chim. Biophys. Acta 562, 41–50.
Sander, M., & Hsieh, T. S. (1983) J. Biol. Chem. 258, 8421–8428.
Tewey, K. M., Chen, G. L., Nelson, E. M., & Liu, L. F. (1984a) J. Biol. Chem. 259, 9182–9187.
Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D., & Liu, L. F. (1984b) Science (Washington, D.C.) 226, 466–468.
Zwelling, L. A., Michaels, S., Erickson, L. E., Ungerleider, R. S., Nichols, M., & Kohn, K. W. (1981) Biochemistry 20, 6553–6563.
Zwelling, L. A., Michaels, S., Kerrigan, D., Pommier, Y., & Kohn, K. W. (1982) Biochem. Pharmacol. 31, 3261–3267.

Ethidium Ion Binds More Strongly to a DNA Double Helix with a Bulged Cytosine Than to a Regular Double Helix†

Jeffrey W. Nelson‡ and Ignacio Tinoco, Jr.*,§

Department of Chemistry and Laboratory of Chemical Biodynamics, University of California, Berkeley, Berkeley, California 94720, and Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received March 18, 1985

ABSTRACT: Thermodynamic parameters for ethidium intercalation were determined for the double helices formed by the oligonucleotides $dCA_6G + dCT_6G$, which form a normal helix, and $dCA_3CA_3G + dCT_6G$, which form a double helix with the middle cytosine bulged outside of the helix. Ethidium intercalation was measured by monitoring the absorbance at 260 and 283 nm as a function of temperature for a number of concentrations of ethidium. The binding to the normal helix occurs equally at all the intercalation sites, with an enthalpy of binding of −8 kcal mol^{-1}, an entropy of binding of −6 eu, and an equilibrium constant at 25 °C of 2.2×10^4 M^{-1}. The binding to the bulged double helix was considerably stronger and is consistent with a model in which the intercalation sites on either side of the bulged base bind 10 times stronger than the other sites. Thus, there are two strong binding sites on the perturbed helix with equilibrium constants for binding of 2×10^5 M^{-1} at 25 °C in addition to five normal sites. Several other binding models were tested but did not fit the data satisfactorily.

Frameshift mutagenesis requires the formation of a structure in which there is an extra nucleotide on one strand of a DNA double helix. This intermediate in the mechanism of mutation is called a bulge. The probability of a frameshift mutation depends on the incorporation of an extra nucleotide into a newly synthesized strand (a plus one mutation) or on the omission of a nucleotide from the new strand (a minus one mutation). Repair of the bulged structure before replication is also important to the mutation process. The stability and conformation of the bulge affect all of these reactions. The role of intercalators in the process of frameshift mutagenesis has been hypothesized for many years (Streisinger et al., 1966). Intercalating molecules are thought to promote frameshift mutagenesis by binding preferentially to bulged structures, thereby stabilizing them and increasing the likelihood that the bulge will become incorporated into the DNA.

Ethidium ion intercalates into a complex consisting of the trinucleoside diphosphate rGpUpG and the dinucleoside phosphate rCpC, forming a mini double helix with the ethidium sandwiched between the two C-G base pairs with the bulged U outside the helix (Lee & Tinoco, 1978). It is also known that ethidium bromide binds more strongly into synthetic RNA polynucleotides that contain a small amount of A·I mismatches (Helfgott & Kallenbach, 1979). These mismatches probably form nonstandard base pairs, and although they are not bulges, the situation is relevant to the bulges. No direct measurement has been done on the magnitude or mechanism of the stabilization of a bulge by ethidium bromide. We previously studied the intercalation of ethidium bromide into the ribooligonucleotides $rCA_5G + rCU_5G$ and the corresponding deoxyribooligonucleotides $dCA_5G + dCT_5G$ (Nelson & Tinoco, 1984). Here, we discuss the extension of the work to the intercalation of ethidium ion into the deoxyribooligonucleotides $dCA_6G + dCT_6G$, which form a normal double helix, and $dCA_3CA_3G + dCT_6G$, which form a double helix with a bulged cytosine in the middle of the helix.

The structure of these normal and bulged double helices has been characterized by NMR,[1] and the thermodynamic stabilities have been studied by optical melting curves (Morden et al., 1983). It was found that the bulged cytosine is out of the helix and the two A·T base pairs on either side of the bulge stack on each other similar to the normal helix. The destabilization caused by the bulge was also determined to be due to a less favorable enthalpy of double-strand formation, with an increase in the standard free energy at 25 °C of approximately 2.9 kcal mol^{-1}.

Materials and Methods

The synthesis of the oligonucleotides is discussed in Morden et al. (1983). The extinction coefficients per mole of strand at 260 nm and 25 °C were estimated from the coefficients for

† This work was supported by National Institutes of Health Grants GM 10840 and CA 24101 and by the U.S. Department of Energy, Office of Energy Research, under Contract 82 ER 60090. J.W.N. was supported by NIH Training Grant CA 09485.

‡ University of Pennsylvania.
§ University of California, Berkeley.

[1] Abbreviations: NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

0006-2960/85/0424-6416$01.50/0

mononucleotides and dinucleoside phosphates (Fasman, 1975). The values for dCA_6G, dCA_3CA_3G, and dCT_6G are 9.1×10^4, 9.8×10^4, and 6.6×10^4 M^{-1} cm^{-1}, respectively. Ethidium bromide was purchased from Sigma; to remove any ethanol present, the ethidium bromide was lyophilized twice with double-distilled water prior to use.

The buffer used throughout this study consisted of 0.2 M NaCl, 0.01 M sodium phosphate buffer, pH 7.0, and 0.1 mM EDTA. Samples were prepared by adding small amounts of concentrated stock solutions of the oligonucleotides and ethidium bromide to the buffer. The buffer was degassed by purging with helium for 3 min prior to preparing the samples.

Samples of the dCA_6G + dCT_6G + ethidium were made up with a nominal strand concentration of 65 μM, with ethidium:strand ratios of approximately 0, 0.6, 0.8, 1.0, and 1.6. Eppendorf 0.5-mL polypropylene microcentrifuge tubes were used to prepare the samples. Tubes were pretreated by rinsing with an ethidium bromide solution to avoid adsorption of ethidium from the samples (Nelson et al., 1984). The actual concentrations were determined from the absorbances at 50 °C, where the oligonucleotides are single stranded and the ethidium is unbound. When the melting process was not finished by 50 °C, the absorbances of single strands and free ethidium at 50 °C were determined by extrapolating the absorbances at higher temperatures. Samples of dCA_3CA_3G + dCT_6G + ethidium were made at a nominal strand concentration of 60 μM, with ethidium:strand ratios of approximately 0, 0.6, 0.9, 1.0, 1.5, and 2.1.

Melting curves were obtained on a Gilford Model 250 UV–vis spectrophotometer, with a Gilford Model 2527 thermoprogrammer. The cuvettes were Teflon stoppered with a path length of 0.1 cm. Data were obtained concurrently at 260 and 283 nm on a Gilford Model 2530 wavelength scanner. The data were collected by an Apple 2e microcomputer interfaced to the instrument and were later transmitted to a VAX 11/780 computer for the data analysis. The temperature range was generally 0–75 °C. The samples were returned to 0 °C after attaining the high temperature to check for evaporation. Changes in absorbance were always less than 2%.

The data analysis was done essentially as described earlier (Nelson & Tinoco, 1984). The same procedure was used to determine the fraction of double strands and the fraction of intercalated ethidium from the absorbances with the extinction coefficients for the single strands, double strands, and free and intercalated ethidium at 260 and 283 nm. The statistical theory presented earlier was used for analyzing the fraction ethidium bound in terms of the equilibrium constant for double-strand formation and the equilibrium constant for intercalation (K_d). A value for the difference in binding between normal and strong sites was chosen, and the best fit to the thermodynamics was determined by a least-squares analysis of the ln K_d vs. $1/T$ plot obtained by fitting the experimental points of several melting curves. Values of the fraction of ethidium bound between 0.15 and 0.85 were used in the analysis. The reduced χ^2 was then calculated for these data from the best fit parameters. The goodness of fit can be determined from either the linear correlation coefficient from the ln K_d vs. $1/T$ plot or the reduced χ^2, and both criteria consistently gave the same results.

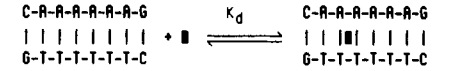
Theory

The statistical theory of ethidium intercalation into oligonucleotide double helices is described in detail in Nelson & Tinoco (1984). The model assumes that ethidium intercalates only between base pairs of the double strands, with nearest-neighbor exclusion. Here, we briefly describe the differences required when bulged double helices are studied.

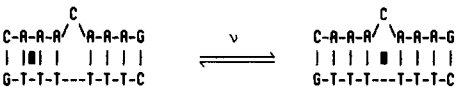
The single-strand to double-helix transition is described by the equilibrium constant K_h:

$$CA_6G + CT_6G \overset{K_h}{\rightleftharpoons} CA_6G{\cdot}CT_6G$$

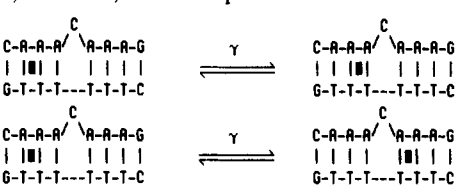
with a similar expression for the bulged double helix. The intercalation of ethidium to the normal helix is described by the equilibrium constant K_d to any of the intercalation sites. It was found earlier that ethidium intercalates into all sites about equally (Nelson & Tinoco, 1984):



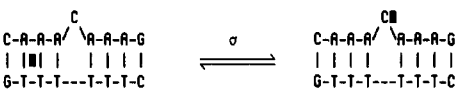
With the bulged double helices, we can define three other equilibrium constants, associated with intercalation at the bulge site, the intercalation sites on either side of the bulge, and binding to the bulge outside the helix. All three can be described as a ratio between binding at a normal site and the perturbed site. For the model assuming a strong site at the bulge, we have, for example



whereas for the model assuming two strong sites surrounding the bulge, we have, for example



and for the model assuming binding to the bulge on the outside of the helix, we have



The partition function, Q, for the double-stranded species is then the sum of the statistical weights of the species with 0, 1, 2, ..., n ethidium ions bound, as described in Nelson & Tinoco (1984):

$$Q = \sum_{i=0}^{n} g_i K_h S^i$$

where $S = K_d$ (free ethidium concentration) and g_i values describe all the arrangements of binding i ethidium ions, taking into account the different affinities of the various sites. For example, assuming the model with two strong sites ($\gamma > 1$, $\nu = 1$, $\sigma = 0$), there are five ways to bind one ethidium into a site with unperturbed binding strength and two ways to bind to a strong site, so $g_1 = 2\gamma + 5$. Similarly, we determine $g_0 = 1$, $g_1 = 2\gamma + 5$, $g_2 = \gamma^2 + 6\gamma + 8$, $g_3 = 2\gamma^2 + 4\gamma + 4$, and $g_4 = \gamma^2$, with $n = 4$. Otherwise, the treatment is identical with that of Nelson & Tinoco (1984).

Results

Ethidium Binding to dCA_6G + dCT_6G. The melting curves at 260 and 283 nm for dCA_6G + dCT_6G with ratios of ethidium:strand between 0 and 1.6 are shown in Figure 1, all at approximately 65 μM strand concentration. The actual concentrations are given in the figure caption. The melting curves are all normalized to 1.0 at 50 °C in order to display them together. The stabilization of the helix is clearly seen from the shifts of the curves to higher temperatures as the

Table I: Extinction Coefficients (M^{-1} cm^{-1}) at 260 and 283 nm[a]

ethidium bromide[b]	$\epsilon_{260} = (1.7 \times 10^4) - 25.5(T - 50)$
	$\epsilon_{283} = (5.1 \times 10^4) - 74(T - 90) + [0.25(\text{concn})^{1/2} + (4.4 \times 10^{-4})](T - 90)^3$
$dCA_6G + dCT_6G$ single strands	$\epsilon_{260} = (1.63 \times 10^5) + 240(T - 50)$
	$\epsilon_{283} = (6.12 \times 10^4) + 44(T - 50)$
$dCA_6G + dCT_6G$ double strands	$\epsilon_{260} = (1.34 \times 10^5) + 270T$
	$\epsilon_{283} = (5.82 \times 10^4) + 35T$
ethidium bromide bound to $dCA_6G + dCT_6G$	$\epsilon_{260} = (1.25 \times 10^4) - 15T$
	$\epsilon_{283} = (2.3 \times 10^4) + 75T$
$dCA_3CA_3G + dCT_6G$ single strands	$\epsilon_{260} = (1.69 \times 10^5) + 180\ (T - 50)$
	$\epsilon_{283} = (6.57 \times 10^4) + 31(T - 50)$
$dCA_3CA_3G + dCT_6G$ double strands	$\epsilon_{260} = (1.40 \times 10^5) + 240T$
	$\epsilon_{283} = (6.37 \times 10^4) - 5T$
ethidium bromide bound to $dCA_3CA_3G + dCT_6G$	$\epsilon_{260} = (1.32 \times 10^4) - 15T$
	$\epsilon_{283} = (2.1 \times 10^4) + 75T$

[a] In 0.2 M NaCl, 0.01 M phosphate buffer, pH 7.0, and 0.1 mM EDTA. Temperature are in degrees Celsius. [b] Data taken from Nelson & Tinoco (1984).

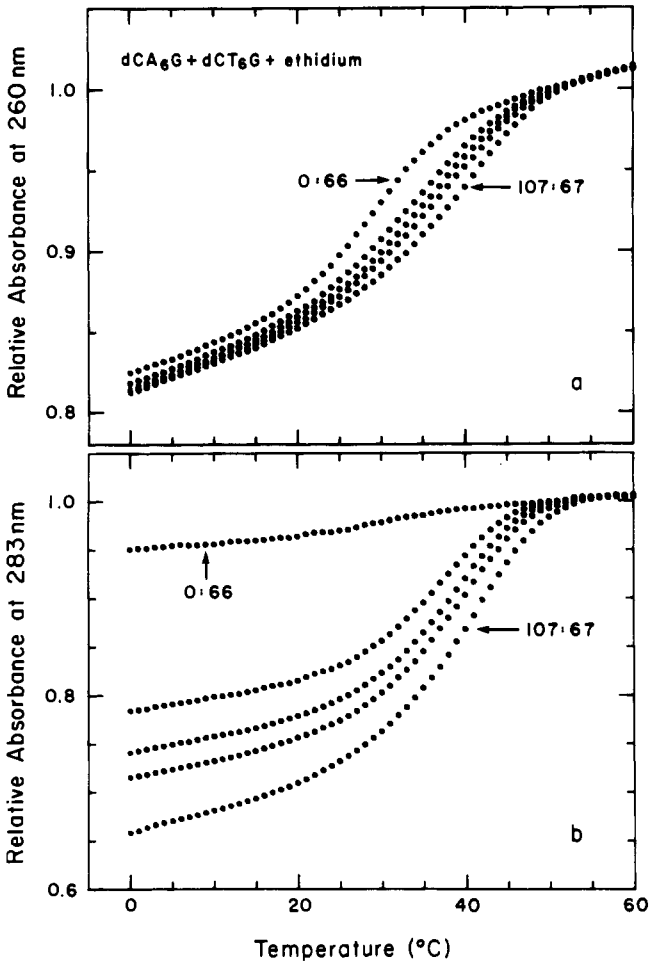


FIGURE 1: (a) Melting curves at 260 nm of $dCA_6G + dCT_6G$ + ethidium, for roughly equal concentrations of strands and increasing ratios of ethidium:strands. The data are all normalized to 1.0 at 50 °C. The concentrations of ethidium:strands were (μM) 0:66, 36:64, 52:68, 64:68, and 107:67. (b) Melting curves at 283 nm.

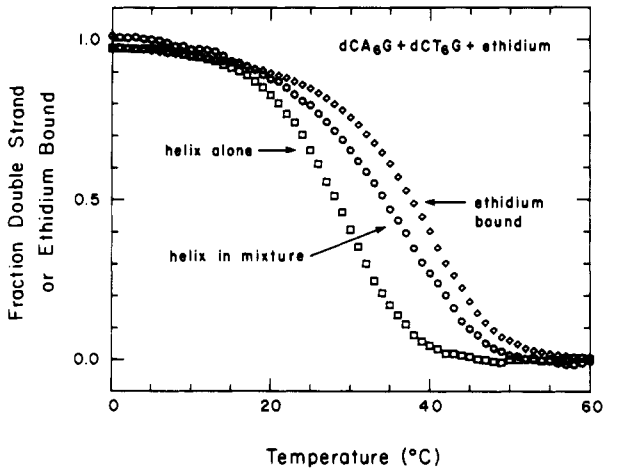


FIGURE 2: Fraction of double strands formed by $dCA_6G + dCT_6G$ + ethidium alone (□), fraction of double strands in a mixture of strands and ethidium (○), and fraction of ethidium bound in the mixture (◇). The concentration of the strands alone was 66 μM. The concentration of the strands in the mixture was 68 μM; ethidium concentration in the mixture was 64 μM.

ethidium concentration increases.

In order to convert the melting curves to fractions of double-helix formation and ethidium binding, the knowledge of eight extinction coefficients, at 260 and 283 nm for the single strands, double helices, free ethidium, and intercalated ethidium, is required. The extinction coefficients depend on temperature. The procedure for determining these is described in Nelson & Tinoco (1984), and they are included in Table I. The extinction coefficients for bound ethidium in the case of $dCA_6G + dCT_6G$ are nearly the same as those determined for $dCA_5G + dCT_5G$. In the case of $dCA_5G + dCT_5G$, the single strands and the double strands absorbed nearly the same at 283 nm. However, with $dCA_6G + dCT_6G$, there is a small decrease in absorbance upon double-helix formation. This causes a slight complication in determining the fraction of ethidium bound, since ethidium binding is not the only contribution to the curve at 283 nm. However, the problem is minimal because of the relatively small change in oligonucleotide absorbance compared to that of the ethidium.

A plot of the fraction of double-strand formation, in the absence and the presence of approximately a ratio of ethidium:strand of 1, is shown in Figure 2, along with the fraction ethidium bound. The ethidium clearly shifts the melting curve of the strands to higher temperature, whereas the curve of the fraction of ethidium bound is shifted to an even higher temperature and is sharper than the double-helix curve.

The thermodynamics of double-helix formation for the $dCA_6G + dCT_6G$ in 1 M NaCl were determined by Morden et al. (1983). Since the stability of the double helices is lower in the 0.2 M NaCl buffer used in these studies, the entropy had to be adjusted. The enthalpy does not change significantly with ionic strength (Nelson et al., 1981). The values used were $\Delta H°_h = -59$ kcal mol^{-1} and $\Delta S°_h = -175$ eu.

The ethidium binding data were fit to the statistical model, assuming all of the binding sites were equivalent. The resulting thermodynamic values were $\Delta H°_d = -8$ kcal mol^{-1}, $\Delta S_d = -6$ eu, and $K_d = 2.2 \times 10^4$ M^{-1} at 25 °C. These agree well with the values determined for ethidium binding to $dCA_5G + dCT_5G$, −9 kcal mol^{-1}, −10 eu, and 2.5×10^4 M^{-1}, respectively (Nelson & Tinoco, 1984).

Table II: Thermodynamics of Ethidium Binding to Normal and Bulged Oligonucleotides[a]

oligomers	binding sites[b]	$\Delta H°$ (kcal mol^{-1})	$\Delta S°$ (eu)	K at 25 °C ($\times 10^{-4}$ M^{-1})
$dCA_5G + dCT_5G$[c]	all sites	−9 ● 3	−10 ± 7	2.5 ● 0.4
$dCA_6G + dCT_6G$	all sites	−8 ± 3	−6 ± 7	2.2 ± 0.4
$dCA_3CA_3G +$ dCT_6G	normal sites	−11 ± 5	−16 ± 10	1.9 ± 0.5
	strong sites	−4 ± 3	12 ± 8	19 ± 4

[a] In 0.2 M NaCl. [b] For the normal helices a model with all sites equal is used; a model with two binding sites on either side of the bulge that are 10 times stronger is used for the bulged helix. [c] Data taken from Nelson & Tinoco (1984).

The errors in the thermodynamic parameters for ethidium binding arise from at least four sources: errors in the thermodynamics of double-helix formation, errors in extinction coefficients and, hence, fraction ethidium bound, random errors in the data, and errors in the model used for fitting the data. The contributions of the first two are directly estimated by reanalyzing the data with different values for the thermodynamics or the extinction coefficients. Using $\Delta H°_h = -56$ kcal mol^{-1} instead of −59 kcal mol^{-1} and adjusting the entropy accordingly changed the enthalpy, entropy, and equilibrium constant for intercalation by −1.0 kcal mol^{-1}, −3.3 eu, and 0.08 $\times 10^4$ M^{-1}, respectively. Decreasing the extinction coefficient for the bound ethidium at 283 nm by 5% changed the values by 1.2 kcal mol^{-1}, 3.9 eu, and 0.31 $\times 10^4$ M^{-1}, respectively.

The contribution of the random errors and the error in the model are manifested in the reduced χ^2 or the correlation coefficient of the fit. The validity of the model can only be evaluated by comparing fits using different models, using the reduced χ^2 and correlation coefficient as criteria. Models were tested that assumed cooperativity between adjacent intercalation sites, two terminal binding sites with different binding constants, and a stronger pyrimidine–purine binding site. None of the alternative models fit the data as well. The reduced χ^2 for a fit should be equal to approximately the square of the estimated experimental error. We estimate the error in the measured fraction ethidium bound to be approximately 0.01, and thus, we expect a reduced χ^2 of about 1×10^{-4}, compared to the observed 1.9×10^{-4}.

From the above considerations, we estimated the errors in the thermodynamic parameters to be ±3 kcal mol^{-1} for the enthalpy, ±7 eu, for the entropy, and ±0.4 $\times 10^4$ M^{-1} for the equilibrium constant. The results are shown in Table II, along with the bulge results discussed later. The results found for ethidium binding to $dCA_5G + dCT_5G$ are also shown in Table II for comparison.

Binding to $dCA_3CA_3G + dCT_6G$. The melting curves for $dCA_3CA_3G + dCT_6G$ at ratios of ethidium:strand between 0 and 2.1 are shown in Figure 3, all at approximately 60 μM strand concentration. The extinction coefficients were determined as before and are shown in Table I. The extinction coefficients for bound ethidium at both 260 and 283 nm were approximately 10% lower in the bulged helix compared to the normal helix. This is presumably due to differences in the structure of the intercalation complex or the distribution of the binding.

The fraction double helix and fraction ethidium bound in the absence and presence of ethidium are shown in Figure 4. By comparing Figures 2 and 4, it is clear that the stabilization of the double strands is greater in the case of the bulged double helix.

The thermodynamics of double-strand formation for the bulged oligonucleotide were obtained from Morden et al. (1984) in a similar manner as described above. The values

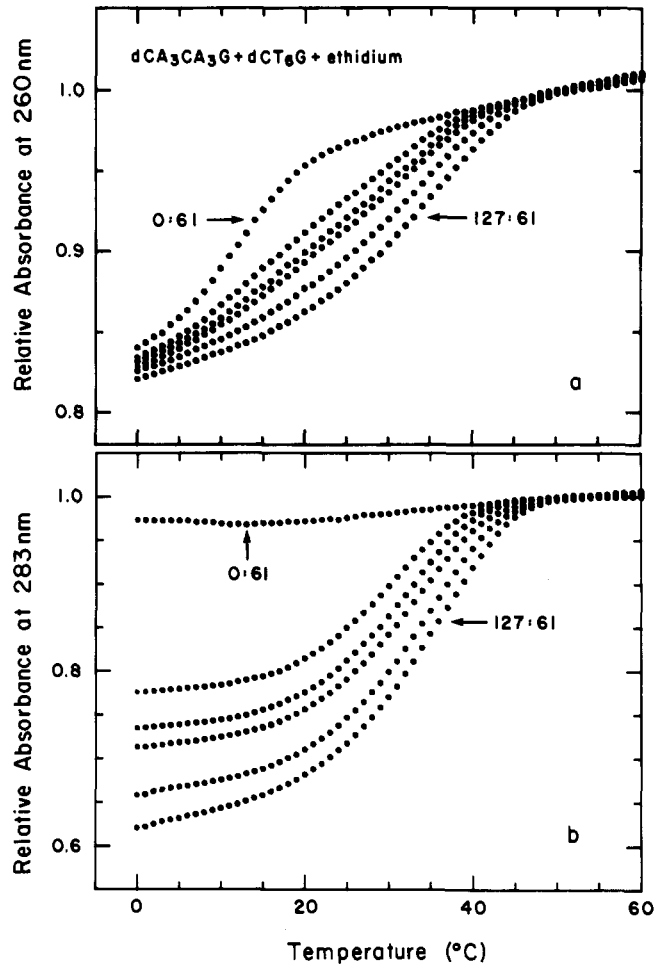


FIGURE 3: (a) Melting curves at 260 nm of $dCA_3CA_3G + dCT_6G$ + ethidium, for roughly equal concentrations of strands and increasing ratios of ethidium:strands. The data are all normalized to 1.0 at 50 °C. The concentrations of ethidium:strands were (μM) 0:61, 36:59, 53:60, 61:63, 94:61, and 127:61. (b) Melting curves at 283 nm.

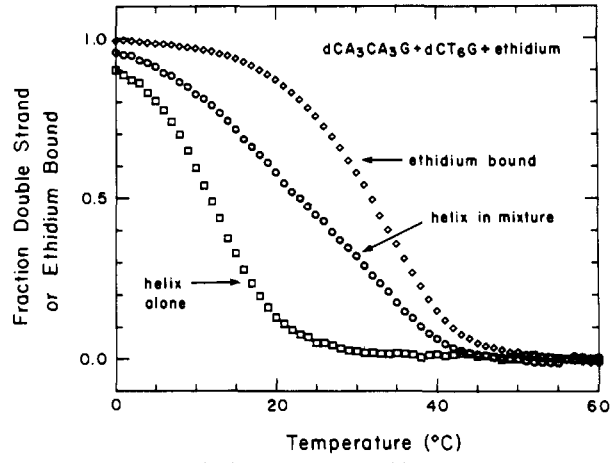


FIGURE 4: Fraction of double strands formed by $dCA_3CA_3G + dCT_6G$ + ethidium alone (□), fraction of double strands in a mixture of strands and ethidium (○), and fraction of ethidium bound in the mixture (◇). The concentration of the strands alone was 61 μM. The concentration of the strands in the mixture was 63 μM; ethidium concentration in the mixture was 61 μM.

used were $\Delta H°_h = -53$ kcal mol^{-1} and $\Delta S°_h = -165$ eu. The data can be fit by assuming that all of the binding sites are equivalent, as was done above, with a resulting equilibrium constant for ethidium binding at 25 °C of 5.7×10^4 M^{-1}, which is 2.6 times larger than that for the normal helix. This underestimates the increased binding to bulges and corresponds

to the result expected if binding studies were carried out on the appropriate polymers containing a comparable population of bulges.

Refinement of the binding mechanism was attempted with several different models. Three models were tested that assumed (1) that the intercalation site at the bulge was ν times stronger than the others, (2) that either one or two ethidium ions could bind to the bulge in solution (external to the helix) with a binding constant of σ times that to the normal site with no effect on the intercalation into the helix, or (3) that the intercalation sites on either side of the bulge are γ times stronger than the others.

The only model that gave a better fit than all sites equal was the one assuming that there are two binding sites γ times stronger on either side of the bulge. In fact, it was the only model in which the reduced χ^2 reached a minimum as γ was increased. Assuming that γ is independent of temperature, and hence all entropic, the best fit occurred with $\gamma = 10$ and $\Delta S°_\gamma = 4.6$ eu, with the resulting parameters $\Delta H°_d = -6$ kcal mol^{-1}, $\Delta S°_d = -2$ eu, and $K_d(25\ °C) = 1.9 \times 10^4$ M^{-1}. Here the subscript d refers to the parameters for the normal binding sites and the subscript γ refers to the incremental values for the special sites. The reduced χ^2 was 0.5×10^{-4}. The fit was very sensitive to the value of γ. For example, changing γ by a factor of 2 increased the χ^2 by 60%.

The fit could be made slightly better, if it is assumed that γ is composed of both entropic and enthalpic contributions. The best fit in this case was $\Delta H°_\gamma = 7$ kcal mol^{-1} and $\Delta S°_\gamma = 28$ eu, with $\gamma = 10$ at 25 °C. The values obtained for the normal sites was $\Delta H°_d = -11$ kcal mol^{-1}, $\Delta S°_d = -16$ eu, and K_d at 25 °C $= 1.9 \times 10^4$ M^{-1}, with a reduced χ^2 of 0.4×10^{-4}. The fit was much less sensitive to the enthalpy of γ than it was to the magnitude of γ itself. The binding constant obtained for the normal sites in the bulged helix are within 15% of the values obtained above for the normal helix, which is very reassuring. The fact that the reduced χ^2 for the bulged helix is smaller than that for the normal helix is interesting and is probably due to the stronger binding to the bulged oligonucleotides. The values for the enthalpy, entropy, and equilibrium constant at 25 °C for binding to the strong sites can be obtained by combining the values for K_d and γ and are −4 kcal mol^{-1}, 12 eu, and 19×10^4 M^{-1}, respectively.

When the model that the binding site located at the bulge is ν times stronger than the others was tested, the reduced χ^2 was found to increase continuously as ν increased. The only way to determine a value for ν was to require that the binding constant and enthalpy for the normal sites were the same as found for the normal helix. When this was done, the value for ν was 40, with $\Delta H°_\nu = 4$ kcal mol^{-1}. The reduced χ^2 was 3.7×10^{-4}, or 9 times greater than that with the previous model.

The model with the ethidium bound to the bulged base exposed to solution behaved similarly. The reduced χ^2 did not reach a minimum, so the constraint that the normal binding sites have the same values as in the normal helix had to be imposed. The best value was with $\sigma = 10$, with $\Delta H°_\sigma = 3$ kcal mol^{-1}. The reduced χ^2 was 2.2×10^{-4}, about 5 times greater than that with the first model. One possible mechanism by which ethidium can stabilize a bulge is the binding of ethidium directly to the base bulged out into the solution. Although our data cannot directly rule out such a mechanism, the poor fit of such a model suggests that it is not significant for ethidium binding to these oligonucleotides.

The errors in the binding constants for the model assuming two strong binding sites on either side of the bulge was estimated in the same way as described above. The errors for binding to the strong sites (γK_d) were smaller than those for the normal sites (K_d), since the product γK_d and the sums $\Delta H°_\gamma + \Delta H°_d$ and $\Delta S°_\gamma + \Delta S°_d$ varied less than the values for the normal sites. The thermodynamic parameters and errors are shown in Table II.

Discussion

The results indicate very clearly that the ethidium binds much more strongly to the bulged oligonucleotide double helix than to the normal double helix. We were expecting to see stronger binding at the bulge site, with little effect at the adjacent sites. Instead, what we observed was most consistent with strong binding at two sites, presumably the two intercalation sites on either side of the bulge. These sites have equilibrium constants for binding ethidium that are 10 times stronger at 25 °C than the other sites.

Although the binding at two sites appears to be stronger than the others, we cannot unambiguously determine the positions of these stronger sites. However, they cannot be within the nearest-neighbor exclusion limit of each other; namely, binding at one cannot prohibit binding to the other. Thus, we proposed that the two strong sites are at the binding sites on either side of the bulge. Although it is possible that the bulge site and the second intercalation site are the strong sites, the fit to the data was significantly worse.

Evidence supporting our proposal was obtained recently by Kean et al. (1985). They studied intercalation into 16S rRNA using (methidiumpropyl-EDTA)–Fe(II), which induces strand cleavage at intercalation sites (Hertzberg & Dervan, 1982, 1984). They found enhanced intercalation on one side of A-746, which is a single-base bulge. This site corresponds to one of our strong sites. It is interesting that they observe stronger binding on only one side of the bulge. The reason for this is not known.

The bulged cytosine was shown by NMR to be outside of the helix (Morden et al., 1983). We were expecting the bulge site to be a strong site since putting an ethidium there would presumably stretch the helix by a distance equal to one base pair, and thus the backbone at the bulge would be extended as in a normal helix. Instead, it seems that the strain is relieved by strong intercalation into the two adjacent sites; in fact, the model fits equally well if it is assumed that there is no binding at the bulge site. It is possible that the extension at the bulge constrains the bulged base to move inside the helix, and in particular, it would be interesting to know whether the bulged base moves inside the helix upon dye intercalation.

It is interesting to consider how the bulge destabilizes the double helix and how the intercalation partially counteracts the destabilization. Morden et al. (1983) found that the bulge destabilization is associated with a 6 kcal mol^{-1} reduction in the enthalpic stabilization of the double helix. Whereas the enthalpy associated with intercalation of ethidium into the double helix is on the order of −8 kcal mol^{-1}, the enthalpy for binding to the strong sites near the bulge is about half as much. Thus, the stabilization of the bulge is associated with a much more favorable entropy of ethidium binding. This is surprising, since any reduction of strain in the backbone would be expected to lead to a more favorable enthalpy of ethidium binding. Perhaps the backbone around the bulge (and at the bulge itself) is very constrained, and intercalation nearby results in a more flexible backbone. Other effects might also be important. For example, the electrostatic effects of the extra phosphate near the bulge site might cause increased

binding near the bulge. Additional counterion release upon intercalation near the bulge might also contribute to the entropy of intercalation.

We can look at the extent of stabilization of the bulged double helix by comparing the products of the equilibrium constant for double-helix formation and the ethidium binding constant for the normal helix with the corresponding equilibrium constant for formation of the bulged double helix and the binding constant to the strong site. For the normal helix at 25 °C, we calculate $K_hK_d = (1.0 \times 10^5)(2.2 \times 10^4) = 2.2 \times 10^9$, whereas for the bulged helix we obtain $(6.2 \times 10^2)(10)(2.2 \times 10^4) = 1.4 \times 10^8$. The difference in free energy between the bulged and normal helices is $-RT \ln [(6.2 \times 10^2)/(1.0 \times 10^5)] = 3.0$ kcal mol^{-1} at 25 °C, whereas for the double helices with one ethidium ion bound the difference is $-RT \ln [(1.4 \times 10^8)/(2.2 \times 10^9)] = 1.6$ kcal mol^{-1}. Thus, the binding of an ethidium ion to one strong site near a bulge cuts the destabilization of the bulge in half. Binding an ethidium to both strong sites makes the difference almost zero. From the thermodynamic results, it is clear that the intercalation of ethidium significantly stabilizes a bulge.

It would be very instructive to study the binding of ethidium into oligonucleotides containing other types of bulged or mismatched bases. For example, in the double helix formed by the self-complementary deoxyoligonucleotide dCGCAGAATTCGCG, the extra adenine base is stacked into the helix (Patel et al., 1982). The extent of ethidium binding to such an extra base could be very different than to the bulged base studied here. Also, in the case of mismatched bases, where there is a base on both strands that presumably forms a nonstandard base pair, it is known that ethidium binding is enhanced (Helfgott & Kallenbach, 1979). One could speculate that the effect should be similar to that observed in the case of the bulged cytosine.

ACKNOWLEDGMENTS

We thank Dr. Neville R. Kallenbach for useful discussions.

Registry No. $dCA_5G + dCT_5G$, 75579-56-5; $dCA_6G + dCT_6G$, 87261-35-6; $dCA_3CA_3G + dCT_6G$, 87261-33-4; ethidium, 3546-21-2.

REFERENCES

Fasman, G. D. (1975) *Biochem., Mol. Biol., 3rd Ed. 1*, 589.
Helfgott, D. C., & Kallenbach, N. R. (1979) *Nucleic Acids Res. 7*, 1011–1017.
Hertzberg, R. P., & Dervan, P. B. (1982) *J. Am. Chem. Soc. 104*, 313–315.
Hertzberg, R. P., & Dervan, P. B. (1984) *Biochemistry 23*, 3934–3945.
Kean, J. M., White, S. A., & Draper, D. E. (1985) *Biochemistry 24*, 5062–5070.
Lee, C.-H., & Tinoco, I., Jr. (1978) *Nature (London) 274*, 609–610.
Morden, K. M., Chu, Y. G., Martin, F. H., & Tinoco, I., Jr. (1983) *Biochemistry 22*, 5557–5563.
Nelson, J. W., & Tinoco, I., Jr. (1984) *Biopolymers 23*, 213–233.
Nelson, J. W., Martin, F. H., & Tinoco, I., Jr. (1981) *Biopolymers 20*, 2509–2531.
Patel, D. J., Kozlowski, S. A., Marky, L. A., Rice, J. A., Broka, C., Itakura, K., & Breslauer, K. J. (1982) *Biochemistry 21*, 445–451.
Streisinger, G., Okada, Y., Emrich, J., Newton, J., Tsugita, A., Terzaghi, E., & Inouye, M. (1966) *Cold Spring Harbor Symp. Quant. Biol. 31*, 77–84.

Coat Formation in Coated Vesicles

Kondury Prasad, Roland E. Lippoldt, and Harold Edelhoch*

Clinical Endocrinology Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Received February 20, 1985

ABSTRACT: The proteins of M_r 100 000–110 000 present in the protein coat of coated vesicles have been shown to facilitate formation of a homogeneous small-size basket (coat) when added to clathrin [Zaremba, S., & Keen, J. H. (1983) *J. Cell Biol. 97*, 1339]. We have prepared this fraction of coat proteins by two different methods and shown that they are very important for the binding of clathrin to uncoated vesicles to form coated vesicles. By labeling the three components (clathrin, 100 000–110 000 proteins, and uncoated vesicles) with different fluorescent markers and analyzing their distribution on sucrose gradients, we have been able to determine the composition of the products formed. In the presence of the 100 000–110 000 fraction of coat proteins, not only does the size distribution of the clathrin basket become uniform but also the rate of polymerization is strongly increased.

Coated pits and coated vesicles are specific structures in the plasma membrane and cytoplasm, respectively, responsible for numerous transport processes either across or between membranes, i.e., receptor-mediated endocytosis (Brown et al., 1983; Ciechanover et al., 1983; Fine & Ockleford, 1984; Gex-Fabry & DeLisi, 1984), secretion (Rothman & Fine, 1980), membrane retrieval (Heuser & Reese, 1973), and transcytosis (Herzog, 1983). The coat surrounding the pits and vesicles is composed of protein, principally clathrin (Pearse, 1975). Clathrin has the shape of a triskelion and forms the framework of the network of polygons (largely pentagons and hexagons) constituting the coat structure (Ungewickell & Branton, 1981).

We have reported (Nandi et al., 1982b) that the coat proteins, dissociated from CVs[1] at pH 8.5 (0.01 M Tris), can be