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The Trapping of a Spontaneously "Flipped-Out" Base from Double Helical Nucleic Acids by Host–Guest Complexation with β-Cyclodextrin: The Intrinsic Base-Flipping Rate Constant for DNA and RNA

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Abstract: β-Cyclodextrin, which forms stable host–guest complexes with purine bases, induces the melting of RNA and DNA duplexes below their normal melting temperatures. α-Cyclodextrin, which does not form stable complexes, has no effect on either RNA or DNA. γ-Cyclodextrin, which forms weaker complexes, has no effect on RNA and a smaller effect than β-cyclodextrin on DNA. The rate of melting is kinetically first-order in duplex and, above about 20 mM β-cyclodextrin, is independent of the β-cyclodextrin concentration with a first-order rate constant, common to both RNA and DNA, of $(3.5 \pm 0.5) \times 10^{-3}$ s^{-1} at 61 °C (DNA) and at 50 °C (RNA). This is taken to be the rate constant for spontaneous "flipping out" of a base from within the duplex structure of the nucleic acids, the exposed base being rapidly trapped by β-cyclodextrin. Like β-cyclodextrin, nucleic acid methyltransferases bind the target base for methylation in a site that requires it to have flipped out of its normal position in the duplex. The spontaneous flip-out rate constant of around 10^{-3} s^{-1} is near the value of k_{cat} for the methyltransferases (ca. 10^{-3} to 10^{-1} s^{-1}). In principle, the enzymes, therefore, need effect little or no catalysis of the flipping-out reaction. Nevertheless, the flip-out rate in enzyme/DNA complexes is much faster. This observation suggests that the in vivo circumstances may differ from in vitro models or that factors other than a simple drive toward higher catalytic power have been influential in the evolution of these enzymes.

Introduction

The marking of bases in nucleic acids by methylation[1−10] is a technique of genetic regulation. The reaction can be effected by enzyme-catalyzed methyl transfer from *S*-adenosylmethionine by enzymes that bind the duplex nucleic acid in a site adjacent to the binding site for *S*-adenosylmethionine. The target base for methylation is removed from its position in the duplex structure and, with considerable distortion of the double-helical duplex structure, is bound into the catalytic site next to the *S*-adenosylmethionine methyl donor.[4−10] In such a structure, the base is said to be "flipped out" of the duplex.

One may consider two simple models for the process by which the target base in the enzyme–nucleic acid complex becomes fully flipped out of the duplex and bound into the enzymic methylation site. On one model, the enzyme catalyzes base-flipping. On the alternative model, the enzyme relies on normal, spontaneous motions of the nucleic acid to provide it with a fully flipped-out target base, which the enzyme then binds into the methylation site.

We report in this paper the probable formation of host–guest complexes with flipped-out nucleic acid bases by β-cyclodextrin, as a probe of the rate of base-flipping in the absence of enzyme interaction. This technique relies on a melting-point depression for double-helical nucleic acids, induced by β-cyclodextrin. Relaxation kinetics of the β-cyclodextrin-induced melting of double-helical nucleic acids can then measure the rate constant for base-flipping.

Host–guest interactions (i.e., base inclusion into the cyclodextrin) are likely to account for the melting-point depression by β-cyclodextrin. α-Cyclodextrin and γ-cyclodextrin, which differ in the diameter of the central cavity (Table 1) and fail to bind nucleic acid bases strongly into their cavities, do not exhibit the melting-point depression observed for β-cyclodextrin.

Results

β-Cyclodextrin Lowers the Melting Temperatures of RNA and DNA in a Manner Not Matched by α-Cyclodextrin or γ-Cyclodextrin. Figure 1 shows how β-cyclodextrin induces

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Table 1. Characteristic Features of α-, β-, and γ-Cyclodextrins[a]

	α	β	γ
number of glucopyranose units	6	7	8
molecular weight (g/mol)	972	1135	1297
central cavity diameter, Å	4.7−5.3	6.0−6.5	7.5−8.3

[a] Loftsson, T.; Brewster, M. E. *J. Pharm. Sci.* **1996**, *85*, 1017−1025.

the melting of DNA and RNA at temperatures below their normal melting temperatures (t_m). The ultraviolet spectrum of a DNA sample composed of poly(dA)−poly(dT) about 240 base pairs in length (t_m about 62.2 °C) is stable for at least 30 min at a temperature of 61.8 °C, but upon addition of 50 mM β-cyclodextrin, the hyperchromicity typical of the loss of helical structure develops over about 8 min (Figure 1a). Similarly, the ultraviolet spectrum of an RNA sample consisting of poly(rA)−poly(rU) and ranging in length from 150 to several thousand base pairs (t_m about 51.9 °C) is stable for at least 30 min at 50.0 °C, but in the presence 50 mM β-cyclodextrin, hyperchromicity again develops over about 8 min (Figure 1b).

Figure 2 shows that the β-cyclodextrin-induced shift in t_m for DNA and RNA is not matched by α-cyclodextrin, which has a smaller central cavity than β-cyclodextrin, or by γ-cyclodextrin, which has a larger central cavity than β-cyclodextrin. DNA, whether alone or in the presence of 50 mM α-cyclodextrin, exhibits similar melting behavior with an average t_m of 62.2 ± 0.1 °C. In the presence of 50 mM β-cyclodextrin, the melting curve is shifted to lower temperatures by about 1.4 °C, the average t_m becoming 60.8 ± 0.1 °C. γ-Cyclodextrin at 50 mM produces a smaller effect with t_m moving down to around 61.7 °C. RNA, whether alone or in the presence of either 50 mM α-cyclodextrin or 50 mM γ-cyclodextrin, melts with an average t_m of 51.9 ± 0.2 °C. Again 50 mM β-cyclodextrin produces a downward shift of t_m, here by about 3.3 °C to 48.6 ± 0.2 °C. Smaller concentrations of β-cyclodextrin produced smaller downward shifts of t_m, for DNA by 0.6 °C at 20 mM and for RNA by 1.8 °C at 25 mM.

β-Cyclodextrin-Induced Melting of RNA and DNA Is a First-Order Relaxation Process. Figure 1 also shows that the melting of both DNA and RNA in the presence of β-cyclodextrin is kinetically a first-order relaxation process with a rate constant independent of the concentration of the duplex nucleic acid and is thus unimolecular in the duplex. The spectrum of the duplex nucleic acids was initially unchanged upon exposure of the duplex to β-cyclodextrin, suggesting that there was no rapid interaction of β-cyclodextrin with the duplex itself.

Rate Constant for β-Cyclodextrin-Induced Melting of RNA and DNA at Saturating Cyclodextrin Concentrations. Figure 3 demonstrates that the melting rate constants for both DNA at 61 °C and RNA at 50 °C, which are less than 10^{-5} s^{-1} in the absence of β-cyclodextrin, rise to a level of (3.5 ± 0.5) × 10^{-3} s^{-1} for concentrations of β-cyclodextrin above about 20−30 mM. The data do not require or support any particular functional form for the β-cyclodextrin-induced increase in the relaxation rate constant, although the 100-fold increase in the rate constant in the presence of 20−30 mM cyclodextrin shows that a saturable process is under observation. If a hyperbolic relationship is employed for illustrative purposes, the data are consistent with a concentration at half-saturation of 2.2 ± 2.1 mM.

Discussion

Induced Melting of RNA and DNA by Host−Guest Complexation. β-Cyclodextrin displays the ability to melt RNA and DNA double helices below their normal melting points, while α-cyclodextrin does not exhibit this property and γ-cyclodextrin exhibits the ability only in attenuated form with DNA and not at all with RNA. Host−guest complexation is therefore a reasonable mechanism for the induced melting process. Adenine can serve as a guest in β-cyclodextrin,[17−19] while α-cyclodextrin has too small a guest cavity to allow full penetration of a purine base.[20−22] Furthermore, γ-cyclodextrin, which has a larger guest cavity than β-cyclodextrin, forms significantly weaker inclusion complexes with purine guests.[21] These properties are suggestive of a host−guest role for β-cyclodextrin, in its reduction of t_m for double-stranded DNA and RNA.

The specificity of β-cyclodextrin in producing the melting effect militates against a destabilizing interaction with the closed, duplex nucleic acid itself as opposed to base-specific interactions with open regions that are undergoing flipping out. The reduction in melting point is also consistent with the general experience that compounds which bind to the duplex, but not to single strands or exposed regions, tend to increase the melting point[12] rather than decreasing the melting point as observed here. The lack of any spectroscopic change when the duplex nucleic acids were exposed to cyclodextrins further suggests that no rapid binding of cyclodextrins to the intact duplex is occurring. This is consistent with the comprehensive work of Hoffman and Bock, who examined a wide array of nucleotides, double-stranded nucleic acids, and single-stranded nucleic acids for spectroscopic changes in the presence of β-cyclodextrin.[17] β-Cyclodextrin was found to interact only with species having exposed bases, not with double-helical nucleic acids.

The kinetics of β-cyclodextrin-induced melting of both RNA and DNA duplexes is independent of the β-cyclodextrin concentration above about 20−30 mM. The rate constant approaches a value of (3.5 ± 0.5) × 10^{-3} s^{-1}, common to both RNA and DNA; this is at least 100-fold larger than the rate in the absence of β-cyclodextrin. No functional dependence of the rate constant on β-cyclodextrin concentration can be deduced from the limited data, except that saturation of the effect of β-cyclodextrin begins to be reached between β-cyclodextrin concentrations of zero and around 6−10 mM. Illustrative use of a hyperbolic relationship generates a concentration of β-cyclodextrin at half-saturation of 2.2 mM.

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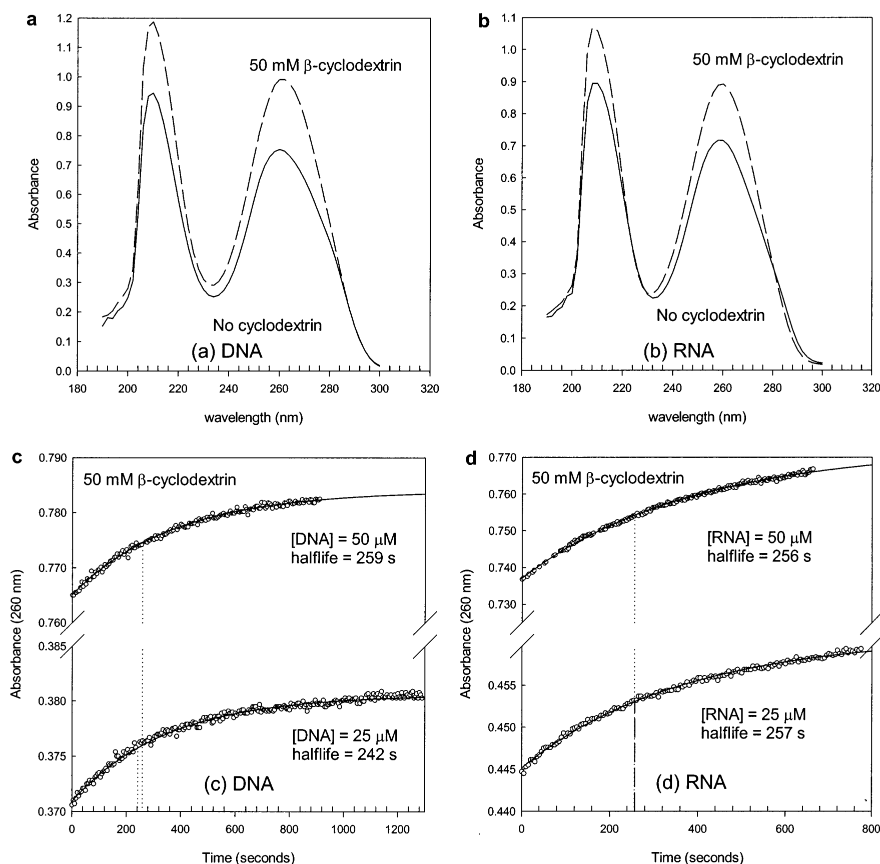

Figure 1. β-Cyclodextrin induces a decrease in nucleic acid melting temperatures. The ultraviolet spectra of DNA (a) and RNA (b) at 50 μM base-pair concentrations are stable for at least 30 min in the absence of β-cyclodextrin (solid lines, 50 mM NaCl, 5 mM Tris, pH 7.0) at temperatures of 61.8 °C for DNA and 50.0 °C for RNA. After exposure to 50 mM β-cyclodextrin for 8 min (dashed lines), both DNA and RNA exhibit the hyperchromicity typical of the helix-to-coil transition. The rates of β-cyclodextrin-induced melting of both DNA (c) and RNA (d) exhibit simple first-order relaxation kinetics with a half-life time independent of the base-pair concentrations.

One may compare this value with the dissociation constants of a wide variety of complexes between β-cyclodextrin and aromatic guests, tabulated by Tee[22] (all of which lie between 0.13 and 67.8 mM). Xiang and Anderson determined the dissociation constant of the complex of free adenosine with β-cyclodextrin at 25 °C to be 75 mM, while it is greater than 1.3 M for α-cyclodextrin.[19] These values are also similar to those in the very extensive tabulation of cyclodextrin binding thermodynamics by Rekharsky and Inoue.[23]

Figure 4 shows a possible kinetic scheme for the β-cyclodextrin-induced melting of nucleic acid duplexes. This scheme envisions the spontaneous flipping out of a base in the helical duplex to generate an unstable, low-concentration, steady-state intermediate that either returns to helix or proceeds to the denatured (coil) form of the nucleic acid. This putative intermediate in the melting reaction is suggested to be the target for cyclodextrin trapping. As also shown in Figure 4, the relaxation constant for equilibration of helix and coil on this model should display saturation with increasing concentrations of cyclodextrin.

Figure 3 shows that some form of saturation occurs, since the rate constant at concentrations of β-cyclodextrin where measurements are possible (>5 mM) is larger than the rate constant in the absence of β-cyclodextrin and is independent of the concentration of β-cyclodextrin. The data do not establish or even suggest a hyperbolic form for the saturation, but this is unimportant because modifications to the model of Figure 4 could generate many functional forms for the concentration dependence. The fact that the phenomenon has reached saturation, regardless of the functional form, is the important observation.

The fact that the host molecule β-cyclodextrin, with an internal cavity that accommodates purine rings well, is capable of inducing both DNA and RNA melting in a saturable manner at temperatures below their normal melting temperatures, while the smaller-cavity α-cyclodextrin and the larger-cavity γ-cyclodextrin have no similar effects, suggests that the induced melting derives from rapid trapping of a normal, low-concentration form of the nucleic acid with a completely exposed base that can be enveloped and captured by β-cyclodextrin. The rate constant at saturation, $(3.5 \pm 0.5) \times 10^{-3}\ \mathrm{s}^{-1}$, should therefore be the rate constant for the nonenzymic flipping-out reaction.

Relationship of Host−Guest Induced Melting of RNA and DNA to Enzymic Nucleic Acid Methylation. As described above, for enzymic methylation of nucleic acid bases, the target base of the nucleic acid must emerge completely from its normal position along the central axis of the duplex and bind in a substrate site of the enzyme. Methyl transfer can then occur, with different enzymes preferring different methylation sites: Transfer to carbon, nitrogen, or oxygen can occur, with C-5 of cytosine and N-6 of adenine being typical.

Allan, Reich, and Beechem[1] have shown by fluorescence-anisotropy relaxation that base flipping out of 14-base-pair DNA

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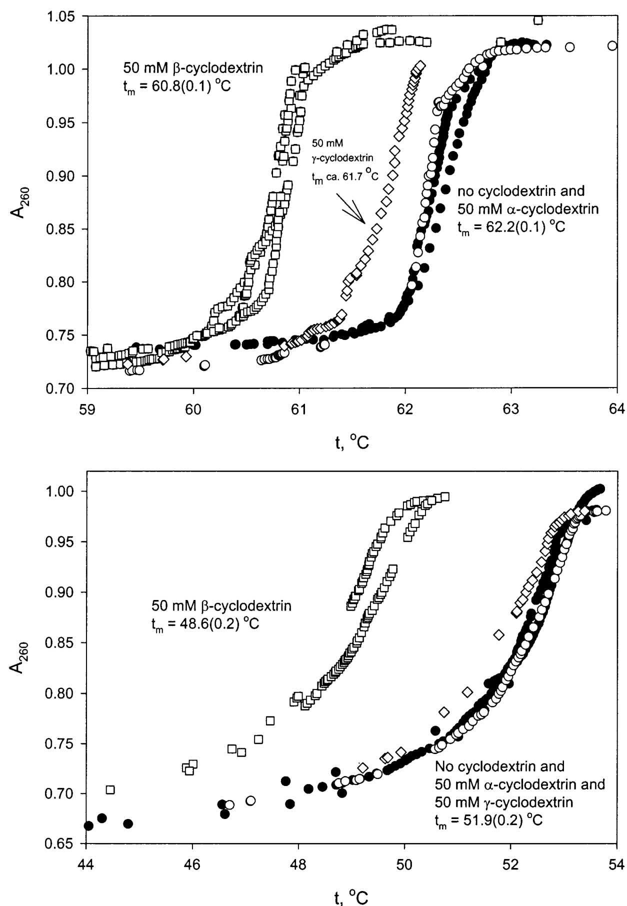

Figure 2. (upper panel) DNA (poly(dA)-poly(dT), 240 base pairs) melts with $t_m = 62.2 \pm 0.1$ °C either alone (filled circles) or in the presence of 50 mM α-cyclodextrin (open circles). Addition of 50 mM β-cyclodextrin (open squares) shifts t_m to 60.8 ± 0.1 °C. Addition of 50 mM γ-cyclodextrin (open diamonds) shifts t_m only to about 61.7 °C. Melting temperatures were estimated from $A_{260}(t)$ by two-state fits[11−14] as described in the Experimental Section, which gave results very similar to estimates from simple interpolation. The computer program MELTSIM,[15] which employs "nearest neighbor" stacking and pairing energies,[16] was used to simulate a melting curve for a 240 base pair poly(dA)−poly(dT) species, with 50 mM Na^+ (as used here). The simulated t_m was 62.85 °C, which compares favorably to the fit data, which yielded a value of 62.2 °C. (lower panel) RNA (poly(rA)−poly(rU), 150 to several thousand base pairs) has $t_m = 51.9 \pm 0.2$ °C, whether alone (filled circles), in the presence of 50 mM α-cyclodextrin (open circles), or in the presence of 50 mM γ-cyclodextrin (open diamonds). Addition of 50 mM β-cyclodextrin shifts t_m to 48.6 ± 0.2 °C.

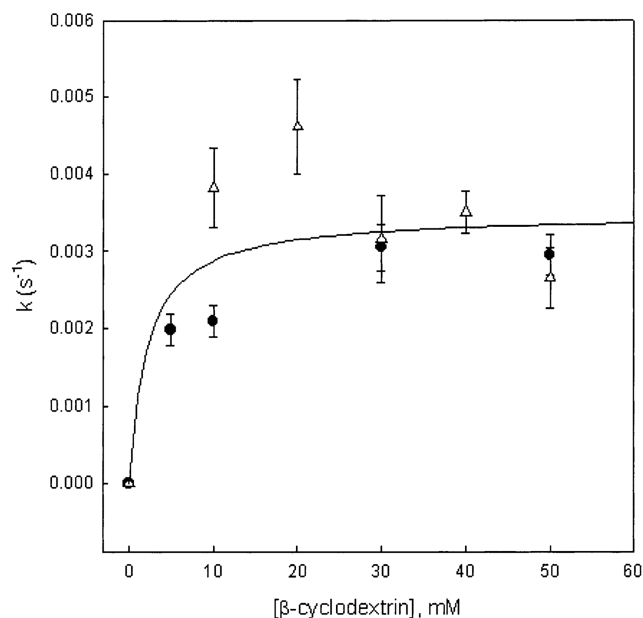

Figure 3. Effect of β-cyclodextrin on the first-order rate constant for melting of DNA (filled circles) and RNA (open triangles). Melting was initiated by addition of 0.1 mL of nucleic acid solution to 0.9 mL of β-cyclodextrin solution at 61 °C (DNA) or 50 °C (RNA) after thermal equilibration of both. The total absorbance change at 260 nm increases with increasing β-cyclodextrin concentration so that data could not be obtained below about 5 mM β-cyclodextrin. The data are consistent with saturation behavior, defining a rate constant for both DNA and RNA of (3.5 ± 0.5) $\times 10^{-3}$ s^{-1} at high concentrations of β-cyclodextrin. An illustrative fit to a hyperbolic saturation function yields a concentration at half-saturation of 2.1 ± 2.2 mM.

duplexes is coupled to binding of the duplex to *Eco*RI methyltransferase and that the flipping rate constant is at least 195 s^{-1}. Thus the *Eco*RI methyltransferase accelerates the flipping out of nucleic acid bases by a factor of at least 5×10^4. This renders the reaction much faster than the overall k_{cat} values of around 10^{-1} to 10^{-3} s^{-1} for various methyltransferases.[24−27] Uracil-DNA glycosylases[28−35] exhibit a larger base-flipping rate constant[36] of 700 s^{-1}.

It is a reasonable question why an enzyme accelerates by so large a factor a process that is already roughly as fast as the rate-limiting step. Possibly, the flipping energetics of in vivo substrates may be different from those in vitro, as a result of differences in nucleic acid properties.[37−41] Nucleic acid "breathing" rate constants, obtained by hydron-exchange studies, are approximately 6−7 orders of magnitude greater than the base-flipping rate constant measured in the current study,[42−50] but the processes involved seem unlikely to correspond to base

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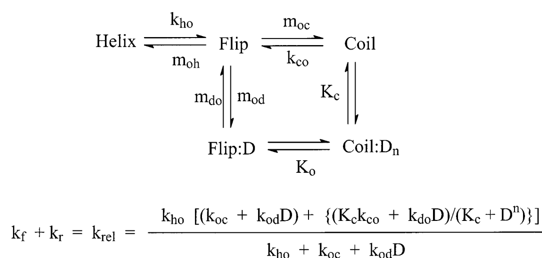

$$k_f + k_r = k_{rel} = \frac{k_{ho}\ [(k_{oc} + k_{od}D) + \{(K_c k_{co} + k_{do}D)/(K_c + D^n)\}]}{k_{ho} + k_{oc} + k_{od}D}$$

Figure 4. Schematic version and steady-state kinetic model of the effect of β-cyclodextrin (D) on the conversion of nucleic acid helical forms (Helix) to the coil forms (Coil). The normal melting reaction is assumed to proceed through a reactive intermediate (Flip) in which a base has been flipped out of the helical structure. Either this base may return and regenerate the helix or the flipped form may complete its unwinding to generate the coil. β-Cyclodextrin is considered to trap the intermediate Flip by formation of a host−guest complex with the flipped-out base to generate a second reactive intermediate (Flip:D) which also can either return to Helix or proceed to a coiled form. The coiled form can logically be supposed to accumulate a large number n of β-cyclodextrin ligands in a rapid-equilibrium reaction. Defining $K_o m_{do} = k_{do}$ and otherwise (except for k_{ho} and k_{co}) defining $k_{xy} = k_{ho} m_{xy}/m_{oh}$, application of the steady-state approximation leads to the relaxation constant for the helix−coil equilibrium as shown. This expression yields a saturation curve with $k_{rel} = k_{ho}$, the rate constant for flipping out, when the β-cyclodextrin concentration D becomes large.

flipping of the sort considered here. In the most general sense, it may simply be the case that considerations other than a drive toward higher catalytic power have been critical in the evolution of base-flipping enzymes.

Experimental Section

Materials. RNA poly(rA)−poly(rU) was purchased from Sigma (catalog no. P-1537) as a mixture of species between 150 and several thousand base-pairs in length. DNA poly(dA)-poly(dT) (approximately 240 base-pairs in length) was purchased from Pharmacia Biotech (catalog no. 27-7860-01). Cyclodextrins α (catalog no. C-4642), β (catalog no. C-4767), and γ (catalog no. C-4892) were purchased from Sigma.

Kinetic Measurements and Equilibrium Spectroscopic Measurements. Kinetic measurements were carried out with a Shimadzu UV-160 UV−vis spectrophotometer interfaced to an IBM-compatible computer or with a Hewlett-Packard 8452A diode array spectrophotometer interfaced to an IBM-compatible computer, after the addition of nucleic acid to a cyclodextrin solution. Nucleic acid duplex solution (100 μL, 5 mM Tris, pH 7.0, 50 mM NaCl) and cyclodextrin solution (900 μL, 5 mM Tris, pH 7.0, 50 mM NaCl) were thermally equilibrated (250 s), the solutions were combined to obtain a solution with a base-pair concentration of 50 μM, and absorbance at 260 nm was recorded as a function of time. The temperature was monitored with an Omega Digicator temperature probe placed in a sample cuvette. Equilibrium spectroscopic measurements were made similarly.

Data Analysis. The relaxation time courses were fitted to a single-exponential function by use of the nonlinear least-squares fitting program within the SIGMA-PLOT program of the Jandel Corporation. The estimation of t_m values from melting curves was made either by fitting the data for $A(t)$ to a simple two-state model for conversion of base-stacked to base-unstacked nucleic acids[11−14] or by interpolation of the data for $A(t)$ to obtain the approximate temperature for half-completion of the total absorbance change. The expression for $A(t)$ on the simplest (stacked/unstacked) model[11] can be written,

$$A(t) = A_f - \{[A_f - A_0]/[1 + \exp\{h[(1/\{t + 273.16\}) - (1/\{t_m + 273.16\})]\}]\}$$

where A_f is the final absorbance at 260 nm at high temperature, A_0 is the initial absorbance at low temperature, h is a fitting parameter, t is the temperature in degrees Celsius, and t_m is the melting temperature in degrees Celsius. For a true two-state model, $h = \Delta H°/R$, where $\Delta H°$ is the enthalpy of conversion of the unstacked to the stacked nucleic acid. Here h is regarded simply as an arbitrary parameter of the fit, although the values obtained were similar to those expected for the systems under observation. The two-state model is not expected to fit these cases well,[11] but the value of t_m appears insensitive to the detailed model, and the estimates obtained in these two ways commonly agreed within a few tenths of a degree.

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