Titration and Temperature-Dependent Properties of Homodinucleoside Monophosphates. Evaluation of Stacking Equilibrium Quotients for Neutral and Half-Ionized ApA, CpC, GpG, and UpU

Naotake Ogasawara and Yasuo Inoue*

Contribution from the Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo, Japan. *Received February 4, 1976*

Abstract: The temperature dependence of the ultraviolet difference spectra of ApA, CpC, and GpG at neutral pH was analyzed by two-state model. From these thermal denaturation data, apparent thermodynamic parameters, $\Delta H°$, $\Delta S°$, or $\Delta G°$, were determined by a method of iterative least-squares treatment. The thermal denaturation data were used together with the overlapping pK values of ApA, CpC, GpG, and UpU to throw light on the degree of stacking for the half-ionized homodinucleotides. The comparison of the intramolecular stacking association quotients between XpX and the corresponding half-protonated species indicated that XpX can be classified into those with a relation of $s_0 \simeq s_1$ and $s_0 < s_1$ at 25 °C, the former including ApA and CpC, while the latter is GpG. The microscopic stacking equilibrium quotients for the half-protonated GpG were evaluated from the data based on the determination of the microscopic ionization constants of GpG at 25 °C. The results indicated that the enhanced stacking observed for $(GpG)^+$ as compared to GpG at neutral pH was due to the protonation on the guanine base in the 3′-linked nucleoside.

It is well known that poly(A)[1] and poly(C) at neutral pH exist in a single-stranded form with stacked bases arranged in a helical fashion.[2-9] Dinucleoside monophosphates are the shortest chain-length oligomers having the ability of such base–base intramolecular stacking interactions. It is also well recognized that single-stranded oligo- and polynucleotides approach the unstacked conformation at high temperatures and stacked conformation is favored at lower temperatures.[2,3,7-22] The lack of cooperativity gives rise to a broad thermal denaturation curve, associated with the gradual diminish of stacking interactions as the temperature is raised from 0 to 85 °C. Among dinucleotides, ApA has been most extensively studied and there have been at least eight experimental determinations[2,13-15,17-19,23,24] of the enthalpy and entropy for the conformational equilibrium $(ApA)_{unstacked} \rightleftharpoons (ApA)_{stacked}$.

It has been suggested and confirmed by experiments that "intramolecular stacking" tends to suppress the ionization of the base residues.[25-27] Since there is a lack of experimental information about stacking properties of half-ionized molecular species of simple homooligonucleotides, we have undertaken the present investigation concerning the effect of ionization on the intramolecular stacking interactions of four homodinucleotides made up of the four principal nucleosides. Measurements of the stepwise ionization constants of ApA, CpC, GpG, and UpU together with thermal denaturation data have enabled an estimation to be made of the values of pK_0 and stacking equilibrium quotients for their half-ionized species. In a previous[28] and the preceding paper,[27] we have already shown that ApA, CpC, and GpG undergo stepwise protonation and determined the apparent pK values of ApA, CpC, GpG, and UpU for the gain and/or the loss of two protons. We have also estimated, merely based on the titration data of homodinucleotides and the component monomers, the extent to which these dimers and their half-ionized form exist in the stacked conformation.[27]

As early as 1965, Warshaw and Tinoco[29] pointed out that the ORD of ApA is the same as that of an equimolar mixture of adenylic acid and adenosine at pH 1 within the experimental certainty. They have concluded from these observations that ApA is unstacked at pH 1. Now, there is a general agreement that doubly protonated dinucleoside monophosphates are unstacked, although the origin of the low pH induced unstacking is not fully understood.[19] However, little has been known concerning the effect of half-ionization of dinucleotide upon the stacking interactions. Furthermore, for this problem, the experimental results based on the pH titration[26] and the theoretical prediction[30] are conflicting; Simpkins and Richards[26] have concluded that the singly ionized form of ApA is unstacked, while Jordan and Sostman[30] have reported the half-protonated dimers are expected to be even more stable than unprotonated ones in stacking mode. In favor of the latter view, Guschlbauer et al.[31] have recently reported that GpU stacks only at low pH. In order to settle the problem of literature discrepancies with regard to the intramolecular stacking interactions of half-protonated homodinucleotides, this and the preceding paper record the determination of the stacking equilibrium quotient for $(XpX)_u^+ \rightleftharpoons (XpX)_s^+$ by means of the two alternative methods.

No information is available on the stacking equilibrium of GpG at neutral pH, and there is a range of values of $\Delta H°$ and $\Delta S°$ for ApA and CpC in the literature. Hence, we have examined the temperature dependence of the optical properties of ApA, CpC, and GpG in aqueous solution. This information, together with values of the overlapping ionization constants determined in the preceding paper,[27] enables us to estimate the stacking equilibrium quotient, s_1, of the homodinucleotides named in the title and to compare the present results with those in the preceding paper.

In this paper, we also present the results of the determination of microscopic stacking equilibrium quotients for GpG.

Experimental Section

Materials. Materials were as given in a preceding paper.[27]

Methods. (a) Thermal Denaturation. The thermal denaturation of XpX at neutral pH was followed spectrophotometrically. Previous studies[18,32] have shown that increasing temperature does not appreciably alter the conformational features nor the absorption spectra of nucleosides and mononucleotides, so that the spectral changes, which occurred upon thermal denaturation of XpX, were measured by the difference spectra obtained for solutions Y mM per residue in XpX vs. Y mM component monomers (RNase T_2 hydrolysate of XpX), both at pH 7 ($0.13 < Y < 0.23$). The advantage of the use of difference spectrophotometry was the increased differences during the thermal denaturation by the use of a higher concentration of XpX. This was particularly so for CpC which showed only small differences in the extinction coefficient (see Figure 1). Since the analysis of the

amplitude ($A_{273.5} - A_{290}$) gave the best fit (smallest rms), in our investigations we have preferred to use amplitude of difference spectra. This eliminates errors due to base-line shifts between readings. The variations of the spectra with temperature were measured on a Hitachi Model 124 spectrophotometer. In order to prevent evaporation the cells used were stoppered securely and placed in the thermostatically controlled cell holder (Komatsu Electronics Inc., Tokyo). Temperature was measured to ±0.2 °C by a standard thermometer connected to a calibrated thermistor inserted in the sample cell. Solutions were stirred during measurements with a magnetic stirring bar to prevent bubbles (solutions were degassed under vacuum before use for spectral measurements). At low temperatures, dry nitrogen gas was introduced through the cell compartment to prevent condensation. Absorbance readings were corrected for volume expansion.

(b) Data Analysis for Determination of $\Delta H°$ and $\Delta S°$. Data analysis was performed by the iterative least-squares method (see Appendix), and actual computations were carried out at the Computer Centre of the University on a HITAC 8800/8700 computer.

Results and Discussion

Analysis of Thermal Denaturation Data for ApA, CpC, and GpG. It is well known that dinucleotides have a strong tendency to form intramolecularly stacked base helices that exhibit either a hypo- or a hyperchromic effect, depending on wavelength, relative to the unstacked form.[25] There are several prior determinations of the values of $\Delta H°$ and $\Delta S°$ for ApA and CpC, but disagreement is seen among these values of various authors. Besides this, no experimental determinations of $\Delta H°$ and $\Delta S°$ for $(GpG)_u \rightleftarrows (GpG)_s$ have been made until now.[33] We have thus reexamined the temperature dependence of the ultraviolet absorption spectra of ApA and CpC together with GpG at neutral pH. Since the rate at which $(XpX)_u$ and $(XpX)_s$ interconvert is slow compared to the time scale of uv spectroscopy, it is possible to observe superposition spectra for the equilibrium mixture at temperatures between 0 and 85 °C. In line with the previous suggestion by Powell et al., the stacking equilibrium can be operationally regarded as a two-state system, and thermodynamic parameters can be extracted by the iterative least-squares computer method of the thermal denaturation profiles.

(a) Effect of Temperature on the Absorption Spectra of XpX. Absorption spectra at a series of temperatures for ApA, CpC, and GpG give excellent isosbestic points. The thermal denaturation spectra of ApA, CpC, and GpG are illustrated as the difference in molar extinction coefficient per base residue for the dimer at a lower and a higher temperature as denoted over the wavelength range 220–300 nm (Figure 1). Uv difference spectra of ApA at different temperatures displayed isosbestic points at 217.5, 229.7, 274.3 nm. The CpC vs. component monomer thermal difference spectra taken over the range 0–85 °C showed that CpC changed progressively to the unstacked form with a clear isosbestic point at 286.6 nm. GpG has exhibited changes in the GpG vs. component monomer difference spectrum on heating which are similar to those observed with CpC; however, the magnitude of change is somewhat less than CpC. The relatively large experimental error in this case renders a precise analysis of data difficult. There is a hyperchromic effect on band(s) above 292 nm as the temperature is raised. This gives a positive feature above 292 nm in the GpG vs. component monomer thermal difference spectrum (Figure 1). We have found a definite isosbestic point at 291.5 nm in a set of the GpG vs. component monomer thermal difference spectra. All of these results are again considered as a strong indication of the validity of the so-called "two-state model" for the stacking-destacking equilibrium system.[18,19,21]

(b) Determination of the Thermodynamic Parameters, $\Delta H°$ and $\Delta S°$, for Stacking Equilibrium, $(XpX)_u \rightleftarrows (XpX)_s$. Consider intensive parameters ϵ_u and ϵ_s of extreme conformers $(XpX)_u$ and $(XpX)_s$ under mobile conformational equilibrium in aqueous solution at neutral pH. At the ith of n temperatures,

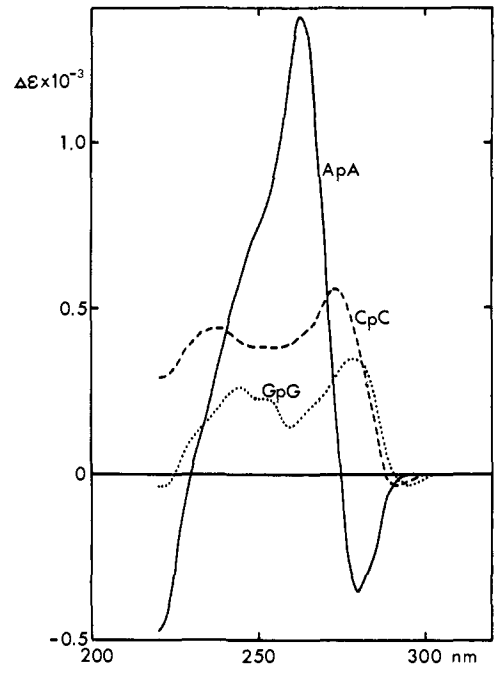


Figure 1. Thermal denaturation spectra of ApA(—), CpC(- - -), and GpG(· · ·) at neutral pH: ApA, $\epsilon_{86.6°} - \epsilon_{24.9°}$; CpC, $\epsilon_{78.0°} - \epsilon_{23.3°}$; GpG, $\epsilon_{78.5°} - \epsilon_{23.3°}$. (Correction for a change in concentration of XpX due to the thermal expansion of water was made.)

the observed parameter, ϵ_i, of the stacking equilibrium system

$$(XpX)_u \rightleftharpoons (XpX)_s \tag{1}$$

reflects an average of ϵ_u and ϵ_s weighted according to the relative populations of the two conformers, f_{ui} and f_{si}. The essence of the two-state model is that ϵ_i can be expressed by means of a linear combination of ϵ_u and ϵ_s with temperature coefficient.

$$\epsilon_i = f_{ui}\epsilon_u + f_{si}\epsilon_s = (1 - f_{si})\epsilon_u + f_{si}\epsilon_s \tag{2}$$

The stacking equilibrium quotient at a given temperature, i, may be written as

$$s_{0i} = \frac{[(XpX)_s]}{[(XpX)_u]} = \frac{f_{si}}{1 - f_{si}} = \frac{\epsilon_u - \epsilon_i}{\epsilon_i - \epsilon_s} \tag{3}$$

or

$$\ln\left(\frac{\epsilon_u - \epsilon_i}{\epsilon_i - \epsilon_s}\right) = -\frac{\Delta H°}{RT_i} + \frac{\Delta S°}{R} \tag{4}$$

Besides the assumption of two optical states, the following assumptions have to be made before the analysis of the thermal denaturation data by the van't Hoff procedure. (1) ϵ_u and ϵ_s are temperature independent, and (2) $\Delta H°$ is also temperature independent, which is equivalent to the standard heat capacity change $\Delta C_p°$ being close to zero for the reaction $(XpX)_u \rightleftarrows (XpX)_s$. Then, the temperature dependence of ϵ_i simply reflects the temperature dependence of f_{ui} and f_{si} at constant $\Delta H°$ and $\Delta S°$. Equation 4 can now be solved, in principle, for unknowns ϵ_u, ϵ_s, $\Delta H°$, and $\Delta S°$ by the use of the thermal denaturation data (see Appendix).

Since ϵ_u and ϵ_s occur above 85 and below 0 °C, these limiting values are not determined directly. Consequently, a method of computing $\Delta H°$ and $\Delta S°$, which does not rely on the ϵ_u and ϵ_s, has to be adopted. In this work, the iterative least-squares method was applied to the analysis of the uv absorption data over the temperature range 0–85 °C. Of several factors which will determine the reliability of the thermodynamic parameters,[21] the accuracy of the experimental readings is most im-

Table I. Thermodynamic Parameters and Stacking Equilibrium Quotients (s_0 at 25 °C) of ApA, CpC, GpG, and UpU

Dimer	$\Delta H°$, kcal/mol (σ)[a]	$\Delta S°$, eu (σ)[a]	s_0 at 25 °C (σ)[a]
ApA	−5.5 (0.30)	−18 (0.95)	1.00 (0.04)
CpC	−4.4 (1.05)	−16 (3.16)	0.63 (0.12)
GpG	−4.5 (0.78)	−17 (2.19)	0.45 (0.10)
UpU[b]	−6.1 (0.7)	−22.2 (1.9)	0.44

[a] The standard deviations (σ) were computed from the root mean square (rms) values between experimental and calculated melting profiles [rms are 2.7×10^{-3}, 4.6×10^{-3}, and 5.1×10^{-3} for ApA, CpC, and GpG, respectively, when differences between the values of the optical (absorbance) parameters for the fully stacked and fully unstacked species are normalized to unity (see also Appendix c)]. [b] Taken from ref 21.

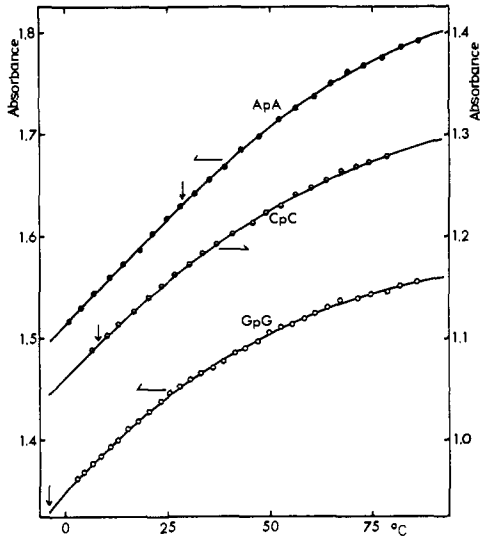


Figure 2. Thermal denaturation of ApA (●; $A_{262.5}$ vs. temperature at 1.27×10^{-4} M), CpC (◒; $A_{273.5} - A_{290}$ vs. temperature at 1.97×10^{-4} M), and GpG (○; $A_{277.5} - A_{295}$ vs. temperature at 2.28×10^{-4} M) at pH 7 and $I = 0.1$. The curves are calculated by the iterative least-squares method [see text and Appendix (a)]. T_m (T_m is the temperature at which 50% of the total increase in extinction due to thermal denaturation is observed) is indicated by an arrow. The spectral extremes for the fully stacked and fully unstacked states are $(A_{262.5})_{unstacked} = 1.881$ and $(A_{262.5})_{stacked} = 1.383$ for ApA; $(A_{273.5} - A_{290})_{unstacked} = 1.375$ and $(A_{273.5} - A_{290})_{stacked} = 0.817$ for CpC; and $(A_{277.5} - A_{295})_{unstacked} = 1.616$ and $(A_{277.5} - A_{295})_{stacked} = 1.046$ for GpG.

portant; when the change in absorbance is small over the temperature range employed, sizable errors would occur. However, in the present investigation, the error was minimized by taking spectra at more than 20 temperatures in the range 0-85 °C. In order to determine the effect of precision in the melting data on the accuracy of the thermodynamic parameters obtained, synthetic ϵ_i values in a temperature range used were processed by the same computer program [see Appendix (b)]. A summary of the average best values of $\Delta H°$, $\Delta S°$, and stacking equilibrium quotient s_0 at 25 °C, for more than two runs, is shown in Table I. The uncertainties are the standard deviations.

Our results for the apparent thermodynamic parameters for ApA seem to be in general agreement with prior determinations ranging from −10 to −4.9 kcal/mol in $\Delta H°$ and from −30 to −17 eu in $\Delta S°$, though the $|\Delta H°|$ and $|\Delta S°|$ values are somewhat lower than the literature values clustering at $|\Delta H°| \simeq 8.5$ kcal/mol and at $|\Delta S°| \simeq 30$ eu. Temperature-absorbance profiles of ApA, CpC, and GpG at the analytical wavelengths are obtained from a series of the XpX vs. component monomer thermal difference spectra and shown in Figure 2, together with curves representing the best fits to the data.

Table II. Stacking Equilibrium Quotient ($s_1 = [(XpX)_s^+]/[(XpX)_u^+]$) and Intrinsic Basic p$K_0$ for ApA, CpC, and GpG at 25 °C[a]

Dimer	s_1	pK_0
ApA	0.93 ± 0.22	$3.61_1 \pm 0.086$
CpC	0.71 ± 0.17	$4.19_8 \pm 0.066$
GpG	0.95 ± 0.13	$2.07_6 \pm 0.042$

[a] The probable errors given for s_1 and pK_0 are based on estimated maximal errors in pK_1, pK_2, and s_0 in $\sigma_{s_1} = 1.15(1 + s_1)[(\sigma_{pK_1})^2 + (\sigma_{pK_2})^2 + (\sigma_{s_0})^2/5.29(1 + s_0)^2]^{1/2}$ and $\sigma_{pK_0} = [(\sigma_{pK_2})^2 + (\sigma_{s_1})^2/5.29(1 + s_1)^2]^{1/2}$.

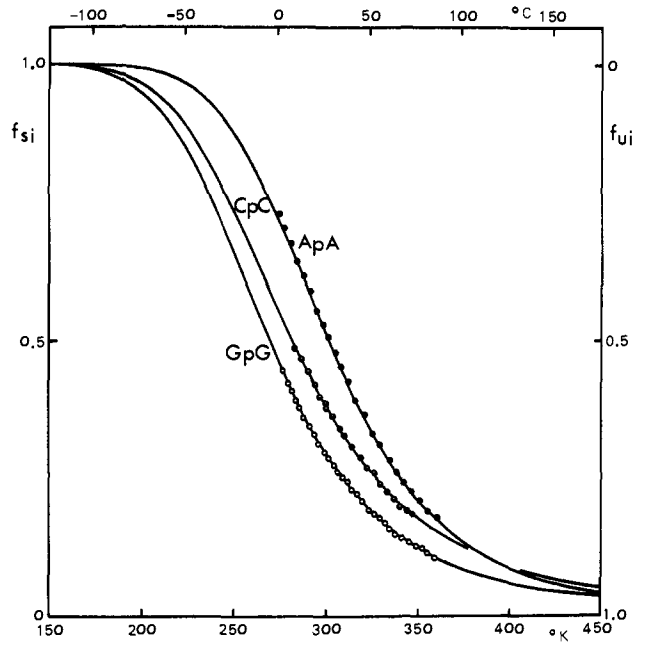


Figure 3. The fraction of stacked bases (f_{si}) as a function of temperature: ApA (●), CpC (◒), and GpG (○). Curves represent the best fits to the data, calculated by the iterative least-squares method.

Evaluation of Values of pK_0 and s_1 at 25 °C from the Data Based on s_0 and Overlapping pK Measurements. At each temperature, the fraction of molecules in the fully stacked state can be estimated using the van't Hoff equations and the $\Delta H°$ and $\Delta S°$ values derived as described above. Figure 3 illustrates the variation of f_{si} of ApA, CpC, and GpG with temperature. Our values of s_0 at 25 °C are listed in Table I, together with relevant data for UpU from the literature.[21] According to Lowe and Schellman[17] and Catlin and Guschlbauer,[21] UpU does stack at 25 °C to some extent. At 25 °C, GpG is also found to exist in a stacked conformation to an appreciable extent. The s_0 values for ApA, CpC, GpG, and UpU are now compared with those estimated from pK measurements.[27] The values of s_0 listed in Table I are somewhat smaller than those obtained by titration experiments though the former has been found to agree, to within an order of magnitude, with the latter.

Based on the ORD and CD studies of ApA, CpC, and GpG, the diprotonated species are considered to be unstacked, i.e., $s_2 \simeq 0$. Thus, we have combined the overlapping basic ionization constant data and the measured values of s_0 and obtained values of s_1 and basic pK_0. The estimated values of s_1 and basic pK_0 at 25 °C are listed in Table II. The basic pK_0 values listed in Table II compare favorably with the corresponding values taken for ApA, CpC, GpG as pK_0 =

Table III. Ionization Constants (as pK) of ApA, CpC, and GpG at 25 °C[a]

Dimer	pK_{u_1}	pK_{u_2}	pK_{s_1}	pK_{su_2}
ApA	$3.91_1 \pm 0.086$	$3.31_1 \pm 0.086$	$3.88_0 \pm 0.13$	$3.34_2 \pm 0.14$
CpC	$4.49_8 \pm 0.066$	$3.89_8 \pm 0.066$	$4.55_1 \pm 0.13$	$4.04_6 \pm 0.13$
GpG	$2.37_6 \pm 0.042$	$1.77_6 \pm 0.042$	$2.70_2 \pm 0.12$	$1.79_7 \pm 0.08$

[a] The probable errors of pK_{u_1}, pK_{u_2}, pK_{s_1}, and pK_{su_2} are estimated from the following relationships: $\sigma_{pK_{u_1}} = \sigma_{pK_{u_2}} = \sigma_{pK_0}$, $\sigma_{pK_{s_1}} = (1/2.3s_0s_1)[5.29s_0^2s_1^2(\sigma_{pK_0})^2 + s_1^2(\sigma_{s_0})^2 + s_0^2(\sigma_{s_1})^2]^{1/2}$, and $\sigma_{pK_{su_2}} = [(\sigma_{pK_0})^2 + (\sigma_{s_1})^2/5.29s_1^2]^{1/2}$.

¼(p$K_{3'\text{-mononucleotide}}$ + p$K_{5'\text{-mononucleotide}}$ + 2p$K_{\text{nucleoside}}$), 3.67, 4.21, and 2.19. We consider these agreements sufficiently good. Though the slightly higher values of pK_0, as compared to the values in Table II, cause s_0 and s_1 to be somewhat greater than those derived from thermal denaturation data, s_1 values which have been estimated on the basis of titration experiments alone, seem to be in reasonable agreement with those given in Table II. It is therefore gratifying to note that conformational free energies, $\Delta G°_{(XpX)_u \rightleftarrows (XpX)_s}$ and $\Delta G°_{(XpX)_u^+ \rightleftarrows (XpX)_s^+}$, evaluated from two approaches are reasonably good.

The s_1 values of ApA and CpC now obtained show that the half-protonated species have stabilities comparable to their un-ionized counterparts. These findings are again in disagreement with the conclusion of $s_1 = 0$ for ApA deduced by Simpkins and Richards.[26] The conclusion reached for ApA and CpC, $s_0 \simeq s_1$, seems to represent a general relationship as judged from other work carried out by our own group on homodimers having methylated bases (Tazawa and Inoue, unpublished results). By contrast, the abnormality ($s_1 > s_0$) found for GpG may be attributed to markedly different overlapping of the two bases in GpG and $(GpG)^+$ (see ref 28 and the next section). The relative stability order, as measured by stacking equilibrium quotients, is found to be $(XpX) \simeq (XpX)^+$ compared with the order $(XpX) < (XpX)^+$ predicted by Jordan and Sostman.[30] Their calculations are purely energetic so that the difference may, in some part, be attributed to a contribution of the enhanced solvation in $(XpX)^+$ to the overall stacking interactions. Similar associations between nitrogenous cations and neutral hydrophobic molecules have been established for both micellar[34,35] and nonmicellar[36,37] systems of quarternary ammonium ions such as cetyltrimethylammonium ion and aromatic compounds and are probably very common.

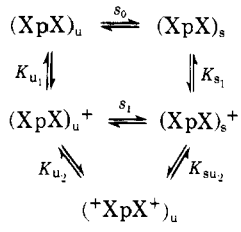
Using the known values of s_0 and the overlapping acidic pK, we have computed $s_{-1} = 0.20 \pm 0.09$ and acidic p$K_0 = 9.37 \pm 0.051$ for GpG. We have used the literature value of s_0 for UpU, and values of s_{-1} and acidic pK_0 have been estimated as $s_{-1} = 0.20 \pm 0.07$ and p$K_0 = 9.23 \pm 0.035$ for UpU. Compared with these pK_0 values we obtained in the preceding paper[27] by the empirical choice of pK_0 = ¼(p$K_{3'\text{-nucleotide}}$ + p$K_{5'\text{-nucleotide}}$ + 2p$K_{\text{nucleoside}}$) values of 9.22 for GpG and 9.19 for UpU.

The principal conclusion from the results is that half-protonation to a homodinucleotide does not reduce the extent of intramolecular stacking interaction,[38] although at present it is not certain what determines the equilibrium conformation of half-protonated stacked dinucleotides. It is hoped to extract thermodynamic parameters for the $(XpX)_u^+ \rightleftarrows (XpX)_s^+$ process by studying the temperature dependence of ionization of homodinucleotides.[39]

Determination of Microscopic Stacking Equilibrium Quotients and Intrinsic Tautomeric Quotients. Site of protonation in XpX is of course expected to influence the stacking of half-protonated dimers. The degree to which half-protonated XpX would exist in $^+$XpX or XpX^+ is, at present, unknown except for $(GpG)^+$. For the specific case of GpG, we have reported[28] the tautomeric quotient K_t (= $[^+GpG]/[GpG^+]$) to be approximately 1.7 at 25 °C. This was obtained by CD and basic pK comparisons with methylated derivatives.[28] The present work, together with the data reported previously for GpG, has enabled an estimation of various microscopic constants involved in stacking and protonation equilibrium schemes.

(a) Ionization Constants, K_{u_1}, K_{u_2}, K_{s_1}, and K_{su_2}. Prototropic and stacking equilibria of XpX can be depicted by Scheme I

Scheme I



provided that doubly protonated dinucleotides are unstacked, i.e., $s_0, s_1 \gg s_2 \simeq 0$. By definition, p$K_{u_1}$ = pK_0 + log 2; pK_{u_2} = pK_0 − log 2; pK_{s_1} = pK_{u_1} − log (s_0/s_1); and pK_{su_2} = pK_{u_2} − log s_1. Since the values of s_0 and s_1 are known for the three homodinucleotides, by applying the above relationships the pK values of completely unstacked and stacked species have been determined and are set out in Table III. The larger percentage of stacked half-protonated GpG is reflected in the higher basic strength (pK_{s_1}) of the stacked GpG relative to pK_{u_1}: if pK_{s_1} > pK_{u_1}, then $s_1 > s_0$; if pK_{s_1} < pK_{u_1}, $s_1 < s_0$.

(b) Microscopic Stacking Equilibrium Quotients, $s_1^{3'}$ and $s_1^{5'}$ of GpG. The empirical stacking equilibrium quotient (s_1) of XpX is composite, and it is related to the true constants of the XpX^+ ($s_1^{3'}$) and the $^+$XpX tautomer ($s_1^{5'}$) by the expressions, $s_1^{3'} = [(XpX^+)_s]/[(XpX^+)_u] = (1 - K_t + 2s_1)/(1 + K_t)$ and $s_1^{5'} = [(^+XpX)_s]/[(^+XpX)_u] = (2s_1K_t + K_t - 1)/(1 + K_t)$. From the K_t value and s_1 value of GpG, the microscopic stacking equilibrium quotients can be calculated to be $s_1^{3'} = 0.44 \pm 0.15$ and $s_1^{5'} = 1.45 \pm 0.21$, the latter being more than three times greater than $s_1^{3'}$, which is almost identical with the s_0 value at 25 °C. This fact favors the hypothesis of anti → syn conversion of the guanosine residue on protonation.[28]

(c) Microscopic Ionization Constants and Tautomeric Quotients of GpG. The pK_{s_1} and pK_{su_2} are now seen to be composite constants, namely, $K_{s_1} = a_H[(XpX)_s]/[[(^+XpX)_s] + [(XpX^+)_s]]$ and $K_{su_2} = a_H[[(^+XpX)_s] + [(XpX^+)_s]]/[(^+XpX^+)_u]$, determined under experimental conditions where it is likely that $(^+XpX)_s$ and $(XpX^+)_s$ are in equilibrium. The true pK of species $(^+XpX)_s$ and $(XpX^+)_s$ can be obtained from p$K_{s_1}^{3'}$ = pK_0 − log $(s_0/s_1^{3'})$; p$K_{s_1}^{5'}$ = pK_0 − log $(s_0/s_1^{5'})$; p$K_{su_2}^{3'}$ = pK_0 − log $s_1^{5'}$; and p$K_{su_2}^{5'}$ = pK_0 − log $s_1^{3'}$. Since we have stipulated that the two sites are equivalent and noninteracting in $(XpX)_u$, it follows that $K_{u_1}^{3'} = K_{u_1}^{5'} = K_{u_2}^{3'} = K_{u_2}^{5'} = K_0$. We have determined the microscopic ionization constants for the stacked conformational isomers of GpG and the results are included in Table IV. These constants involve only one species and its conjugate acid and can therefore be used for purpose of molecular interpretation. In the stacked GpG conformation the difference between p$K_{s_1}^{5'}$ and p$K_{s_1}^{3'}$

Table IV. Summary of Ionization Constants and Stacking and Tautomeric Equilibrium Quotients of GpG at 25 °C

Overall constants	Microscopic constants
Ionization constants (composite: s + u; 3′ + 5′) $pK_1 = 2.50$; $pK_2 = 1.48$	Ionization constants (composite: s + u) $pK_1^{3'} = 2.07$; $pK_1^{5'} = 2.30$; $pK_2^{3'} = 1.68$; $pK_2^{5'} = 1.91$
Ionization constants (composite: 3′ + 5′) $pK_{s_1} = 2.70_2 \pm 0.12$; $pK_{su_2} = 1.79_7 \pm 0.08$; $pK_{u_1} = 2.37_6 \pm 0.042$; $pK_{u_2} = 1.77_6 \pm 0.042$	Ionization constants $pK_{s_1}^{3'} = 2.06 \pm 0.18$; $pK_{s_1}^{5'} = 2.58 \pm 0.12$; $pK_{su_2}^{3'} = 1.91 \pm 0.13$; $pK_{su_2}^{5'} = 2.43 \pm 0.19$; $pK_{u_1}^{3'} = pK_{u_1}^{5'} = pK_{u_2}^{3'} = pK_{u_2}^{5'} = pK_0 = 2.07_6 \pm 0.042$
Stacking equilibrium quotient (composite: 3′ + 5′) $s_1 = 0.95 \pm 0.13$	Stacking equilibrium quotients $s_0 = 0.45 \pm 0.10$; $s_1^{3'} = 0.44 \pm 0.15$; $s_1^{5'} = 1.45 \pm 0.21$
Tautomeric equilibrium quotient (composite: 3′ + 5′) $K_t = 1.7 \pm 0.3$	Tautomeric equilibrium quotients $K_{ts} = 3.3 \pm 1.8$; $K_{tu} = 1.0$

is 0.52 pK units, considered to be due solely to the conformational stabilization of the stacked $^+$GpG species relative to the stacked GpG^+. This could be rationalized on the basis of a preferred geometry of right-handed (syn)$^+$Gp(anti)G which would be especially suited for intramolecular overlapping of the two bases.

Microscopic stacking equilibrium constants also enabled the microscopic tautomeric quotients, K_{tu} and K_{ts}, of completely stacked and unstacked species of half-protonated GpG to be obtained from the following relations: $K_{tu} = [(^+XpX)_u]/[(XpX^+)_u] = (s_1^{3'} - s_1)/(s_1 - s_1^{5'}) = K_{u_1}^{3'}/K_{u_1}^{5'}$ and $K_{ts} = [(^+XpX)_s]/[(XpX^+)_s] = (s_1^{3'} - s_1)s_1^{5'}/(s_1 - s_1^{5'})s_1^{3'} = K_{s_1}^{3'}/K_{s_1}^{5'}$. We have arrived at the conclusion that GpG undergoes preferred 3′-linked nucleoside base protonation. This conclusion was apparently backed by both CD and pK evidence,[28] but exact analysis of the geometry of half-protonated GpG is beyond the present scope of conformational analysis.

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Appendix

(a) Extraction of Thermodynamic Parameters from the Thermal Denaturation Data.[18,40-43] As a result of the assumptions made in text, the conformational equilibrium quotient s_{0i}, which is given by $(\epsilon_u - \epsilon_i)/(\epsilon_i - \epsilon_s)$, is related to $\Delta H°$ and $\Delta S°$ by the familiar expression (eq 4). In solving eq 4 we consider solution values of the unknowns to be those which make the sum of the squared residues (Σ_{res}) of the molar extinctions a minimum

$$\sum_{res} = \sum_{i=1}^{n} (\epsilon_i - \epsilon_i^{calcd})^2 \quad (5)$$

where ϵ_i^{calcd} may be written as

$$\epsilon_i^{calcd} = \frac{\epsilon_u - \epsilon_s}{1 + \exp[(-\Delta H°/RT_i) + (\Delta S°/R)]} + \epsilon_s \quad (6)$$

We adopted the well-known Newton–Raphson iterative least-squares approach[43] to determine values of the unknowns that result in a minimization of Σ_{res} and, hence, in the solution of eq 4. If the difference between the observed and calculated extinctions for the ith one ($\epsilon_i - \epsilon_i^{calcd}$) is approximately linearly related to corrections to the four unknowns, ΔU_s (s = 1, 2, 3, 4), according to

$$(\epsilon_i - \epsilon_i^{calcd}) \simeq \sum_{s=1}^{4} \frac{\partial \epsilon_i^{calcd}}{\partial U_s} \cdot \Delta U_s \quad (7)$$

for $i = 1, 2, \ldots, n$, ΔU_s may be obtained by a method of least squares, which, after application to the initial values of the unknowns, ^0U_s, provides new values for unknowns, ^1U_s, and for ϵ_i^{calcd} that will reduce Σ_{res}. These new values of ϵ_i^{calcd} are returned to eq 7 and the process is repeated and continued until all values of ΔU_s are less than 0.001 units. Thus, a practical minimization of Σ_{res} is realized.

If the calculated experimental parameters, ϵ_i^{calcd}, are assumed to be linear functions of the unknown parameters, U_s, small changes in the unknown parameters, ϵU_s, result in linear changes in the calculated parameters, $\Delta\epsilon_i^{calcd}$. Thus, one may write

$$\Delta\epsilon_i^{calcd} \simeq \frac{\partial \epsilon_i^{calcd}}{\partial U_s} \cdot \Delta U_s \quad (8)$$

where U_s refers to any particular unknown parameter. When $\Delta\epsilon_i^{calcd} = \epsilon_i - \epsilon_i^{calcd}$, ΔU_s becomes the correction to the unknown, U_s. In addition

$$\Delta U_s = {}^1U_s - {}^0U_s \quad (9)$$

where ^0U_s is the value of the sth unknown in a given iteration and ^1U_s is the corresponding value in the next iteration. One would like to find changes in all the unknown parameters such that ϵ_i^{calcd} are brought into coincidence with ϵ_i, i.e.

$$\frac{\partial}{\partial U_s} \sum_{i=1}^{n} (\epsilon_i - \epsilon_i^{calcd})^2 = -2 \sum_{i=1}^{n} (\epsilon_i - \epsilon_i^{calcd}) \left(\frac{\partial \epsilon_i^{calcd}}{\partial U_s}\right) = 0$$

$$\sum_{s=1}^{4} \frac{\partial \epsilon_i^{calcd}}{\partial U_s} \cdot \Delta U_s = \Delta\epsilon_i^{calcd} \quad (i = 1, 2, \ldots, n) \quad (10)$$

where the number of unknowns is 4 (ϵ_u, ϵ_s, $\Delta H°$, and $\Delta S°$) and n is the total number of observed parameters, ϵ_i. The above equations of condition can be written in matrix notation

$$\mathbf{DU} = \epsilon \quad (11)$$

where $\mathbf{D}$ is the $n \times 4$ matrix of partial differentials with elements $\epsilon_{is} = \partial\epsilon_i^{calcd}/\partial U_s$ obtained by differentiating eq 6

$$\frac{\partial \epsilon_i^{calcd}}{\partial \epsilon_u} = \frac{1}{1 + \exp[(-\Delta H°/RT_i) + (\Delta S°/R)]}$$

$$\frac{\partial \epsilon_i^{calcd}}{\partial \epsilon_s} = 1 - \frac{1}{1 + \exp[(-\Delta H°/RT_i) + (\Delta S°/R)]}$$

$$\frac{\partial \epsilon_{i\,calcd}}{\partial \Delta H°} = \frac{\epsilon_u - \epsilon_s}{RT_i} \cdot \frac{\exp[(-\Delta H°/RT_i) + (\Delta S°/R)]}{[1 + \exp[(-\Delta H°/RT_i) + (\Delta S°/R)]]^2}$$

$$\frac{\partial \epsilon_i^{calcd}}{\partial \Delta S°} = -\frac{\epsilon_u - \epsilon_s}{R} \cdot \frac{\exp[(-\Delta H°/RT_i) + (\Delta S°/R)]}{[1 + \exp[(-\Delta H°/RT_i) + (\Delta S°/R)]]^2}$$

$\mathbf{U}$ is the four-dimensional vector of corrections to the unknowns and ϵ is the n-dimensional vector of the residuals in the molar extinction coefficients. Standard least-squares procedure for minimizing Σ_{res} is to form the normal equations

$$\mathbf{D^T DU} = \mathbf{D^T}\epsilon \quad (12)$$

which are solved to yield the corrections to the unknown parameters, $\mathbf{U}$.

$$\mathbf{U} = (\mathbf{D^T D})^{-1}\mathbf{D^T}\epsilon \quad (13)$$

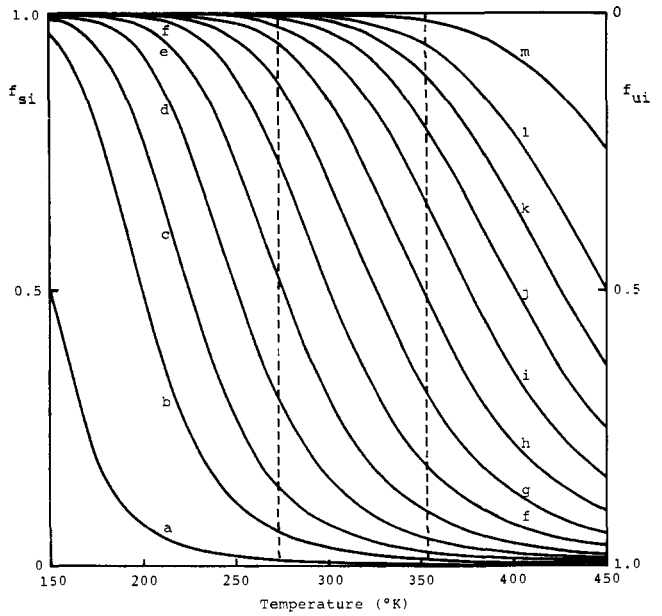


Figure 4. Normalized melting profiles for various values of $\Delta H°$ with a fixed value of $\Delta S°$ (−20 eu): a, $\Delta H° = -3.0$ kcal/mol; b, $\Delta H° = -4.0$ kcal/mol; c, $\Delta H° = -4.5$ kcal/mol; d, $\Delta H° = -5.0$ kcal/mol; e, $\Delta H° = -5.5$ kcal/mol; f, $\Delta H° = -6.0$ kcal/mol; g, $\Delta H° = -6.5$ kcal/mol; h, $\Delta H° = -7.0$ kcal/mol; i, $\Delta H° = -7.5$ kcal/mol; j, $\Delta H° = -8.0$ kcal/mol; k, $\Delta H° = -8.5$ kcal/mol; l, $\Delta H° = -9.0$ kcal/mol; m, $\Delta H° = -10.0$ kcal/mol. The dashed lines are upper and lower temperature limits between which ϵ_i may be determined in the present experiments.

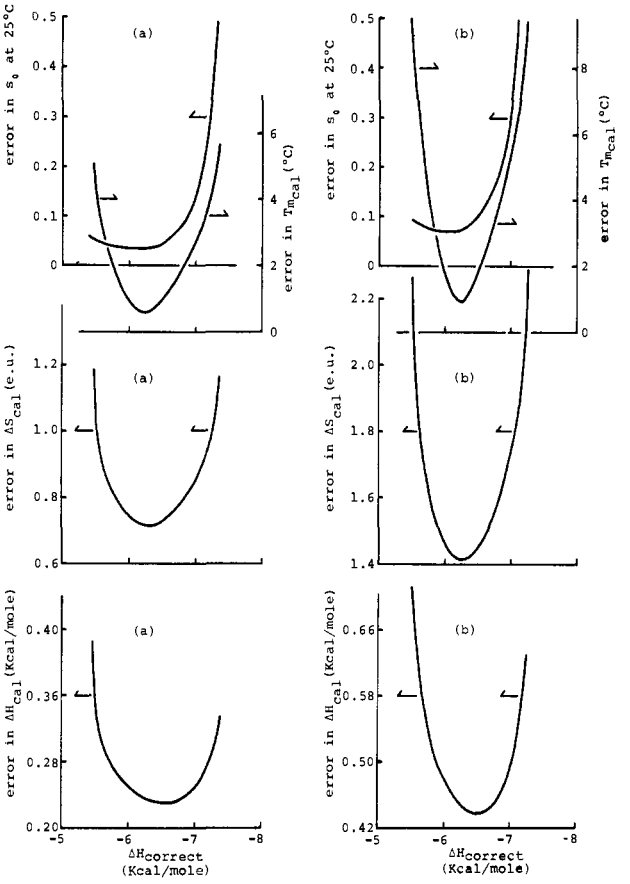


Figure 5. Variations in probable errors in the calculated parameters $\Delta H°_{calcd}$, $\Delta S°_{calcd}$, and s_0 at 25 °C and $T_{m(calcd)}$ as a function of the standard deviation (σ) in synthetic ϵ_i values obtained for various values of $\Delta H°$ with a constant $\Delta S°$ (−20 eu). Synthetic data were processed by our program in the temperature range 0–85 °C: (a) $\sigma = 2.5 \times 10^{-3}$; (b) $\sigma = 5.0 \times 10^{-3}$.

The corrections are applied to the unknowns and the iterative loop is repeated until the rms (root mean square) changed by less than 10^{-7} in the last cycle.

(b) Reliability of Results. To test the reliability of the results obtained by the present approach, computation of the errors associated with the parameters was made by introducing synthetic data containing Gaussian distributed random errors for a series of assumed values of the parameters into the present computer program. We have applied standard deviations ranging from 2×10^{-3} to 5×10^{-3} to construct synthetic data in solving eq 4 with assumed values of the parameters, because the smallest root mean square (rms) values in fitting of experimental and calculated melting profiles have been found to be in this range after several (less than 10) iterative cycles (see footnote *a* in Table I). In Figure 4 typical normalized melting curves are shown for different values of $\Delta H°$ with $\Delta S°$ = 20 eu. Because the temperature range over which ϵ_i is determined is limited (0–80 °C), in intervals of about 3 °C, synthetic ϵ_i values obtained for various values of $\Delta H°$ and $\Delta S°$ in this temperature range were processed by our program. In order to demonstrate the effect of precision in ϵ_i on the accuracy of the solutions of eq 4, the probable errors in the calculated $\Delta H°$, $\Delta S°$, s_0 at 25 °C, and T_m are plotted against the correct $\Delta H°$ for systems in which a fixed value of $\Delta S°$ (−20 eu) has been assigned (Figure 5). It was again found that for the synthetic values of ϵ_i having $2 \times 10^{-3} < \sigma < 5 \times 10^{-3}$, whenever the iterations converged, the estimates of the parameters were reliable and the probable errors in the calculated parameters are acceptable.[18,42] In line with the previous suggestions,[18,21,42] it was found that the probable errors in the parameters are generally smallest for systems in which the T_m is in the experimental temperature range.

(c) Definition of Microscopic Stacking Equilibrium Quotients and Ionization Constants. Equilibria in the half-protonated XpX can be treated as Scheme II. The overall tautomeric quotient, K_t, and the microscopic stacking equilibrium quotients, $s_1^{3'}$ and $s_1^{5'}$, may be expressed by

Scheme II

$$\begin{array}{ccc} [(^{+}\mathrm{XpX})_u] & \overset{s_1^{5'}}{\rightleftharpoons} & [(^{+}\mathrm{XpX})_s] \\ & K_t \Big\Updownarrow & \\ [(\mathrm{XpX}^{+})_u] & \overset{s_1^{3'}}{\rightleftharpoons} & [(\mathrm{XpX}^{+})_s] \end{array}$$

$$K_t = \frac{[^{+}\mathrm{XpX}]}{[\mathrm{XpX}^{+}]} = \frac{[(^{+}\mathrm{XpX})_u] + [(^{+}\mathrm{XpX})_s]}{[(\mathrm{XpX}^{+})_u] + [(\mathrm{XpX}^{+})_s]} = \frac{1 + s_1^{5'}}{1 + s_1^{3'}} \quad (14)$$

$$s_1^{3'} = \frac{[(\mathrm{XpX}^{+})_s]}{[(\mathrm{XpX}^{+})_u]} \quad (15)$$

$$s_1^{5'} = \frac{[(^{+}\mathrm{XpX})_s]}{[(^{+}\mathrm{XpX})_u]} \quad (16)$$

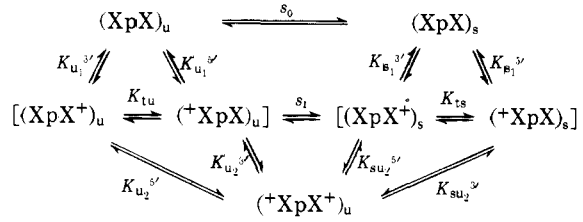
Because $s_1 = [(\mathrm{XpX})_s^{+}]/[(\mathrm{XpX})_u^{+}] = [[(^{+}\mathrm{XpX})_s] + [(\mathrm{XpX}^{+})_s]]/[[(^{+}\mathrm{XpX})_u] + [(\mathrm{XpX}^{+})_u]] = \frac{1}{2}(s_1^{3'} + s_1^{5'})$, we obtain eq 17 and 18.

$$s_1^{3'} = \frac{[(\mathrm{XpX}^{+})_s]}{[(\mathrm{XpX}^{+})_u]} = \frac{1 - K_t + 2s_1}{1 + K_t} \quad (17)$$

$$s_1^{5'} = \frac{[(^{+}\mathrm{XpX})_s]}{[(^{+}\mathrm{XpX})_u]} = \frac{2s_1K_t + K_t - 1}{1 + K_t} \quad (18)$$

Similarly, for the equilibria summarized by Scheme III, microscopic ionization constants for the fully stacked and unstacked XpX are defined as

Scheme III



$$K_{s_1}{}^{3'} = \frac{a_H[(XpX)_s]}{[(XpX^+)_s]} \qquad K_{s_1}{}^{5'} = \frac{a_H[(XpX)_s]}{[(^+XpX)_s]}$$

$$K_{su_2}{}^{3'} = \frac{a_H[(^+XpX)_s]}{[(^+XpX^+)_u]} \qquad K_{su_2}{}^{5'} = \frac{a_H[(XpX^+)_s]}{[(^+XpX^+)_u]}$$

and $K_{su_1}{}^{3'} = K_{u_1}{}^{5'} = K_{u_2}{}^{3'} = K_{u_2}{}^{5'}$.

References and Notes

(1) Abbreviations: nucleosides are specified by the usual symbols, A (adenosine), C (cytidine), G (guanosine), or U (uridine). The protonation to the 3′- and 5′-linked nucleoside base in a homodinucleoside monophosphate, XpX, is denoted by $^+$XpX and XpX$^+$, espectively, and to both nucleoside bases by $^+$XpX$^+$; poly A = polyriboadenylic acid; poly C = polyribocytidylic acid; CD = circular dichroism; ORD = optical rotatory dispersion.
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(33) As a referee has pointed out, the reason why GpG was not done before is probably the aggregative nature of GpG. We also believe that earlier difficulties were due to the presence of aggregates in the prepared solution. We found that the preheating at 60–70 °C for 30 min was effective to dissociate the aggregates,[28] and no significant amount of aggregates was re-formed in the resulting solutions during the period of the pK measurements at 25 °C and the thermal denaturation experiments. Without this treatment, our titration and melting data cannot be obtained.
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(38) We have learned that Topal has reached the same conclusion from studying the titration and optical (CD, NMR, hypochromicity) properties of CpC (M. D. Topal, Ph.D. Thesis, New York University, 1974). We thank a referee for drawing our attention on this point.
(39) The values of ΔH° and ΔS° for the stacking process in (CpC)$^+$ have been determined by M. D. Topal. The results indicate that stacking interactions are also solvophobic (enthalpy driven–entropy opposed; $\Delta H^\circ = -4.7$ kcal/mol, $\Delta S^\circ = -17.6$ eu).
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Catalysis of Decarboxylation of Nitrobenzisoxazolecarboxylic Acid and of Cyanophenylacetic Acid by Modified Polyethylenimines

Junghun Suh, Ioannis S. Scarpa, and Irving M. Klotz*

Contribution from the Department of Biochemistry and Molecular Biology and from the Department of Chemistry, Northwestern University, Evanston, Illinois 60201.

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Abstract: Modified polyethylenimines have been prepared containing apolar lauryl groups and fully quaternized amine nitrogens. These derivatives catalyze markedly the decarboxylation of nitrobenzisoxazolecarboxylate and of cyanophenylacetate. The kinetics of decarboxylation fit equations analogous to those of enzymic kinetics. The preequilibrium binding constant is in the range of 10^4–10^5 M^{-1} and the catalytic constant, k_2, is 10^{-2}–10^{-3} s^{-1}. In terms of the turnover number, k_2, the reaction at a catalytic site on the polymer is about 1300 times faster than the spontaneous reaction in water.

It has been shown by Kemp and Paul[1] that the decarboxylation of benzisoxazolecarboxylicacids is very markedly accelerated in an aprotic solvent, as contrasted to water. An apolar, non-hydrogen-bonding environment evidently stabilizes the charge-delocalized transition state of the benzisoxazole carboxylate anion and thereby accelerates the rate of decarboxylation. Further application of this concept to aqueous micelles by Bunton and Minch[2] has revealed substantial ac-