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DETERMINATION OF MICROSCOPIC IONIZATION CONSTANTS AND MICROSCOPIC STACKING EQUILIBRIUM QUOTIENTS OF ADENYLYL(3′→5′)ADENOSINE

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Using the basic ionization constants for a pair of isomers, m¹ÂpA and Apm¹Â, and the measured values for the overlapping pK values of ApA, the microscopic ionization constants and intramolecular stacking quotients for the monoprotonated ApA were estimated. The results indicate that, in contrast to the case of GpG, ApA did not exhibit preferential protonation on either site of 3′- and 5′-linked nucleoside bases and neither enhanced nor diminished stacking was observed for ÂpA and ApÂ as compared to ApA.

1. Introduction

This extension of earlier work [1,2] on the microscopic ionization of adenylyl(3′ → 5′)adenosine (ApA) and on the microscopic intramolecular stacking association of monoprotonated adenylyl dimers (ÂpA and ApÂ) was undertaken in order to ascertain the influence of the protonation effect on stacking association. Among dinucleoside monophosphates known as the shortest chain-length oligomers having the ability of intramolecular base-base stacking interactions, ApA has been most extensively studied and there have been already a number of experimental determinations [2–10] of the enthalpy and entropy for the conformational equilibrium $(ApA)_u \rightleftharpoons (ApA)_s$. In a previous [2] and a recent paper [11], we determined the most reliable [12] stacking association quotient for ApA. We have also shown that homoribodinucleoside monophosphates including ApA undergo stepwise protonation and determined the macroscopic ionization constants. However, no information was available on microscopic ionization and miscropic stacking equilibrium quotients for homodimers until we reported those values for GpG [1,2]. It is the intention of this communication to report the corresponding results of studies that were designed to extend the characterization of monoprotonated ApA molecules by preparation of m¹ÂpA and Apm¹Â and by determination of the stacking quotients using ultraviolet thermal denaturation spectral measurements and titrations.

Abbreviations: nucleosides are specified by the usual symbols, A (adenosine) or G (guanosine). ApA, adenylyl(3′ → 5′)adenosine; ÂpA and ApÂ, monoprotonated ApA at either a 5′ or 3′ terminal base; $(ApA)^+$, ÂpA plus ApÂ; ÂpÂ, doubly protonated ApA; m¹ApA, N_1-methyladenylyl(3′→5′)adenosine; Apm¹A, adenylyl(3′→5′)-N_1-methyladenosine. In $(ApA)_s$ and $(ApA)_u$, s and u denote stacked and unstacked conformations, respectively.

2. Experimental section

2.1. Materials

m¹ApA and Apm¹A were prepared by partial methylation of ApAp with dimethyl sulfate, followed by column chromatographic separation with Dowex 1X2, and enzymatic dephosphorylation of m¹ApAp and Apm¹Ap with alkaline phosphatase

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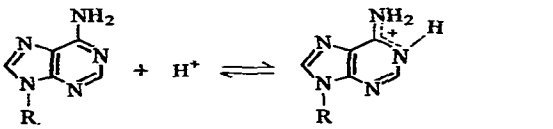
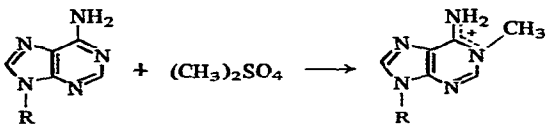
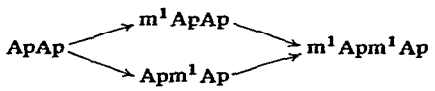
[11]. ApAp is believed to undergo protonation and methylation on the same nitrogen atom under the conditions used, and ApAp is thus methylated simultaneously at nuclear-nitrogen atoms of the 5′ and 3′ termini, the end product being m^1Apm^1Ap as depicted in scheme 1. The purity of m^1ApA and





R = ribosyl residue



Scheme 1.

Apm^1A thus obtained was checked by paper chromatography and thin-layer chromatography and ascertained by the formation of m^1Ap and A, and Ap and m^1A both in a 1 : 1 ratio when treated with RNase T_2. The molar extinction coefficients of $m^1\hat{A}pA$ and $Apm^1\hat{A}$ (at pH 5.5) were determined from hypochromicity measurements at 258 nm upon the above hydrolysis (hypochromicities at 258 nm are 10% at 25°C and pH 5.5 for both compounds) to be 2.61×10^4 M^{-1} cm^{-1}. The extinction coefficients of m^1A and A residues were taken from the literature [13]. For $m^1\hat{A}pm^1\hat{A}$, ϵ_{258} was also determined to be 2.70×10^4 M^{-1} cm^{-1}.

2.2. Methods

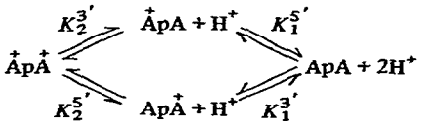
In the present work, methods and calculations followed closely those described previously [1,2,14–16].

3. Results and discussion

In a previous paper [11], we took two approaches to determine stacking equilibrium quotients for the equilibria, $(m^1\hat{A}pA)_u \rightleftharpoons (m^1\hat{A}pA)_s$ and $(Apm^1\hat{A})_u \rightleftharpoons (Apm^1\hat{A})_s$, one involving measurements of the temperature dependence of an intensive property and the other, measurements of the stepwise basic ionization constants of the dimers and pK values of the component monomers. In this paper, we present the results of the determination of microscopic ionization constants and microscopic stacking equilibrium quotients for ApA.

3.1. Protonation of ApA and model compounds

The specific site of protonation on nucleic acid bases differs for various nucleosides. The most basic position in adenosine is N_1. Protonation may be expected to occur at the same sites in the dinucleoside monophosphate (ApA); however, equilibria may exist involving nonprotonated, monoprotonated, and diprotonated forms in ApA. The course of ionization of the two bases in ApA can be illustrated as in scheme 2.



Scheme 2.

Protonation to adenosine shifts the longest wavelength band at 259.5 nm slightly (by ≈ 2.5 nm) to shorter wavelength. Replacement of the N_1 proton by a methyl group causes a similar shift by almost the same amount. Based on these findings and the structural similarities between $m^1\hat{A}$ and protonated adenosine residue ($\hat{A}$), it was expected that $m^1\hat{A}pA$ and $Apm^1\hat{A}$ would be useful as model compounds for the specifically monoprotonated species of ApA involved in the equilibria (scheme 2 and fig. 1).

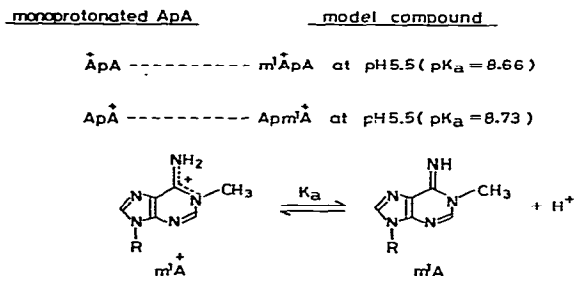


Fig. 1. Model compounds for monoprotonated ApA and ionization of the m¹A residue. R, ribosyl residue.

3.2. Determination of microscopic ionization constants and tautomeric equilibrium quotient, $K_t = [\hat{A}pA]/[Ap\hat{A}]$

The macroscopic ionization constants for ApA may be defined [11,14] as

$$K_1 = \frac{a_H[ApA]}{[\hat{A}pA] + [Ap\hat{A}]} \quad (1)$$

and

$$K_2 = \frac{a_H([\hat{A}pA] + [Ap\hat{A}])}{[\hat{A}p\hat{A}]} \quad (2)$$

By applying the least-squares computer method to the spectroscopic pH-titration data, we estimated [11] the apparent constants, K_1 and K_2 (as pK), to be $pK_1 = 3.91 \pm 0.02$ and $pK_2 = 3.05 \pm 0.07$. These values are obviously composite, and the overall constants, K_1 and K_2, can be related to the individual, or microscopic, ionization constants defined in scheme 2 by the expression

$$\frac{1}{K_1} = \frac{1}{K_1^{3'}} + \frac{1}{K_1^{5'}} \quad (3)$$

$$K_2 = K_2^{3'} + K_2^{5'} \quad (4)$$

The tautomeric equilibrium quotient, K_t, being constant regardless of the pH, is also given by

$$K_t = \frac{K_1^{3'}}{K_1^{5'}} = \frac{K_2^{3'}}{K_2^{5'}} \quad (5)$$

From the structural similarities of m¹ÂpA and Apm¹Â to ÂpA and ApÂ, respectively, it was expected that the basic ionization constants of ÂpA and ApÂ ($K_2^{3'}$ and $K_2^{5'}$) would be approximated by the second ionization constants of m¹ÂpA and Apm¹Â, respectively, as was the case for GpG [1].

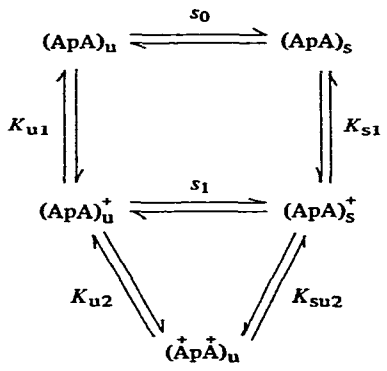
Using a spectrophotometric method, the apparent pK values for m¹ÂpA and Apm¹Â were determined [11]: for m¹ÂpA, $pK = 3.41 \pm 0.03$, and for Apm¹Â, $pK = 3.36 \pm 0.03$. With these values, the equilibrium quotient, K_t, and the microscopic ionization constants could then be calculated by means of the above equations. The results are included in table 1. The K_t value of 0.89 suggests that the monoprotonated form with a proton on the base at a 3′ terminus only slightly preponderates over that with a proton on the adenine base at a 5′ terminus. In contrast to the present results, from circular dichroic spectral and basic pK comparisons with methylated derivatives [1], the predominant form is GpG and we estimated the degree of predominance as $[\hat{G}pG]/[Gp\hat{G}] = 1.7$–$2.0$ at 25°C.

3.3. Determination of microsopic stacking equilibrium quotients and intrinsic tautomeric quotients

The site of protonation in a given homodimer, XpX, is of course expected to influence the stacking of monoprotonated dimers. The degree to which monoprotonated XpX would exist in $\hat{X}pX$ or $Xp\hat{X}$ is now known for $(ApA)^+$ and $(GpG)^+$. The present work, together with the thermal denaturation data reported previously [11,16] for ApA, has enabled an estimation of various microscopic constants involved in stacking and protonation equilibrium scheme. The results can be compared with the corresponding values for the known specific case of GpG.

3.3.1. Ionization constants, K_{u1}, K_{u2}, K_{s1} and K_{su2}

Prototropic and intramolecular stacking equilibria of ApA can be depicted by scheme 3 provided that doubly protonated ApA is unstacked, i.e., $s_0, s_1 \gg s_2 \simeq 0$. By definition, $pK_{u1} = pK_0 + \log 2$; $pK_{u2} = pK_0 - \log 2$; $pK_{s1} = pK_{u1} - \log(s_0/s_1)$; and $pK_{su2} = pK_{u2} - \log s_1$; where K_0 is the intrinsic ionization constant for each site of the two bases in the fully unstacked ApA. Since the values



Scheme 3.

of s_0 ($= 1.10 \pm 0.01$), pK_1 and pK_2 are all known for ApA, the following equations may be used to afford the values of s_1 and pK_0.

$$pK_1 = (pK_0 + \log 2) - \log\frac{1+s_0}{1+s_1} \quad (6)$$

$$pK_2 = (pK_0 - \log 2) - \log(1+s_1) \quad (7)$$

With these values the pK values of completely unstacked and stacked species were determined for ApA as follows: $pK_{u1} = 3.94 \pm 0.04$; $pK_{u2} = 3.34 \pm 0.04$; $pK_{s1} = 3.88 \pm 0.08$; $pK_{su2} = 3.36 \pm 0.08$.

3.3.2. *Microscopic stacking equilibrium quotients ($s_1^{3'}$ and $s_1^{5'}$) of ApA*

The empirical stacking equilibrium quotient (s_1) of ApA is composite, and it is related to the true constants of the $\overset{+}{A}pA$ ($s_1^{3'}$) and $Ap\overset{+}{A}$ ($s_1^{5'}$) by the expressions:

$$s_1^{3'} = \left[(\overset{+}{A}pA)_s\right] / \left[(\overset{+}{A}pA)_u\right] = (1 - K_t + 2s_1)/(1 + K_t) \quad (8)$$

and

$$s_1^{5'} = \left[(\overset{+}{A}pA)_s\right] / \left[(\overset{+}{A}pA)_u\right] = (2s_1K_t + K_t - 1)/(1 + K_t) \quad (9)$$

From the K_t and s_1 values of ApA, the microscopic stacking quotients can be calculated to be $s_1^{3'} = 1.07 \pm 0.38$ and $s_1^{5'} = 0.84 \pm 0.34$, both being more or less identical within the experimental errors. This point is also distinctly in contrast to the case of GpG [2].

3.3.3. *Microscopic ionization constants and tautomeric quotients of ApA*

pK_{s1} and pK_{su2} are now seen to be composite constants determined under experimental conditions where it is likely that $(\overset{+}{A}pA)_s$ and $(Ap\overset{+}{A})_s$ are

Table 1

Summary of ionization constants and stacking and tautomeric quotients of ApA and GpG at 25°C and ionic strength, 0.1 (data for GpG from ref. 2)

	ApA	GpG
(A) Overall constants		
(a) Ionization constants (composite: s+u; 3′+5′)		
pK_1	3.91±0.02	2.51±0.03
pK_2	3.05±0.07	1.49±0.03
(b) Ionization constants (composite: 3′+5′)		
pK_{s1}	3.88±0.08	2.70±0.12
pK_{su2}	3.36±0.08	1.80±0.08
pK_{u1}	3.94±0.04	2.38±0.04
pK_{u2}	3.34±0.04	1.78±0.04
(c) Stacking quotient (composite: 3′+5′)		
s_1	0.95±0.16	0.95±0.13
(d) Tautomeric quotient (composite: s+u)		
K_t	0.89±0.10	1.7 ±0.3
(B) Microscopic constants		
(a) Ionization constants (composite: s+u)		
$pK_1^{3'}$	3.63±0.10	2.07±0.07
$pK_1^{5'}$	3.58±0.10	2.30±0.10
$pK_2^{3'}$	3.38±0.07	1.68±0.09
$pK_2^{5'}$	3.33±0.07	1.91±0.06
(b) Ionization constants		
$pK_{s1}^{3'}$	3.62±0.16	2.06±0.18
$pK_{s1}^{5'}$	3.51±0.18	2.58±0.12
$pK_{su2}^{3'}$	3.73±0.18	1.91±0.13
$pK_{su2}^{5'}$	3.62±0.16	2.43±0.19
pK_0	3.64±0.04	2.08±0.04
(c) Stacking quotients		
s_0	1.10±0.01	0.45±0.10
$s_1^{3'}$	1.07±0.38	0.44±0.15
$s_1^{5'}$	0.84±0.34	1.45±0.21
(d) Tautomeric quotients		
K_{ts}	0.79±0.43	3.3 ±1.8
K_{tu}	1.0	1.0

in rapid equilibrium. The true pK of species $(\dot{A}pA)_s$ and $(ApA^{\cdot})_s$ can be obtained from the following relationships:

$$pK_{s1}^{3'} = pH - \log\frac{[(ApA)_s]}{[(\dot{A}pA)_s]} = pK_0 - \log(s_0/s_1^{3'}) \quad (10)$$

$$pK_{s1}^{5'} = pH - \log\frac{[(ApA)_s]}{[(ApA^{\cdot})_s]} = pK_0 - \log(s_0/s_1^{5'}) \quad (11)$$

$$pK_{su2}^{3'} = pH - \log\frac{[(\dot{A}pA)_s]}{[(\dot{A}pA^{\cdot})_u]} = pK_0 - \log s_1^{5'} \quad (12)$$

and

$$pK_{su2}^{5'} = pH - \log\frac{[(ApA^{\cdot})_s]}{[(\dot{A}pA^{\cdot})_u]} = pK_0 - \log s_1^{3'} \quad (13)$$

Since we have stipulated that the two sites are equivalent and noninteracting in $(ApA)_u$, it follows that $K_{u1}^{3'} = K_{u1}^{5'} = K_{u2}^{3'} = K_{u2}^{5'} = K_0$. We have determined the microscopic ionization constants for the stacked conformational isomers of ApA and the results are summarized in table 1 together with those for GpG for comparison.

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 $pK_{s1}^{5'}$ and $pK_{s1}^{3'}$ is 0.52 pK units, and this was considered [2] to be due solely to the conformational stabilization of the stacked GpG species relative to the stacked GpG. By contrast, a much smaller difference is observed for a pair of isomers, $(\dot{A}pA)_s$ and $(ApA^{\cdot})_s$, indicating that the conformational stability is almost the same in these fully stacked monoprotonated ApA. Microscopic stacking equilibrium constants also enabled the microscopic tautomeric quotients, K_{tu} and K_{ts}, of completely stacked and unstacked species of monoprotonated ApA to be obtained from the following relationships:

$$K_{tu} = \frac{[(\dot{A}pA)_u]}{[(ApA^{\cdot})_u]} = (s_1^{3'} - s_1)/(s_1 - s_1^{5'}) = K_{u1}^{3'}/K_{u1}^{5'} \quad (14)$$

and

$$K_{ts} = \frac{[(\dot{A}pA)_s]}{[(ApA^{\cdot})_s]} = (s_1^{3'} - s_1)s_1^{5'}/(s_1 - s_1^{5'})s_1^{3'} = K_{s1}^{3'}/K_{s1}^{5'} \quad (15)$$

Previously [2], we arrived at the conclusion that GpG undergoes preferred 3'-linked nucleoside base protonation, and at present we have concluded that this is not the case with ApA.

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