Proton magnetic resonance spectroscopic analysis of diadenosine 5',5'''-polyphosphates

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Certain diadenosine 5',5'''-polyphosphates are potent inhibitors of ADP stimulated platelet aggregation, acting possibly via competitive ADP-receptor binding. ^{1}H NMR studies of a series of such compounds where the number of phosphate groups between adenosine groups was varied from 2 to 6 were performed to analyze possible preferred solution conformations and to define structure-activity relations. Relative to mononucleotides ADP and ATP, chemical shifts of adenosine proton resonances in diadenosine polyphosphate analogs are upfield shifted suggesting base stacking. This effect is greatest for AP2A and AP3A. Coupling constants of ribose ring proton resonances support the idea of an anti-base-ribose ring conformation, and $^3J_{H5'\text{-}P}$ values suggest a preferred gauche H–C–O–P structure. In all cases, NMR parameters for AP2A are near-limiting values for a static base stacked conformation. Increasing the number of phosphate groups between adenosine moieties tends to weaken this interaction.

Nuclear magnetic resonance; Diadenosine polyphosphate; Inhibitor of platelet aggregation; ADP; ADPreceptor

1. INTRODUCTION

Human platelets store diadenosine 5',5'''-p^1-p^4-tetraphosphate (AP4A) in dense granules at a concentration higher than in any other cellular compartment (about 0.4 nmol/mg protein, or about 1% of the ATP content). The stored AP4A appears to be metabolically inert. Thrombin-treated platelets release AP4A along with other stored nucleotides, including ADP and an analog dinucleotide, diadenosine 5',5'''-p^1,p^3-triphosphate (AP3A) [2]. The precise physiological role of AP4A has not been defined, but it has been associated with a variety of cellular metabolic events [3] and has been proposed to play a role in platelet physiology. AP4A is a potent inhibitor of ADP-induced platelet aggregation [4] and has even been shown to reverse the aggregation process [5,6]. Of all the diadenosine polyphosphate (APnA with $n = 2-6$) examined, AP4A was found to be the most potent inhibitor of ADP-induced platelet shape change and to bind aggregin – a putative ADP-receptor (R.N. Puri and R.W. Colman, unpublished results) on the platelet surface. Since AP4A has been shown to competitively inhibit ADP-induced platelet aggregation, it has been suggested that it may do so by competitively binding the ADP membrane receptor [7]. The possible function of this and similar dinucleotides for the process of platelet aggregation is, therefore, of high interest.

In this present ^{1}H NMR (500 MHz) study, we investigate a series of diadenosine 5',5''' polyphosphates, i.e. AP2A, AP3A, AP4A, AP5A and AP6A, in order to see any conformational effect of varying the number of phosphate groups positioned between the two adenosines.

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Abbreviations: NMR, nuclear magnetic resonance; rf, radio frequency; FID, free induction decay; 2D-NMR, two dimensional-NMR; COSY, correlated spectroscopy; EDTA, ethylene diaminatetraacetic acid; AP2A diadenosine 5',5'''-p^1,p^2-diphosphate; AP3A, diadenosine 5',5'''-p^1,p^3-triphosphate; AP4A, diadenosine 5',5'''-p^1,p^4-tetraphosphate; AP5A, diadenosine 5',5'''-p^1,p^5-pentaphosphate; AP6A, diadenosine 5',5'''-p^1,p^6-hexaphosphate; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate

2. METHODS AND MATERIALS

Diadenosine 5',5'''-polyphosphates (AP2A, AP3A, AP4A, AP5A and AP6A), ADP and ATP were purchased from Sigma Chemical Co. and were used without further purification. With the exception of AP5A and AP6A, these samples were at least 95% pure as judged by ^{1}H NMR spectral analysis. Other chemicals used were reagent grade.

In order to normalize nucleotide concentrations for NMR studies, dinucleotide concentrations were 3 mM and mononucleotide concentrations were 6 mM. Concentrations were determined by UV absorbance measurements. The solutions contained 0.01 M sodium phosphate buffer (p^{2}H 6.9, not corrected for isotope effects), EDTA at 1 mM to chelate paramagnetic species, and 0.1 M NaCl to maintain a constant ionic strength. The samples were lyophilized once from 99.8% ^2H_2O and dissolved in 99.9% ^2H_2O. The p^{2}H was adjusted by adding microliter increments of NaO^2H or ^{2}HCl to a 0.6 ml sample.

^{1}H NMR spectra were recorded in the Fourier mode on a GE GN-Omega-500 spectrometer (500 MHz for protons). The solvent deuterium signal was used as a field-frequency lock. All chemical shifts are quoted in parts per million (ppm) downfield from sodium 4,4-dimethyl-4-silapentane sulfonate (DSS).

Two-dimensional (2D) COSY spectra [8,9] were accumulated in ^2H_2O as 512 time incremented, 1024 point spectra with 96 transients

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per FID. Data processing was done on a Sun 3/160 computer with software supplied by GE on the spectrometer. FIDs were first multiplied by a Lorentzian-Gaussian transformation or shifted sine-bell function, and data sets were zero-filled to 1024 in the evolution dimension. The $^1H^2HO$ solvent resonance was suppressed by direct irradiation for 1 s prior to application of the first 90° NOESY pulse.

3. RESULTS AND DISCUSSION

Fig. 1 shows a series of conventional 500 MHz proton NMR spectra for ADP, AP2A, AP3A, AP4A, AP5A, and AP6A. Aside from apparent chemical shift differences, spectra look similar. Specific ribose ring proton resonance assignments were made via 2D-COSY spectral analysis as shown, for example, with AP2A in Fig. 2. In all cases, the H1′ doublet resonance (not shown in the conventional NMR spectrum insert of Fig. 1) was, as usually observed, the most deshielded, followed by the H2′, H3′, H4′, and H5′/H5″ resonances. The H5′/H5″ resonances demonstrate second-order coupling since their geminal coupling constants are on the order of their chemical shift differences [10]. The sharp, singlet adenine ring proton resonances, i.e. H2 and H8 (data not shown), were assigned on the basis of previous NMR studies [11]; the H8 resonance is the more deshielded. Additional unidentified proton resonances are observed in spectra for AP5A and AP6A (marked with an 'X' in Fig. 1). Based on chemical shifts and COSY spectral analysis, these are most likely due to the presence of some AP2A and AP4A impurities present in the longer phosphate chain samples.

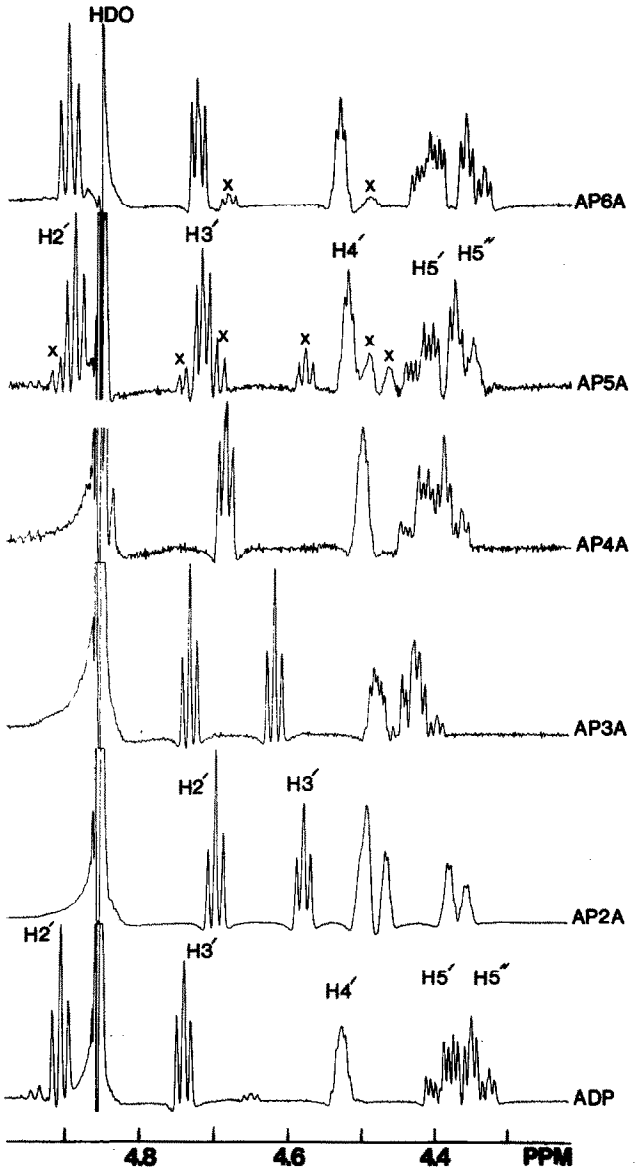

Fig. 1. Proton NMR spectra of diadenosine polyphosphates. 500 MHz proton NMR spectra (303 K) are shown as labelled. Samples (3 mM for AP2A, AP3A, AP4A, AP5A and AP6A, and 6 mM for ADP and ATP) were dissolved in ^2H_2O solutions containing 0.01 M sodium phosphate buffer (p^2H 6.9), 0.1 mM EDTA, and 0.1 M NaCl. 256 transients were accumulated per FID which was multiplied by a Lorentzian-Gaussian function prior to Fourier transformation. The $^1H^2HO$ solvent resonance was suppressed by direct irradiation for 1s at an appropriate rf power prior to a 90° observation pulse. A delay of 4 s was included between acquisitions to allow for recovery of z-magnetization.

At the concentrations studied, chemical shifts were nucleotide concentration independent. Above 20 mM in nucleotide concentration, however, strong downfield chemical shifts were observed for AP4A and AP5A, but not until higher concentrations for other polyphosphate nucleotide samples. These concentration-dependent chemical shift changes are the result of some type of intermolecular nucleotide interactions, probably via base stacking.

In Fig. 3, chemical shifts of resonances are plotted versus the number of phosphates separating the two adenosines. At the origin are plotted the values for the

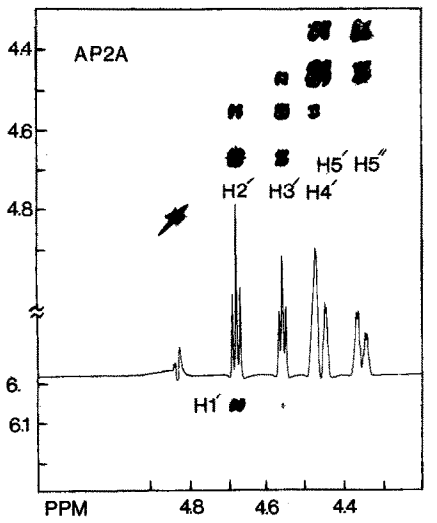

Fig. 2. 2D-NMR COSY contour plot. The ribose ring proton spectral region from a 2D-COSY contour plot is shown for AP2A with a 1D-NMR spectral trace at the bottom of the figure. Experimental details are given in section 2, and solution conditions are as described in the Fig. 1 legend.

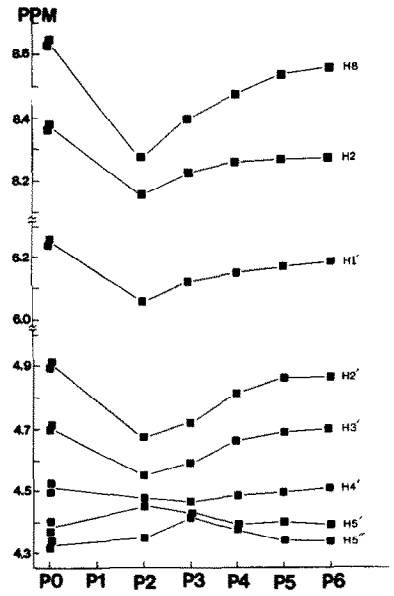

Fig. 3. Plots of chemical shifts versus number of phosphate groups. The chemical shifts of resonances are plotted versus the number of phosphate groups separating adenosine groups. At the origin is plotted those values for standard shifts of resonances in ADP and ATP. Solution conditions are as given in the Fig. 1 legend.

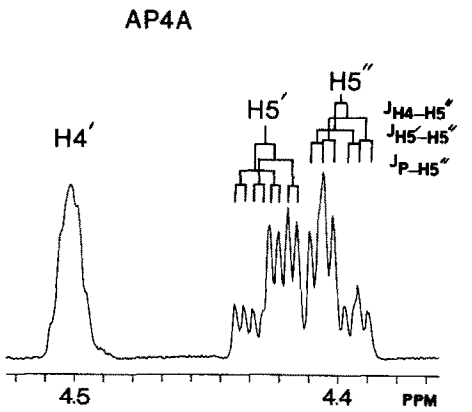

Fig. 4. Proton NMR 5,5'-ribose resonance region in AP4A. An expansion from Fig. 1 of the 5,5'-ribose proton resonance region in AP4A is shown to exemplify second order spectral analysis. ^2J and ^3J-coupling are indicated by lines as labelled.

ATP en ADP standards. A one-phosphate group separation was not studied. Relative to ADP and ATP, upfield chemical shifts for adenine ring protons H2 and H8 are noted for each diadenosine derivative. The largest shift difference is found for AP2A. Such upfield chemical shifts are normally explained by considering base stacking of the two adenine rings [11]. Giessner-Prettre and Pullman [12] calculated the ring current for two stacked adenines lying in parallel planes separated by a distance of 3.4 Å normally found for stacked bases from X-ray diffraction data. Obviously, their study was on a static system and ours deals with small, fluctuating molecules whose resonance chemical shifts are weighted averages over a number of structures. In the simplest case, one can consider two states in equilibrium: a closed, base-stacked state and an open, upstacked state. Interestingly, relative to ATP/ADP, ring proton chemical shift differences for AP2A are 0.375 ppm for H8 and 0.225 ppm for H2 and approach the maximum values calculated for the static, closed state [12] suggesting that AP2A exists predominantly in solution in the stacked state. If in this simplified two-state picture, we assume that this chemical shift difference represents a 100% base-stacked state, then AP4A, for example, with a shift of 0.1 ppm would have a fractional base-stacked population of about 0.5. Increasing the number of phosphate groups between the bases, therefore, tends to decrease the stacked population; this value is observed (Fig. 3) to plateau off at about 20–30% with AP6A.

Chemical shift differences of ribose protons also generally show upfield ring current shift effects. H4' and H5'/H5'' resonances are at least shifted, and H5'/H5'' resonances even show small downfield shifts for AP2A and AP3A. These protons are probably positioned more perpendicular to the ring plane where deshielding effects are observed [11].

Coupling constants, ^3J and ^2J, for ribose ring proton resonances are given in Table I. These were derived from analysis of COSY and conventional NMR spectra. In most cases, J-values were readily derived with the exception of H4' and H5'/H5'' resonances which display second-order effects. In this case, J-values were estimated as exemplified in Fig. 4 for AP4A. In general, ^3J-values are decreased in diadenosine 5',5''-polyphosphates relative to standard ADP and ATP which give the same ^3J-values. $^3J_{H1'-H2'}$ and

Table 1

Diadenosine polyphosphate coupling constants (units of Hz)

	$J_{H1'-H2'}$	$J_{H2'-H3'}$	$J_{H3'-H4'}$	$J_{H4'-H5'}$	$J_{H4'-H5''}$	$J_{H5'-H5''}$	$J_{P-H5'}$	$J_{P-H5''}$
ADP	5.7	5.3	3.9	6.3	4.4	12.5	3.1	4.4
ATP	6.0	5.5	4.2	6.3	4.4	12.5	3.1	4;4
AP2A	4.9	5.9	4.3	2.5	2.5	11.3	1.3	1.9
AP3A	4.6	5.0	4.7	7.5	3.8	11.3	2.5	4.4
AP4A	5.4	?	3.9	6.9	5.0	11.3	3.1	3.8
AP5A	6.0	5.0	4.0	6.3	3.8	11.9	3.1	3.8
AP6A	6.0	5.3	3.8	6.3	3.8	12.5	3.1	4.4

$^3J_{H3'-H4'}$ show extreme values in AP3A, while the remaining ^3J-values are extreme in AP2A. A $^3J_{H1'-H2'}$ value of about 6 Hz is consistent with a $C_{2'}$-endo-$C_{3'}$ exo ribose ring conformation normally observed with purine bases in the anti-conformation [12,13]. The noted decrease in $^3J_{H1'-H2'}$ with the diadenosine derivatives might suggest a shift towards the $C_{3'}$-exo state [13] due to base stacking.

The proton-phosphorus coupling constant, $^3J_{HP}$, in H-C-O-P compounds has been studied [14]. 1-phenyltrimethylene phosphate exists in a fixed conformation where the axial 1-proton is in a gauche relation with respect to the O-P bond around the C-O bond; $^3J_{HP}$ was found to be 1.5 Hz. The *trans*$^3J_{HP}$ coupling constant was estimated to be about 28 Hz [14]. The $^3J_{HP}$ coupling constants observed for adenosine compounds in this study are all less than 4.5 Hz indicating the predominance of the gauche conformation. Interestingly for AP2A, $^3J_{HP}$ is 1.3–1.9 Hz which is at the limiting value found for the fixed gauche conformation [14] supporting the ideal that AP2A exists in a more fixed, less fluctuating conformation relative to the other diadenosine derivatives.

Regarding biological activities, AP4A and AP5A are the most potent in inhibiting ADP-stimulated platelet aggregation possibly via competitive ADP-receptor binding [7]. While correlation of structural elements with antagonist activity does not produce a clear picture for the biologically active structural state, some conclusions can be made: (1) the active state cannot be one which involves *only* base stacking since the most stable base stacked structure, AP2A, is least active; and (2) the number of phosphates separating the bases plays a critical role in determining structure and activity. It may be that base stacking and polyphosphate conformation is critical to activity, or it could be that the distance between the adenosine groups as determined by the number of phosphates is critical to activity. The nature of interactions of diadenosine polyphosphates with unsolubilized and solubilized intact platelets by biochemical and NMR methods is under investigation. The results will be reported in a future communication.

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