Biophysical Chemistry 3 (1975) 234–240

EFFECT OF DNA BASE COMPOSITION ON THE INTERCALATION OF PROFLAVINE. A KINETIC STUDY

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Received 21 November 1974

The effect of DNA base composition on the kinetics of the association between DNA and proflavine has been investigated using the temperature jump relaxation method. It is found that, regardless of the G + C base composition the results fit a two step mechanism, the second of which exhibits characteristics of intercalation of proflavine into DNA. However, the two equilibrium constants corresponding to these steps, K_I and K_{II}, depend on the nature of the DNAs. The constant K_I is found to be an order of magnitude greater for *M. lysodeikticus* DNA (72% G + C) than for calf thymus DNA (48% G + C). Increasing G–C content thus appears to favor the intermediate non-intercalated complex of proflavine with DNA. Methylation of *M. lysodeikticus* DNA with dimethyl sulfate, preferentially yielding N_7 methyl guanine as the modified base, again leads to an apparent two step mechanism, with the value of K_I unchanged with respect to untreated DNA, while the affinity of proflavine for the intercalated complex measured by the value of K_{II} increases for methylated DNA.

1. Introduction

The interaction between DNA and acridine dyes has been extensively studied. Many articles have already been devoted to the study of the interaction between DNA and acridines. Acridines such as proflavine can interact with DNA through two distinct modes of binding: a weak binding and a strong binding. The first structural model for the strong binding mode was proposed by Lerman [1]. In this model proflavine is intercalated between two base pairs, the planar dye being parallel to the base pairs. The base pairs of the binding site remain perpendicular to the helix axis, but they have to move 3.4 Å apart to allow the intercalation of the dye. Different studies have provided several lines of evidence which are consistent with the intercalation process. These have been reviewed by Blake and Peacocke [2] and by Gale et al. [3]. Recently also the X-ray structure of a crystalline complex of ApU with amino acridine has been determined [4], in which the dye molecule is in fact intercalated according to the proposed scheme.

Although many acridines have been shown to be intercalated in DNA, they exhibit substantive differences with respect to their biological properties, for example in their action as mutagens [3]. Therefore, if there is any relation between intercalation complexes and biological effects, some specific interactions must occur at the level of the strong binding modes.

No definitive preferential binding of proflavine with one type of base (or pairs of the DNA) have so far been found [5–7]. However, two physical properties of the complexes are dependent upon base composition of DNA: fluorescence and viscosity; Weill [8] found that from the point of view of fluorescence, two types of binding sites on DNA can be distinguished: one a fluorescent binding site and the other a binding site in which the fluorescence of the dye is quenched. These are, according to Thomes et al. [9], located respectively in (AT) rich and (GC) rich regions of DNA. Moreover, when the guanine residues are methylated (on the N_7), the bound dyes near the methylated guanine are found to remain fluorescent [10].

Viscosity measurements on the DNA–proflavine complexes have shown that the length increase of the DNA due to the binding of proflavine is also a function of the base composition [11]. The increment in length of DNA–proflavine complexes decreases as the percentage of (G–C) base pairs increases. N_7 methylated DNA behaves as a (AT) rich DNA [10] in this respect.

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In order to investigate the specificity of interactions between proflavine and DNA, we have carried out a kinetic study, by *T*-jump experiment, of the binding of proflavine to a (GC) rich DNA and to the same DNA chemically methylated, and we have compared our results to those obtained on (AT) rich DNA.

2. Materials and methods

The temperature-jump apparatus was purchased from Messanlagen Studiengeselschaft mbH, Göttingen, Germany and is similar to that described by Eigen and de Maeyer [12]. The relaxation process was followed by recording at a fixed wavelength (430 nm) the time dependent variation of the optical transmission of the solution after a fast rise of temperature. The signal was recorded on a Tektronix oscilloscope and in order to improve the signal-to-noise ratio a multichannel signal averager was employed (Memoscope RE 10). Temperature jumps of about 5°C were produced by a 20 KV capacitor discharge giving a heating time of about 5 μs.

Absorption spectra were determined on a Cary 15 recording spectrophotometer.

All solutions were prepared in a standard buffer consisting of 0.1 M sodium chloride, 0.01 M sodium acetate, 10^{-4} M EDTA, pH 5.

Kinetic experiments were performed at a constant ratio of $P/D = 20$ (D being the concentration of dye, and P the concentration of phosphate); the amount of proflavine bound per base pair, r, was found equal to 0.08.

In this work we have used *M. lysodeikticus* DNA (72% G + C). The purification and methylation of DNA samples have been described elsewhere [13]. For the sake of convenience we shall abbreviate *Micrococcus lysodeikticus* DNA as M.L. DNA and methylated *Micrococcus lysodeikticus* DNA as M.L. Meth DNA.

Proflavine was a gift of Dr. M. Charlier and was purified according to the method of Weill and Calvin [14].

To avoid any viscosity effect on the kinetic parameters, DNA was degraded with a MSE ultrasonicator as previously described [11]. The sedimentation constants S_{20} of the sonicated DNA samples were between 7 and 8.2 in standard sodium citrate buffer.

The amount of bound proflavine per base pair (r) was determined from the visible absorption spectra of the DNA–proflavine solutions [2]. We used the procedure of Li and Crothers [15] for the determination of the molar extinction coefficient (visible absorption band) ϵ_B of bound proflavine. We found $\epsilon_B = 15440\ \text{cm}^{-1}\ \text{M}^{-1}$ at $\lambda = 430$ nm, irrespective of DNA base composition.

3. Results

The relaxation signals of the M.L. DNA–proflavine and M.L. Meth DNA–proflavine complexes display three distinct rates (fig. 1): (1) A fast stage which is characterized by a retrogression point, that is, the trace goes rapidly below the base line and then increases. This increase may be described by a single relaxation time (τ_r) of about 20 microseconds. The value of this relaxation time is independent of the concentrations as can be seen in fig. 2. (2) An intermediate stage which can be analyzed with a single relaxation (τ_1) in the range of a few tenths of a millisecond. Fig. 3 shows the observed dependence of this relaxation time on concentration, with data for M.L. DNA and M.L. Meth DNA at different temperatures. Since $1/\tau_1$ has a linear dependence upon the concentration of free base pairs and uncomplexed proflavine, this parameter can be assigned to a bimolecular step. It is worthwhile noting that methylation has no effect upon the variation of $1/\tau_1$ with concentration. This result is important and we shall discuss it later. (3) A final slow stage appears also to be monoexponential (τ_2). The small amplitude of this relaxation time (fig. 4), both in the case of M.L. DNA and M.L. Meth DNA, did not allow us to establish the variation of τ_2 with concentration. Nevertheless in

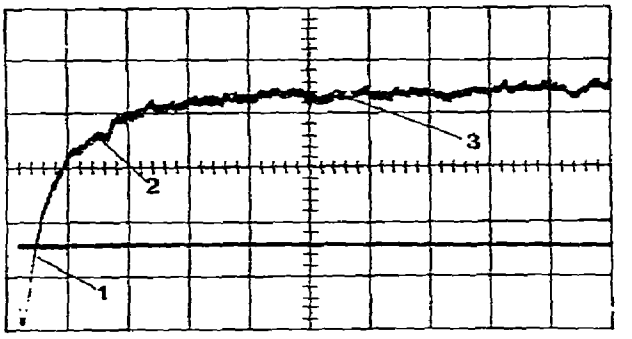

Fig. 1. Typical relaxation signal. Proflavine–M.L. DNA complex. $\bar{C}_D + \bar{C}_F = 0.62 \times 10^{-3}$ M. Time scale: 200 μs/cm. Intensity scale: 10 mV/cm. Temperature (to): 12°C.

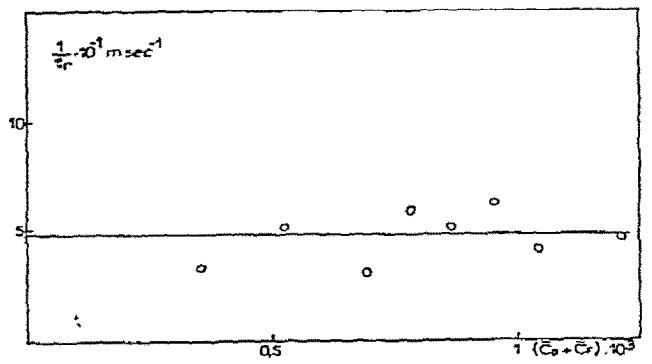

Fig. 2. Variation of the reciprocal of the relaxation time τ_r with $(\bar{C}_D + \bar{C}_F)$. Proflavine–M.L. Meth DNA (with 60% of guanine residues methylated) complex. Temperature (to): 12°C.

the most favorable case with respect to the signal-to-noise ratio we have been able to estimate the value of τ_2 which is several milliseconds for both M.L. DNA and M.L. Meth DNA.

Li and Crothers [15] have assigned the faster relaxation time (τ_r) to a dependence of the extinction coefficient of proflavine upon temperature. Recently Dourlent et al. [16] have shown that τ_r may arise in fact from an orientation effect of the DNA molecules in the electric field generated in the Tjump during the discharge of the capacitor. We have found by electric birefringence measurements that the orientation relaxation time of complexed sonicated DNA molecules is in the range of the value of τ_r [17], in agreement with the interpretation of Dourlent et al. Thus we conclude that τ_r is independent of the two other relaxation times.

The results concerning the two remaining relaxation processes may be interpreted in terms of a two step mechanism similar to that proposed by Li and Crothers [15] for the intercalation of proflavine in calf thymus DNA.

$$\text{M.L. DNA} \\ \text{M.L. Meth DNA} + \text{proflavine} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C_I \rightleftharpoons C_{II}, \qquad (1)$$

where the first step equilibrates more rapidly than the second step. C_I and C_{II} denote two different nucleic acid–dye complexes, C_{II} being the intercalation complex. According to this mechanism there are two relaxation times and the following relation applies for the fast relaxation time.

$$1/\tau_1 = k_{12}(\bar{C}_D + \bar{C}_F) + k_{21}, \qquad (2)$$

where $\bar{C}_D$ and $\bar{C}_F$ are the final equilibrium concentrations of free base pairs and uncomplexed dye respectively. Although it was not possible to find the dependence of the slow relaxation time (τ_2) upon concen-

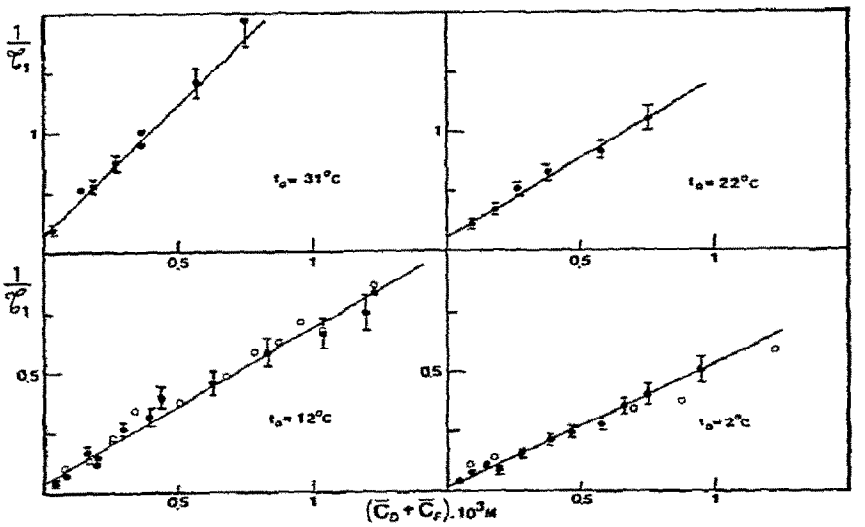

Fig. 3. Variation of the reciprocal of the relaxation time ($\times 10^{-1}$ msec^{-1}) with $(\bar{C}_D+\bar{C}_F)$ at different temperatures. (●) Proflavine-M.L. DNA complex. (○) Proflavine-M.L. Meth DNA complex. The data were fitted to eq. (12) by a simple least-squares procedure.

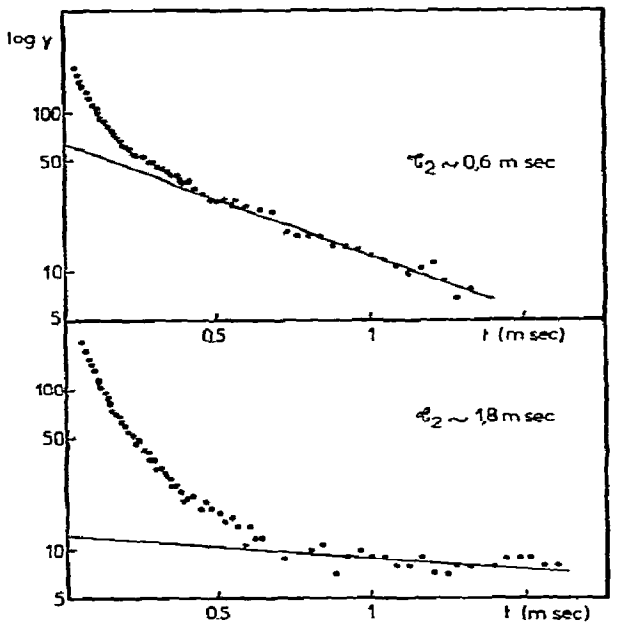

Fig. 4. Typical semi-logarithmic analysis of a relaxation signal. Upper part: proflavine–calf thymus DNA complex. Lower part: proflavine–M.L. DNA complex. $(\bar{C}_D + \bar{C}_F) = 1.23 \times 10^{-3}$ M. Temperature (to): 12°C.

tration it seems reasonable to assign τ_2 to the intercalation process (the value of τ_2 is in the same range as the slow relaxation time obtained by Li and Crothers in the case of Calf thymus DNA).

The kinetic constants k_{12}, k_{21} and the corresponding equilibrium constant $K_I = k_{12}/k_{21}$ are summarized in table 1. These values are derived from the slopes and the intercept of the lines in fig. 5 using the relation (2). The thermodynamic and activation parameters are given in table 2; these are determined from the typical Arrhenius plots shown in fig. 5.

Although the accuracy is poor, one can nevertheless see that H^*_{12} for M.L. DNA is twice the value of this parameter for calf thymus DNA, H^*_{21} is the same for both DNAs.

4. Discussion

First, it must be emphasized that all our experiments were performed at large P/D values, which means that we are dealing primarily with the strong binding. The results in table 1 show that the equilibrium constants K_{II} which describe the distribution of bound proflavine between the two complexes C_I and C_{II} are about 2 and

Table 1
Equilibrium and kinetic constants of proflavine binding to DNA. Solvent = 0.1 M NaCl, 10^{-2} M Na acetate, pH 5, 10^{-4} M EDTA

	t (°C)	k_{12} (M^{-1} s^{-1})	k_{21} (s^{-1})	K_I (M^{-1})	K_{II}	K_{app} (M^{-1})
M.L. DNA	6	0.99×10^7	0.224×10^3	4.4×10^4	1.6	11.4×10^4
	17	1.26×10^7	0.48×10^3	2.6×10^4	2.38	8.8×10^4
	27	2.6×10^7	1.22×10^3	2.1×10^4	2.2	6.8×10^4
	36	4.5×10^7	1.31×10^3	3.4×10^4	0.59	5.4×10^4
M.L. Meth DNA	6	0.99×10^7	0.224×10^3	4.4×10^4	–	–
	17	1.3×10^7	0.43×10^3	3×10^4	4	14×10^4
Calfthymus DNA	10 [a]	2×10^7	3.5×10^3	5.6×10^3	13.6	8.2×10^4
	17	1.8×10^7	4.4×10^3	4.2×10^3	14	8×10^4
	25 [a]	2.8×10^7	1.3×10^3	2.2×10^3	16	4×10^4

[a] Data from Schmechel and Crothers [25]: solvent 0.2 M NaCl, pH = 6.9.

K_{app} is determined at equilibrium and is defined by the following relation:

$$K_{app} = (C)/(C_F)\,(ADN),$$

where (C) is the concentration of bound proflavine, (C_F) is the concentration of free proflavine and (ADN) is the concentration of free binding sites of the DNA. All these constants were calculated assuming a binding model where each base pair is a potential binding site. The values of K_{II} for M.L. DNA and M.L. Meth DNA have been evaluated using the relation

$$K_{app} = K_I(1 + K_{II}).$$

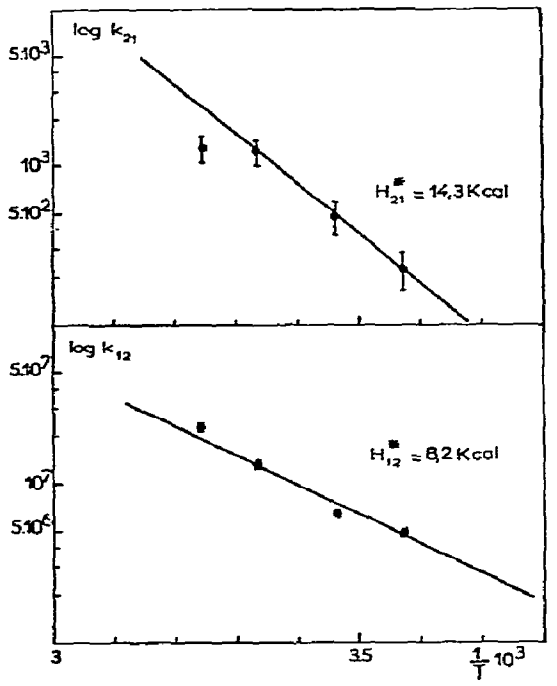

Fig. 5. Plot of the decimal logarithm of the rate constants k_{12} and k_{21} against reciprocal temperature to obtain activation energies. Proflavine–M.L. DNA complex.

14 for M.L. DNA and calf thymus DNA respectively. This means that an increase in the percentage of (GC) base pairs favors the formation of the intermediate complex C_I. The system, M.L. DNA–proflavine, where one has about 50% of the bound proflavine in the state of complex C_I thus becomes very interesting for the study of the characteristics of this complex.

Table 2
Energies of the interaction between proflavine and DNA (kcal/mole)

	H^*_{12}	H^*_{21}	H^0_{12}	H^0_{23}	TS^0_{12}	TS^0_{23}
M.L. DNA	8.2	14.6	−6	1	−0.5	−0.5
calf thymus DNA[a)]	4	14	−9.8	2	−5.4	3.4

H^*_{ij} is the activation energy for transformation from states i to j and H^0_{ij} and S^0_{ij} are the thermodynamic standard enthalpy and entropy changes for the same reaction. These quantities were measured in the temperature range 6 to 36°C, in 0.1 M NaCl, 10^{-3} M Na acetate, pH = 5. All DNA concentrations are expressed in terms of concentrations of base pairs.

a) Data from Schmechel and Crothers [25]: solvent 0.2 M NaCl, pH = 6.9.

The spectroscopic behavior in the visible region of a solution of proflavine in presence of M.L. DNA is identical to the one observed in presence of calf thymus DNA or M.L. Meth DNA [10] (maximum absorption at 460 nm for the bound proflavine, isosbestic point at 455 nm). This implies that the visible absorption spectra of complex C_I is essentially the same as that of the intercalation complex C_{II}.

Moreover, we have previously found [11] that the length increment of DNA on binding of proflavine decreases as the percentage of (GC) base pairs increases. The mechanism [1] can explain this result if one assumes that the complex C_I does not contribute to the increase in length of the DNA molecule. With this assumption, for small values of r, the following relation holds:

$$L/L_0 = 1 + [1/(1 + K_{II})]r, \qquad (3)$$

where L and L_0 are the contour lengths of DNA in the presence and in the absence of dye respectively. Using K_{II}, the value obtained by kinetic measurements, one finds that the coefficient of r in relation (3) is equal to about 0.65. From viscosity measurements on the other hand we have determined that the slope of the line L/L_0 versus r is equal to 0.55. The agreement between the two values is satisfactory, especially if one takes into account the uncertainty in K_{II}. Thus, it seems reasonable to conclude that the complex C_I does not contribute to lengthening of DNA, or if so, only weakly.

In a comparative study by electric dichroism of the systems calf thymus DNA–proflavine and M.L. DNA–proflavine it was shown that the mean orientation of the dye ring in complex C_I appears to be parallel to the planes of the base pairs [17]. While the visible absorption spectrum and structural features (no lengthening of DNA, orientation of the dye ring parallel to the base pairs) of the complex C_I seem to be independent of the nature of DNA, on the other hand the energies involved in the formation of this complex depend on the DNA base composition. The equilibrium constant K_I for proflavine binding to M.L. DNA is roughly an order of magnitude greater than that for proflavine binding to calf thymus DNA. Thus GC base pairs stabilize complex C_I. By contrast, the affinity of proflavine for the

intercalation complex C_{II} is about the same for calf thymus DNA and M.L. DNA (the values of the product $K_I K_{II}$ are equal for these two DNAs).

The intercalation complex has been shown to be strongly anticooperative [18]. In order to obtain information about the cooperativity of the complex C_I we have calculated binding curves based on the mechanism

M.L. DNA + proflavine $\overset{K_I}{\rightleftharpoons} C_I$, $\overset{K_{II}}{\rightleftharpoons} C_{II}$

which is thermodynamically equivalent to mechanism (1), and fit them to the experimental binding curves. To perform our calculation we have represented the DNA molecule as a double-stranded homopolymer with each base pair acting as a potential binding site. For complex C_{II} we have selected the site exclusion model [18] while for the complex C_I we have assumed that two adjacent bound proflavines may interact with an interaction coefficient k which is related to the standard free energy of the interaction process $(\Delta G^0)_{in}$ according to the following relation:

$$k = \exp\left[-(\Delta G^0)_{in}/RT\right] .$$

This coefficient k serves as the variable parameter in our binding model. Fig. 6 shows that the binding curve calculated with $k = 1$ fits the experimental points nicely, suggesting that C_I can be considered an essentially non-cooperative complex. This result is interesting in that complex C_I exhibits an intermediate behavior between the anti-cooperative intercalation complex, C_{II}, and the electrostatic complex seen at low P/D ratios ("weak binding mode") which is very cooperative.

Concerning the methylated DNA, one can see from table 1 that methylation appears to have no effect on the kinetic constants k_{12} and k_{21}. This result is rather surprising since methyl groups might be expected to disturb the hydration shell and thereby the electrostatic potential of DNA (methylation occurs preferentially on the N_7 of guanine located in the large groove and 7 Me G bears a positive charge). The fact that methylation has no influence on the formation of the complex C_I, argues that, proflavine in the first step of the reaction (1) approaches G·C pairs in M.L. DNA via the small groove. Such an interpretation is consistent with the results of Li and Crothers [15], who found differences

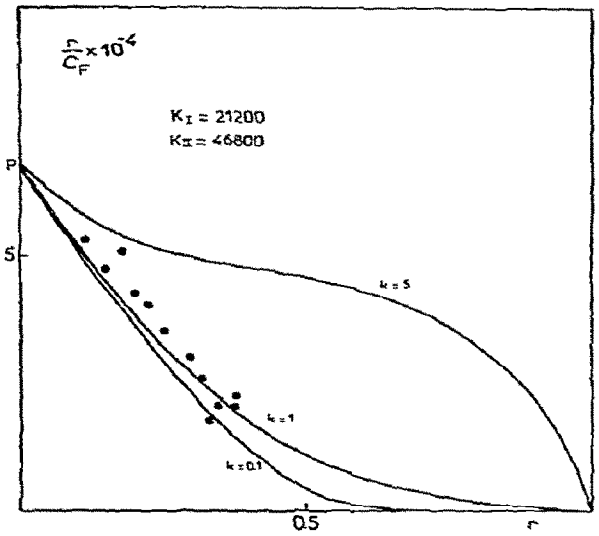

Fig. 6. Calculated isotherms. The lines are theoretical for different k values (see text), $K_{II} = 46800\ M^{-1}$, $K_I = 21200\ M^{-1}$. (•) Experimental points corresponding to the binding of proflavine on M.L. DNA.

in the values of the kinetic and equilibrium constants of the first step between calf thymus DNA and T_2 DNA in which some cytosines located in the small groove are glucosylated.

One may also note from table 1 that in marked contrast to the equilibrium constant K_I, K_{II} depends upon methylation: K_{II} equals 2 and 4 for M.L. DNA and M.L. Meth DNA respectively. Thus methylation of M.L. DNA favors formation of the intercalation complex C_{II}. Proflavine exhibits a greater affinity for the intercalation complex C_{II} in M.L. Meth DNA than M.L. DNA, since the product $K_I K_{II}$ is increased on methylation.

5. Conclusion

This study shows that the two-step mechanism postulated for intercalation of proflavine with DNA holds irrespective of DNA base composition. However, the equilibrium constants K_I and K_{II} are different from one DNA to the other. Let us examine now the possible consequences of this base composition effect.

It is known that the apparent quantum yield of fluo-

rescence of bound proflavine is a function of DNA base composition: it decreases when the (GC) content of DNA increases [9]. Furthermore, we have shown that methylation of DNA leads to an increase of the intensity of fluorescence of bound proflavine [10]. If we compare the changes of fluorescence intensity to the changes of the equilibrium constant K_{II}, it can be seen that the fluorescence intensity increase parallels an increase of the equilibrium constant K_{II}. One might then ask whether or not the fluorescence behavior of bound proflavine results from the existence of the two complexes C_I and C_{II}, the fluorescence of proflavine in complex C_I being quenched owing to specific interactions between proflavine and (GC) base pairs. In addition specific interactions could stabilize complex C_I in the vicinity of (GC) base pairs in accord with the larger value of K_I of M.L. DNA as compared to calf thymus DNA. We have no experimental indication concerning the exact nature of such interactions, but the existence of ligand-base hydrogen bonding is one possibility. Mataga et al. [19,20] have shown that hydrogen bonds affect the fluorescence of electron systems such as acridines and aminoacridines.

Finally, a biological role for the complex C_I cannot be ruled out. Schreiber and Daune [21] have noticed that within the limits of the coding ambiguity mutations induced by proflavine in the gene of bacteriophage T_4 lysozyme [22–24] could be interpreted as having their origin on (GC) base pairs. If this is so, possible sequence related effects of proflavine in mutagenesis may ultimately arise from the affinity of the C_I complex for G·C sequences, leading to errors in replication or transcription of these regions.

Acknowledgement

We wish to thank Dr. N.R. Kallenbach for his help with the calculations of the theoretical binding curves.

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