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Base Stacking and Unstacking As Determined from a DNA Decamer Containing a Fluorescent Base†

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Received January 9, 1990; Revised Manuscript Received March 26, 1990

ABSTRACT: Time-resolved fluorescence decay of a single-stranded DNA decamer d(CTGAAT5CAG), where d5 is the fluorescent base 1-(β-D-2′-deoxyribosyl)-5-methyl-2-pyrimidinone, was measured and analyzed at several temperatures. The d5 base in the decamer is resolved into three states according to their fluorescence decay lifetime characteristics and temperature dependence of their associated amplitudes: fully extended and completely unstacked state, loosely associated state, and fully stacked state. These states are in slow exchange compared to their fluorescence decay rates. The population of the fully extended and completely unstacked state is small and decreases further with increasing temperature. The loosely associated state, whose fluorescence can still be efficiently quenched by other DNA bases, occupies a large portion of the conventionally defined unstacked state. Stacking enthalpy and entropy for the d5 base with thymine or cytosine bases in the DNA decamer are calculated to be −6.6 kcal/mol and −22 cal/mol·K, respectively. This work shows that fluorescent bases in DNA can be useful to the study of local conformations of bases.

Base stacking interactions are one of the driving forces for helix formation in double-stranded nucleic acids (Saenger, 1984). Although considerable effort has been made to quantitate the stacking interactions in ribonucleotides and to determine the number of states available for the bases, there is at present no consensus value for stacking enthalpy (Petersheim & Turner, 1983) and the issue of the number of states that a base can adopt during stacking and unstacking processes is not clearly understood (Powell et al., 1972; Lee & Tinoco, 1977; Baker et al., 1978; Reich & Tinoco, 1980; Olsthoorn et al., 1981). Thermodynamic parameters for stacking interactions are usually measured by UV absorption (Petersheim & Turner, 1983) or by NMR chemical shifts (Lee & Tinoco, 1977) based on a two-state model, or measured by calorimetry (Breslauer & Sturtevant, 1977). These methods can provide information about the macroscopic states of nucleobases but are limited in their ability to describe the local states of individual bases. These limitations are due either to sensitivity or to base motions occurring on the time scale of measurements.

Helix to coil transitions in DNA are usually monitored by UV hypochromicity changes in the DNA bases, which are interpreted as effects resulting from the disruption of stacked states and formation of unstacked states. This type of measurement can quantitate overall transitions in DNA structure but sheds little light on the local states of individual bases. NMR experiments on proton exchange show that base pairs open and close at finite rates, but recent results (Reid, 1987) question the lifetimes of the base-pair open states. Comparison of UV melting results with those from NMR proton exchange indicates that the open state in NMR may not be the unstacked state as assumed in UV hypochromicity (Benight et al., 1988). Thus the nature of the local states of bases at varying temperatures still remains to be accurately described.

Fluorescence emissions of DNA bases are sensitive to local environments and thus provide some information about the nature of local states. However, the fluorescence of the four

† Supported in part by grants to T.M.N. from NSF (U.S.-Sweden Cooperative Science Program Grant INT-8713453), NIH (Fogarty Senior International Fellowship Grant 1 F06 TW01332 and CA 41368), and PHS (Grant S7RR05403-28) and by grants to L.W.M. from NIH (GM 37065) and NSF (DMB 8904306).

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common DNA bases in duplex DNA can be characterized by extremely low quantum yields and short lifetimes (Nordlund, 1988; Georghiou et al., 1985); consequently, their fluorescence decays are difficult to determine accurately. N^1,N^6-ethenoadenine (ϵA) derivatives are highly fluorescent and have been used to study the fluorescence decay of stacked bases by Kubota et al. (1983a,b). These studies show that the fully extended and completely unstacked ϵA generally occupies only a very small fraction of the population of possible states and this fraction decreases further with increasing temperature. 2-Aminopurine is a fluorescent base that can form hydrogen bonds with thymine when incorporated into DNA and exhibits multiexponential decays (Nordlund et al., 1989). Again, the population of the fully extended and unstacked form is very small and decreases with increasing temperature. These studies suggest that the nature and variety of local states of the bases are much more complicated than might be initially assumed.

In the present work, we have used a fluorescent-base-containing DNA decamer, d(CTGAAT5CAG), which is largely single stranded under our experimental conditions, to study base stacking interactions involving 1-(β-D-2′-deoxyribosyl)-5-methyl-2-pyrimidinone (d5) with common DNA bases. The pyrimidinone d5 is a modified thymine derivative (Volz & Tamm, 1978; Gildea & McLaughlin, 1989; Connolly & Newman, 1989) but does not appear to base pair with dA. We now show that the predominant portion of the unstacked bases is actually in close vicinity to bases such that their fluorescence can still be efficiently quenched. The fully extended and completely unstacked state appears to be unfavorable compared with the loosely associated state.

Materials and Methods

The synthesis of 1-(β-D-2′-deoxyribosyl)-5-methyl-2-pyrimidinone (d5) and the DNA decamer d(CTGAAT5CAG) has been described elsewhere (Gildea & McLaughlin, 1989). The decamer was dissolved in 0.1 M KCl, 10 mM Tris, 0.1 mM EDTA (pH 7.5) to a 1-cm absorbance value of ~0.9 at 260 nm.

Steady-state fluorescence emission and excitation spectra were obtained from a Perkin-Elmer MPF-66 fluorescence spectrophotometer at ambient temperature (~20 °C). A PE3000 microcomputer was used to record the spectra. The UV melting curve was obtained with a Gilford 250 absorbance spectrophotometer.

Fluorescence decay data were collected at magic angle with the time-resolved single-photon counting apparatus in the Department of Chemistry, University of Rochester. A Nd–YAG laser was mode locked at 76 MHz and the IR output was frequency doubled to pump a Coherent Model 700 dye laser with R6G dye. The repetition rate was reduced to 3.8 MHz by a Coherent Model 7220 cavity dumper. The dye laser output at 600 nm was frequency doubled to 300 nm to excite the d5 base in the DNA decamer. The instrument response was ~90 ps (full width at half maximum). Emission was detected at 380 nm or higher and a Schott WG360 filter was used to cut off the light at shorter wavelengths. Fluorescence was detected by a Hamamatsu multichannel plate photomultiplier tube (R1564U-07), and a Nucleus Personal Computer Analyzer card residing in an IBM XT was used for data collection. Each data set contained 2000 points.

The data analysis of fluorescence decays was performed at two levels. First, we used a sum of exponentials to fit each individual decay curve at one temperature to obtain the amplitudes and lifetimes of each component by nonlinear least-squares routines (Thomas et al., 1980). Second, we assumed that these components exchange and the exchange rate constants and the decay constants are of the form

$$k_{ij}(T) = \alpha_{ij} \exp\left(-\frac{\Delta H_{ij}^*}{RT}\right) \quad (1)$$

$$k_i(T) = \alpha_i \exp\left(-\frac{\Delta H_i^*}{RT}\right) \quad (2)$$

where k_{ij}'s are exchange rate constants and k_i's are decay rates. These rates, together with the initial conditions, were used to compute the amplitudes and lifetimes. These calculated values could then be compared with the fitted ones at each temperature. The differences at all temperatures were minimized by nonlinear least-squares method. This is essentially a global analysis approach used previously (Knutson et al., 1983), which is useful to systematically analyze data collected under different conditions (Beechem & Brand, 1986). We are, in effect, using the amplitude and lifetime parameters extracted from the decay data as "data" that the multistate model must fit with its thermodynamic parameters. For a given set of kinetic parameters (eqs 1 and 2) and initial conditions, the resulting amplitudes and lifetimes uniquely determine the shape of the decay. Thus, fitting the real decay data and fitting the amplitudes and lifetimes should give qualitatively the same picture of the system. Fitting real decay data by using global analysis should give more precise results and discriminate different models since the number of data points in the analysis is much larger (in our case, 36 amplitude and lifetime parameters vs 6 × 2000 fluorescence data points). However, fits to the (fitted) amplitudes and lifetimes can be performed quickly and then can be used as initial guesses to analyze the real data globally. What we have employed in the present report can be considered an intermediate step for global analysis. In our case, the fits by this step were sufficient for us to obtain the information regarding the exchange rates such that further analysis was not necessary.

The UV melting curve of the decamer was used to estimate the fraction of normal stacked bases, stacking enthalpies, and entropies assuming a two-state model as follows. The absorbance at temperature T is

$$A(T) = \frac{a \exp(-\Delta H/RT + \Delta S/R)}{1 + \exp(-\Delta H/RT + \Delta S/R)} + b \quad (3)$$

where $a \approx (\epsilon_s - \epsilon_u)C_T$ and $b \approx \epsilon_u C_T$ are assumed to be temperature independent, ϵ_s and ϵ_u are the molar absorbances of stacked and unstacked bases, respectively, and C_T is the total concentration of bases. a, b, ΔH, and ΔS were used as adjustable parameters to fit the measured absorbance by nonlinear least-squares methods (Powell et al., 1972; Dewey & Turner, 1979).

Results

Steady-State Fluorescence and Melting of d(CTGAAT5CAG). Figure 1 shows the steady-state fluorescence excitation and emission spectra of the decamer d(CTGAAT5CAG) at ~20 °C. The absorption peak of d5 base is separated from that of normal DNA bases such that d5 can be preferentially excited. Its fluorescence is much stronger than that of normal DNA bases and can be easily detected. Thus, as a complement to 2-aminopurine (Nordlund et al., 1989), it appears to be convenient pyrimidine probe for studying conformations of DNA.

The melting curve of the decamer in 0.1 M KCl is shown in Figure 2A. Within the temperature range (0–80 °C), there is no cooperative transition. Since the melting temperature

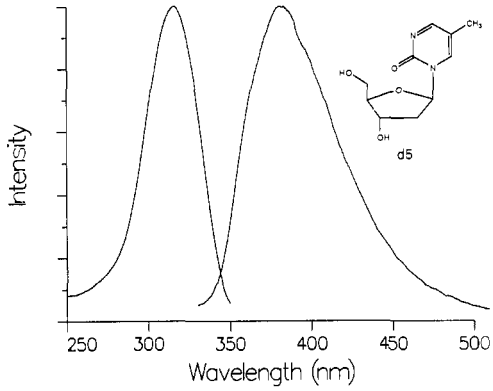


FIGURE 1: Fluorescence excitation and emission spectra of d-(CTGAAT5CAG). For emission spectrum (right), the excitation wavelength was set a 280 nm. For excitation spectrum (left), the emission was set at 400 nm. Inset: structure of d5.

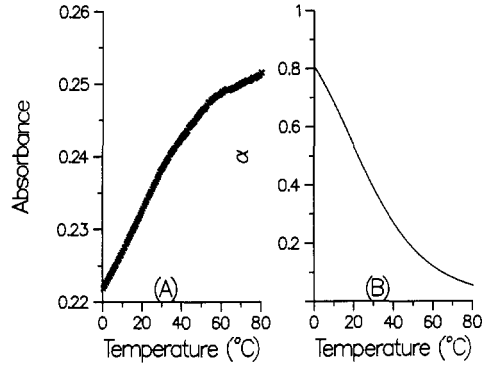


FIGURE 2: (A) Melting curve (×) of d(CTGAAT5CAG) in 0.1 M KCl, 10 mM Tris, 0.1 mM EDTA (pH 7.5) measured at 280 nm with a 0.5-cm cell and with heating rate of 1 °C/min (150 data points). (—) Fitted absorbance. (B) Fraction of stacked bases calculated from the fits in (A).

of the unmodified decamer d(CTGAATTCAG) is ~40 °C and d5 does not appear to form hydrogen bonds with adenine (Gildea & McLaughlin, 1989), the T_m of d(CTGAAT5CAG) will be lower than 40 °C. It is likely that this decamer is mainly single stranded. CD spectra of the decamer at 15 and 50 °C are similar (Gildea and McLaughlin, unpublished results), suggesting similar structures at the two temperatures. In Figure 2A, there is no evidence for cooperative melting from 5 to 15 °C so that single strand is probably the main character of this decamer in this temperature range. Thus we have a DNA decamer that is single stranded within the temperatures of our fluorescence decay measurements. This provides a good reference for studying a DNA double helix.

The d5 Base in the Decamer Can Be Time-Resolved into Three States. Fluorescence decay curves at two temperatures are shown in Figure 3. As temperature rises, the fluorescence of d5 in the decamer decays faster. The decay and fitted curves at 15 °C together with their difference and autocorrelation of the weighted residuals are shown in Figure 4. The decay data at all temperatures can be adequately represented by the sum of three exponentials with reduced χ^2 ranging from 1.1 to 1.4. The fitted results are shown in Table I.

We show in the following that three-exponential fits are appropriate for the system.

(a) If we use a two-exponential fit, which resembles a two-state model as used in most absorption measurements, the reduced χ^2's are larger than those from three-exponential fits, the residuals show clear structure (nonrandom residuals), and the corresponding autocorrelations indicate the two-exponential fits are not adequate. Furthermore, the population of the

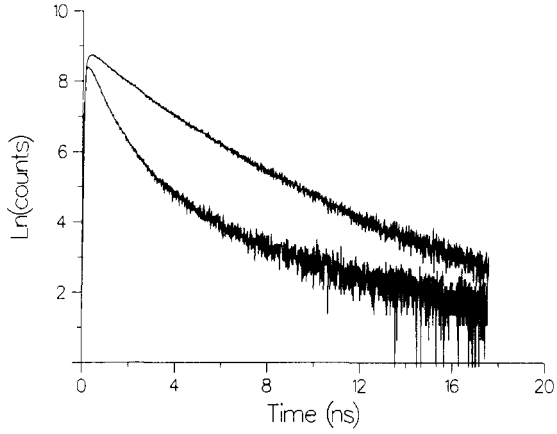


FIGURE 3: Fluorescence decays of d5 in d(CTGAAT5CAG) at 5.5 °C (upper curve) and at 38.5 °C (lower curve). Data were collected at 9.7 ps per channel.

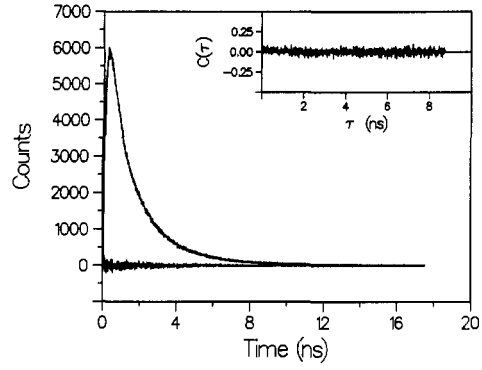


FIGURE 4: Fluorescence decay (fitted and measured) of d5 decamer at 15.5 °C. The decay is fitted with three exponentials (see Table I). $\chi_2 = 1.16$. The difference between the fitted and measured decays is also plotted. inset: autocorrelation of the weighted residuals.

Table I: Fluorescence Decay Amplitudes and Lifetimes of d(CTGAAT5CAG)[a]

T (°C)	a_1	τ_1 (ns)	a_2	τ_2 (ns)	a_3	τ_3 (ns)
5.5	0.1966	0.573	0.6834	1.950	0.1201	3.85
9.5	0.2659	0.696	0.6577	1.859	0.0764	3.95
15.5	0.3122	0.427	0.6302	1.520	0.0576	3.97
20.0	0.3761	0.387	0.5763	1.354	0.0476	4.09
29.0	0.4529	0.319	0.5130	1.077	0.0341	4.01
38.5	0.4955	0.268	0.4784	0.857	0.0261	3.96

[a] Relative percentage of errors of a_1, a_2, and a_3 are less than 5%. The standard deviations for τ_1 and τ_2 are ~0.01 ns and for τ_3 ~0.2 ns.

component with a longer lifetime, which is presumably the "unstacked" state, decreases with increasing temperature, which in turn leads to a positive enthalpy of "stacking". We thus lean to a model with more than two states.

(b) The possibility of a model with an extra, nearly non-fluorescent state (Kubota et al., 1983a) besides the three states corresponding to the three exponentials can also be discounted. In steady-state fluorescence measurements, the intensity is proportional to the product of concentration (amplitude) and lifetime. For a system with a mixture of fluorophores or a fluorophore in different fluorescence states, a state with a large population but with a short lifetime may contribute little to the total intensity and thus can be easily buried in the uncertainties of the measurements. On the other hand, in time-resolved fluorescence decay measurements, amplitudes and lifetimes are determined separately. The amplitude of a component with very short lifetime but large population can still be determined quite accurately, since both amplitudes and lifetimes determine the decay shape. If there is a fast decay

component in our sample, it will enter the decay curve roughly as a stray light component. In data analysis, if we use a stray light correction term (Schurr et al., 1990)

$$I(t) = A_s\delta(t) + \sum a_i \exp(-t/\tau_i)$$

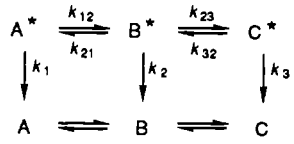
where $I(t)$ is the time-dependent fluorescence intensity, $\delta(t)$ is the delta function, which is used to approximate the fast decay, and A_s is the amplitude of the fast decay, then we can obtain the amplitude of this component, which is proportional to its population. The inclusion of this term in our data analysis of d5 data does not improve the quality of data fits (not shown) and gives the same reduced χ^2. A_s is within random noise range. Actually, the fits with three exponentials are already good enough as shown in Figure 4 so that addition of one extra component is not necessary and is probably not meaningful.

(c) The values of the three lifetimes are well separated so that a fit by the method of lifetime distributions (Mērola et al., 1989) will give three distinctive distributions whose averages are close to the fitted lifetimes using sum of exponentials. Thus, although we cannot distinguish a discrete state from a distribution of states centered near the same lifetime value, we can clearly define three different states or distributions.

(d) A superposition of continuous quenched states might lead to a nonexponential decay. However, a stretched exponential or similar type of decay needs at least four exponentials to approximate (not shown). Though we cannot rule out distribution models, the three-state model fits the data with a minimum of parameters and the results make physical sense.

Multistate Kinetics and Thermodynamics. The longest lifetime of d5 in the decamer (cf. Table I) is about equal to that of free d5 base itself, which exhibits a single-exponential decay with a lifetime of 4 ns. The other two components have shorter lifetimes apparently due to neighboring base quenching. Thus, d5 exists in three states in the decamer on the time scale of its fluorescence decay. We are interested in the following aspects.

(1) Since we can resolve three states, the exchange rates between states must be slower than those corresponding decay rates of the excited states. If we lay out a dynamic picture of the three states



then the resulting decay can be described by three exponentials with lifetimes being functions of exchange rate constants and decay rate constants. The measured lifetimes differ by a factor of 5 for two distinct states. It thus might be possible that some exchange rates, such as those between state 1 and state 2 in Table I, are comparable to the decay rate of state 3. We might then be able to extract from temperature dependence some dynamic information such as the exchange rate constants of the system.

(2) In DNA melting experiments, it is always assumed that DNA bases move from a stacked to an unstacked state as DNA helix melts, i.e., bases are in two states, either stacked or unstacked. However, fluorescence decay measurements of base stacking almost always show three (Kubota et al., 1983a,b) or more (Nordlund et al., 1989) states. In addition, recent work on proton exchange and UV melting shows that the open state in NMR is probably not the unstacked state in melting experiments (Benight et al., 1988). Thus, the states of bases are probably more complicated than a two-state model. It is important to further understand the local states of bases.

Table II: Thermodynamic Parameters Determined from Amplitude Ratios

ratio	ΔH (kcal/mol)	ΔS (cal/mol·K)	correlation coeff[a]
a_2/a_1	−6.6 ± 0.7	−21.6 ± 2.3	0.98
a_1/a_3	12.2 ± 1.4	45.4 ● 4.8	0.97
a_2/a_3	5.6 ± 0.8	23.8 ± 2.8	0.96
$a_2/(a_1 + a_3)$	−4.7 ± 0.3	−15.4 ± 1.1	0.99

[a] Correlation coefficient for linear fit of ln ratio vs $1/T$.

The amplitudes and lifetimes in Table I are generally complicated functions of the initial conditions, exchange rate constants, and decay rate constants. The exchange rate constants cannot be much larger than the corresponding decay rates, but if they are not too low, then the final amplitudes and lifetimes as determined from experiments can be different from the initial populations and decay rate constants, respectively. It is therefore necessary to estimate the range of the exchange rate constants.

Fits to the data in Table I using eqs 1 and 2 invariably return to α and ΔH^* values such that k_{ij}'s are much smaller than k_i's for many different initial guesses. Thus it is likely that the exchange rates are indeed much smaller than the decay rates. In this case, the decay process for the three states effectively decouples from the exchange process so that only the ratios of k_{ij}'s (equilibrium constants) can be determined, while individual rate constants cannot be determined. The fluorescence decays so fast that the exchanges are negligible on the time scale of our measurements even though the exchange rates determine the population of each state. Thus, the amplitudes are approximately equal to the populations of the states while the measured lifetimes are about equal to each state's lifetimes.

The results above can also be expected by direct inspection of the data in Table I. While τ_3 is temperature independent, τ_1 and τ_2 change by a factor of 2 as temperature rises from 5 to 38 °C. All plots of ln τ vs $1/T$ give simple linear relations. Since τ_i's are complicated nonlinear functions of decay rates and exchange rates if they are comparable in magnitude, the simple temperature dependence of τ_1, τ_2, and τ_3 suggests that the exchange rates make negligible contributions to the observed lifetimes of d5.

From the amplitude ratios at different temperatures, we can determine the thermodynamic parameters that govern the exchanges between states for the d5 base. Results are shown in Table II. Further discussion is in the following section.

The state transitions of normal DNA bases in the d5-containing decamer can be obtained by analyzing the melting curve by use of a two-state model. Nonlinear fitting of eq 3 to the data in Figure 2A gives the calculated fraction of the stacked state as shown in Figure 2B. The fitted enthalpy is −10.3 (±0.3) kcal/mol and the entropy is −34 (±1) cal/mol·K (with a = −0.039 and b = 0.253, corresponding to a hypochromicity of ~15% at 280 nm). These errors together with the fitted fraction of stacking should be treated with caution, since in the fit, only a slightly higher converged sum of the squares of the differences (a slightly worse fit to the melting curve) between the calculated and measured absorbances can lead to the uncertainties of the estimated enthalpy and entropy as much as 100% (while the converged or minimized enthalpies and entropies are about the same) and the measured melting data here (150 data points) do not provide a strict discrimination. The fraction of stacked base as in Figure 2B is, at best,

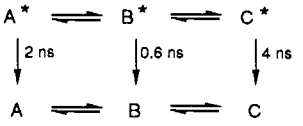
with 30% relative error so that only a qualitative comparison with d5 base stacking can be made, which we will discuss in the following section.

Discussion

Fully Extended and Completely Unstacked State. The d5 base incorporated into a DNA decamer exhibits three fluorescence decay lifetimes instead of one when it is free. This indicates that d5 is in three states or three clusters of states in DNA. Since d(CTGAAT5CAG) is mainly in single-stranded form, it is reasonable to assume base stacking interaction in solution is the dominant factor for the observed fluorescence quenching. Stacking itself may not be the cause of quenching, as we will discuss. The component with the longest lifetime, equal to that of free d5 base, is most likely the state with the d5 base fully extended into solution and completely unstacked since its fluorescence is not quenched by DNA bases. This component, however, cannot be equated to the unstacked state measured by absorption techniques such as UV melting or CD, since not only is its population surprisingly small, but also it decreases with increasing temperature. At 38 °C, its population is already below 3% (cf. Table I). Thus, it is likely that a large portion of the so-called unstacked base as determined or assumed in other methods is actually in the near vicinity of neighboring bases and consequently its fluorescence can still be efficiently quenched.

An intermediate question regarding the small fraction of the completely unstacked d5 base is how large this fraction will be for normal DNA or RNA bases. It is unlikely to be very large. While one may argue that the small fraction of completely unstacked base in ϵA might be due to the addition of an extra ring to the adenine base so that some peculiar properties are produced, it is not convincing that this is the case for 2-aminopurine and 5-methyl-2-pyrimidinone base. The latter two have similar structures compared to their unmodified bases (adenine and thymine). In fact an amino group is simply moved from the 6-position in adenine to the 2-position in 2-aminopurine. Therefore their solvations and interactions with other bases are expected to be similar, and yet the fully extended and completely unstacked fraction of 2-aminopurine in DNA decamer is very small (Nordlund et al., 1989). Fluorescence quenching defines a certain type of interaction. By the criterion of this interaction, the fully extended and completely unstacked fraction of ϵA, 2-aminopurine, and d5 is very small. Similarly, the fully extended and completely unstacked fraction of normal bases in DNAs or RNAs above their melting points is probably not large.

Assignment of Fully Stacked and Loosely Associated States. If we examine the temperature dependence of the amplitudes of components 1 and 2 in Table I, it is clear that the population of component 1 increases with increasing temperature while that of component 2 decreases. Since stacking is a favorable process at low temperatures as the enthalpy is negative (Saenger, 1984), it is likely that component 2 is the stacked form while component 1 is the loosely associated form. Thus the assignment of the three states can be shown as



where A* (a_2 in Table I) represents a fully stacked state or a cluster of stacked states, B* (a_1) represents a cluster of loosely associated states, and C* represents the fully extended and completely unstacked state. B is in such a state or a cluster of states that it is still very close to other bases. The B and C states together could be defined as the unstacked state. If we plot the concentration ratio (A/B + C) vs temperature, we obtain the apparent overall stacking enthalpy. Table II shows the value to be −4.7 kcal/mol. This is the average of the stacking interactions of d5 with the neighboring thymine and cytosine bases. The real stacking/unstacking transition of d5 base is probably that between A and B states. The enthalpy of stacking for d5 is thus actually −6.6 kcal/mol. It is interesting to note that depending on how we define stacked and unstacked states, the stacking enthalpy and entropy can change substantially in their final values.

Table II also shows that for d5 the transition from completely unstacked to loosely associated state is entropically favorable, which is the primary driving force for the overwhelming population of the loosely associated state over the completely unstacked state, and positive enthalpy for state C to state B explains the decrease of the completely unstacked state population at high temperature. It is known that nucleic acid three-dimensional structure is not driven by hydrophobic interactions (Alvarez & Biltonen, 1973). Base stacking is thus unlikely due to hydrophobic forces. However, when a base is completely unstacked in water solution it is hydrated, while when it is completely stacked it is in a dehydrated state. The process from completely unstacked state to loosely associated state is probably associated with dehydration (positive enthalpy) and release of water molecules (positive entropy). The general tendency that higher temperature results in lower population of the fully extended and completely unstacked form in ϵA (Kubota et al., 1983a), 2-aminopurine (Nordlund et al., 1989), and d5 base here implies that dehydration is probably responsible.

Fully Stacked State of the d5 Base. One may ask whether the assignments of states are correct in terms of fluorescence decay lifetime characteristics. We assign the fully stacked state with an intermediate lifetime compared to the completely unstacked and loosely associated states. This seems at first not a good choice since intuitively we would think that a stacked base can efficiently quench d5 fluorescence so that the stacked state should decay faster or perhaps be nonfluorescent. A nonfluorescent stacked state was proposed by Kubota et al. (1983a) to explain the differences of fluorescence quantum yields measured and calculated from amplitudes and lifetimes of ϵA. Although vertical base stacking may provide maximum stacking interaction, there is no experimental or theoretical evidence that this is the configuration where excitation can efficiently transfer from a fluorescent base to a nonfluorescent one. In fact, it is not even clear whether stacking itself is the direct cause of quenching, though it certainly provides a condition for this process. For example, some DNA intercalating dyes such as ethidium and proflavine have similar stacking interactions with DNA bases (Quadrifoglio et al., 1974) while their fluorescence properties are totally different. Proflavine fluorescence is quenched when bound to DNA while ethidium fluorescence is enhanced (Steiner & Kubota, 1983). In the work of Kubota et al. (1983a), the discrepancy in fluorescence quantum yields may be due to a component with a lifetime below their instrument resolution by flash lamp excitation. As stated in Results, the existence of a nonfluorescent d5 state is unlikely, or if it exists, its population is below our detection capability. It is thus unlikely that there is an extra stacked state besides what we have proposed.

If we compare the fluorescence-derived fraction of d5 stacked state in the decamer (a_2 in Table I) with the absorption-derived fraction (Figure 2B), it is clear that at low

temperature the fractions are comparable while the fraction from absorption is smaller at high temperatures. This is not surprising, since the enthalpy obtained from UV melting is always higher than that from other methods (Dewey & Turner, 1979). The fact that there is no cooperative transition in the d5-containing decamer implies that the stacking of the d5 base with other bases is different. The d5 base is in position 7 (5′ to 3′) in the decamer so that it is three bases away from one end and six from the other. Yet it has such a large effect that little or no duplex forms. It is difficult to rationalize that a deficiency of hydrogen bonds alone is responsible for the observed single-stranded character of the sequence (increasing the sequence length from 10 to 12 will allow helix formation in some cases; Connolly & Newman, 1989). It is likely that the loss of hydrogen-bonding interactions coupled with the stacking interactions is responsible for the properties of the d5-containing decamer. The inability of d5 to form two normal hydrogen bonds will cause its equilibrium position in the d5-containing decamer to be different from that of the thymine base in the unmodified decamer. This in turn will cause changes in base stacking patterns, since bases in single- or double-stranded DNAs are more or less stacked over each other under normal conditions (Saenger, 1984). One local perturbation, such as the change of the equilibrium position of d5 in the decamer relative to that of thymine, will cause other bases along the same strand to shift their equilibrium positions. In other words, base–base stacking interactions can transmit a local perturbation to other bases, which can lead to less stability of hydrogen bonds of other base pairs. This is probably the important contributing factor to the observed single-stranded character of the d5-containing decamer. The characteristics of the d5-containing decamer indicate that the structural stabilities and perhaps dynamics of DNA are not exclusively local due to neighboring base–base interactions. Base stacking was implicated as the primary factor for the stiffness of DNA (Hagerman, 1988). The motions of one base can be coupled to neighboring bases by both backbone motions and stacking interactions so that on some time scales the collective motions of DNA bases can make a substantial contribution to measured dynamic variables. In fact, collective torsional motions of longer DNAs were proposed (Barkley & Zimm, 1979; Allison & Schurr, 1979) and confirmed (Thomas et al., 1980, Millar et al., 1980) on the time scale of nanoseconds. The results of the d5-containing decamer show that local perturbation (substitution of thymine by d5) can affect the overall behavior of DNA strand. Thus there are structural bases for both local motions and collective motions in DNA.

Loosely Associated State. Why the loosely associated state exhibits the shortest lifetime is difficult to answer, partly because the nature of quenching by stacking is not well understood. The temperature dependence of τ_1 is the same as that of τ_2 (cf. Table I). This indicates that the dynamic quenching mechanisms are the same for the fully stacked and loosely associated states. Earlier work (Ferguson et al., 1973) has shown that many aromatic sandwich dimers exhibit much longer fluorescence lifetimes compared to their respective monomer lifetimes at low temperature in glass states when molecular motions are almost absent. By analogy the fluorescence lifetime of the fully stacked state may be longer than that of the free base, were there no local motion of bases. On the other hand, the loosely associated state would exhibit a fluorescence lifetime of the free base. Given a similar dynamic quenching mechanism for the two states, we would observe a shorter lifetime for the loosely associated state. It is also possible that the loosely associated state has a greater number of degrees of freedom, some of which involve favorable quenching configurations, and the interchanges within the cluster of states are very fast compared with fluorescence decay rates.

We emphasize that the loosely associated state B is not likely a particular portion of the fully extended and completely unstacked population with rotational orientations favorable for quenching since the rotational time of the extended base is probably close to that of the free base, which is of the order of several hundred picoseconds, and the whole population of rotamers will behave as a single-exponential decay in our measurements. Even though this state may be regarded as in the vicinity of other bases, it is impossible at the present time to define a geometical distance from which the quenching occurs.

The loosely associated state we identify here should correspond to the unstacked state measured or assumed in other techniques since the population of the fully extended and completely unstacked state is typically below 10% at all temperatures and hence will not make much contribution to the detected signals by other methods. In UV melting, once a base is out of the range where hypochromicity occurs, its status cannot be identified. In NMR experiments, chemical shifts of base protons in dimers at high temperatures are still away from those of monomers (Lee & Tinoco, 1977), which can be explained either as the contribution from the fraction of stacked bases or from the possibility that two bases may not be stacked but are also not far away, so that there are still some chemical shift changes due to interactions. Similarly, CD spectra at high temperatures may also come from two contributions. The unstacked state up to now has been conceptually defined but experimentally not clearly identified. Our results show that the "unstacked" state assumed in hypochromism may be unstacked but it is not free from interactions with other bases. The "free" state can indeed be observed, but oligomer bases rarely take on this conformation, even at high temperature.

In summary, we show here that d5 base in a DNA decamer exhibits three exponential decays, which are assigned to three components in equilibrium: the stacked state, the loosely associated state, and the fully extended and completely unstacked state. The fully extended and completely unstacked state occupies only a very small fraction of all the possible states of d5 and the major portion of the unstacked base is in the close vicinity of other DNA bases where its fluorescence is still efficiently quenched. The exchanges between states are slow compared to their fluorescence decay rates so that the amplitude ratios are related to their thermodynamic equilibrium constants. From the temperature dependence of the ratios we obtain the stacking enthalpy and entropy of d5 with thymine or cytosine to be −6.6 kcal/mol and −22 cal/mol·K, respectively. This work shows that fluorescence decay measurements of fluorescent bases in DNA can provide useful information about the local conformations of bases.

ACKNOWLEDGMENTS

We thank Professor D. H. Turner, Department of Chemistry, University of Rochester, for the use of his melting apparatus, A. Peritz for measuring the melting curve, and M. Prichard for help in time-resolved fluorescence measurements.

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Anomalous Gel Migration of DNA Oligomers Containing Multiple Conformational Junctions†

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Received November 28, 1989; Revised Manuscript Received March 15, 1990

ABSTRACT: We have previously shown that a short 16 base pair DNA oligomer can accommodate a B-Z conformational junction [Sheardy, R. D., & Winkle, S. A. (1989) *Biochemistry 28*, 720-725]. Results from ^{1}H NMR studies indicated that only three base pairs were involved in the junction and that one of these base pairs was highly distorted. Being interested in the nature of this distortion, we constructed DNA oligomers which have the potential to contain multiple B-Z junctions for polyacrylamide electrophoretic studies. We report that the mobilities displayed by these molecules through acrylamide gels in the absence and presence of cobalt suggest that these molecules run shorter than they actually are. This anomalous migration may be due to structural/dynamic properties of the DNA helix manifested by the periodic disortions of the potential B-Z junctions.

The interest in the biophysical properties of unusual DNA structures has been steadily increasing over the past few years. Investigations have focused on the relationship between sequence and conformation for specific DNA segments. One particular area of interest is the study of DNA molecules that contain bends which give rise to global curvature of the DNA molecules. It has been shown that DNA which contains tracts of oligo(dA)-oligo(dT) exhibits bending or curvature (Levene & Crothers, 1983; Wu & Crothers, 1984; Hagerman, 1984, 1985, 1986; Levene et al., 1986; Koo et al., 1986; Rice & Crothers, 1989; Cacchione et al., 1989). One experimental

†Supported by NSF Grant DMB-8996232 (R.D.S.) and by a Provost's Summer Faculty Fellowship (S.A.W.).

0006-2960/90/0429-6514$02.50/0 © 1990 American Chemical Society