Local Dynamics in DNA by Temperature-Dependent Stokes Shifts of an Intercalated Dye

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Abstract: For the first time, the static and dynamic properties of the interior of DNA have been measured through their effects on the Stokes shift of an intercalated dye. Fluorescence excitation and emission spectra of acridine orange (AO) intercalated in DNA have been measured from 100 to 320 K in a 3:1 glycerol−aqueous-buffer mixture. The solvent dependence of the excitation spectrum shows that AO is sensitive to the polarizability of its local environment but is insensitive to the local polarity. The interior of DNA provides a highly polarizable environment, similar to simple aromatic solvents. The Stokes shift of AO results from movements of neighboring groups that change the effective cavity size of the dye. A large portion of the Stokes shift in DNA can be frozen out at low temperature, as it can be in solution. This result shows that the interior of DNA has the diffusive and viscous dynamics characteristic of a fluid, rather than the purely vibrational dynamics of a crystal. At high viscosity, the rate of these dynamics is linked to that of the bulk solvent. We argue that the dye is sensing the movement of the DNA, and we propose that, at high viscosity, the rate of DNA motion is limited by the rate of solvent motion. The potential for extending these measurements to low solvent viscosities with ultrafast spectroscopy is very good.

Introduction

Many experiments have investigated the large amplitude tumbling, flexing, and bending of DNA on slow time scales.[1−4] Few have looked at the localized, low-amplitude dynamics on the scale of individual nucleotides. These dynamics are important for at least two reasons. Chemical reactions involving DNA depend on local interactions with the reacting site. In addition, the long-range mechanical properties of DNA have their origin in the dynamics of molecule-sized segments, and an understanding of the sequence dependence of these properties must start at a molecular length scale. Similar dynamics in simple liquids have been extensively probed by using the electronic transitions of dissolved dye molecules.[5−13] This paper reports the first step in applying similar techniques to probe the dynamics of DNA. The results show that the interior of DNA has the diffusive, viscous dynamics more typical of a fluid, rather than the purely vibrational, elastic dynamics typical of a crystal.

Dynamics in condensed materials can be broadly classified as diffusive or vibrational. All motions in a crystal are purely vibrational and can be attributed to independent harmonic oscillators (phonons). The motions are rapid, normally subpicosecond, and the resulting macroscopic mechanical properties are elastic. The rate of the dynamics is essentially temperature independent. In more disordered materials, such as typical liquids, movement of one group or molecule is dependent in a complex fashion on the movement of many other groups, and the dynamics become diffusive. The material is viscous on a macroscopic scale, and many motions become slow. As with the viscosity of liquids, the rate of diffusive dynamics slows dramatically at low temperatures and may even be frozen out if the material enters a glassy phase. A number of experiments have shown that the dynamics of proteins are largely diffusive[14,15] and may even display a glass transition.[16−21] DNA is often thought to be more rigid and structured than a protein, but clearly it is not as structured as a molecular crystal. Where DNA dynamics reside on a scale of liquidlike to solidlike is uncertain. The primary goal of this paper is determining whether DNA dynamics are diffusive or vibrational.

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In many systems, including DNA, the nature of local dynamics is central to determining the rates of chemical reactions. The key time scale for chemistry is the subpicosecond time needed for bond breaking and formation.[22−24] If the environmental dynamics are vibrational, neighboring groups can move rapidly to accommodate changes in structure as the reaction barrier is crossed. If the dynamics are diffusive, much of the reorganization of the environment that appears in the product cannot occur quickly enough to be effective in lowering the barrier height of the reaction. In the limiting case of reactions involving large amplitude motion (e.g., isomerizations), the rate of reaction varies directly with the viscosity of the environment.[25,26] A special and widely studied chemical reaction is electron transfer. It is widely recognized that the rate of electron transfer is directly dependent on the magnitude and rate of reorganization of the environment.[13,24,27]

These general features of dynamics play an integral role in the function of DNA. For example, the dynamic nature of DNA has been implicated as a control point for protein recognition. In the conventional lock and key mechanism, rigid structural features determine protein/DNA interactions, but recent results indicate that the interaction is often more allosteric than structural.[28−31] Additionally, charge transfer through DNA may be extremely fast and may control oxidative damage propagation and repair.[32−37] The short-range flexibility and mechanical properties of DNA are also important to its function. In several cases, crystal structures show that transcription factor proteins induce an extraordinary degree of DNA bending over a few base pairs, when they bind to their target sequence.[38−40] Protein-induced DNA bending is correlated with regulatory functions in other systems, including damage recognition.[41−48] DNA repair enzymes can completely swing out a damaged base, an extreme distortion at the nucleotide level.[49−51] To perform these functions, DNA needs structural flexibility, which originates in the movement of individual bases, sugars, and phosphate groups.

A number of techniques have been used to look at DNA dynamics at various time and length scales, but information on localized dynamics and short time scales has been the most difficult to obtain. A number of different NMR techniques have been used to extract dynamical information on DNA,[52−59] and this work has been reviewed in detail.[1,60] In general, the "overall" dynamics corresponding to the rigid-body tumbling motion of an oligomer is well characterized by NMR. In many cases, the total amplitude of motion due to all "internal" motions can be found and is typically in the range 5−30°.[52−57,59] These internal motions comprise a variety of motions, including both collective, long-range bending and twisting of the helix and local motions of the bases and backbone. These motions are typically rapid enough to be strongly averaged in an NMR experiment, but in favorable cases the average effective correlation time can be estimated to be in the range of tens to hundreds of picoseconds.[52,53,57] Although it is not generally possible to decompose the average amplitude and time scale into the contributing components, the fact that there is substantial variation in these parameters between different sites suggests that local motions are important contributors.[56,59]

A similar situation exists with time-resolved fluorescence depolarization experiments. These experiments are highly sensitive to overall reorientation of the DNA structure and, with care, information on long-range bending and twisting can be obtained.[4,61−70] A very small and rapid initial depolarization has been attributed to local motion of the dye on the ∼100 ps time scale.[4,62−65]

These experiments lead to the general conclusion that DNA has local motions of significant amplitude that are on the picosecond time scale in aqueous solution. These times are too

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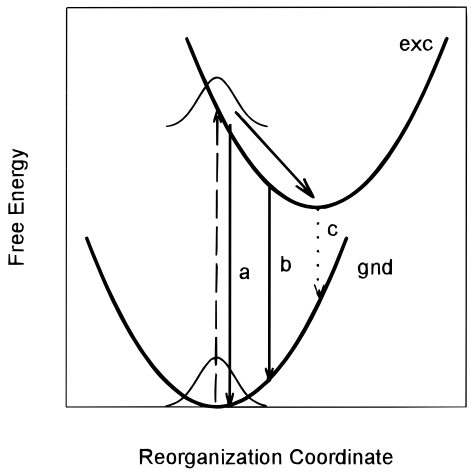

Figure 1. Schematic illustration of the Stokes-shift experiment. Optical excitation transfers the equilibrium distribution of molecules from the ground state to the excited state without reorganizing the environment (dashed arrow). With time, the environment reorganizes to lower the energy of the system. The fluorescence frequency decreases as the reorganization proceeds (a, b, c). If the solvent reorganization is slower than the excited-state lifetime, the fully relaxed Stokes shift (c) is never observed.

slow for purely vibrational motion, suggesting that they may have a diffusive character. Other experiments also support this picture. X-ray[71] measurements show significant amplitude of movement about the equilibrium structure of DNA. Molecular dynamics simulations consistently find motion of bases and backbone segments on the tens to hundreds of picosecond time scales.[72−75] Calorimetry has seen features similar to a glass transition in DNA.[76,77] Measurements of absorption line widths of an intercalated dye show deviations from solid-state theories at high temperature, suggesting the existence of strong anharmonicity, as is seen in liquids.[78] In all of these experiments, it is not yet clear if motion occurs throughout a continuum of conformations or a finite set of substates.[79−83]

Measurement of the fluorescence Stokes shift of a probe molecule is a powerful method to measure reorganization of the probe's local environment. This method has been used extensively in the measurement of solvation dynamics in liquids.[5−13] The concept is illustrated in Figure 1. The reorganization of the local DNA environment is represented schematically by a single coordinate. Because of changes in the chromophore's properties (charge distribution, polarizability, size, etc.), the excited-state energy minimizes at a different value of the reorganization coordinate compared to the ground state. Absorption occurs from molecules equilibrated to a distribution about the ground-state minimum. If there is no environmental relaxation, the fluorescence occurs at the same frequency as the absorption, and there is no Stokes shift (Figure 1a). If the environment relaxes completely, the fluorescence is red-shifted, and the full equilibrium Stokes shift is observed (Figure 1c). If the environment is partially relaxed, a corresponding fraction of the full Stokes shift is observed (Figure 1b).

In this paper, we will use these ideas to measure dynamics by varying the temperature and observing the steady-state Stokes shift.[9−12] In the steady-state fluorescence spectrum, the fluorescence lifetime acts as a time gate. Ninety percent of the total fluorescence occurs in the range of 0.1−2.3 times the fluorescence lifetime. If the environmental reorganization is faster than this range, the steady-state Stokes shift is close to the equilibrium Stokes shift. If the relaxation is slower than this range, the full Stokes shift does not appear in the steady-state measurement.

Acridine orange (AO), an intercalating dye, was used to probe the interior of DNA. The steady-state Stokes shift of AO was measured as the DNA was cooled in a cryogenic solution. At high temperature, the dynamics are expected to be faster than the AO fluorescence lifetime, and the equilibrium Stokes shift will be observed. If diffusive dynamics are present, they will slow at low temperature and the Stokes shift will be reduced as they become slower than the fluorescence lifetime. If the dynamics are purely vibrational, they will not slow, and there will be little change in the steady-state Stokes shift as the temperature is lowered.

The results show that a substantial portion of the DNA dynamics are diffusive. The temperature dependence of the Stokes shift is most simply accounted for by assuming that the dynamics of the DNA are coupled with movement of the solvent. To help interpret the Stokes-shift measurements, the solvent dependence of the AO excitation and Stokes shifts was also studied. The optical properties of AO show little effect from the polarity of the environment but are sensitive to the local polarizability. These results indicate that the "reorganization" being monitored by AO is primarily a shift in the positions of its neighboring base pairs and not movement of the charged backbone or counterions nor reorientation of polar groups.

The Stokes shift experiments presented here provide an excellent complement to existing methods for measuring DNA dynamics. Existing techniques are most sensitive to motions that are slow, large amplitude, and long ranged. Rapid, low amplitude localized motions appear as perturbations on overall tumbling and internal collective dynamics and are difficult to quantify. In contrast, Stokes shifts are highly sensitive to local motions, weakly sensitive to collective internal motions, and completely insensitive to overall tumbling. Although the current experiments focus on nanosecond time scales, the extension to picosecond or even femtosecond time scales is straightforward with modern time-resolved fluorescence techniques.[5−7]

Experimental Section

Acridine orange hydrochloride (AO) is a classic intercalating dye (Figure 2).[84] The structure of the AO−DNA intercalation complex is known from X-ray crystallography,[85,86] and AO has a binding constant

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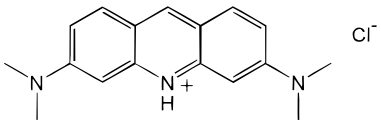

Figure 2. Structure of acridine orange hydrochloride.

of $\sim 10^4$ M^{-1} to DNA.[87,88] Its binding is largely sequence-independent, although a slight preference for GC base pairs has been noted.[89] Unlike the similar proflavine,[90] AO does not show sequence-dependent fluorescence quenching.[91] Thus, AO fluorescence samples DNA structures in a relatively unbiased fashion.

Fluorescence excitation and emission spectra were taken at AO concentrations of 4.65 μM (glycerol−buffer) or 1.00 μM (nonaqueous). The invariance of the spectra at higher concentrations confirmed that reabsorption effects on the spectra are negligible at these concentrations. Acridine orange forms dimers and higher aggregates at sufficiently high concentration.[88,92,93] Spectra were routinely checked for changes at a 10× dilution to ensure that aggregation was not important in our samples.

All the nonaqueous solvents were dried over molecular sieves. Trace amounts of water in the solvents as received altered the spectra, presumably due to water clustering around the AO ion. The protonation state of AO in the neat nonaqueous solvents was unpredictable. Fortunately, the absorption spectrum of the unprotonated AO is unmistakably different from the protonated form. To ensure that the AO was fully protonated, 5 μL of trifluoroacetic acid was added to each 1 mL of unbuffered sample (except that concentrated HCl was used in the neat water sample).

Calf thymus DNA, sodium salt (Sigma) was purified by phenol−chloroform extraction.[94] A concentrated stock solution of the purified DNA (ϵ_{260} = 6600 M^{-1} cm^{-1}) was prepared using 10 mM phosphate buffer (pH 7.2). Sample solutions were prepared in 3:1 (v:v) glycerol−buffer. Once prepared, the DNA solutions were allowed to equilibrate for 24 h before measurements were taken.

The relative amounts of dye and DNA were chosen on the basis of a titration experiment. The excitation maximum of AO has a definite red shift when intercalated in DNA (Figure 3). DNA was titrated into a glycerol−buffer solution of AO until the AO excitation spectrum stopped changing. At this ratio of concentrations, the AO is fully bound, and the AO is sufficiently dilute along the DNA helix to prevent interaction of the AO molecules. On the basis of these titration experiments, DNA was used at a concentration of 1 mM (nucleotide), where the nucleotide-to-dye ratio is 200:1. Other experiments also indicate that the AO molecules act independently at this concentration.[95,96] The excitation maximum of AO intercalated in DNA remained shifted at low temperatures, indicating that binding is not reduced under those conditions.

The low-temperature experiments were conducted in a coldfinger cryostat cooled by flowing liquid N_2. Samples were sealed at atmospheric pressure in glass cells, attached to the coldfinger, and the cryostat was evacuated. A silicon-diode sensor and a heater on the coldfinger controlled the temperature to ± 1K. To prevent thermal gradients due to blackbody radiation, the cells were surrounded by a copper heat shield, except for the beam paths. A separate sensor was mounted directly on the sample to measure its temperature.

Because dye rotation rates vary among our samples, fluorescence spectra were collected at the magic-angle polarization, which removes reorientation effects. To avoid fluorescence line narrowing at low temperature, excitation and detection band-passes were kept broad, and the center wavelengths were placed high in the vibronic manifold, where spectral congestion produces nonselective excitation. Emission spectra were recorded with excitation at 490 nm and emission and excitation band-passes of 4 and 8 nm, respectively. Excitation spectra were recorded with detection at 530 nm and emission and excitation band-passes of 8 and 4 nm, respectively. Excitation spectra were corrected for lamp variations with a Rhodamine B quantum counter. Emission spectra are uncorrected, except those used for the quantum yield measurement. Those were corrected relative to a fluorescein standard.[97] Absorption spectra of AO correspond well to the fluorescence excitation spectra and are considered equivalent throughout the paper.

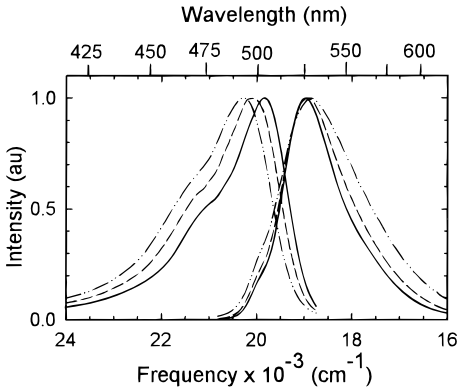

Figure 3. Room-temperature excitation (left) and emission (right) spectra of acridine orange in various solvents: water (dot−dashed), 3:1 glycerol−buffer (dashed), and intercalated in DNA in 3:1 glycerol−buffer (solid). The excitation maximum and Stokes shift show a clear sensitivity to the nature of the environment.

When an asymmetric spectrum is broadened, its maximum shifts as well. To avoid including this effect in our measurements, a convolution procedure was used to measure relative shifts. The narrowest excitation and emission spectra of AO available were taken as reference spectra. They were convolved with a Gaussian and matched to the other spectra to give a relative shift. These values are reported as absolute wavelengths by adding the peak wavelengths of the reference spectra.

Fluorescence lifetimes were measured by exciting AO at 470 nm using a nitrogen-pumped dye laser. The fluorescence emission at 518 nm was isolated by a monochromator and detected by a PMT. A sampling oscilloscope digitized the PMT signal and averaged 1000 waveforms for each sample. The averaged waveform was fit to a single-exponential decay convolved with the instrument response function.

Solvent-Dependent Results

Figure 3 shows the excitation and emission spectra of AO in three different environments: aqueous buffer, 3:1 glycerol−aqueous buffer, and intercalated into DNA. The spectra show a clear sensitivity to the environment. The excitation spectrum in DNA is red-shifted and narrowed relative to that of the aqueous solution. The emission spectrum is also narrowed, but its peak shifts only slightly. As a result, the Stokes shift in DNA is significantly smaller than it is in aqueous solution.

We examined the spectra of AO in a number of different solvents to determine which property of the environment affects the absorption and Stokes shifts (Table 1). A number of different properties of the environment can be responsible for spectral shifts depending on the properties of the electronic states of the specific solute. In the most widely recognized interaction mechanism, the solute has a large change in dipole moment between the ground and excited electronic states, and its spectra are sensitive to the local electric field generated by the

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Table 1. Spectral Properties of Acridine Orange and Various Solvent Parameters: Maximum of the Fluorescence Excitation (λ_{ex}) and Emission (λ_{em}) Spectra, Steady-State Stokes Shift, Polarity [$E_T(30)$], Static Dielectric Constant (ϵ_0), and Index of Refraction (n)

solvent	λ_{ex} (nm)	λ_{em} (nm)	Stokes shift (cm^{-1})	$E_T(30)^a$	$\epsilon_0{}^b$	n^b
water	492.4	529.8	1434	63.1	78.54	1.332
formamide	496.0	527.4	1200	55.8	109	1.446
ethanol	490.2	521.6	1228	51.9	24	1.359
benzyl alcohol	501.4	526.4	947	50.4	13.1	1.540
acetonitrile	494.4	527.0	1251	45.6	35.96	1.342
dimethyl sulfoxide	496.0	536.2	1512	45.1	46.40	1.476
aniline	505.4	529.8	911	44.3	6.89	1.590
dimethylformamide	495.0	531.4	1384	43.2	37.24	1.427
benzyl cyanide	502.8	528.0	949	42.7	19	1.523
acetone	494.4	526.4	1230	42.2	20.7	1.357
acetophenone	502.4	528.4	979	40.6	17.39	1.540
pyridine	495.0	532.4	1419	40.5	12.3	1.510
1,1,2,2-tetrachloroethane	501.8	519.4	690	39.4	8.2	1.493
1,2-dibromoethane	504.0	525.4	808	38.3	4.78	1.538
ethyl acetate	493.8	525.4	1218	38.1	6.02	1.370
glycerol	498.2	527.0	1097		42.5	1.470
glycerol−buffer	497.8	527.8	1142			
DNA	504.4	528.0	886			

[a] Ref (100). [b] Ref (98).

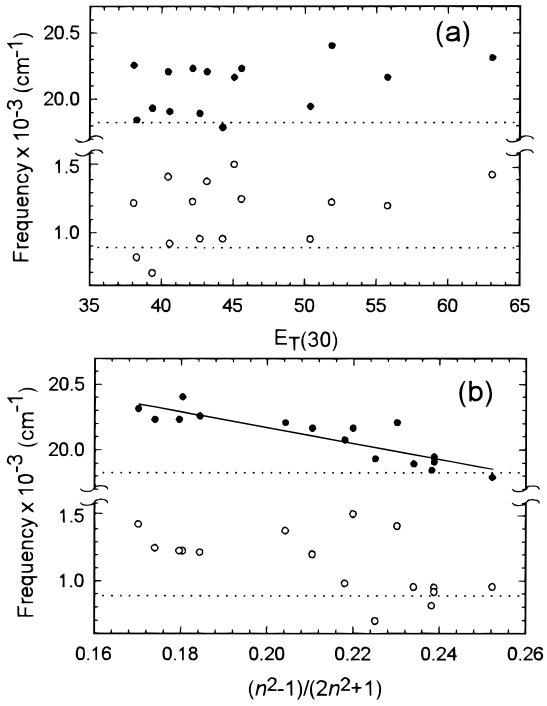

Figure 4. (a) Excitation maxima (●) and Stokes shift (○) of AO in various solvents vs the $E_T(30)$ polarity scale. There is no correlation with solvent polarity. (b) Excitation maxima (●) and Stokes shift (○) vs a polarizability scale represented by a function of the index of refraction n. A correlation of excitation maxima with polarizability is found (solid line). The dashed lines show the values of the excitation maximum and Stokes shift in DNA.

environment. In this case, both the excitation and Stokes shifts should correlate with the "polarity" of the environment.[5,6,13,99] Figure 4a shows the excitation maximum and Stokes shift of AO in a number of solvents plotted against $E_T(30)$, a popular empirical measure of solvent polarity.[100] There is no correlation for either the excitation maximum or Stokes shift. Plots (not shown) against solvent π^* values, another empirical polarity scale,[101] or the appropriate function of the dielectric constant[99] also show no correlation. We note that attempts to use similar dipole-based models to explain solvent-dependent spectra of proflavine, which is very similar to AO, also could not find a consistent explanation of the data.[102] These results show that AO has little change in dipole moment upon excitation and is not sensitive to the local electric field produced by the environment.

A second possibility is that the AO has a different polarizability in its excited state than in its ground state. The resulting changes in dispersion (van der Waals) interactions with the environment would shift the excitation transition, and the excitation maximum would correlate with the polarizability of the solvent as measured by the appropriate function of the solvent index of refraction.[99] Figure 4b shows that such a correlation does exist. Thus, AO spectra are primarily affected by changes in dispersion interactions with the environment. Because the transition is red-shifted in more polarizable solvents, the excited state has a higher polarizability and stronger dispersion interactions than the ground state, in accord with general expectations.

By comparison with these various solvents, the portions of the DNA affecting the AO can be inferred. The excitation and Stokes shifts of DNA in AO are shown as dotted lines in Figure 4. Judging from the excitation shift at the top of Figure 4b, AO senses a high polarizability environment, comparable to aromatic solvents such as aniline or acetophenone. The sugars, phosphates, and solvent are all low polarizability, so the interaction with AO must be dominated by the highly polarizable, aromatic base pairs. This conclusion is reinforced by the close proximity of the base pairs to the π and π^* orbitals involved in the AO transition. In comparison, the backbone is relatively distant and placed near the nodes of the π and π^* orbitals. Furthermore, the dispersion interaction drops off rapidly as $1/r$.[6] The majority of the interaction will be with the nearest base pairs. Thus, the reorganization that is sensed by AO in DNA is primarily movement of the base pairs flanking the intercalation site.

Table 1 and Figure 4b show that specific interactions are not important for AO. Solvents that are hydrogen-bond donors or aromatics do not show significant deviations from the general trend, indicating that hydrogen bonding or specific π-stacking interactions do not affect AO's electronic states to a significant degree.

The last potential interaction mechanism is change in the repulsive interactions between AO and its neighboring groups. There is no readily available measure for these interactions, and they may be responsible for some of the deviations from the polarizability correlation in Figure 4b. Because of their very short range, repulsive interactions would also be confined to the base pairs in contact with the AO.

A general outline of the reorganization process now can be drawn. Because dispersion interactions dominate AO's interaction with its environment, the Stokes shift arises from a "nonpolar" reorganization.[8,103] Nonpolar reorganization is characterized by movement of neighboring groups inward or outward relative to the chromophore. In contrast, "polar" reorganizations are dominated by rotations of neighboring groups. Repulsive interactions move groups inward or outward,

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and to the extent that they are present, they also cause a nonpolar reorganization.

Recent theoretical work provides a simple picture of the processes occurring in nonpolar reorganizations.[8,104] In the ground state, the chromophore occupies a cavity within its environment whose radius is determined by the competition between attractive dispersion interactions and repulsions of the molecular cores. Upon excitation, the attractive dispersion interactions increase, creating an instantaneous force pulling the surroundings inward. At short times, all environments behave elastically. The sudden increase in attractive forces launches acoustic waves (phonons) into the environment, resulting in a subpicosecond contraction of the AO cavity and a "phonon-induced" Stokes shift in the emission spectrum. This component of the Stokes shift is not only very rapid, but its rate is insensitive to the viscosity of the environment, and it is not frozen out in a low-temperature liquid or glass. These dynamics are vibrational in nature.

This initial contraction leaves a degree of strain in the environment. In a solid, this strain is never relieved and there is no further increase of the Stokes shift with time. In a fluid, viscous flow acts on a longer time scale to relieve the strain and allows an additional increase in cavity size and a further increase in the Stokes shift. This "structural" component of the Stokes shift is diffusive in nature, and its rate is directly proportional to the viscosity of the environment. At low temperatures and high viscosities, this component freezes out as the high viscosity liquid becomes a glass. Thus solids have only vibrational dynamics, whereas liquids have both vibrational and diffusive components to their dynamics. The temperature dependence of the Stokes shift in DNA will be examined in the next section to determine which of these cases is closest to describing DNA.

A final feature of the solvent-dependent studies that must be considered is the magnitude of the Stokes shifts. In polar reorganizations, both the excitation and Stokes shifts are expected to correlate with solvent polarity.[5,13,99] However, for a nonpolar reorganization, only the excitation shift is expected to correlate well with the index of refraction, as seen in Figure 4b.[99] In the nonpolar case, the magnitude of the Stokes shift is determined by both the strength of the dispersion interaction and by the change in cavity size permitted by the molecular repulsions and environmental rigidity. Because several factors combine to determine the magnitude of the Stokes shift, it is not surprising that a clear trend does not emerge for the Stokes shifts with solvent polarizability in Figure 4b.

However, it is clear that the magnitude of the Stokes shift in DNA is within the range of values seen in simple liquid solvents at room temperature. Apparently, the structural constraints of DNA do not restrict reorganization to a substantial extent. This conclusion is not surprising given that the movements involved in nonpolar reorganizations are small, typically a fraction of an intermolecular distance.[104]

Temperature-Dependent Results

Existence of Diffusive Relaxation in DNA. The temperature dependence of the steady-state Stokes shift of AO in a glycerol−aqueous buffer mixture is typical for supercooled liquids (Figure 5, open symbols).[9−12] At high temperatures, the viscosity of the mixture is low, and the full equilibrium Stokes shift, including the viscosity-sensitive diffusive component, is attained within the fluorescence lifetime. The liquid has a relatively large coefficient of thermal expansion, so there is a modest slope to the temperature dependence resulting from changes in solvent density ("liquidlike" lines).

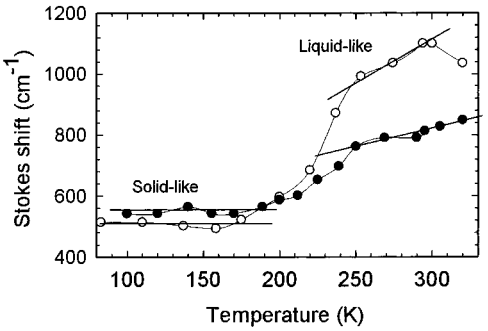

Figure 5. Comparison of acridine orange Stokes shifts intercalated in DNA (●) and free in solution (○) (3:1 glycerol−buffer). In the high-temperature region (liquidlike), the full equilibrium Stokes shift is obtained. At low temperatures (solidlike), diffusive relaxation is frozen out and only the Stokes shift due to vibrational dynamics develops within the fluorescence lifetime.

At low temperature, the solvent is frozen into a glass or has a high viscosity. Only the viscosity-independent vibrational component of the Stokes shift can form within the fluorescence lifetime, and the diffusive component is effectively frozen out. Because the thermal expansion coefficient of the glass is low, the Stokes shift has a flat temperature dependence ("solidlike" line).

The Stokes shift undergoes a steep transition between these two limits at intermediate temperatures as the rate of the structural component of the Stokes shift passes the fluorescence lifetime. This transition is characteristic of a diffusive component to the relaxation.

The temperature-dependent Stokes shift of AO intercalated in DNA is also shown in Figure 5. The same three regions, including the transition region, are present. Although the magnitude of the change is a factor of 2 smaller in DNA than in solution, the transition is still readily apparent. The AO senses a significant diffusive relaxation of its immediate surroundings.

At low temperatures, where the diffusive component is frozen out, the Stokes shift in DNA is actually a bit larger than in solution. The low-temperature Stokes shift consists of an internal ("inner sphere") component due to the vibronic structure of the AO plus the phonon-induced reorganization due to vibrational dynamics of the DNA. Because the vibronic component is independent of the environment, the magnitude of the vibrational component is as large or larger in DNA than it is in solution. The magnitude of the vibrational component is linked to the high-frequency or short-time compressibility of the environment,[104] so the short-time compressibility of DNA is similar to that of a molecular glass.

Rate of DNA Reorganization. We now address the question of the rate of DNA reorganization and more specifically whether the reorganization rate seen in the interior of DNA is different from that seen in free solution. An initial comparison is facilitated by rescaling the two sets of data in Figure 5 to give the diffusive Stokes shift the same magnitude in each set. The solidlike component has been subtracted off, and the remaining Stokes shift in DNA has been multiplied by 2.06 to bring the two high-temperature regions into coincidence. The results are presented in Figure 6, which shows that the transition regions in DNA and in solution overlap. The diffusive component

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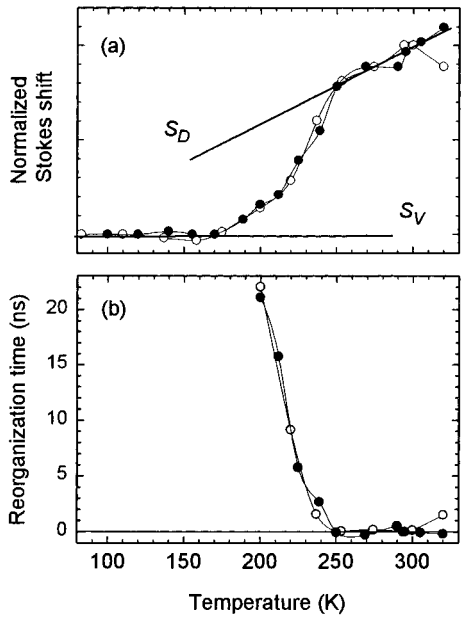

Figure 6. (a) Stokes shifts in DNA (●) and in a glycerol−buffer mixture (○) normalized to the magnitude of the transition region, suggesting that the reorganization rates are similar in both. (b) Calculated reorganization times including corrections for variations in the fluorescence lifetime, confirming that the reorganization in DNA and in solution are indistinguishable.

freezes out over the same temperature range, suggesting that the rates of reorganization in DNA and in solution are very similar.

A more rigorous comparison can be made by examining reorganization rates. Within the transition region, the rate of DNA reorganization can be inferred from the fraction of the total diffusive Stokes shift that is observed. The steady-state Stokes shift S_{ss} consists of the vibrational component S_v and the fraction f of the total diffusive component S_D that occurs within the fluorescence lifetime.

$$S_{ss}(T) = S_v + f(T)\, S_D(T) \tag{1}$$

Starting with the fits shown in Figure 6b, the magnitude of the vibrational component is taken as a constant at higher temperatures, and the magnitude of the diffusive component is linearly extrapolated to lower temperatures. With these extrapolations, the fraction of the diffusive Stokes shift which develops within the fluorescence lifetime τ_f was determined. If the reorganization is characterized by a single-exponential time constant τ_r, then

$$\tau_r = \tau_f\left(\frac{1}{f} - 1\right) \tag{2}$$

Even if the relaxation is not single exponential, eq 2 still gives a useful measure of the average reorganization time.

From this calculation, it is apparent that the comparison in Figure 6a is only valid if the fluorescence lifetimes of AO in solution and in DNA are the same throughout the transition region. To check this assumption, we measured the lifetimes at 298 and 173 K. At 298 K, the lifetime in solution (6.1 ± 0.5 ns) is slightly faster than that in DNA (8.3 ± 0.5 ns). This result is consistent with our observation that the relative quantum yield in solution is slightly lower in DNA (0.85 ± 0.1). At low temperature, the fluorescence lifetime in DNA (7.9 ± 0.5 ns) does not change, within the experimental error. However in solution, the lifetime increases slightly at low temperature (7.2 ± 0.5 ns).

Although there are discernible differences in the fluorescence lifetimes, they are not large enough to change the conclusion that reorganization rates are the same in DNA and in the surrounding solution. Figure 6b shows the reorganization times calculated using eq 2 and incorporating the fluorescence lifetime measurements. An average lifetime was used for AO in DNA, because the measurements at high and low temperature were the same within the estimated error. In glycerol−buffer solution, there is a real temperature dependence, but it is only slightly larger than our error estimate. We used a simple linear interpolation with temperature to account for this small effect.

As expected, the reorganization time is unmeasurably short above 250 K, where it is much faster than the fluorescence lifetime, and unmeasurably long below 200 K, where it is much slower than the fluorescence lifetime. However, in the transition region, where meaningful values are obtained, the reorganization times in DNA and in solution are indistinguishable.

These rates can be compared to dielectric relaxation rates. Solutions of DNA show a characteristic dielectric relaxation time τ_D in aqueous solution that is near 10 ns.[105−108] It has been attributed to the rate of fluctuation of the counterion atmosphere along the DNA chain, although other explanations have also been advanced. If dielectric relaxation were involved in the Stokes shift of the AO, the rate should be given by the longitudinal dielectric time τ_L.[6] The longitudinal time can be derived from the Debye time, if the high- and low-frequency dielectric constants of the DNA and counterion atmosphere are known, but unfortunately, they are not. However, in other systems, the ratio τ_D/τ_L is never greater than 100,[6] so the expected Stokes shift time is >100 ps in room-temperature aqueous solution. The increased viscosity of our glycerol solvent relative to an aqueous solvent and the further increase in viscosity at low temperature would slow the ionic diffusion in our systems. Although substantial uncertainties remain, it appears that the dynamics observed in dielectric experiments are too slow to correspond to the dynamics we observe. This is consistent with our conclusion that AO is not sensitive to the local electric fields in DNA.

Discussion

The results in this paper show that the interior of DNA experiences diffusive dynamics, and in some respects, can be regarded as fluidlike. However, this conclusion must be limited to small amplitude motions. Experiments measuring large amplitude movement such as rotation present a picture of DNA as locally rigid. However, small amplitude motions also dominate many chemical processes such as crossing over a sharp activation barrier or charge-transfer processes. When these processes occur within DNA, it is important to recognize the diffusive aspects of the local DNA dynamics.

The results also show that the diffusive dynamics of the DNA are linked to the dynamics of the bulk solvent. A simple explanation would be that the interior of DNA is not well-shielded from long-range interactions with the bulk solvent and that the dynamics seen here are just an attenuated measurement

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of the bulk solvent and not of the DNA proper. Although this conclusion would be an important statement about the conditions inside DNA, it is not attractive in view of what is known of DNA solvation structure and the range of solvent interactions.

The solvent around a DNA helix is perturbed out to a distance of ∼10 Å from the helix surface by a high concentration of counterions.[109,110] However, it is unlikely that AO senses even this perturbed solvent region. We argued above that AO primarily interacts with the flanking base pairs through van der Waals interactions. Even if our assignment of the interaction mechanism is incorrect, it is known that with a relatively long-ranged dipole−dipole interaction, 85% of the Stokes shift is contributed by the first solvation shell.[111] This scenario would still limit the interaction almost entirely to the DNA proper and the solvent in the DNA grooves. Both X-ray experiments and computer simulation indicate that the solvent in the DNA grooves is strongly structured and its dynamics are highly perturbed relative to bulk solvent.[72,112] Furthermore, X-ray structures of AO intercalated in DNA show that it is well-shielded from contact with even this portion of the solvent, and that the existing contacts are along the edge of the AO where the π and π^* states involved in the transition have a node.[85,86] Despite all these indications that the AO is well-shielded from the bulk solvent, the magnitude of the diffusive solvation is reduced by only a factor of 2 upon intercalation. Finally, the red shift of the AO excitation spectrum in DNA is characteristic of stronger interactions with the environment, not weaker. In view of all these facts, it is unlikely that the diffusive reorganization observed for AO in DNA comes directly from the bulk solvent.

This conclusion must be reconciled to the result that the AO Stokes shift dynamics are identical to the Stokes shift dynamics in the bulk solvent. The results of time-resolved fluorescence depolarization experiments are also important in this context. In room-temperature solution, these experiments have detected a weak relaxation with a time constant of ∼100 ps, which is attributed to localized motion of the intercalated dye.[4,63,64,69] It is reasonable to assume that these motions are similar to those reflected in the Stokes shift. These dynamics were found to be viscosity independent over a range of relatively low viscosities.[64] In comparing to our results, which show a strong viscosity dependence to the DNA dynamics, it is important to remember that our conclusions are confined to the transition region from 170 to 250 K, where the viscosity is very high and the dynamics are on the nanosecond scale.

We propose that the AO is only indirectly coupled to solvent motion and that the solvent motion becomes rate-limiting at high viscosity. Although the AO transition is only sensitive to movement of its neighboring base pairs, the movement of these bases also requires movement of portions of the helix exposed to the solvent. For example, the AO might be directly sensitive to a propeller twist of the base pairs, but the twisting might also involve movement of the backbone, which would in turn require movement of the solvent. At the high solvent viscosities of our experiments, the solvent movement is rate limiting, and the AO relaxation is slow and viscosity dependent. In room-temperature aqueous solution, such as in the fluorescence depolarization experiments, the solvent relaxation time is very rapid ($\lesssim$1 ps).[113] The rate of DNA motion is limited by its internal dynamics and is independent of viscosity.

Although this is a simple explanation consistent with our data, more rigorous tests of this proposal can be made. With time-resolved fluorescence techniques, the rates of the Stokes shift can be measured directly. Time-resolved Stokes shift measurements on the picosecond and even the femtosecond time scale[6,13] are well established and can be applied to the system developed here. With these techniques, the experiments can be extended to picosecond times and low viscosity solvents. With these methods, the dynamics of DNA can be followed to the low viscosity of physiological conditions. Molecular dynamics simulations suggest that the internal dynamics of the DNA will limit motion to the 10−100 ps time range under these conditions.[72−75] Experiments to test these ideas are currently being implemented.

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