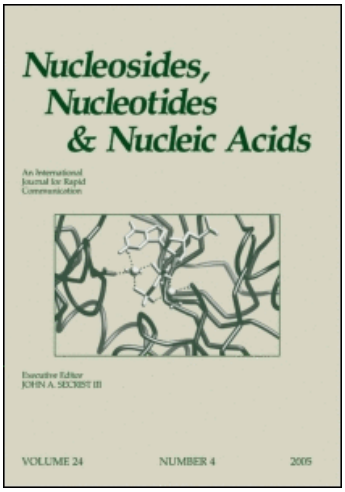

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Thermodynamic and NMR Study of Proton Complex Formation of 2'-Deoxyadenylyl-(3'→5')-2'-Deoxyadenosine in Aqueous Solution

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THERMODYNAMIC AND NMR STUDY OF PROTON COMPLEX FORMATION OF 2'-DEOXYADENYLYL-(3'→5')-2'-DEOXYADENOSINE IN AQUEOUS SOLUTION

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ABSTRACT

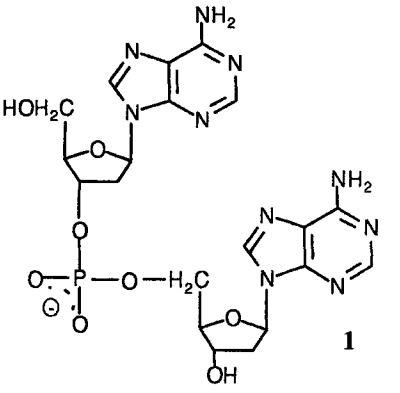
A thermodynamic and proton NMR study has been carried out in order to characterize the behaviour of 2'-deoxyadenylyl-(3'→5')-2'-deoxyadenosine towards the protonation. Conformational changes occurring following two protonation steps are discussed.

INTRODUCTION

Hydrophobic, stacking and electrostatic forces are responsible for high specificity in interactions between macromolecules in biological systems[1,2]. These weak non-covalent interactions are also thought to be responsible for both the folding tendency of aryl and alkyl moieties in synthetic organic molecules[3-5] and the preferential disposition of nucleic bases in simple models of nucleotides[6].

Recently, enthalpy and entropy changes associated with protonation of linear and cyclic peptides have been used, together with NMR data, to study relationships between non-covalent interactions associated with the side-chain conformational changes and the proton complex formation[7,8].

Extending this approach, we have now studied conformational changes which 2'-deoxyadenylyl-(3'→5')-2'-deoxyadenosine (d(ApA)) (**1**) undergoes following protonation. This molecule has been investigated by other researchers[9-12], and fully characterized in the unprotonated form, in which it exists at neutral pH[9-11], while, as regards the relevant protonated species, only



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their protonation constants are known and determined spectrophotometrically[12]. Here we report the thermodynamic parameters concerning the two steps of protonation, determined by potentiometry and direct calorimetry. While separated enthalpic and entropic contributions had been reported for homoribodinucleotides[12-15], no data were up today available for homodeoxyribodinucleotides. The acquisition of these thermodynamic parameters, in addition to ^{1}H NMR and CD investigations, allowed us to characterize the mono- and di-protonated species of d(ApA).

EXPERIMENTAL

Synthesis and purification of 2'-deoxyadenylyl-(3'→ 5')-2'-deoxyadenosine.

The synthesis of 2'-deoxyadenylyl-(3'→5')-2'-deoxyadenosine (d(ApA)) was carried out using the phosphotriester method, following substantially the procedure of Stawinski et al.[16].5'-O-(4,4'-dimethoxytriphenylmethyl)-N^6-benzoyl-2'-deoxyadenosine-3'-O-(2-chlorophenyl) phosphate triethylammonium salt, 3'-O-benzoyl-N^6-benzoyl-2'-deoxyadenosine and 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole were available by Merck.

The condensation product (mixture of the two expected diastereomers) was purified by preparative liquid chromatography (PLC) on silica gel (63-200 μm, Merck) eluting with a gradient of methanol in chloroform/pyridine (97:3) from 0 to 2 % v/v.

The fully protected deoxydinucleotide was detritylated according to Gait et al.[17] and the product (mixture of the two diastereomers) was purified by PLC on silica gel, using a gradient of methanol in chloroform from 0 to 3 % v/v.

Treatment of this product with concentrated ammonia[16] gave the fully deprotected deoxydinucleotide, which was first purified on a DEAE cellulose column (carbonate form) eluting with a linear gradient of triethylammonium bicarbonate (pH 7.5) from 0 to 0.25 M[18]. Pooled fractions were further purified by preparative HPLC (Partisil-10 ODS Whatman; 0.9 x 25 cm) using a linear gradient of CH_3CN in 0.1 M triethylammonium acetate (TEAA, pH 7.0) from 0 to 30 % in 40 minutes. Fractions containing the desired d(ApA) were checked for their purity by analytical HPLC and were then lyophilized to completely remove TEAA.

The triethylammonium salt of d(ApA) was then converted into the potassium one by passing through a column of Dovex-50W (K^+).

The purity of d(ApA), potentiometrically checked, was higher than 99 %.

Thermodynamic measurements

Chemicals.- HNO_3 and KOH stock solutions were prepared by diluting concentrated ampoules (Merck). The ionic strength of all solutions was adjusted to 0.1 mol dm^{-3} by adding KNO_3 (Merck). Twice-distilled water and grade A glassware were used throughout.

Potentiometric measurements.- The potentiometric measurements were carried out by means of a fully automated apparatus, which made use of Metrohm equipment (burette, E665; meter, E 654;

combined microelectrode, EA 125) and was controlled by an IBM compatible PC. All experiments were carried out at 25.0 ± 0.2° C using 2.5 ml thermostated cells. All solutions were magnetically stirred and maintained under an atmosphere of inert nitrogen, previously bubbled through 0.1 mol dm^{-3} KNO_3 solutions.

The electrode was standardized on the $pH = -\log C_H^+$ scale by titrating HNO_3 with CO_2-free KOH.

Solutions (4-5 ml) containing the ligand (0.001-0.002 mol dm^{-3}) were titrated with standard KOH from pH 2.3 to pH 4.5. The solutions were freshly prepared to avoid hydrolysis of the dinucleotide; experiments of different duration gave identical results, thus excluding decomposition phenomena.

Calorimetric measurements.- The calorimetric measurements were performed at 25.000 ± 0.001°C using a Tronac 450 isoperibolic calorimeter equipped with a 25 cm^3 titration Dewar.

Solutions containing the ligand (0.003-0.004 mol dm^{-3}) were titrated with standard HNO_3. The titration data, corrected for all non-chemical energy terms determined in separate experiments, were refined to obtain the final $\Delta H°$ of each system, using the computer program DOEC (see Calculations).

Calculations.- The calculations concerning the calibration of the electrode system, E_j, as well as the slope, were performed using the computer program ACBA[19], which refines the parameters of an acid-base titration by using a non linear least-squares method minimizing the function $U = \Sigma(V_{i,exp} - V_{i,calc})^2$, where V_i is the volume of titrant added. The program SUPERQUAD[20], which minimizes the error-square sum based on measured electrode potentials, was used to handle all other data.

The calorimetric data were treated by using the computer program DOEC[21]. The distribution diagram were obtained by means of the computer program DISDI[22].

Spectroscopic measurements

NMR. - ^1H NMR spectra were recorded in D_2O on a Bruker AC-250 spectrometer at 250.13 MHz. Chemical shifts were measured in ppm, tetramethylammonium chloride (TMA; $\delta = 3.18$ ppm) being used as internal reference. Coupling constants were accurately determined by means of spin decoupling experiments.

Solutions containing d(ApA) at a concentration of 0.005 mol dm^{-3} in D_2O were added with appropriate amounts of 0.1945 M DCl, dictated by the computer program DISDI[22], in order to obtain the highest degree of formation of the species L^-, HL and H_2L^+ (FIGURE 1).

Deuterated d(ApA) at H-8 protons was obtained by heating a sample of the dinucleotide in D_2O at 80°C for 4 h.

All spectra recorded in the presence of manganese(II) were carried out at different $MnCl_2$ concentrations ranging from 0 to 1.4 X 10^{-4} mol dm^{-3}.

CD. - CD spectra were recorded on a Jasco J-600 spectropolarimeter. Calibration of the CD instrument was performed with a solution of isoandrosteron in dioxane ($\Delta\varepsilon = 3.31$ mol^{-1} cm^{-1} at

TABLE 1. The log K values and thermodynamic parameters for the protonation equilibria of d(ApA) (L^-) and AMP (L'^{2-}) at 25° C and I = 0.1 mol dm^{-3} (KNO_3).

Reaction	log K	ΔG° (kcal mol^{-1})	ΔH° (kcal mol^{-1})	ΔS° (cal mol^{-1} deg^{-1})
$L^- + H^+ = HL$	3.73 (1)	-5.09 (1)	-2.68 (7)	8.1 (2)
$HL + H^+ = H_2L^+$	2.88 (2)	-3.93 (2)	-1.77 (9)	7.2 (3)
$L^- + 2H^+ = H_2L^+$	6.61 (2)	-9.02 (2)	-4.45 (9)	15.3 (3)
$HL' + H^+ = H_2L'$	3.78	-5.15	-4.3	2.8

[a] This work. [b] Ref. 22. [c] Standard deviations are given in parentheses.

304 nm). The circular dichroism spectra were recorded at room temperature on freshly prepared aqueous solution of the ligand (0.0001 mol dm^{-3}). Results are reported in terms of θ (molar absorbtion coefficient).

The above cited thermodynamic and spectroscopic measurements were carried out using d(ApA) concentrations ≤ 0.005 mol dm^{-3} in order to minimize intermolecular effects on the measurements[10,11].

RESULTS AND DISCUSSION

The values of log K, ΔG°, ΔH° and ΔS° for the protonation of d(ApA) (**1**) are reported in TABLE 1, together with the corresponding values of the N-1 protonation of adenosine 5'-monophosphate (AMP)[23]. As can be seen, in the investigated pH range, two protonation steps are observed, and thus three different species can be identified (L^-, HL e H_2L^+). The distribution diagram in FIGURE 1 (c_L = 0.005 mol dm^{-3}) shows that L^- is the prevailing species at neutral pH, the protonations occurring at acidic pH.

Log K values are in good agreement with those previously determined by spectrophotometry[12], while no value of the separate enthalpic and entropic contributions can be found in the literature to be compared with those reported here. The similarity of the values of the two protonation constants suggests that they involve similar groups and, in fact, it is known that the atoms which undergo protonation are the N-1 nitrogen atoms of the two adenine rings[24], the difference observed being influenced by the statistical factor. If one looks at the separate thermodynamic contributions for both the steps, the exothermicity is too small and the entropic

contribution is too favourable in comparison with the corresponding values reported for AMP; this may be ascribed to the co-occurrence of conformational events.

^{1}H NMR data concerning the three species are summarized in TABLES 2 and 3. As regards L^-, our results agree with literature data. Fang et al.[9] ascertained that L^- has an anti-anti right handed conformation, stabilized by a stacking interaction between the bases, and Altona et al.[10], by referring to the pseudorotational model[25-27], showed that L^- exists in two different conformations: 70% as *S,S* and 30% as *S,N*. Our data (TABLE 4), obtained by following the latter procedure, agree with those reported by these authors.

HL and H_2L^+ are here characterized for the first time. Signal attributions of ^{1}H NMR spectra of these two species were achieved by means of spin decoupling experiments as concern the deoxyribosilic protons, whereas H-2 and H-8 proton resonances were distinguished by recording the spectra of the H-8 deuterated dinucleotide at the different pH values.

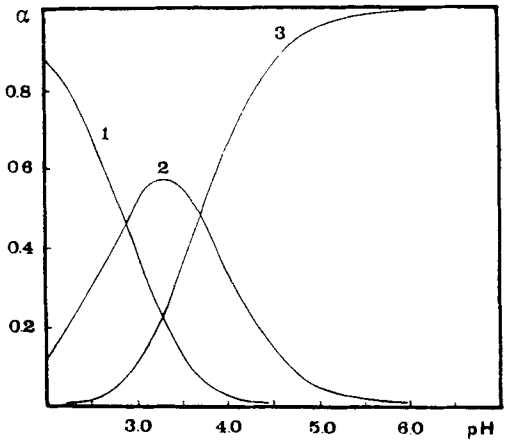

Figure 1. Species distribution diagram for rhe dinucleotide d(ApA); 1) H_2L^+; 2) HL; 3) L^-.

In order to assign the aromatic proton resonances of the d(Ap) and d(pA) moieties, and in order to investigate the syn ↔ anti equilibrium, ^{1}H NMR spectra of these species have been recorded in the presence of increasing concentrations of manganese(II) ion. This paramagnetic ion is known to interact with phosphate oxygens, giving rise to an enhancement of the spin-spin relaxation with the consequent signal broadening of the nearby atoms[28]. It should be mentioned here that it is difficult to observe NOEs for dinucleotides[29] and, in addition, it is impossible to perform any other kind of NMR measurement, for which long experimental times are required. It is well known that d(ApA) easily undergoes depurination process under acidic conditions.

It is convenient, at first, to analyze the results concerning H_2L^+. In this species it is observed the selective broadening of both the H-8 protons, thus suggesting a syn conformation for the d(Ap) moiety, and an anti conformation of the d(pA) one. The downfield H-8 resonance is attributable to d(pA) proton which, also in this conformational situation, is the nearest of the two H-8 protons to the deshielding phosphate group. The d(Ap) H-2 signal, which in L^- species is more upfield than the d(pA) H-2 one, due to the shielding effect of the five-membered adenine ring d(pA), in H_2L^+ species become almost coincident with that of d(pA) moiety. Diprotonation of d(ApA) causes also a downfield shift of all proton resonances of deoxyribosilic rings, as expected, due to a charge effect. But, in particular, the H-2' and H-2" protons of the d(Ap)

Table 2. Proton chemical shifts (δ) at 250 MHz of the L^-, HL and H_2L^+ species of the dimer d(ApA).

Atom	L^-	HL	H_2L^+
H-2' d(Ap)	2.17	2.43	2.69
H-2'' d(Ap)	2.48	2.60	2.69
H-2'' d(pA)	2.51	2.56	2.62
H-2' d(pA)	2.79	2.83	2.88
H-5' d(Ap)	3.72	3.73	3.73
H-5'' d(Ap)	3.72	3.73	3.73
H-5'' d(pA)	4.13	4.12	4.12
H-5' d(pA)	4.16	4.17	4.17
H-4' d(Ap)	4.22	4.22	4.22
H-4' d(pA)	4.24	4.26	4.27
H-3' d(Ap)	4.79	4.82	4.87
H-3' d(pA)	4.79	4.79	4.80
H-1' d(Ap)	6.09	6.23	6.40
H-1' d(pA)	6.28	6.40	6.51
H-2 d(Ap)	7.87	8.15	8.38
H-8 d(Ap)	8.00	8.23	8.42
H-2 d(pA)	8.05	8.22	8.40
H-8 d(pA)	8.32	8.46	8.56

TABLE 3. Coupling constants (J in Hz) of d(ApA).

	Atoms	L^-	HL	H_2L^+
d(Ap)	1'2'	9.4	8.5	6.8
	1'2''	5.6	6.0	6.8
	2'2''	-14.0	-14.0	0
	2'3'	5.6	5.5	4.2
	2''3'	0	2.3	4.2
	3'4'	1.6	2.2	2.6
	4'5'	3.7	3.7	4.0
	4'5''	3.7	3.7	4.0
	3'P[a]	---	---	---
d(pA)	1'2'	6.9	6.7	6.5
	1'2''	6.9	6.7	6.5
	2'2''	-14.0	-14.0	-14.0
	2'3'	7.2	6.7	6.3
	2''3'	3.7	4.5	4.3
	3'4'	2.8	3.2	3.2
	4'5'	2.4	2.4	2.5
	4'5''	3.0	3.0	3.0
	5'5''	-12.0	-12.0	-12.0
	4'P	2.8	2.8	2.8
	5'P	4.1	4.1	4.0
	5''P	3.0	3.0	3.0

[a] Undetectable, owing to the HOD overlap.

TABLE 4. Percentages of S conformer in d(ApA).

	L^-	HL	H_2L^+
d(Ap)	90-100% S	75-80% S	65-75% S
d(pA)	65-70% S	65-70% S	65-70% S

residue show a remarkable $\Delta\delta$ (TABLE 2) not ascribable to a charge effect only. Furthermore (see TABLE 4) the sugar pucker of d(Ap) changes markedly; the *N* form percentage increases just to equal that of the d(pA) ring and the H-2' and H-2" protons, which in L have chemical shifts significantly different each other, in H_2L^+ become equivalent (see TABLES 2 and 3). This would be in accordance to the flexibility gained by this deoxyribose ring and could be due to the changed shielding effects exerted on these protons by the phosphate oxygens and by the purine base, now in syn conformation. These conformational changes could also account for the trend of H-3' proton resonances. In fact in L^- species these proton signals overlap, probably due to a shielding effect which opposites and equals the expected deshielding effect of the phosphate on d(Ap) H-3' proton. In H_2L^+ species, owing to the gained flexibility of the molecule, these opposite effects do not compensate and, as a consequence, d(Ap) and d(pA) H-3' protons have different chemical shifts.

The thermodynamic data agree with the spectroscopic ones. The thermodynamic parameters pertinent to the overall diprotonation process of the dinucleotide show an enthalpic contribution less favourable by 4.15 kcal mol^{-1} and an entropic contribution more favourable by 9.7 cal mol^{-1} $degree^{-1}$ compared to the double of the relevant values obtained following the protonation of the monomer AMP (see Table 1). If during the diprotonation of d(ApA) a stacking interaction is broken, its thermodynamic contribution will be subtracted. Recently, it has been reported[30] that such interaction is enthalpically favoured and entropically unfavoured; so the above mentioned differences may be ascribed to the absence of the stacking interaction in H_2L^+.

Thus in the diprotonated species the stacking interaction would not occur, owing to the electrostatic repulsion between the charged protonated rings. In this situation, all the molecule gains conformational freedom; thus the d(Ap) adenine ring can assume a syn conformation, and the adjacent sugar ring is nomore forced in a specific pucker (TABLE 4).

As regards HL species, it is more difficult to follow an analogous analysis. In fact, as can be seen in FIGURE 1, this species never exceeds 60 % of the total concentration of the dinucleotide; namely it is impossible to observe the HL species alone.

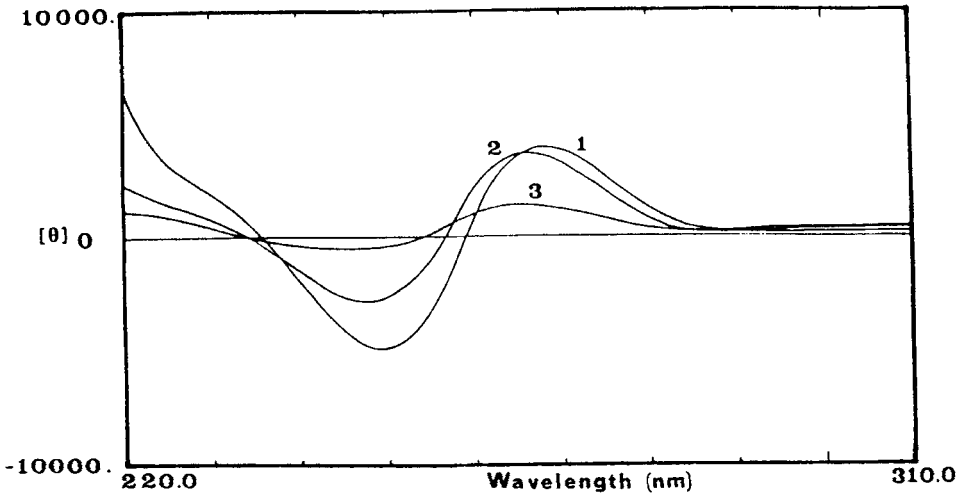

Figure 2. Circular dichroism of d(ApA); 1) L^-; 2) HL; 3) H_2L^+.

Moreover, the differences between the chemical shift values of aromatic protons of HL species and the relevant protons of L^- species are not straightforward for a preferential protonation of one of the two bases. Actually, as previously ascertained for the analogous ribodinucleotide ApA[15], also HL species of d(ApA) could exist as a mixture of the d(Ap) N-1 protonated and the d(pA) N-1 protonated species; both adenine residues, in fact, have comparable basicity, as can be seen by the log K values, which are very close (TABLE 1).

On the other hand, all dinucleoside monophosphates give rise, in solution, to a rapid equilibrium involving stacked and destacked conformers[31,32]. While in experimental conditions we used, L^- species is quite totally stacked and H_2L^+ is quite totally unstacked, each of the two HL species seems to exist as an equilibrium mixture of the two stacked and unstacked conformers[15].

Hence ^{1}H NMR spectrum of HL species will arise from the sum of the contributions of the various components of the equilibrium mixture; in addition to L^- and H_2L^+ species (see FIGURE 1), which probably compensate each other, the d(Ap) N-1 protonated species and the d(pA) one (each as stacked and unstacked conformer) are present. So, chemical shift values and coupling constants observed are intermediate between those of L^- and H_2L^+ species (see TABLES 2 and 3). The d(Ap) sugar pucker also shows an intermediate *N* form percentage (TABLE 4).

The thermodynamic parameters indicate that the formation of HL species is less enthalpically favoured and more entropically favoured with respect to AMP protonation

(TABLE 1), while with respect to the formation of the quite totally unstacked H_2L^+ species the enthalpic contribution is more favoured. This is in agreement with a partial destacking of the bases.

The CD spectra of the three species investigated are reported in FIGURE 2 and entirely agree with the above results. As can be seen, the intensity of the CD bands reduces significantly, passing from L^- to H_2L^+ species. This is a clear evidence of a progressive unstacking of the bases[31,32], following the two protonation steps of the dinucleotide.

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