/nucleotide ratio used in the H-D exchange measurements it is likely that EthDi is bound to the double stranded regions. This would mean that the presence of triple stranded structure at the contact of double strands is able to accelerate the exchange of EthDi bound to the double stranded regions. In triple stranded regions, the protons involved in Hoogsteen base pairing exchange faster than those involved in Watson Crick pairing (12).

DISCUSSION

EthBr elicits a clear preference for the B form of the polymer and induces the Z → B transition at a relatively low value of the ratio bound dye/base-pair. Interestingly, it seems that adriamycin also induces the Z → B transition at a low dye/base-pair ratio (33). EthDi also binds to the Z form of poly[(dG-m^5dC)]. It does not exhibit a clear preference for the B form and consequently the Z → B transition is only observed at a high dye/base-pair ratio which corresponds to the change in binding mode from bisintercalation to monointercalation. In addition the fluorescence properties of EthDi are very similar when the dye is bound to the Z or to the B form of the polymer. Energy transfer experiments suggest that its binding occurs also by intercalation. Therefore EthDi could represent an useful fluorescence probe for the study of both the Z and B form of DNA. The structural dynamic fluctuations of EthDi bound to Z or B DNA could then be compared. The H-D exchange kinetics of bound EthDi is five times slower on the Z form than on the B form of poly[(dG-m^5dC)]. However the addition of Mg^{++} necessary to stabilize the Z form could have caused an increase of the dissociation rate of the EthDi poly[(dG-m^5dC)] complex. If the duplex dissociation rate k_{-1} increases, the second term of equation 2 can then become larger than k_b. Should this be the case, the exchange would then reflect the rate of dissociation of the complex and no longer the dynamic fluctuation of the DNA-dye complex. However, the similar binding constants of EthBr to poly[(dG-dC)] in Z and B buffers (table 1) excludes this possibility.

It was proposed that the hydrogen exchange behavior of the double helix is controlled by the appearance of "melted" open regions of base pairs which propagate up and down the helix as solitons (1). If this were the case, it is expected that such a large fluctuation will also induce the hydrogen exchange of a bound ligand when the fluctuation encounters

Table 4 : Comparison of mean exchange rate constants for EthDi bound to nucleic acid (measured at 25°C) and nucleic acid imino proton exchange rate constants.

	EthDi bound to nucleic acid $\bar{k}$ (s^{-1}) 25°C	Nucleic acid imino protons k (s^{-1}) 25°C (a)	Ref.
B DNA	4-7	5-6	(34)
poly(dA-dT)	3.1	4	(34)
tRNA	2	1.5-5	(11)
poly(A).poly(U)	0.5	2	(11)
Z DNA	0.6	0.007	(b)

a) Values reported in the literature have been obtained at various temperatures. In order to compare them, they have been standardized to 25°C assuming an activation energy of 16 Kcal/mol.
b) Ramstein, J. (unpublished results).

the ligand on the helix. According to this model, a strong coupling between the hydrogen exchange behavior of DNA and of DNA bound ligands is expected.

However, recent NMR studies (34) do not support such a model. They rather favor the idea that DNA hydrogen exchange is limited by random opening of single base pairs. Such limited fluctuations at the level of a base pair might still be sufficient to induce a structural fluctuation of a neighboring ligand. Therefore, it is of interest to look for a correlation between the behavior of the two types of exchange at the level of the nucleic acid and of the ligand.

The largest difference in the hydrogen exchange kinetics of two different DNAs is between Z and B DNA (9). The Z DNA EthDi complex exchanges slower than the B DNA complex. But the difference is smaller than that between B and Z DNA hydrogen exchange rates. As already discussed, this cannot result from the increase of the EthDi dissociation rate in presence of Mg^{++}. The differences between the hydrogen exchange kinetics of other nucleic acids are not as large as those between Z and B DNA. Still, significant variations are observed. In table 4, the rate constants of hydrogen exchange reported in the literature for different

nucleic acids are compared to the H-D exchange rates measured for the corresponding EthDi complexes. Although the number of cases is small, it can be noted that the rates of exchange for the nucleic acid imino protons and the bound EthDi can be ranged in the same order. The results obtained with poly(A). 2 poly(U) are also of interest. They could suggest that the dynamic properties of a complex could be altered at distance by a telestability effect as already suggested (35). It is also notable that the activation energies associated with the nucleic acid and the EthDi complex exchange rates are similar. It must also be pointed out that it is the H-D exchange of the EthDi amino protons which leads to the fluorescence variation. Therefore, the dynamic structural fluctuations which are recorded by fluorescence could be more directly related to fluctuations in the environement of the EthDi amino groups. Such fluctuations would more likely reflect the dynamic of the phosphate sugar backbone than that of the base pairs.

It has recently been suggested (37) that transient base-pair unstacking is involved in the association or the dissociation of an intercalating drug from DNA. Such a DNA structural fluctuation which is accompanied by a modification of the phosphate sugar backbone geometry might also be involved in the exchange of the EthDi amino protons.

In conclusion, we think that the hypothesis of a correlation between the processes leading to the dynamic structural fluctuation of nucleic acids and nucleic acids bound ligands is worthy of consideration.

ACKNOWLEDGMENTS

Support from the Université Paris VI, UER de Biochimie, Fondation pour la Recherche Medicale Francaise, et Association pour le Développement de la recherche sur le Cancer are gratefully acknowledged. The authors thank Dr S. Brown for helpfull discussions and Mrs J. Couprie for her excellent technical assistance.

REFERENCES

1. ENGLANDER, S.W. and KALLENBACH, N. R. (1983) Quaterly Rev. Biophysics 16, 521-655.
2. MARKOVITS, J., RAMSTEIN, J., ROQUES, B.P. and LE PECQ, J.B. (1983) Biochemistry 22, 3231-3237.
3. WAHL, P.H., PAOLETTI, J. and LE PECQ, J.B. (1970) Proc. Natl. Acad. Sci. USA 65, 417-421.
4. LE BRET, M. (1978) Biopolymers 17, 1939-1955.
5. BARKLEY, M.D. and ZIMM, B.H. (1979) J. Chem. Phys. 70, 2991-3007.
6. ALLISON, S.A. and SCHURR, J.M. (1979) Chem. Phys. 41, 35-59.

7. PARDI, A., MORDEN, K.M., PATEL, D.J. and TINOCO, Jr. I. (1983) Biochemistry 22, 1107-1113.
8. PATEL, D., PARDI, A. and ITAKURA, K. (1982) Science 216, 581-590.
9. RAMSTEIN, J. and LENG, M. (1980) Nature 288, 413-414.
10. RAMSTEIN, J. and BUCKINGHAM, R.H. (1980) Proc. Natl. Acad. Sci. USA 78, 1567-1571.
11. JOHNSTON, P.D. and REDFIELD, A.G. (1981) Biochemistry 20, 3996-4006.
12. HAYASHI, Y., NAKANISHI, M., TSUBOI, M., FUKUI, T., IKEHARA, M., TAZAWA, I. and INOUE, Y. (1982) Biochim. Biophys. Acta 698, 93-99.
13. GAUGAIN, B., BARBET, J., OBERLIN, R., ROQUES B.P. and LE PECQ, J.B (1978) Chemistry 17, 5071-5078.
14. WELLS, R.D., LARSON, J.E., GRANT, R.C., SHORTLE, B.E. and CANTOR, C.R. (1970) J. Mol. Biol. 54, 465-497.
15. BEHE, M. and FELSENFELD, G. (1981) Proc. Natl. Acad. Sci. USA 78, 1619-1623.
16. MARQUARDT, D.W. (1963) J. Soc. Ind. Appl. Math. 11, 431-441.
17. LE PECQ, J.B. and PAOLETTI, C. (1967) J. Mol. Biol. 27, 87-106.
18. Mc GHEE, J.D. and Von HIPPEL, P.H. (1974) J. Mol. Biol. 86, 469-489.
19. MULLER, W. and CROTHERS, D.M. (1975) Eur. J. Biochem. 54, 267-277.
20. MARKOVITS, J., GAUGAIN, B., BARBET, J., ROQUES, B.P. and LE PECQ, . J.B. (1981) Biochemistry 20, 3042-3048.
21. MULLER, W., CROTHERS, D.M. and WARING, M.J. (1973) Eur. J. Biochem 39, 223-234.
22. LAMBERT, B. and LE PECQ, J.B. (1984) Biochemistry 23, 166-176.
23. POHL, F., JOVIN, T.M., BAEHR, W. and HOLBROOK, J.J. (1972) Proc. Natl. Acad. Sci. USA 69, 3805-3809.
24. VAN DE SANDE, J. and JOVIN, T.M. (1982) EMBO J. 1, 115-120.
25. SHAFER, R.H., BROWN, S.C., DELBARRE, A. and WADE, D. (1984) Nucleic Acids Res. 12, 4679-4690.
26. REINHARDT, C., ROQUES, B.P. and LE PECQ, J.B. (1982) Biochem. Biophys. Res. Commun. 104, 1376-1385.
27. GAUGAIN, B., BARBET, J., CAPELLE, N., ROQUES B.P. and LE PECQ, J.B. (1978) Biochemistry 17, 5078-5088.
28. DELBARRE, A., GOUREVITCH, M.I., GAUGAIN, B., LE PECQ, J.B. and ROQUES, B.P. (1983) Nucleic Acids Res. 11, 4467-4482.
29. LAGRAVERE, C., MALFOY, B., LENG, M. and LAVAL, J. (1984) Nature 310, 798-800.
30. MANDAL, C., ENGLANDER, S.W. and KALLENBACH, N.R. (1980) Biochemistry 19, 5819-5825.
31. MARKOVITS, J., ROQUES, B.P. and LE PECQ, J.B. (1979) Anal. Biochem. 94, 259-264.
32. LE PECQ, J.B. (1971) Methods of Biochemical Analysis 20, 41-86.
33. CHEN, C.W., KNOP, R.H. and COHEN, S.S. (1983) Biochemistry 22, 5468-5471.
34. ASSA-MUNT, N., GRANOT, J., BEHLING, R.W. and KEARNS, D.R. (1984) Biochemistry 23, 944-955.
35. BURD, J.F., WARTELL, R.M., DODGSON, J.B. and WELLS, R.D. (1975) J. Biol. Chem. 250, 5109-5113.
36. SCHWARZ, G. (1968) Rev. Modern Phys. 40, 206-218.
37. GANDECHA, B.M., BROWN, J.R. and CRAMPTON, M.R. (1985) Biochem. Pharmacol. 34, 733-736.