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A stopped-flow H−D exchange kinetic study of spermine−polynucleotide interactions

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ABSTRACT

The rates of H-D exchange for imino and amino protons in adenosine, calf thymus DNA, poly (dA-dT), poly(dG-dC), and poly (dG-me^{5}dC) were determined using stopped flow kinetic methods in the presence of various concentrations of Tris, imidazole, Mg^{2+}, and spermine in citrate buffer (pH 7, 25°C). CD spectroscopic studies showed that all polynucleotides always remain in the B-form under these conditions. An increase in the concentration of Tris and imidazole from 5 μ M to 20 mM caused an increase in the rates of exchange of both fast-exchanging imino and slow-exchanging amino protons. The limiting rates of exchange at infinite concentrations of catalysts were found to be different for fast (31-57 sec^{-1}) and slow (1-2 sec^{-1}) exchanging protons. These results indicate that imino and amino protons of B-DNA exchange asymmetrically from two different open states as observed for Z-DNA.

An increase in the concentration of spermine from a ratio of 1:50 to 1:2 of positive charge/phosphate decreased the rate of exchange of imino protons of calf-thymus DNA, poly(dG-dC), and poly(dG-me^{5}dC), but increased the rate of exchange of the imino protons of poly(dA-dT) without affecting the exchange rate of the amino protons of any of the polynucleotides. These results are interpreted in terms of possible spermine-induced change of conformations of oligonucleotides of specific sequence that has been suggested by theoretical model building studies.

INTRODUCTION

The polyamines putrescine, spermidine, and spermine are low molecular weight polycations found in all living cells that have been linked with cellular growth and differentiation (1,2). The binding of polyamines to nucleic acids affects various aspects of nucleic acid structure, conformation, and interactions with other molecules (3-12). One of the most significant effects is the observation that micromolecular concentrations of spermine can stabilize the left-handed Z conformation (13-16) of poly(dG-me^{5}dC) at physiologic (17) or lower (11,18) salt concentrations. While some features of the polyamine-DNA interaction may be explained in terms of nonspecific electrostatic binding (4), based on the counterion condensation theory of Manning (19), there are certain facits of this binding that indicate a substantial degree of base and/or sequence specificity (9-12). Early theoretical studies (20-22) considered binding of spermine in the minor groove of double helical DNA while a recent energy minimization and computer graphics

analysis of spermine-oligonucleotide complexes carried out in our laboratory indicates that spermine has a preference for binding in the major groove (23).

The conformations of polynucleotides, the flexibility of their backbones, and the stabilities of specific base-pairs have a pronounced effect on the rate of exchange of amino and imino protons of bases (for review see 24). Cross and his coworkers (25,26) first introduced the stopped-flow UV spectroscopic technique to measure the rate of exchange of amino protons of adenosine for deuterium. This method has been used to study the exchange rate of protons in DNA (27) and polynucleotides (28). While NMR has the advantage of permitting direct assignment of exchange processes with specific protons (29), the stopped-flow technique allows measurement of the slowly exchanging amino protons. There is increasing evidence that polynucleotide conformation has a pronounced effect on proton exchange rates. For example, the rate of exchange of both the fast-exchanging imino and the slow-exchanging amino protons decreases dramatically because of the B to Z transition, as determined by both NMR and stopped-flow studies (30-33).

Given the known effects of polyamines on DNA conformation and the known dependence of conformation on H-D exchange kinetics, we have investigated the possible effects of spermine on H-D exchange kinetics in polynucleotides. Results show that exchange depends on sequence-specific changes in the dynamics of DNA structure that correlate well with our theoretical model for DNA-spermine interactions (23).

MATERIALS AND METHODS

Reagents and solutions

Poly(dA-dT), poly(dG-dC), and poly(dG-me^{5}dC) (Pharmacia P-L Biochemicals, Milwaukee, WI), calf-thymus DNA, deuterium oxide, Na-cacodylate (Sigma Chemical Co, St. Louis, MO), and spermine (Calbiochem, La Jolla, CA) were obtained from commercial suppliers. All chemicals were reagent grade and quartz double distilled water was used as the solvent.

Spermine and $MgCl_2$ were dried overnight in a vacuum dessicator, weighed, and dissolved in the appropriate buffer to prepare solutions of desired concentration. DNA and polynucleotides were dissolved in the desired buffer containing 0.1 mM EDTA and dialysed extensively against the same buffer that did not contain EDTA. The concentrations of nucleic acids in solutions were determined from their UV absorbance using reported molar extinction coefficients (34-36).

UV and CD spectroscopic studies

UV spectra of DNA and polynucleotides were recorded in 50 mM NaCl, 1 mM Tris-HCl (pH=7.02) at room temperature using a Gilford model 2600 UV and visible spectrophotometer coupled with a Hewlett Packard model 7225B plotter. CD spectra were

recorded at 60°C in either 15 mM Na-citrate, 5 mM NaCl (pH=7.02) (citrate buffer) or 50 mM NaCl, 1 mM Na-cacodylate (pH=7.02) (cacodylate buffer) using a Jasco J-500 spectropolarimeter coupled with a DP-500 data processor and IBM personal computer for data collection and storage.

Stopped-flow kinetic studies

Samples for the stopped-flow proton-deuterium exchange kinetic measurements were prepared by filtering solutions twice through 22 micron pore-size Millipore filters, after which they were degassed overnight in a vacuum dessicator at room temperature. Kinetic studies were carried out by recording the decrease in the absorbance at 285 nm of approximately 2.0 A_{260} units/ml of polynucleotide in aqueous citrate buffer mixed with an equal volume of the same buffer made in deuterium oxide (pD=7.02) at 25 $\pm$ 0.1°C in a Durrum D-100 rapid kinetic system coupled with a North Star Horizon computer (On Line-Instrument Systems, Jefferson, GA) for data collection, storage, and analysis. The same concentration of Tris and imidazole were present in buffers prepared in either H_2O or D_2O, and the pH or pD of buffers were adjusted after the addition of bases; pD was determined as the pH_{meter} + 0.4 (37). After allowing for a mixing dead-time of 2 msec., 250 data points were collected within periods of 2 to 30 sec for different samples. Ten sets of data were collected in each experiment and the mean of the observations was fitted to one or more expontentials and a base line using a nonlinear regression program based on the Marquadt-Levenberg algorithm that can fit up to three expotentials and a base line. Rate constants obtained from 6 different experiments were used to determine the average rates and standard deviations of each data point reported here.

RESULTS

UV spectra of poly(dA-dT) and poly(dG-dC) in the presence of different concentrations of spermine are shown in Figure 1. For these experiments, stock solutions of spermine were prepared by adding appropriate amounts of polynucleotides to match the concentrations of those used to produce control spectra. These stocks were added to polynucleotides to avoid the dilution of polynucleotide solution by addition of spermine. The UV absorbance of poly(dA-dT) decreased and that of poly(dG-dC) increased with increasing concentration of spermine in the range in which no change in A_{320}, which is indicative of polynucleotide aggregation, was observed. Increasing the concentration of spermine further caused an increase in the absorbances of both polynucleotides because of aggregation. No immediate aggregation of polynucleotides took place up to a ratio of 1 spermine:4 base-pairs in buffer containing 50 mM Na^+. However, there was evidence for aggregation of poly(dG-dC) but not poly(dA-dT) when polynucleotides were incubated with spermine at that ratio for 24 hr at 4°C at identical ionic strengths.

The CD spectra of poly(dG-me^5dC) in the presence and absence of different

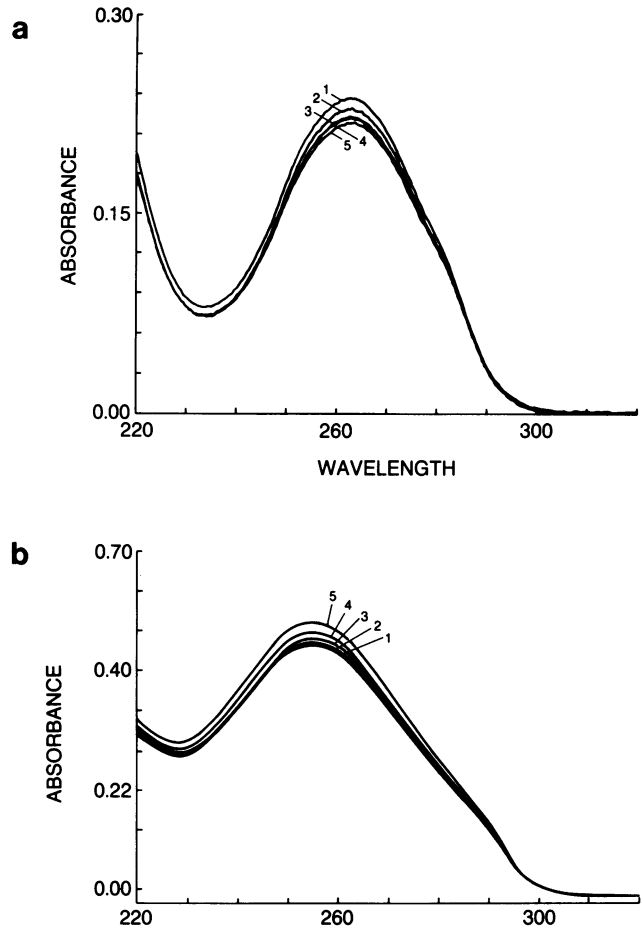

Figure 1. UV spectra of poly(dA-dT) (a) and poly(dG-dC) (b) (approximately 40 μ M nucleotide phosphate) with or without various concentrations of spermine in 50 mM NaCl, 1 mM Tris, and 0.1 mM EDTA (pH 7.2) at room temperature immediately after addition of (1) 0, (2) 2.5 μ M, (3) 5 μ M, (4) 7.5 μ M, and (5) 10 μ M spermine.

concentrations of Mg^{2+} and spermine in citrate and cacodylate buffers are shown in Figure 2. The concentration of Mg^{2+} and spermine required for the B to Z transition of poly(dG-me^{5}dC) in cacodylate buffer was comparable to the concentrations required for the transition in Tris-HCl buffer reported by Behe and Felsenfeld (17). Surprisingly, a 50 fold higher concentration of Mg^{2+} was required to cause the same transition in citrate buffer. The same concentration of spermine used in cacodylate buffer does not appear to affect the conformation of poly(dG-me^{5}dC) in citrate buffer. Similar concentrations of

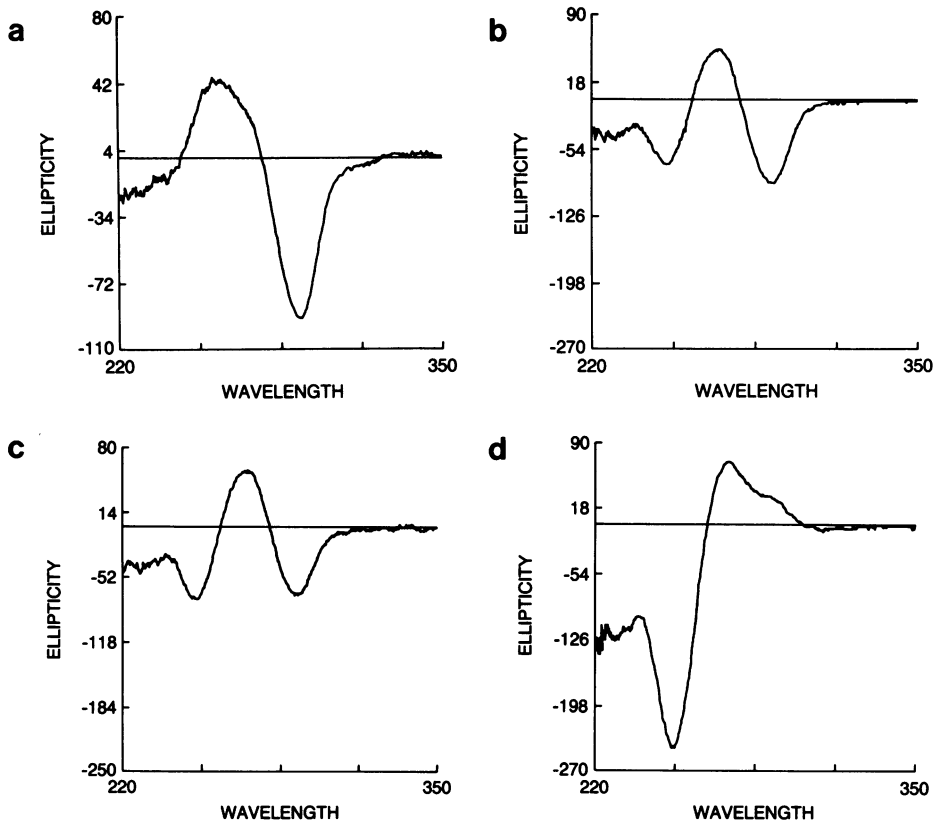

Figure 2. CD spectra of poly(dG-me^{5}dC) (approximately 20 μ M nucleotide phosphate) at 60°C in (a) 50 mM NaCl, 1 mM cacodylate, 2 mM Mg^{2+}, pH 7; (b) 15 mM citrate, 5 mM NaCl, 50 mM Mg^{2+}, pH 7.0; (c), 50 mM NaCl, 1 mM cacodylate, 2.5 μ M spermine, pH 7.0; (d), 15 mM citrate, 5 mM NaCl, 2.5 μ M spermine, pH 7.0.

Mg^{2+} and/or spermine did not affect the CD spectrum of poly(dG-dC) in any of the buffers.

Before studying the effects of spermine on H-D exchange kinetics of the various polynucleotides, we studied the effects of the bases imidazole and Tris on H-D exchange. Typical profiles of the change in A_{285} of polynucleotides with time after mixing with the buffer prepared in D_2O are shown in Figure 3(a). Rate constants for the H-D exchange of calf thymus DNA and polynucleotides were calculated by fitting data to two exponentials and a base line for polynucleotides in general, one exponential and a base line for adenosine, and three exponentials and a base line for poly(dG-dC) and poly(dG-me^{5}dC) in the absence of any catalyst. A representative fitted curve is shown in Figure 3(b). The

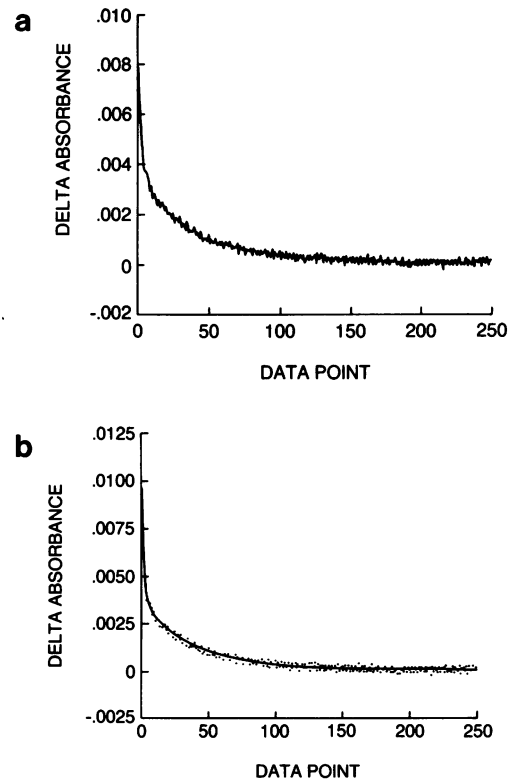

Figure 3. (a) A representative stopped-flow kinetic profile of the decrease in absorbance at 285 nm of poly(dA-dT) (approximately 2.0 A_{259} units) in 15 mM citrate, 5 mM NaCl and 50 μ M Tris, pH 7.0, at 25 ± 0.1°C within 10 sec of mixing with the same buffer made in D_2O.

(b) The plot of the curve obtained by fitting the data points in (a) to two exponentials and a base line.

values of the rate constants thus obtained are close to those of calf thymus DNA (27) and poly(dA-dT) (31) determined under comparable conditions. Nakanishi et al. (27) reported a four-fold lower value of the exchange rate of poly(dA-dT) in approximately 0.4 M Na^+. Salt-induced stabilization of the double strand may cause this difference in the exchange rate. In our system, an approximately 1.5-fold decrease in the exchange rates of both poly(dA-dT) and poly(dG-dC) was observed when the NaCl concentration was increased from 50 to 100 mM (unpublished results).

Plots of the rate constants for both the fast and slow phases of the reaction for poly(dA-dT) and poly(dG-dC) at various concentrations of Tris and imidazole in citrate buffer are shown in Figure 4. It is evident that both the fast and the slow phases of the

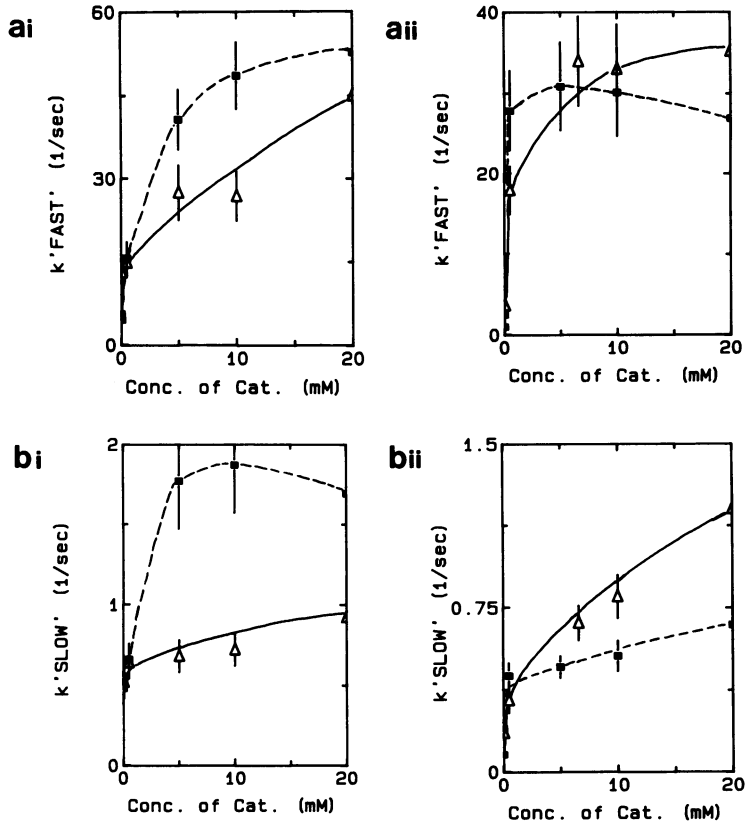

Figure 4. Plot of the fast (a) and slow (b) rate constants for H-D exchange of (i) poly(dA-dT) and (ii) poly(dG-dC) vs. concentrations of Tris (△) and imidazole (■) in 15 mM citrate, 5 mM NaCl, pH 7.0, at 25 ± 0.1°C.

reaction increased with an increase in the concentrations of both Tris and imidazole. Plots of the reciprocals of the exchange rates of the fast and the slow phases of the reaction against the reciprocals of concentrations of Tris and imidazole in the range of 0 to 5 mM were found to be linear. Extrapolation of these plots to infinite catalyst concentration gives the limiting values of the rate of exchange, which are listed in Table 1.

Results of the effect of spermine on H-D exchange kinetics of adenosine, calf-thymus DNA, poly(dA-dT), poly(dG-dC), and poly(dG-me^{5}dC) are shown in Figure 5. It is clear that the exchange behavior of adenosine is unaffected by addition of spermine. However, an increase in the concentration of spermine causes the fast phase of the H-D exchange to decrease for all polynucleotides except poly(dA-dT), in which the fast phase

TABLE I

Rate constants for the fast (k_{fast}) and slow (k_{slow}) phases of the H-D exchange kinetics of poly(dG-dC) and poly(dA-dT) at infinite concentration of Tris and imidazole

Sample	$1/k_{fast}$ (sec)	k_{fast} (sec^{-1})	r	$1/k_{slow}$ (sec)	k_{slow} (sec^{-1})	r
Tris-d(C-G)	0.029 ± 0.001	34.5	0.99	0.72 ± 0.20	1.4	0.87
Tris-d(A-T)	0.018 ± 0.001	55.6	0.99	0.99 ± 0.11	1.0	0.92
Imidazole-d(C-G)	0.032 ± 0.001	31.3	0.96	1.4 ± 0.19	0.71	0.86
Imidazole-d(A-T)	0.019 ± 0.001	52.6	0.99	0.47 ± 0.01	2.1	0.99

r = correlation coefficient.

increased after a small initial decrease. On the other hand, increasing concentrations of Mg^{2+} decreased the exchange rate of the fast exchanging protons of all the polynucleotides (data not shown). The slow phase of the exchange reaction remains more or less unaffected by increasing concentrations of both spermine and Mg^{2+}. All reactions were carried out in citrate buffer, which does not catalyze the exchange reaction. Moreover, the concentration of spermine and Mg^{2+} used did not cause a B to Z transition of any of the polynucleotides in citrate buffer, as determined by CD spectroscopy (see Figure 2).

These experiments show that catalysts such as Tris and imidazole enhance the rate of both the fast and slow phases of the exchange reaction of all polynucleotides studied, while Mg^{2+} decreased the fast rates of exchange for all polynucleotides. In contrast, spermine has different effects on the fast phase of the exchange in poly(dA-dT) and poly(dG-dC).

DISCUSSION

The UV spectra of poly(dA-dT) and poly(dG-dC) (Figure 1) show that minor changes in base-stacking take place upon addition of spermine and that the changes depend on base sequence. Moreover, aggregration induced by spermine is most pronounced for poly(dG-dC) (data not shown). Although it is known that Z-DNA aggregates more readily than B-DNA, and that poly(dG-dC) but not poly(dA-dT) can undergo a transition from B- to Z-DNA under certain conditions (17), the B to Z transition of poly(dG-dC) was neither expected nor was it observed under our experimental conditions (Figure 2). The relatively higher affinity of spermine for GC-rich than for AT-rich DNA (13) could be responsible

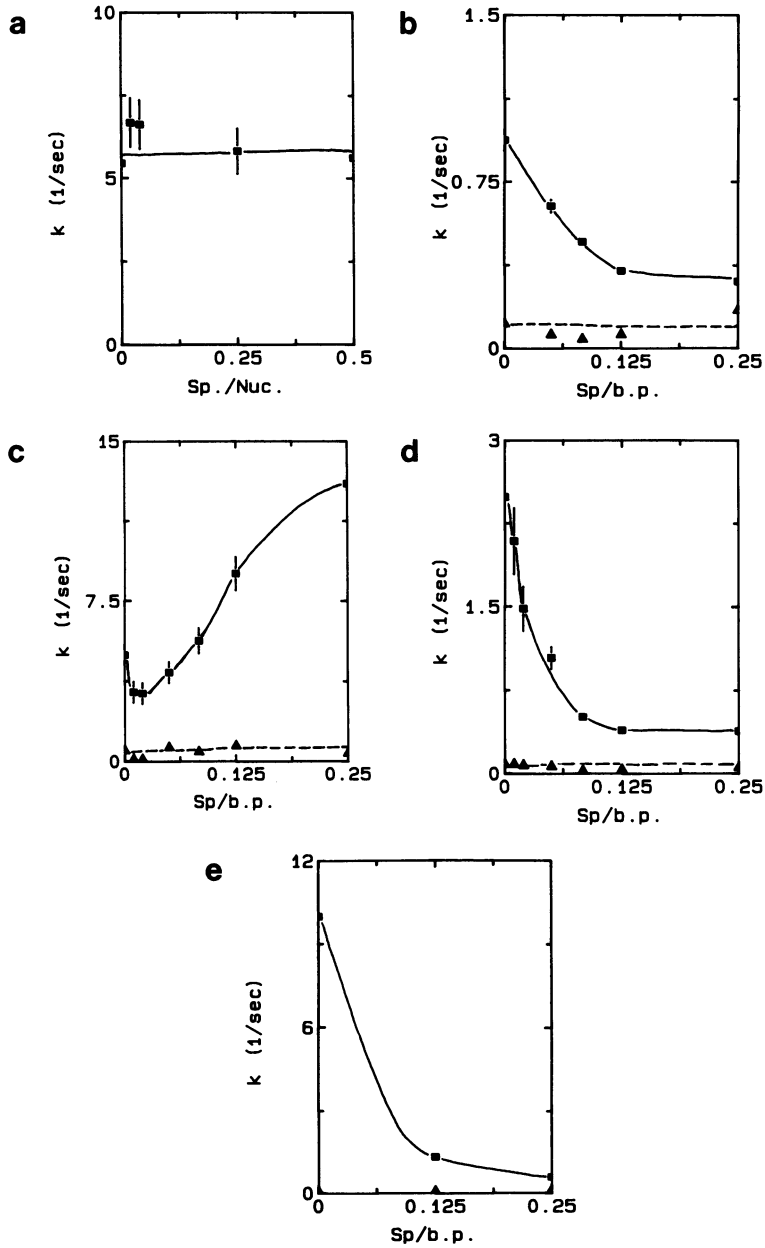

Figure 5. Plot of the fast (■) and slow (▲) rate constants of H-D exchange from (a) adenosine (only one rate constant could be monitored); (b) native calf thymus DNA; (c), poly(dA-dT); (d) poly(dG-dC), and (e) poly(dG-me^{5}dC) in 15 mM citrate and 5 mM NaCl, pH 7.0, at 25 ± 0.1°C.

for the greater extent of saturation and preferential effect on the aggregration of poly(dG-dC).

Because no evidence for non-electrostatic interactions between spermine and nucleic acids has been observed to date (40), it is difficult to find any reason for the sequence specificity in the spermine-nucleic acid interaction. However, recently reported (41) theoretical calculations of the electrostatic interaction between Cro-repressor protein and both specific and non-specific DNA sequences showed that the protein is able to induce a bend in the specific operator sequence only due to the charge distribution on the surface of the polymer and the electrostatic forces of interaction. These results suggest the possibility that spermine may have a specific effect on the conformation of certain base sequences solely on the basis of the charge distributions along spermine and the nucleic acid chain.

We have no explanation for the inhibitory effect of citrate buffer on the B to Z transition of poly(dG-me^{5}dC) (Figure 2). The possibility that the chelating effect of citrate may be removing polyvalent ions from solution (42) can be ruled out in as much as the concentration of Mg^{2+} required to induce the transition in citrate buffer was about 7-fold higher than the concentration of citrate. This phenomenon, however, provided the opportunity to study the H-D exchange kinetics of different polynucleotides in the B-form both in the presence and the absence of spermine.

H-D exchange kinetics are usually interpreted in terms of the following mechanism first proposed by Teitelbaum and Englander (43,44).

$$\text{closed} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \text{open} \xrightarrow{k_{ch}} \text{exchanged} \qquad (1)$$

This model is based on the hypothesis that exchange cannot occur directly from the closed form of the double helix because exchangable protons are inaccessible to solvent molecules. A conformational change to an "open" state must occur before exchange can take place. Rate constants for the opening and closing reactions are k_{op} and k_{cl}, respectively, and their ratio $K_{eq} = k_{op}/k_{cl}$ is the equilibrium constant for the formation of the open state. The rate constant k_{ch} for proton transfer is directly proportional to the concentration of catalyst. When $K_{eq} < 1$, the measured rate constant for exchange can be written as

$$k_{ex} = k_{op} \cdot k_{ch}/(k_{cl} + k_{ch}) \qquad (2)$$

which has hyperbolic dependence on the concentration of catalyst.

Two limiting forms of Eq. 2 depend on the relative magnitudes of k_{cl} and k_{ch}. When exchange from the open state is fast compared to the formation of the closed state ($k_{ch} > k_{cl}$), $k_{ex} = k_{op}$, the system is open-limited and the rate of exchange is a direct measure of the rate of opening. In the other case, the open $\rightleftharpoons$ close equilibrum is fast

relative to the rate of exchange ($k_{cl} > k_{ch}$) and the system is in the pre-equilibrium regime. The limiting equation for this case is $k_{ex} = K_{eq}\ k_{ch}$, and the exchange rate depends linearly on catalyst concentration.

Results for the effect of imidazole and Tris on the H-D exchange rates indicate that both fast and slow exchange processes in poly(dA-dT) and poly(dG-dC) are significantly enhanced by increasing catalyst concentration. Although direct assignment of exchange processes to specific protons is not possible with UV detection, much evidence exists to indicate that imino protons exchange more rapidly than amino protons (24). Therefore, for exchange in poly(dA-dT), the imino and the adenine amino protons have been assigned to the fast and slow exchange rates, respectively (38). In terms of the mechanism discussed above, we can conclude that in all cases open-limited behavior is not observed, except perhaps at high catalyst concentrations. For the fast process in poly(dA-dT), the limiting exchange rates at infinite concentrations of imidazole and Tris (Table 1) appear to be qualitatively similar to and are in fair agreement with opening rates for oligodeoxyribonucleotides measured by NMR (45). The slow exchange rate for poly(dA-dT) is different for the two catalysts. This difference may be the result of an interaction with the polynucleotide (28). In their early work on poly(dA-dT), Teitelbaum and Englander (43) found that catalysts had little effect on the fast exchange process. However, these rates were at the limit of detection of the tritium exchange method. Our results show that imidazole has a larger effect than Tris on the slow rate of exchange, which is similar to results reported by Teitelbaum and Englander (44). A catalytic effect of Tris on the rate of exchange of imino protons of a synthetic oligonucleotide reported recently by Braunlin and Bloomfield (46) is also comparable to the results reported here.

We found that poly(dG-dC) has three distinctly different exchange rates, similar to poly(rG)·poly(rC) found by Teitelbaum and Englander (44). As in the case of poly(rG)·poly(rC), these rates may be assigned tentatively to the imino, the cytosine amino, and the guanine amino protons in decreasing order of magnitude. As the concentration of catalysts is increased, the rates of exchange of all protons are generally increased, but the exchange rate of the guanine amino proton increases more rapidly than the rate for the cytosine amino proton and quickly reaches a value where their exchange rates are no longer distinguishable. This result is different than the observation reported by Teitelbaum and Englander (44), who found that cytosine amino protons of poly(rG-rC) exchange at a faster rate in the presence of Tris at a lower pH. Different rates of exchange for the imino and amino protons were found at infinite concentrations of Tris and imidazole (Table 1), a condition at which $k_{ex} = k_{op}$. It seems likely that different protons of B-DNA exchange asymmetrically from two different open states, a finding reported for exchange in Z-DNA (33).

In the concentration range used, spermine exhibited no catalytic effect on the rate

of exchange of adenosine amino protons (Figure 5[a]). This was also confirmed by the finding that the slow phase of the exchange reaction of DNA and polynucleotides, which is highly susceptible to general base catalysis (28,29), does not increase in the presence of spermine. Therefore, either k_{op} or k_{cl}, or both, must be affected by spermine in order to induce the changes observed in the exchange rates of polynucleotides (Figure 5).

The decrease in k_{op} of calf-thymus DNA, poly(dG-dC), and poly(dG-me^{5}dC) in the presence of increasing concentrations of spermine and Mg^{2+} reported here could decrease the K_{eq} and thereby stabilize the closed form, which parallels the effect of these cations on the thermal stabilization of the double helix. The magnitude of the reduction in rates and the saturation values obtained for spermine, however, are different for different polymers and are also different from results obtained with Mg^{2+} (data not shown). It is also possible that spermine can affect k_{cl} as well; further studies will be necessary to calculate the individual values of k_{op} and k_{cl} before anything can be said conclusively about this process.

Spermine has little or no effect on the exchange rate of the guanine amino protons, but the rates of exchange of the imino and the cytosine amino protons decrease rapidly. The decrease in the rates of exchange for the cytosine amino protons makes it kinetically indistinguishable from the rates of exchange for guanine. Because cytosine amino protons are in the major groove of the helix, the rapid decrease of their exchange rate could be the result of preferential binding of spermine on the major groove as observed in the crystal structure of a dodecanucleotide (10) and predicted from the results of theoretical model building and energy minimization studies carried out in our laboratory (23).

The increase in the imino proton exchange rate of poly(dA-dT) with increasing spermine concentration (Figure 5[c]) may indicate that a change in the conformation of the polynucleotide takes place that increases the accessibility of imino protons of the residues to solvent. Using NMR techniques, which have the advantage of allowing direct identification of exchanging protons, it was found that spermine and/or spermidine cause an increase in the exchange rate of adenine imino protons in a synthetic oligodeoxyribonucleotide of defined sequence (47) and of the imino protons involved in hydrogen bonding in A·U and G·U base pairs of yeast $tRNA^{phe}$ (48). We could not take advantage of this method because the concentration of polynucleotide required for NMR studies is too high to prevent its aggregation after spermine addition. However, results of both NMR and stopped-flow studies suggest that despite the overall effect of polyamines on the stability of the helix, as seen by the enhancement of T_m (5), polyamines may cause a local destabilization of specific sequences containing A-T base pairs.

In the classical B-DNA structure, each imino proton hydrogen bond in poly(dG-dC) is protected from solvent by two hydrogen bonds involving amino protons in both the major and minor grooves. Imino protons involved in the hydrogen bond in poly(dA-dT), however,

are protected from solvent only in the major groove. It is possible that the binding of spermine on the major groove of poly(dA-dT) introduces a bend in the macromolecule, as found in theoretical model building studies (23). As a consequence of this bend, the minor groove of the helix expands, which causes the imino protons to be more exposed to solvent and leads to the observed enhancement of the exchange rate. The same model predicts that binding of spermine causes a similar change in the conformation of poly(dG-dC). However, in the latter case such bending may not induce sufficient strain to break the hydrogen bonds in the minor groove. Therefore, for poly(dG-dC) and poly(dG-me^{5}dC), spermine may act like any other polyvalent cation such as Mg^{2+} and decrease the exchange rate by causing a general stabilization of the helix.

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