Ap_4A AND Ap_5A PREFER FOLDED UNSTACKED CONFORMATIONS AT pH 4–5, IN SHARP CONTRAST WITH Ap_2A AND Ap_3A

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1. Introduction

Acid-soluble nucleotides have been implicated as metabolic signals in regulation and coordination of intracellular functions. Their metabolic lability, which allows their pool sizes to fluctuate rapidly in response to changes in extracellular conditions, enables this class of compounds to act in intracellular mediation of environmental changes [1]. Diadenosine 5′,5‴-P^1, P^4-tetraphosphate (Ap_4A) has recently been suggested, based on its high metabolic lability and its pool sizes which were found directly related to the proliferative activity of mammalian cells, to act as a positive growth signal [2]. This nucleotide is readily formed in a cell free protein synthesizing system as a product of the back reaction of the amino acid activation step [3]. Its intracellular pool, however, is small and fluctuates widely in response to extracellular conditions which affect growth [2]. Addition of Ap_4A to permeabilized G_1-arrested baby hamster kidney cells yielded initiation of DNA replication in the resting cells [4]. A recent study has shown highly specific binding of Ap_4A to mammalian DNA polymerase α which was suggested to account for the ability of Ap_4A to trigger DNA replication [5]. The conformation of Ap_4A in solution is of primary importance in determining its mode of interaction with proteins or DNA. Proton magnetic resonance studies of adenine nucleotides of the type $A(5')P_n(5')A$ (n=2–5) suggest preferred stable intramolecularly stacked conformations for all of these compounds at physiological temperatures and pD 7. Ap_4A and Ap_5A alone, at pD 4–5, assume a unique 'folded' unstacked conformation as their preferred conformation. Space filling models indicate that a chain of 4 or 5 phosphate residues is ideal for this conformation.

2. Materials and methods

PMR studies of Ap_nA (n=2–5) and Ap_n (n=1–4) and ^{31}P NMR studies of Ap_4A and Ap_5A were conducted. Ap_3A and Ap_4A were prepared by the procedure in [6]. Ap_5A was purchased from Boehringer Mannheim. AMP, ADP, ATP, Ap_4 and diadenosine 5′,5‴-P^1,P^2-pyrophosphate (Ap_2A) were purchased from Sigma Chemical Co. All the nucleotides were used as their sodium salts. Solutions of the nucleotides were prepared at concentrations of 0.01 M at 6 different pD values. At pD 1.1 and 2.1 KCl–DCl buffers were used; at pD 4.1 and 5.1 succinic acid–NaOD buffers were used; for PMR studies at pD 7.1 and 8.1 KD_2PO_4–NaOD buffers were used. For ^{31}P NMR studies at pD 7 tris(hydroxymethyl) aminomethane buffer was used. The pD values were calculated by adding 0.4 to the pH values [7]. Ionic strengths of solutions were <0.18, except in the experiments in which ionic strength was raised to 2.0 by the addition of anhydrous NaCl. Solute purity and stability were monitored prior to and following the recording of NMR spectra using thin layer chromatography on PEI-cellulose plates eluted with 1 M Li Cl. PMR spectra were obtained using 5 mm OD sample tubes in a Varian CFT-20 spectrometer adapted for protons, operating at 80 MHz. Temperature control was maintained to ± 1°C by a Varian variable temperature acces-

sory. Acetic acid-d_1 was used as an internal standard [8]. ^{31}P NMR spectra were obtained using 10 mm OD sample tubes on a Bruker WH-90 multinuclear spectrometer operating at 36.43 MHz in the proton noise decoupled mode (through the courtesy of Dr A. Redfield, Brandeis University).

3. Results and discussion

Resonances monitored were H-2 and H-8 of the adenine ring, H-1′ of the ribose, and the phosphorus of the middle and end phosphate moieties of Ap_4A and Ap_5A. PMR results plotted as the change in chemical shifts for Ap_4A compared with AMP are shown in fig. 1. Similar curves were obtained for Ap_5A, with the greatest downfield chemical shifts occurring at pD 5 rather than pD 4. Chemical shifts of H-8 and H-2 of Ap_nA (n=2–5) relative to AMP at 35°C are listed in table 1.

At pD 1 and 2 the chemical shifts for both the H-2 and H-8 resonances of Ap_4A and Ap_5A are similar to those of AMP ($\Delta\delta \sim 0$), with the exception of H-8 of Ap_4A. The two adenine rings of Ap_4A and Ap_5A thus appear to have chemical environments almost equivalent to those for adenine in AMP. An extended conformation for these molecules in which the rings do not interact significantly with each other or with the phosphate chain is thus likely to be preferred at low pD values. At pD 7 significant upfield shifts are seen. These shifts are interpreted as evidence for stacking of the two adenine rings with each other. The adenine isoshielding curves in [9] were used, along with the upfield shifts of H-2 and H-8, to propose the stacked conformation shown in fig. 2.

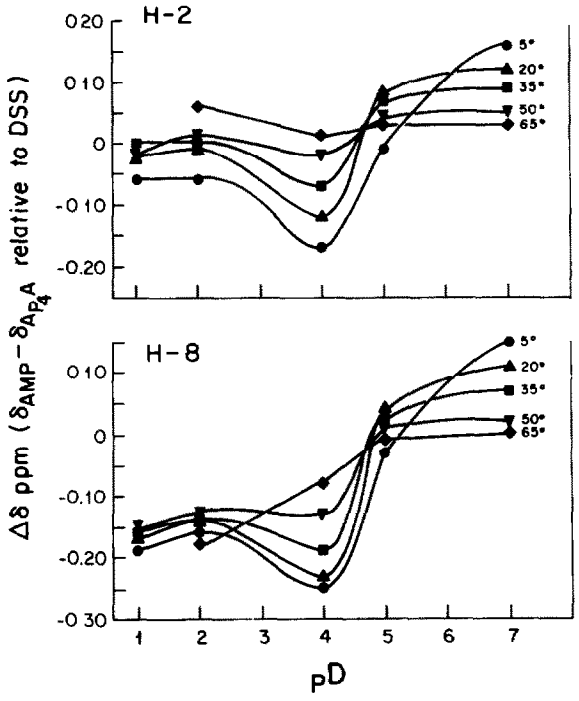

Fig. 1. Plot of $\Delta\delta$ as a function of pD and temperature for Ap_4A ($\Delta\delta = \delta_{AMP} - \delta_{Ap_4A}$).

It is clear from fig. 1 that the stacking is disrupted as the temperature is gradually increased from 5–65°C.

At pD 4 for Ap_4A and pD 5 for Ap_5A there are striking downfield shifts for both H-2 and H-8 (fig. 1, table 1). Downfield shifts are evidence for deshielding, and in these molecules phosphate deshielding was suspected. However, for deshielding to occur the phosphate group must be close to the ring protons, since

Table 1
$\Delta\delta$ ($\delta_{AMP} - \delta_{Ap_4A}$) at 35°C

pD	Ap_2A		Ap_3A		Ap_4A		Ap_5A	
	H-2	H-8	H-2	H-8	H-2	H-8	H-2	H-8
2	0.05	0.08	0.06	0.05	0.00	−0.13	0.00	0.01
4	0.15	0.21	0.06	0.07	−0.07	−0.19	−0.13	−0.07
7	0.20	0.27	0.14	0.16	0.09	0.07	0.07	0.01

Values of $\Delta\delta$ at pD 7 were calculated using δ_{AMP} at pD 5.2 (since AMP is a dianion at pD 7 and a monoanion at pD 5.2)

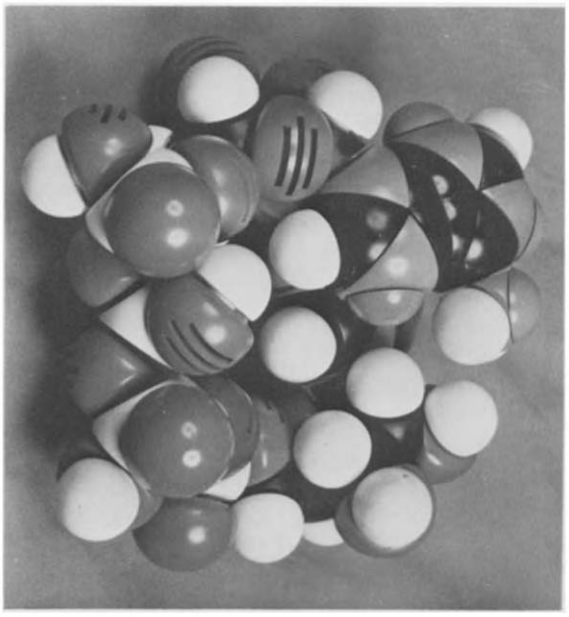

Fig. 2. CPK model of ring-stacked conformation of Ap_4A (predominates at pD 7).

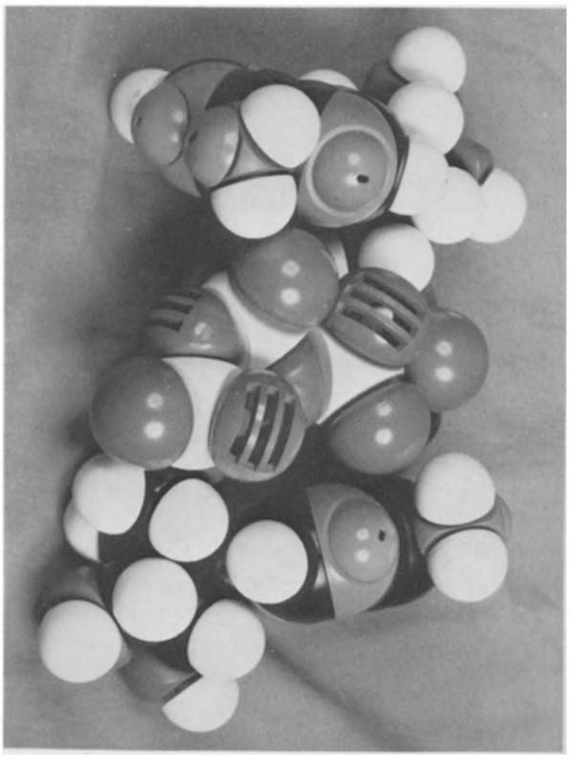

Fig. 3. CPK model of 'folded' unstacked conformation of Ap_4A (predominates at pD 4).

phosphate deshielding is believed to proceed via an electrostatic mechanism [10]. To eliminate the possibility that the increased number of phosphate groups alone was responsible, Ap_n (n=2–4) were studied under identical conditions to Ap_4A and Ap_5A. Although the second and third phosphate groups caused some increase in the downfield shifts of H-8 and H-2 in the mononucleoside polyphosphates over those in AMP, these increases were only about 0.02–0.04 ppm. In the dinucleoside polyphosphates, on the other hand, H-8 is shifted downfield by 0.25 ppm and H-2 is shifted downfield by 0.17 ppm relative to AMP at 5°C.

The possibility of a preferred 'folded' unstacked conformation at pD 4 for Ap_4A and pD 5 for Ap_5A, stabilized by an electrostatic interaction between the negative charge of the phosphate groups and the partially positively charged ring, was confirmed by raising the ionic strength of the solutions to 2.0, a 25-fold increase. At this ionic strength the downfield shifts for H-2 and H-8 disappeared. Whereas Ap_2A and Ap_3A do not have long enough phosphate chains to allow such 'folded' conformations, and do not show downfield shifts relative to AMP, CPK models of Ap_4A and Ap_5A verify the possibility of such conformers (fig. 3).

In addition to the changes in chemical shifts of the adenine H-2 and H-8 resonances, changes in the ribose H-1′ resonances support our proposed changes in conformation. Decreases in the glycosidic torsion angle (χ_{CN}) have been suggested to account for upfield shifts of H-1′ resonance peaks [11,12]. At 80°C and pD 7 both Ap_4A and Ap_5A are assumed to be in extended conformations (see fig. 1). At pD 4 (35–5°C), Ap_4A shows upfield shifts (0.10–0.18 ppm) for H-1′ relative to pD 7 and 80°C. Similarly, Ap_5A shows upfield shifts (0.10–0.22 ppm) at pD 5 (35–5°C) relative to pD 7 and 80°C. Thus, the extended conformation at pD 7 and 80°C no longer exists at pD 4–5 (35–5°C) and the glycosidic torsion angle decreases as Ap_4A and Ap_5A assume the 'folded' conformation.

Finally, ^{31}P NMR results confirm our proposed

conformations for Ap_4A and Ap_5A. As the pD was lowered from 7–5 a change of 0.5 ppm was noted in the phosphorus resonance peak of the middle phosphates of Ap_5A, further evidence that the molecule changes from the stacked to 'folded' unstacked conformer. In Ap_4A the shift of the middle phosphates is even more striking, 1.5 ppm downfield as the stacked to 'folded' transition occurs. Further, a change in coupling constant of 3 Hz was found for Ap_4A as the pD was lowered from 7–4 at 5°C. Again, this suggests a change in conformation as demonstrated by a change in ester bond torsion angles.

Thus, Ap_4A and Ap_5A have been shown to prefer 3 different conformations in solution, according to pH and temperature. At pD 7 and 35°C a ring-stacked conformation predominates; at pD 4 or 5 a 'folded' unstacked conformation, in which the phosphate chains are shielded on both sides by the adenine rings, predominates; at low pH an extended conformation predominates.

Acknowledgements

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