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A ^{31}P-NMR STUDY OF MONO- AND DIMAGNESIUM COMPLEXES OF ADENOSINE 5′-TRIPHOSPHATE AND MODEL SYSTEMS

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Summary

^{31}P-NMR chemical shifts and spin-lattice relaxation times of ATP (adenosine 5′-triphosphate), ribose 5′-triphosphate and tripolyphosphate show closely similar behaviour in aqueous solution at pH 7.5 on titration with Mg^{2+}. The results are interpreted in terms of formation of 1 : 1 and 2 : 1 (dimagnesium) complexes with Mg^{2+} bound exclusively to the triphosphate chain. Stability constants for these complexes are reported. It is suggested that the predominant form of the 1 : 1 complexes has Mg^{2+} bound in tridentate manner (via non-bridging oxygen) to the α, β and γ phosphorus atoms; whilst that of the 2 : 1 complexes has each Mg^{2+} bound in bidentate manner, one to the α and β, and the other to the β and γ, phosphorus positions.

Introduction

Adenosine 5′-triphosphate (ATP) is the most ubiquitous compound to participate in metabolic processes involving energy storage, utilization and transfer. Because magnesium is often an essential cofactor in such processes, the structure of $MgATP^{2-}$ is of considerable interest, and a large amount of literature has accrued dealing with the application of NMR to the problem, which is complicated by the likelihood of rapid interconversion of coordination isomers. The structural relationship between the complexes $MgATP^{2-}$ and Mg_2ATP is also of interest since the latter apparently does not bind to at least two MgATP-activated enzymes, viz. nitrogenase [1] and yeast hexokinase [2]. In spite of this, the predominant structure of $MgATP^{2-}$ in aqueous solution has not been adequately defined, and very little work has been done on the complex Mg_2-

ATP. In this paper, we suggest a most stable structure for each of these complexes, on the basis of ^{31}P chemical shift patterns, spin-lattice relaxation times and stability constants (K_d^{-1}).

Materials and Methods

ATP was obtained as the disodium salt from Boehringer. It was passed down a Chelex 100 column (50—100 mesh, Na^+ form) to remove traces of paramagnetic metal ions. The concentration of nucleotide in the eluate was measured by ultraviolet spectrophotometry and adjusted by lyophilisation to give a stock solution of 30 mM. Tris-HCl buffer (50 mM) was added and the pH adjusted to 7.5 using an EIL 7050 pH meter. Magnesium sulphate was obtained as AnalaR grade from B.D.H.

Tripolyphosphate was obtained as the sodium salt from Sigma. The quality of spectra obtained from this compound removed the need for further purification. A stock solution of 20 mM was prepared, buffered with 50 mM Tris-HCl to pH 7.5.

Ribose monophosphate was obtained as the barium salt from Sigma. Ribose 5′-triphosphate was synthesized essentially according to the method of Horecker et al. [3]. Modifications to the method include the use of a linear salt gradient and the removal of the salt, after lyophilisation, by passing the ribose triphosphate down a Sephadex G-10 column. Concentrations were measured by the phloroglucinol reaction [4], and were then adjusted to give a stock solution of 20 mM.

The stability constants of the 1 : 1 complexes at 298 K were measured by a standard spectrophotometric method [5].

^{31}P chemical shifts were obtained by using Jeol PFT—100 and Jeol FX-90Q spectrometers operating at 40.486 MHz and 36.20 MHz, respectively. Spin-lattice relaxation measurements were obtained using a $180°$-τ-$90°$ pulse sequence. The probe temperature was continually monitored with a Comark digital thermometer and remained constant at 298 ± 1 K.

Results and Analysis

The stock triphosphate solutions, buffered with 50 mM Tris-HCl to pH 7.5, were titrated with 500 mM $MgSO_4$. The pH remained constant during the titration. On increasing R (the ratio of the metal to triphosphate concentration) from 0 to 1, large downfield NMR shifts of 2.5, 2.5 and 3.5 ppm were observed for the β phosphorus nuclei of ATP, ribose 5′-triphosphate and tripolyphosphate, respectively (Fig. 1). Smaller downfield shifts of 0.33 and 0.75 ppm were observed for the α and γ phosphorus nuclei, respectively, of ATP and ribose 5′-triphosphate. One doublet for both the α and γ nuclei of tripolyphosphate was observed and this shifted 0.52 ppm downfield. For ATP, the coupling constants $J_{\alpha\beta}$ and $J_{\beta\gamma}$ had values of 20 Hz in the absence of Mg^{2+}, and both decreased to 15 Hz on adding metal up to $R = 1$. For ribose 5′-triphosphate the respective values were again 20 Hz and 15 Hz, and with tripolyphosphate values of 22 Hz and 16 Hz were observed.

Whereas the doublets due to P_α and P_γ remained sharp throughout the titra-

tions with Mg^{2+} ($R = 0$ to 14), the P_β triplet broadened and subsequently sharpened again within the range $R = 0$ to 1. Broadening of the P_β triplet occurred with all three triphosphates within the range $R = 0$ to 1 (being previously noted in the case of ATP [6]), and was very similar for ATP and RTP. The intermediate broadening was much more pronounced with tripolyphosphate, reaching an estimated line-width of 30 Hz and in this case precluding accurate shift measurement over most of the range $R = 0$ to 1. The cause is probably exchange broadening due to dissociation of the 1 : 1 magnesium-triphosphate complex, whereby P_β nuclei experience alternately the chemical shifts of the free triphosphate and of the 1 : 1 complex. The process is clearly rapid enough to give a sharp averaged spectrum (the 'fast exchange limit') in the case of P_α and P_γ, but the averaging is slightly incomplete for P_β on account of the greater chemical shift difference between the free and bound states. The enhanced broadening and slower exchange in the case of tripolyphosphate is to be expected from the tighter binding of the magnesium-tripolyphosphate ($MgTPP^{3-}$) complex (see below) and consequent higher activation energy for its dissociation. However, there is an asymmetry in the onset of broadening, which in each case reaches a maximum before $R = 0.25$ rather than $R = 0.5$ as expected, and this might be due to exchange between coordination isomers. For this reason we do not deduce rate constants at this time, and are investigating the matter further.

In the range of $R = 1$ to 14, the resonances for the three nuclei in all cases shifted to high field relative to their positions at $R = 1$. The shifts from $R = 1$ to 14 for the α, β and γ signals of ATP were 0.63, 0.72 and 0.93 ppm; and for ribose 5′-triphosphate were 0.63, 0.74 and, 0.91 ppm, respectively. The α, γ resonance of tripolyphosphate shifted by 1.20 ppm and the β signal by 1.25 ppm. Throughout this range, the coupling constants remained constant and no line-broadening was observed.

The results in the low R regions $R = 0$ to 2 for ATP have previously been discussed by Tran-Dinh et al. [6]. In agreement with these earlier studies, the results here for chemical shift, with the turning point in the titration curves of all phosphorus atoms at $R = 1$, are indicative of tight 1 : 1 complexation of Mg^{2+} to the polyphosphate chains of ATP, ribose 5′-triphosphate and tripolyphosphate. The behaviour of the titration curves beyond the point $R = 1$ might be brought about in the following ways; self association of molecules stimulated by the presence of metal; the 2-fold increase in ionic strength; or the formation of 2 : 1 (dimagnesium-triphosphate) complexes. For ATP it is thought that self-association occurs by a ring-stacking mechanism involving the adenine moiety. However, it is known [7] that self association causes a small shift of the ^{31}P resonances to low field, and this coupled with the identical behaviour of ribose 5′-triphosphate means that self association cannot account for the observed data. In order to evaluate the effect of ionic strength on the ^{31}P chemical shifts, the spectrum of an equimolar solution of the compounds and Mg^{2+} was obtained before and after the addition of sufficient sodium chloride to give an identical dilution factor, and commensurate ionic strength, to that at $R = 14$. The increase in ionic strength induced small shifts to low field. We conclude therefore that the resonances behave as observed due to the formation of 2 : 1 complexes.

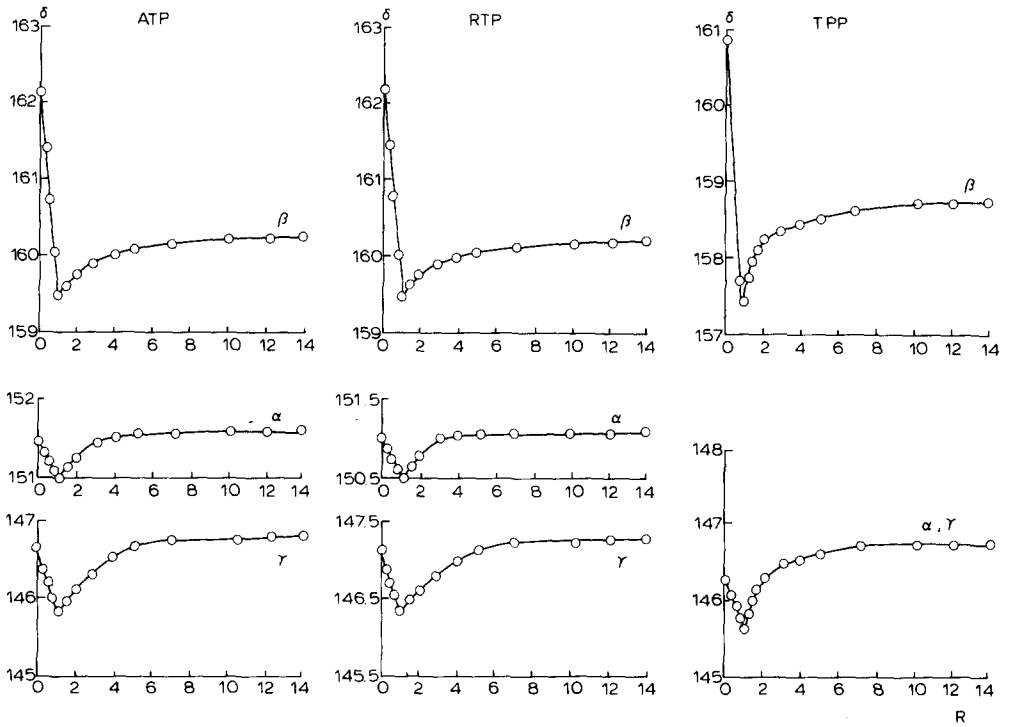

Fig. 1. Observed ^{31}P chemical shifts δ (ppm to high field from external trimethylphosphite) vs. molar ratio R of Mg^{2+} to triphosphate. RTP, ribose 5'-triphosphate; TPP, tripolyphosphate.

Binding in the 1 : 1 complexes is too tight to enable stability constants to be found from the NMR data of Fig. 1. However, the stability constants for formation of the 2 : 1 dimagnesium complexes can be obtained by a theoretical fitting of the experimental titration curves. In order to do this, values of the stability constants for the 1 : 1 complexes were required. This value has not been previously obtained in the case of ribose 5'-triphosphate. The stability constant for $MgATP^{2-}$ varies considerably with the experimental conditions and is notably dependent on the buffer used. In view of this, and the lack of a constant for the magnesium-ribose 5'-triphosphate complex $MgRTP^{2-}$, it was decided to measure the K_d^{-1} values for all three compounds under identical conditions of pH, temperature and buffer concentration. The constants were determined by the spectral changes of 8-hydroxyquinoline [5]. The results are given in Table I. The value obtained for tripolyphosphate is in good agreement with that obtained previously [8]. The stability constant for ATP, which is the same as for ribose 5'-triphosphate, is within the range of values previously obtained [9].

The observed resonance position for any of the signals arising from the compounds under conditions of rapid chemical exchange can be written

$$\delta^i_{obs} = \sum_s p_s \, \delta^i_s \qquad (1)$$

where $i = \alpha$, β and γ and, for example, s = ATP^{4-}, $MgATP^{2-}$ and Mg_2ATP. Un-

TABLE I

CONSTANTS FOR MAGNESIUM TRIPHOSPHATE SPECIES AT 298 K

RTP, ribose 5′-triphosphate; TPP, tripolyphosphate.

Species	K_d^{-1} (M^{-1})
$MgATP^{2-}$	$(53 \pm 5) \cdot 10^3$
$MgRTP^{2-}$	$(59 \pm 5) \cdot 10^3$
$MgTPP^{3-}$	$(625 \pm 30) \cdot 10^3$
Mg_2ATP	33 ± 3
Mg_2RTP	40 ± 3
Mg_2TPP^-	110 ± 9

der our experimental conditions, small quantities of other chemical species such as $MgHATP^-$ and $HATP^{3-}$ may be present, but inclusion of their equilibria had little effect upon the overall result. p denotes mole fraction and δ chemical shift relative to an arbitrary reference.

For a trial value of K_d^{-1} for the reaction $Mg + MgL \rightleftharpoons Mg_2L$ the equilibrium distribution of species in solution was calculated using a computer programme. The concentration data and trial chemical shift values for the 1 : 1 and 2 : 1 complexes then gave the time-averaged chemical shifts through application of the fast exchange Eqn. 1. The process was repeated for various values of the equilibrium constant and chemical shifts, and agreement between calculated and experimental titration curves optimized by minimizing the sum of the squared deviations between calculated and experimental data. The optimal values obtained at 298 K for all three compounds are given in Tables I and II. The final formation constant for Mg_2ATP, 33 M^{-1}, is in reasonable agreement with published values, Burton [10] having reported a value of 70 M^{-1} at 298 K and Frey et al. [11] a value of 59 M^{-1} at 288 K. The stability constant for Mg_2-RTP has not been previously obtained. The value of 110 M^{-1} obtained for Mg_2TPP^- agrees well with the value of 135 M^{-1} at 298 K found by a pH titration [8].

^{31}P spin-lattice relaxation times (T_1) were also measured as a function of magnesium-to-triphosphate ratio for all three systems, on titration of the stock solutions with 500 mM $MgSO_4$ as before (Fig. 2). Unlike the chemical shift

TABLE II

CHEMICAL SHIFTS

Values are given in ppm to high field from external trimethylphosphite. RTP, ribose 5′-triphosphate; TPP, tripolyphosphate.

Species	P_α	P_β	P_γ
ATP^{4-}	151.47	162.20	146.62
$MgATP^{2-}$	150.97	159.32	145.78
Mg_2ATP	151.79	160.45	146.99
RTP^{4-}	151.02	162.23	147.15
$MgRTP^{2-}$	150.51	159.35	146.32
Mg_2RTP	151.32	160.50	147.54
TPP^{5-}	146.25	160.87	146.25
$MgTPP^{3-}$	145.60	157.25	145.60
Mg_2TPP^-	146.85	158.75	146.85

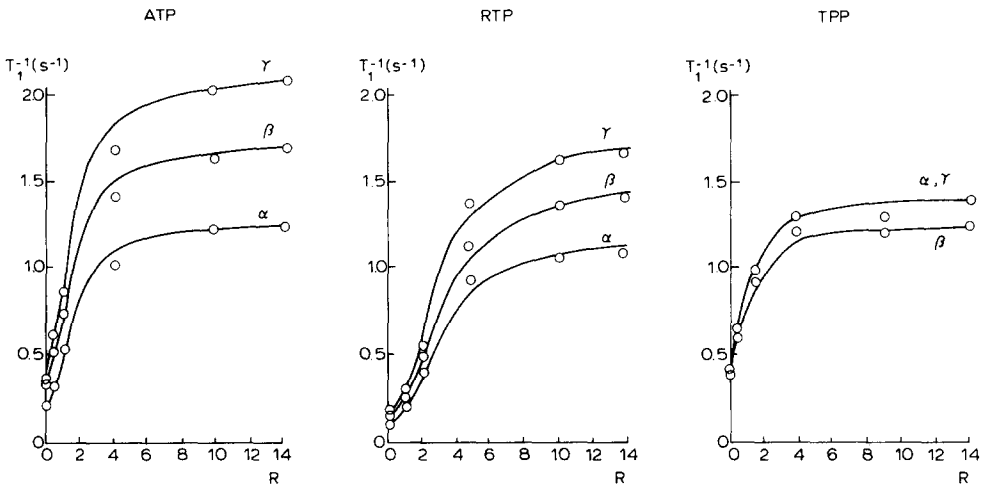

Fig. 2. ^{31}P Spin-lattice relaxation rates (T_1^{-1}) vs. molar ratio R of Mg^{2+} to triphosphate. RTP, ribose 5'-triphosphate; TPP, tripolyphosphate.

TABLE III

SPIN-LATTICE RELAXATION TIMES

RTP, ribose 5'-triphosphate; TPP, tripolyphosphate.

Species	T_1(s)		
	P_α	P_β	P_γ
ATP^{4-}	4.76	2.86	2.63
$MgATP^{2-}$	2.22	1.43	1.23
Mg_2ATP	0.77	0.57	0.46
RTP^{4-}	6.67	5.56	5.26
$MgRTP^{2-}$	4.76	3.70	3.23
Mg_2TRP	0.87	0.69	0.55
TPP^{5-}	2.63	2.78	2.63
$MgTPP^{3-}$	1.14	1.37	1.14
Mg_2TPP^-	0.68	0.73	0.68

curves there is no change in direction at $R = 1$, and the general form indicates a progressive shortening of T_1 in each case from the uncomplexed triphosphate through the 1 : 1 complex to the 2 : 1 complex. As the measurement was of time-averaged relaxation times it was again possible to apply the fast exchange Eqn. 1 and to calculate curves to fit the experimental data, using the same computer programme as before. With the K_d^{-1} values of Table I, it was possible to reproduce the experimental behaviour satisfactorily (see Fig. 2) using T_1 values as given in Table III.

Discussion

Structure of the magnesium complexes

First we consider which part of the triphosphate molecules is responsible for

binding the metal. From the virtually unaltered stability constant on going from ATP to ribose 5′-triphosphate, and almost identical magnitude of chemical shift changes up to $R = 1$, it is clear that binding of the magnesium in the 1 : 1 complex with ATP does not involve the adenine moiety. This is in contrast with earlier proposals but in agreement with recent views [12,13]. The case of Mg^{2+} therefore contrasts with that of Zn^{2+}, Cd^{2+} and Ag^{+}, where interaction with the adenine ring is likely in solution [13]. Far less literature has appeared concerning the structure of the 2 : 1 complex, and structures proposed hitherto involve the binding of the second cation either to the adenosine moiety alone or to both this and the polyphosphate chain [11]. By using the arguments outlined above, viz. the closely similar stability constants for the 2 : 1 complex for ribose 5′-triphosphate and ATP, and the virtually identical patterns of chemical shifts in the region $R = 1$ to 14, it is possible to state that the binding of the second magnesium also does not involve the adenine moiety. On further removal of the ribose moiety to leave tripolyphosphate, binding of both the first and second magnesium ions is tighter. This is readily explained in terms of the extra negative charge on the P_α position (from published pK_a values [14] the phosphate chain in nearly fully ionized at the pH used). The chemical shift data for tripolyphosphate are very similar to those for ribose 5′-triphosphate and ATP, and we conclude that only the triphosphate chain is involved in Mg^{2+} binding in all three cases.

The precise nature of the binding to the phosphate moiety is more problematical. Indeed, a mixture of rapidly exchanging coordination isomers may well be present, in which case our conclusions will refer to the predominant form in aqueous solution at 25°C. Recent studies by Jaffe and Cohn [15] and by Ramirez and Marecek [12] have made it clear that the relative size of phosphorus chemical shift displacements cannot be correlated with sites of metal binding. Specifically, the maximal displacement of the P_β shift on forming the 1 : 1 $MgATP^-$ complex does not necessarily indicate binding or preferential binding to the β position. Rather, ^{31}P chemical shifts are sensitive to changes in local conformation and bond angles [12,16]. The close similarity of the chemical shift vs. composition curves, on titration of ATP, ribose 5′-triphosphate and tripolyphosphate with Mg^{2+}, indicates a similar binding pattern and conformation, with regard both to the 1 : 1 and to the 2 : 1 complexes, for each of the three triphosphates. Moreover, the sharp reversal of chemical shift displacements at 1 : 1 molar composition, for all three ^{31}P nuclei in all three triphosphates, indicates a different predominant phosphate conformation in the 1 : 1 complex from that of either the uncomplexed triphosphate or the 2 : 1 complex. Furthermore, it suggests that the conformation in the 2 : 1 complex is closer to that of the uncomplexed triphosphate than that of the 1 : 1 complex.

Consider first the case of the 1 : 1 complexes. The difference in pK_a values between $ATPH^{3-}$ and $MgATPH^-$ is reasonable evidence that the P_γ position, carrying the remaining acidic hydrogen, is involved in binding to the metal in the 1 : 1 complex. Furthermore, the tighter binding of Mg^{2+} to ATP^{4-} as compared to PO_4^{3-} suggests binding via more than one P atom. Ramirez and Marecek [12] have suggested that the 1 : 1 $MgATP^{2-}$ complex has Mg bound (via non-bridging oxygen atoms) predominantly to the P_α and P_γ positions (although they also consider the possibility of equilibrium, rapid on an NMR

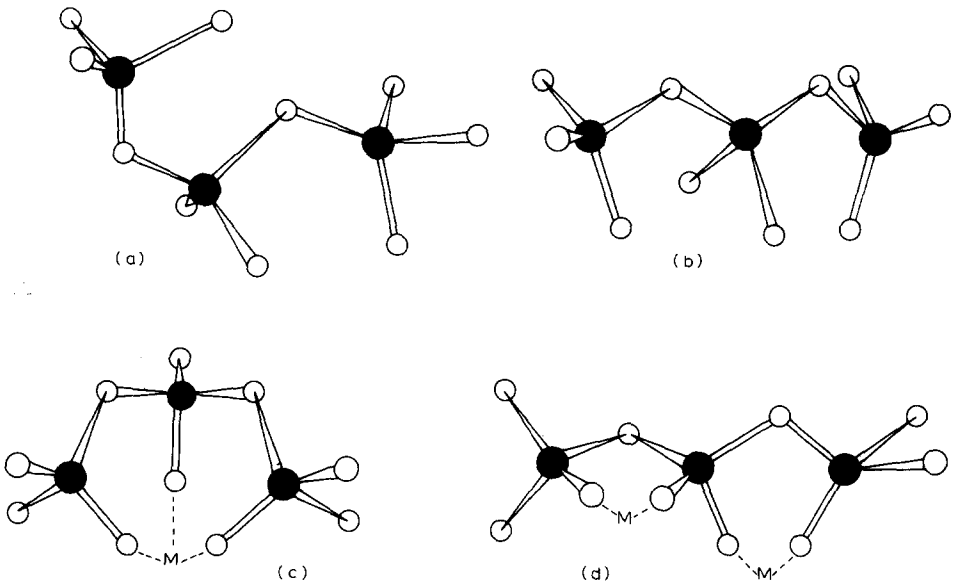

Fig. 3. Triphosphate chain conformations (adenine and ribose moieities not shown). (a) and (b), probable conformations for uncomplexed triphosphate chains in aqueous solution (see text); (c) suggested predominant structure for a 1 : 1 Mg^{2+}-triphosphate complex; (d) suggested predominant structure for a 2 : 1 dimagnesium-triphosphate complex.

time scale, between more than one structure). We support their view that interaction involves P_α and P_γ, but suggest that on steric grounds a further interaction with P_β is likely, giving a tridentate complex as in Fig. 3c. Further to their evidence, we note that (i) the conformation is then most dissimilar from the staggered chain conformations of Fig. 3 a, b. The latter are likely for the uncomplexed triphosphates, since they achieve greater separation between the negatively charged oxygen atoms (the *anti-gauche* conformation of Fig. 3a occurs in the solid state for certain complexes of ATP, and the *anti-anti* conformation of Fig. 3b similarly for tripolyphosphate [17]. (ii) Cleland and coworkers have shown that 1 : 1 complexes Cr(III)ATP [18] and Co(III)ATP [19] are found in aqueous solution as a mixture of kinetically stable coordination isomers, with tridentate forms predominating over bidentate forms.

With regard to the 2 : 1 complexes, we suggest that the structure shown in Fig. 3d is predominant. This is the only possible scheme in which the two magnesium ions are each bound to two phosphate positions, one to $P_\alpha P_\beta$ and the other to $P_\beta P_\gamma$, (thereby modifying the proposed binding of the first Mg^{2+}). There are two pieces of evidence that indirectly support this. Firstly, the proposed binding scheme requires a major change in conformation from that of the 1 : 1 complex, with some restoration towards the extended chain forms of Fig. 3 a, b (and incidentally providing a reasonable separation between the bound magnesium ions). This is consistent with our chemical shift results. In the case of P_α and P_γ, the shifts of the uncomplexed triphosphate are fully restored in the 2 : 1 complex, suggesting a very similar conformational environment. In the case of P_β, however, only partial restoration occurs. This is consistent with a more strained conformation for P_β as expected from Fig. 3d in com-

parison with Fig. 3 a, b. Secondly, there is consideration of stability constants. Typical room-temperature values for the interaction of a single Mg^{2+} with a doubly-charged monophosphate are $K_d^{-1} = 89$ or 63 M^{-1} ($Mg^{2+} + AMP^{2-}$). Our present values for stability of the 2 : 1 triphosphate complexes from (330 to 110 M^{-1}) are similar to the above, and might seem to suggest binding of the second magnesium ion via one phosphate position only. However, the latter scheme should lead to substantially weaker binding than observed, due to repulsion between the two bound magnesiums. On the assumption that this will not be too different from that between free magnesium ions, we estimate the energy of repulsion at a separation of 5 Å (as in our model, Fig. 3 d) in an aqueous environment to be 12 kJ · (g atom pair)$^{-1}$. This is equivalent to two orders-of-magnitude decrease in K_d^{-1} for removal of the second Mg^{2+} from the complex. (Even at maximum possible separation of opposite ends of a fully extended triphosphate chain, we would still predict one order-of-magnitude decrease in K_d^{-1}). Furthermore our model implies a weakening in binding of the first magnesium ion on reorganisation to bind the second Mg^{2+}, and this would be reflected in a further decrease in the K_d^{-1} of the 2 : 1 complex. The observed K_d^{-1} value for the 2 : 1 complex is therefore higher than is compatible with binding of the second Mg^{2+} via a single phosphate position.

Finally, we may note that the difference of some three powers of ten between the observed K_d^{-1} values for the 1 : 1 and 2 : 1 triphosphate complexes (i.e. a difference in ΔG^0 values of about 18 kJ · mol^{-1}) is compatible with the above ion repulsion and reorganisation energy terms plus an unevaluated entropy term. We could also note that the observation of coincident resonances from P_α and P_γ in tripolyphosphate cannot be taken unsupported as evidence for symmetrical binding in the 1 : 1 or 2 : 1 complexes, since fast exchange involving unsymmetrical sites could produce the same result.

The ^{31}P spin-lattice relaxation times given in Table III suggest involvement of the whole length of the triphosphate chain on binding to Mg^{2+}, and the curve fitting in Fig. 2 supports the K_d^{-1} values given for the 2 : 1 complexes. Shortening of T_1 values on binding to Mg^{2+} is to be expected from restriction of segmental motion of the chain, causing a lengthening of the correlation time for dipolar relaxation. The further shortening of T_1 values on binding a second magnesium ion can at least be rationalized in terms of our binding scheme for the complexes. First, the rotational correlation time for the extended and conformationally rigid 2 : 1 structure of Fig. 3 d will be longer than that of the more compact 1 : 1 structure of Fig. 3c. Furthermore, the T_1 value reported for the 1 : 1 complexes is probably a weighted average value with contributions from more flexible didentate structures.

Cleland, Mildvan and co-workers ([20] and references therein) have extensively studied the systems Cr(III)ATP and Co(III)ATP, in which the coordination isomers are kinetically stable. ^{31}P chemical shift data were obtained for the diamagnetic Co(III) system [19]. For a number of kinase enzymes Cr(III)ATP is an active substrate in lieu of $MgATP^{2-}$, but in each case activity was restricted to a $\beta\gamma$-bidentate isomer rather than the predominant $\alpha\beta\gamma$-tridentate forms, even though the latter usually bind as well or better to the enzyme [18, 21]. By analogy it was inferred that the active substrate in vivo is $\beta\gamma$-bidentate $MgATP^{2-}$. On the basis of the binding data for the Cr(III)ATP complexes it

might be postulated that generally the tridentate $MgATP^{2-}$ form (in our analysis the predominant isomer in solution) is the form that binds to enzymes, whereupon it then isomerises to an active β,γ isomer. Some support for this postulate is given by our proposed linear structure for the Mg_2ATP form. This does not bind to at least two enzymes, nitrogenase and yeast hexokinase, and therefore presumably its linear conformation is unsuitable for binding. The β,γ structure is more linear than the tridentate structure and this may also not be favourable for binding.

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