Enhanced Stability of Ternary Metal Ion/Adenosine 5'-Triphosphate Complexes.[1] Cooperative Effects Caused by Stacking Interactions in Complexes Containing Adenosine Triphosphate, Phenanthroline, and Magnesium, Calcium, or Zinc Ions

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Abstract: The stability constants of the mixed-ligand complexes of ATP, 1,10-phenanthroline (phen), and Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, or Zn^{2+} (M^{2+}) have been determined by potentiometric titration. Changes in the stabilities are quantified by $\Delta \log K_M$ = $\log K^{M(phen)}_{M(phen)(ATP)}$ − $\log K^{M}_{M(ATP)}$, corresponding to the equilibrium $M(ATP)^{2-} + M(phen)^{2+} \rightleftharpoons M(phen)(ATP)^{2-} + M^{2+}$. For the Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, and Cu^{2+} systems $\Delta \log K_M$ is 0.6, 0.4, 0.22, 0.12, and 0.05, respectively. The stability enhancement in the Ca^{2+} and Mg^{2+} systems has also been determined more precisely by ultraviolet-difference spectroscopy, $\Delta \log K_M = 0.53$ and 0.55, respectively. All these ternary complexes are much more stable than expected on statistical grounds; in fact, the observation of *positive* values for $\Delta \log K_M$ means that ATP^{4-} binds more tightly to $M(phen)^{2+}$ than to M^{2+}. The formation of stacked adducts between phen and adenosine or ATP has been observed by difference spectroscopy and by ^{1}H NMR and the stability of these adducts has been determined. ^{1}H NMR of the phen/ATP^{4-}/Mg^{2+} and phen/ATP^{4-}/Zn^{2+} systems confirms the presence of stacking in the ternary complexes. This stacking causes the increased stability of the ternary Mg^{2+} and Ca^{2+} complexes.

As many enzymes require activation by metal ions[3] and as this often involves a ternary enzyme/substrate/metal ion complex,[4] ternary metal ion complexes have received considerable attention in recent years.[5] Ternary Cu^{2+} complexes are the best studied group,[6] and relatively little work has been done on ions of other transition metals,[7-9] the lanthanides,[10] zinc,[8,11] cadmium,[12] mercury,[13] aluminium,[14,15] and lead.[15] Although ternary Ca^{2+} complexes have been reported,[15,16] the group I and II metal ions have largely been ignored. These metal ions play major biochemical roles,[17,18] for example, Na^+ and K^+ in nerves,[17] Ca^{2+} in muscles, and Mg^{2+} in energy transfer and storage as high-energy phosphates.[3] Yet despite the requirement[3] of Mg^{2+} by most enzymes which use ATP[19] and which involve ternary enzyme/ATP/Mg^{2+} complexes,[20] no studies of the stability of low molecular weight ternary complexes containing Mg^{2+} and ATP have been reported, although the temperature-jump method has been used in a kinetic study[21] of ternary complexes containing 8-hydroxyquinoline, Mg^{2+}, and ATP.

Measurement of stability constants of ternary complexes of ATP, a transition metal ion, and 2,2'-bipyridyl,[22] or tryptophan[23] showed that these complexes are rather stable. Stacking between the purine moiety of ATP and bipyridyl or tryptophan in the ternary complexes $Zn(ATP)(bpy)^{2-}$ and $Zn(ATP)(trp)^{3-}$ has been observed by ^{1}H NMR.[23,24] Spectrophotometric studies[22-24] indicate that adducts between a purine derivative and bipyridyl or tryptophan even occur in the absence of a metal ion, although these binary adducts are rather weak. Similar stacked complexes have been observed with manganese(II),[23] cobalt(II),[24] nickel(II),[24] copper-(II),[22-24] and zinc(II),[23,24] but Mg^{2+} and Ca^{2+} were not studied owing to the low stability of their complexes with bipyridyl. We now report evidence for the existence of, and the presence of stacking in, ternary complexes containing Mg^{2+} or Ca^{2+} and ATP; we used 1,10-phenanthroline as the second ligand as its complexes with group II metals are more stable. We also measured the stabilities of some complexes of Mn^{2+}, Cu^{2+}, or Zn^{2+} for comparison; Zn^{2+} is particularly important as it offers the possibility of studying the stacking interaction by ^{1}H NMR.

Experimental Section

Materials. Metal(II) perchlorates (purum) were obtained from Fluka AG, Buchs, Switzerland, and metal(II) nitrates (pro analysi) from Merck AG, Darmstadt, Germany; the concentrations of the metal stock solutions were determined by titration with EDTA. $NaClO_4$ and 2,2'-bipyridyl (purissimum) were also from Fluka AG; adenosine, 1,10-phenanthroline hydrate (p.a.), D_2O (99.75%), $NaNO_3$ (p.a.), and 10% tetramethylammonium hydroxide solution were from Merck AG. The disodium salt of ATP was from Serva Feinbiochemica GMBH, Heidelberg, Germany (for specifications see ref 22).

Determination of Equilibrium Constants by Potentiometric Titrations. Under our experimental conditions $[H_2(phen)]$ is insignificant ($pK^{H}_{H_2(phen)} = -1.6$).[25] The acidity constant $K^{H}_{H(phen)}$ was determined from pairs of automatic titrations under N_2 of aqueous solutions (25 mL) containing 0.0018 M $HClO_4$ and $NaClO_4$ ($I = 0.1$) in the presence and absence of 0.0014 M phen with 0.05 M NaOH (25 °C), performed with a Metrohm potentiograph E 336 and a UX glass electrode.

The conditions for the determination of $K^{M}_{M(phen)}$ were the same as for the acidity constant, but $NaClO_4$ was partly replaced by $M(ClO_4)_2$ to give Ca^{2+} or Mg^{2+}:phen = 26:1, and Mn^{2+}:phen = 5:1 or 9:1. Titrations of solutions without phen were used as a basis for the evaluation. $K^{M}_{M(phen)}$ was calculated considering the species $H_2(phen)^{2+}$, $H(phen)^+$, phen, M^{2+}, and $M(phen)^{2+}$.[26] As our values for $K^{H}_{H(phen)}$ and $K^{Mn}_{Mn(phen)}$ agree well with those of Anderegg,[27] we used his values of the Cu^{2+} and Zn^{2+} phenanthroline 1:1 and 1:2 complexes in our calculations for the mixed-ligand systems phen/Cu^{2+}/ATP and phen/Zn^{2+}/ATP.

The stability constants of the binary and ternary ATP^{4-} complexes were measured and calculated in the way described recently,[24] except that for Cu^{2+} and Zn^{2+} $[M^{2+}] = 0.0012$ M. For Mg^{2+}, Ca^{2+}, or Mn^{2+} $[M^{2+}] = 6 \times 10^{-4}$ M, and the titrations were carried out with 0.025 M NaOH. Under these conditions, and with Mn^{2+}:phen:ATP = 1:1:1, and Ca^{2+} or Mg^{2+}:phen:ATP = 1:2:1 and 1:3:1 no precipitate formed. $K^{M}_{M(ATP)}$ and $\beta^{M}_{M(phen)(ATP)}$ were calculated as before.[24] In the mixed-ligand systems containing Mg^{2+} or Ca^{2+} the degree of formation of the ternary complex $M(phen)(ATP)^{2-}$ was low, 2-12% for Mg^{2+} and 2-5% for Ca^{2+}; this accounts for the relatively large errors (cf. Table I).

Determination of the Stability Constants of 1,10-Phenanthroline-Purine Adducts by Spectrophotometric Measurements. Absorbance spectra were recorded with a Varian Techtron spectrophotometer

Table I. Logarithms of the Stability Constants (25 °C; $I = 0.1$; NaClO$_4$) of the Ternary Complexes and of the Binary Complexes M(ATP)$^{2-}$ and M(phen)$^{2+}$, Determined by Potentiometric Titrations[a,b] or Spectrophotometry[b]

M^{2+}	Log $K^M_{M(ATP)}$	Log $K^M_{M(phen)}$	Log $\beta^M_{M(phen)(ATP)}$	Log $K^{M(phen)}_{M(phen)(ATP)}$	Log $K^{M(ATP)}_{M(ATP)(phen)}$	Δ log K_M
None					1.19 ± 0.02[c,e]	
Ca^{2+}	3.88 ± 0.02	1.11 ± 0.02	5.63 ± 0.18	4.52	1.75[g]	+0.6[d]
		1.11 ± 0.02			1.64 ± 0.10	+0.53[e]
Mg^{2+}	4.24 ± 0.02	1.45 ± 0.02	6.10 ± 0.12	4.65	1.86[g]	+0.4[d]
		1.47 ± 0.02			2.02 ± 0.02	+0.55[e]
Mn^{2+}	4.91 ± 0.02	4.01 ± 0.01	9.04 ± 0.04	5.03	4.13[g]	+0.12[d]
Zn^{2+}	5.10 ± 0.03	6.55[f]	11.87 ± 0.04	5.32	6.77[g]	+0.22[d]
Cu^{2+}	6.03 ± 0.03	9.25[f]	15.33 ± 0.02	6.08	9.30[g]	+0.05[d]

[a] Acidity constants of ATP: $pK^H_{H_2(ATP)} = 4.06$ (from M. M. T. Khan and A. E. Martell, *J. Am. Chem. Soc.*, **88**, 668 (1966); see also C. F. Naumann, B. Prijs, and H. Sigel, *Eur. J. Biochem.*, **41**, 209 (1974)), $pK^H_{H(ATP)} = 6.42 ± 0.01$. [b] The errors given are three times the standard error of the mean value, or the sum of the probable systematic errors, whichever is the larger. [c] Log $K^{(aden)}_{(aden)(phen)} = 1.33 ± 0.05$ (cf. footnote e). [d] By potentiometric titration. [e] By spectrophotometry. [f] $T = 20$ °C, $I = 0.1$, NaNO$_3$.[27] [g] Calculated from log $\beta^M_{M(phen)(ATP)}$ and log $K^M_{M(ATP)}$ using eq 5.

(Model 635) connected to a Honeywell recorder (Model 196). One cell in the reference beam contained equimolar metal perchlorate and ATP^{4-} and the other contained phenanthroline; one cell in the sample beam contained the mixed system and the other water. All four cells contained NaClO$_4$ to maintain $I = 0.1$, and also 10^{-3} M phosphate buffer[28] to stabilize the pH ($\sim$8), which was measured with a Metrohm potentiometer E 353 B or E 510 using a UX glass electrode. The constants for the binary phen/adenosine and phen/ATP^{4-} systems were determined using the same conditions, but omitting Mg^{2+} or Ca^{2+}; similarly in the experiments on the binary Mg^{2+}/phen or Ca^{2+}/phen systems, ATP^{4-} was absent.
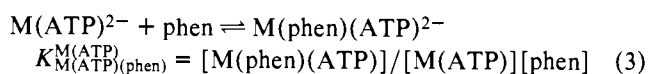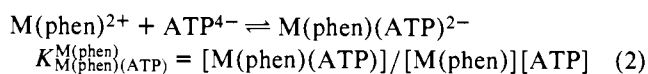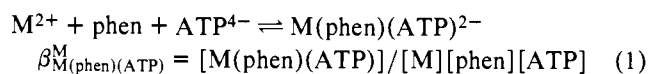
As the absorbance of the reference solution of phen is rather high, a low concentration (10^{-4} M) was used so that stray light did not affect the measured absorbance. As the total absorbance of the reference solutions was kept below 1.0, the differential absorbance is usually small, 0.01 to 0.1 A. Considerable care is then required in the choice of cells, the preparation and filtration of solutions, and the measurement of cell blanks. However the use of such dilute solutions has the advantage that stray light, which has been observed to have a marked effect on extinction coefficients obtained by difference spectroscopy,[24] and which can also affect the value of the stability constant somewhat,[29] is minimized. A slit width setting of "1.0 nm" was used: changing the setting only altered the noise level.

The stability constants were calculated by the Benesi-Hildebrand method.[30] The weak self-association[31] of nucleosides or nucleotides was negligible.

^{1}H NMR Measurements. ^{1}H NMR spectra were recorded on D$_2$O solutions using a Varian Anaspect EM-360 spectrometer (60 MHz) or a Bruker WH-90 FT spectrometer (90.025 MHz) using the center peak of the tetramethylammonium triplet as reference; all chemical shifts were converted to a trimethylsilylpropanesulfonate reference by adding 3.188 ppm. The use of sodium 3-(trimethylsilyl)propanesulfonate as reference in the presence of Zn(phen)$^{2+}$ is unreliable as hydrophobic interactions between the trimethylsilyl group and the aromatic moiety shift the trimethylsilyl resonance considerably.[32] The pD values were obtained by adding 0.4 to the pH meter reading.[33]

Results and Discussion

Equations 1–3 define the stability constants for the mixed-ligand systems. The overall stability constant $\beta^M_{M(phen)(ATP)}$ obtained from the titration results is related to the constants $K^{M(phen)}_{M(phen)(ATP)}$ and $K^{M(ATP)}_{M(ATP)(phen)}$ by eq 4 and 5, respectively.

$$M^{2+} + phen + ATP^{4-} \rightleftharpoons M(phen)(ATP)^{2-}$$

$$\beta^M_{M(phen)(ATP)} = [M(phen)(ATP)]/[M][phen][ATP] \quad (1)$$

$$M(phen)^{2+} + ATP^{4-} \rightleftharpoons M(phen)(ATP)^{2-}$$

$$K^{M(phen)}_{M(phen)(ATP)} = [M(phen)(ATP)]/[M(phen)][ATP] \quad (2)$$

$$M(ATP)^{2-} + phen \rightleftharpoons M(phen)(ATP)^{2-}$$

$$K^{M(ATP)}_{M(ATP)(phen)} = [M(phen)(ATP)]/[M(ATP)][phen] \quad (3)$$

$$\log K^{M(phen)}_{M(phen)(ATP)} = \log \beta^M_{M(phen)(ATP)} - \log K^M_{M(phen)} \quad (4)$$

$$\log K^{M(ATP)}_{M(ATP)(phen)} = \log \beta^M_{M(phen)(ATP)} - \log K^M_{M(ATP)} \quad (5)$$

One way[34] to quantify the increase or decrease in the stability of mixed-ligand complexes compared with the binary complexes is through eq 6 and 7.[6] Equation 6, the comparison

$$\Delta \log K_M = \log K^{M(phen)}_{M(phen)(ATP)} - \log K^M_{M(ATP)} \quad (6)$$

$$= \log K^{M(ATP)}_{M(ATP)(phen)} - \log K^M_{M(phen)} \quad (7)$$

of the coordination of ATP to free M^{2+} and to M(phen)$^{2+}$ for the *experimental* determination of Δ log K_M, is more appropriate for Cu^{2+} and Zn^{2+} for which the phen complex is more stable than that of ATP; eq 7, the comparison of the coordination of phen to free M^{2+} and to M(ATP)$^{2-}$, is more useful with Mg^{2+} and Ca^{2+} which form fairly stable ATP complexes but only weak phen complexes.

Although Δ log K_M is the same as the equilibrium constant for eq 8, the low value of Δ log K_M ($\sim$0 ± 0.5) for most sys-

$$M(ATP)^{2-} + M(phen)^{2+} \rightleftharpoons M(phen)(ATP)^{2-} + M^{2+} \quad (8)$$
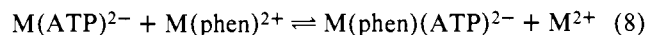
tems[2,6] means that very high concentrations of M(ATP)$^{2-}$ and M(phen)$^{2+}$ would be required for direct measurement of Δ log K_M.

Potentiometric Determination of the Stability of the Ternary Metal Ion/ATP/Phenanthroline Complexes. The stability constants for the ternary complexes M(phen)(ATP)$^{2-}$ (M = Ca, Mg, Mn, Cu, Zn) and those of M(phen)$^{2+}$ (M = Ca, Mg, Mn) are given in Table I. The redetermined values for the binary ATP complexes agree well with those reported earlier;[35] in any experiment on a nucleoside 5'-triphosphate designed to determine Δ log K_M, it is preferable that the stability constants of the binary and ternary systems be determined on the same batch of ligand under identical conditions as experimental differences can cause errors[36] large enough to mask Δ log K_M.

Since more coordination positions are available for bonding by the first ligand to a hydrated metal ion than for the second ligand, Δ log K_M is expected to be negative. Consideration of the statistics for the coordination of two different bidentate ligands to an octahedral (oh) coordination sphere leads to an expected value of Δ log $K_{oh} = -0.4$; for any other stereochemistry, e.g., the distorted octahedron of Cu^{2+}, Δ log K is even lower (cf. ref 6). Negative values for Δ log K_M are usually observed, and are often about the same as expected statistically, in the absence of electronic effects[6] or of specific interactions between the two ligands.[2,23] For all the metal ions we used the ternary complexes are more stable than expected; for Mg^{2+} and Ca^{2+} this is strikingly so. However, the weakness of these complexes and the resultant low formation degree (see Experimental Section) render Δ log K_M somewhat uncertain. The increase in stability observed here is larger (Δ log K_M is more positive) for those metals (Mg^{2+} and Ca^{2+}) which form weaker
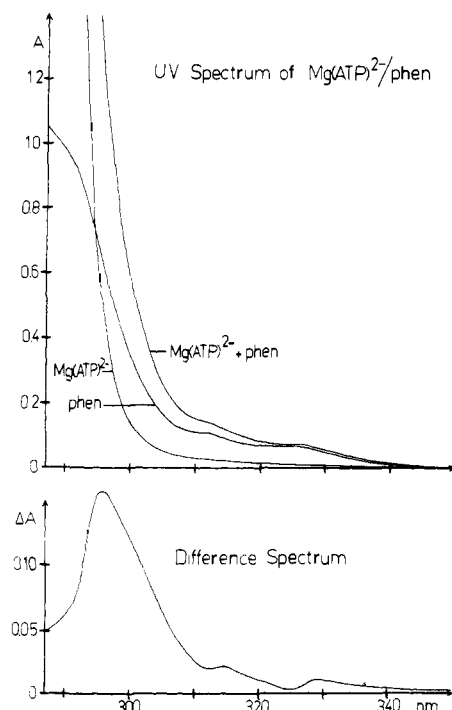
Figure 1. Ultraviolet absorption spectra of a mixture of Mg(ATP)$^{2-}$ (0.02 M) and phenanthroline (10^{-4} M), of Mg(ATP)$^{2-}$ (0.02 M), and of phenanthroline (10^{-4} M). Difference spectrum of a mixture of Mg(ATP)$^{2-}$ (0.02 M) and phenanthroline (10^{-4} M) measured against Mg(ATP)$^{2-}$ (0.02 M) and phenanthroline (10^{-4} M) in separate cuvettes.

complexes, than for Mn^{2+}, Cu^{2+}, and Zn^{2+}, which form more stable complexes. This is probably due to an interaction between the metal and N(7) of ATP in the binary complexes M(ATP)$^{2-}$ which enhances their stability somewhat;[36] NMR studies on the ternary complexes indicate that this interaction is absent.

Spectrophotometric Determination of the Stability of Binary Phenanthroline/Purine Derivative and Ternary Phenanthroline/ATP/Metal Ion Complexes. For the weaker complexes of Mg^{2+} and Ca^{2+} the low formation degree of the ternary complex makes the stability constant determination by potentiometric titration less accurate; conversely, determination by spectrophotometric measurement becomes easier.

The difference spectrum of Mg(phen)$^{2+}$ measured against phen shows peaks at 291 and 270.5 nm and subsidiary maxima at 326 and 312 nm; for Ca^{2+}/phen the spectrum is similar. The reciprocal of the differential absorbance at any of these wavelengths, plotted against the reciprocal of [Mg^{2+}], gives log $K^{Mg}_{Mg(phen)}$ = 1.47; log $K^{Ca}_{Ca(phen)}$ = 1.11 was obtained in the same way: these values agree very well with those obtained by potentiometric titration. The ternary system is more complicated, but if ATP^{4-} is completely complexed by Mg^{2+} or Ca^{2+}, and assuming that only the equilibrium M(ATP)$^{2-}$ + phen $\rightleftharpoons$ M(phen)(ATP)$^{2-}$ is affected by the changing concentrations, the Benesi–Hildebrand method is usable. Thus, measurement of the difference spectrum of a mixture of M(ATP)$^{2-}$ and phen against M(ATP)$^{2-}$ and phen in separate cuvettes, with varying [M(ATP)$^{2-}$], and evaluation by the Benesi–Hildebrand method give the stability constants. These, and the values of $\Delta \log K_M$, are summarized in Table I. The values of $\Delta \log K_M$ are large for both Mg^{2+} and Ca^{2+}, and agree well with the less precise results obtained by potentiometric titrations.

The spectrum of the ternary mixture Mg(ATP)$^{2-}$/phen, when compared with that of the components Mg(ATP)$^{2-}$ and phen, shows only a slight shift to lower energy, which is re-

sponsible for the observed small difference spectrum (Figure 1). A similar slight shift and the resulting difference spectrum are also observed in the binary systems aden/phen and ATP^{4-}/phen; the Benesi–Hildebrand plots for these binary systems are also linear, indicating 1:1 complex formation with log $K^{(aden)}_{(aden)(phen)}$ = 1.33 and log $K^{(ATP)}_{(ATP)(phen)}$ = 1.19. In these binary systems no interaction other than aromatic stacking is likely and stacking has already been suggested[22] for the aden/bpy and ATP^{4-}/bpy systems. As the reference beam absorbance in the earlier work[22] on bipyridyl (8 × 10^{-4} M) was extremely high ($A_{293} \simeq 6$) the difference spectra of the aden/bpy system were remeasured using a lower bipyridyl concentration (10^{-4} M); a Benesi–Hildebrand plot gave log $K^{(aden)}_{(aden)(bpy)}$ = 1.3, in good agreement with the earlier value of 1.36 ± 0.06, although the absorption maximum was shifted and the difference extinction coefficient at the maximum (ϵ_{298} ~2000) is much higher than that found[22] earlier (ϵ_{293} 720 ± 160). The stabilities of the binary stacked adducts (aden)-(phen) and (aden)(bpy) are thus very similar, as are (phen)-(ATP)$^{4-}$ and (bpy)(ATP)$^{4-}$. The concentration of the stacked adduct between ATP and phenanthroline is increased considerably by the bridging of the phosphate chain of ATP^{4-} and the N-donor atoms of phen by coordination of Mg^{2+} or Ca^{2+} simultaneously.

For other ternary systems, e.g., with Zn^{2+} and Cu^{2+}, accurate determination of the stability of the ternary complexes with phenanthroline by spectrophotometric measurements is extremely difficult, as such a complicated series of equilibria occur (see the discussion of the NMR spectra of the ternary systems).

^{1}H NMR Studies of the phen/ATP^{4-} and Related Binary Systems. As we wished to use ^{1}H NMR to confirm stacking in these systems, the known self-association of ATP^{4-} had to be considered. Although self-association causes a small shift in the NMR spectrum of adenosine, the stability constant (log K = 0.6) is very low.[31] We also find that the NMR spectrum of ATP^{4-} is almost independent of concentration at [ATP^{4-}] ≤ 10^{-2} M; at higher concentrations considerable changes occur.[37] The concentration dependence of the ^{1}H NMR spectrum of phenanthroline is much more pronounced,[37] and thus shifts of the phenanthroline resonances are less useful as a quantitative indicator of stacking.
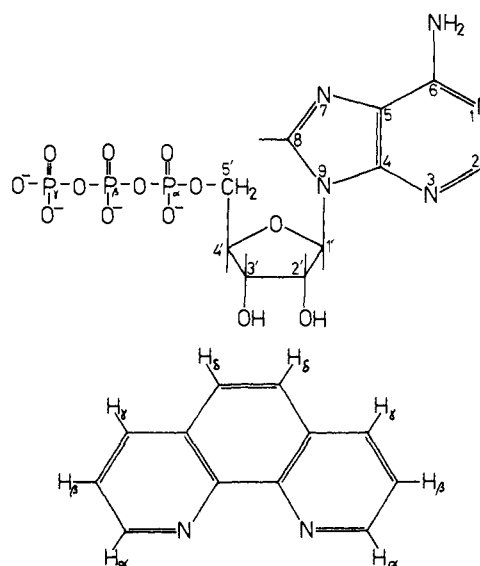
The adenine protons H(2) and H(8) and the ribose protons H(1′), H(2′), H(3′), and H(4′) of adenosine and of ATP^{4-} are shifted upfield in the presence of phenanthroline (Table II). The spectra of ATP^{4-}, and of equimolar ATP^{4-}/phen, are shown in Figure 2. The upfield shifts of these protons are far
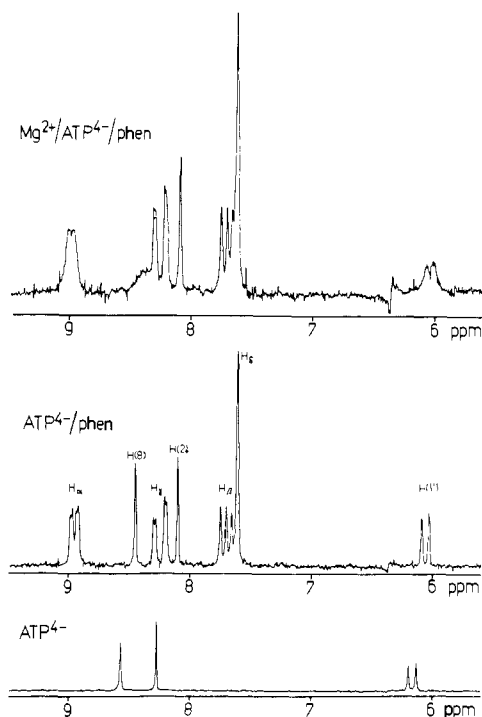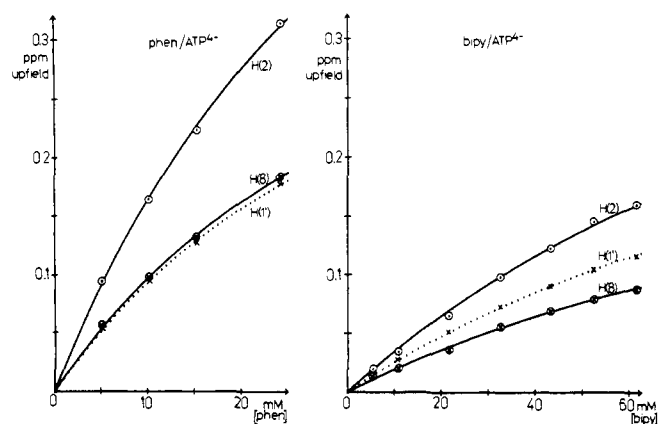
Figure 2. ^{1}H NMR spectra run on a Bruker WH-90 FT spectrometer at 90.025 MHz at 27 °C, I = 0.1 (NaNO$_3$ in D$_2$O), pD = 7.4, measured relative to (CH$_3$)$_4$N$^+$NO$_3^-$ (0.01 M) and converted to parts per million relative to sodium trimethylsilylpropanesulfonate using the chemical shift of (CH$_3$)$_4$N$^+$NO$_3^-$, δ = 3.188 ppm: of ATP^{4-} (0.01 M); ATP^{4-} (0.01 M) and phen (0.01 M); and ATP^{4-} (0.01 M), phen (0.01 M), and Mg^{2+} (0.004 M).

Figure 3. Upfield shifts of the resonances of H(1') (X), H(2) (○), and H(8) (⊗) of ATP^{4-} (0.005 M) in the presence of increasing concentrations of phenanthroline and of bipyridyl, compared with the resonance positions in the same concentration of ATP^{4-} itself (90.025 MHz; 27 °C; I = 0.1; NaNO$_3$; pD = 8.5). The curves shown are the computer calculated best fit of the experimental data.

greater than are observed in the presence of the same concentration of bipyridyl, although this also shifts H(2), H(8), H(1'), and H(2') somewhat (Table II).

Although the low solubility of phen limits the concentration range usable in a measurement of the stability constant of (phen)(ATP)$^{4-}$ by NMR, computer fitting (Figure 3) of the upfield shifts of H(1'), H(2), and H(8) of ATP^{4-} as [phen] is increased gives a stability constant of log $K_{(ATP)(phen)}^{(ATP)}$ = 1.45 ± 0.07 (27 °C; I = 0.1; NaNO$_3$ in D$_2$O). This is significantly higher than the spectrophotometric value, log K = 1.19 ± 0.02; the difference may be due to the presence of self-stacked ATP moieties at the high concentrations necessary for spectrophotometric measurement, or to the resulting high ionic strength. Similar measurement of the upfield shift of the resonances of ATP^{4-} as [bpy] is increased (Figure 3) gives a stability constant log $K_{(ATP)(bpy)}^{(ATP)}$ = 0.91 ± 0.14 (27 °C; I = 0.1; NaNO$_3$ in D$_2$O) which agrees well with the value found[22] by spectrophotometric measurement (0.91 ± 0.22). The limiting values of the upfield shifts of H(1'), H(2), and H(8) in the phen/ATP^{4-} system, 0.455, 0.803, and 0.472 ppm, are larger than those observed for bpy/ATP^{4-}, 0.357, 0.496, and 0.268 ppm, respectively. This larger shift with phen is to be expected as the effects of the ring currents in phen are to some extent additive,[38] although the relative orientation of the adenine ring system and the aromatic heterocyclic ring system also have an effect. The main cause of the much larger upfield shift with phen compared with bpy (see Table II and Figure 3) is the higher concentration of the stacked adduct resulting from the larger stability constants.

At 35 °C, using a Varian EM 360, the shifts of H(1'), H(2), and H(8) in the phen/ATP^{4-} system were smaller than at 27 °C, corresponding to a lower formation degree for (phen)-(ATP)$^{4-}$ of ~10% compared with 19% at 27 °C. At 35 °C, therefore, the stability constant must be lower, log $K_{(phen)(ATP)}^{(phen)}$ ≃ 1.15 compared with log $K_{(phen)(ATP)}^{(phen)}$ = 1.45 at 27 °C. Similar

Table II. ^{1}H NMR Chemical Shifts Observed for the Stacking between the Purine Moiety of Adenosine or of ATP^{4-} and 2,2'-Bipyridyl or 1,10-Phenanthroline

| Proton | Upfield shift[a] (ppm) in the system | | | |
	aden/ bpy	aden/ phen	ATP^{4-}/ bpy	ATP^{4-}/ phen[b]
Adenine moiety				
H(2)	0.041	0.196	0.031	0.163
H(8)	0.029	0.143	0.015	0.104
Ribose moiety[c]				
H(1')	0.030	0.111	0.029	0.091
H(2')	0.034	0.070	0.016	0.059
H(3')	0.013	0.030	0	0.029
H(4')	0.010	0.032	0.009	0.013
H(5')[d]	{0.008	0.028	0	0
	{0.011	0.024	0	0

[a] Measured in 10^{-2} M solution in D$_2$O on a Bruker WH 90 FT spectrometer at 90.025 MHz at 27 °C, I ≃ 0.1 (NaNO$_3$), pD ~8.5 using (CH$_3$)$_4$N$^+$NO$_3^-$ (2.5 × 10^{-3} M) as reference, compared with the resonances of adenosine or of ATP^{4-} alone, under the same conditions. [b] Measured at pD ~8. [c] The ribose resonances were assigned as in I. Feldman and R. P. Agarwal, *J. Am. Chem. Soc.*, **90**, 7329 (1968). [d] The two protons H(5') are inequivalent.

decreases in the upfield shift occur for the other systems; the small changes precluded accurate measurements.

It is thus clear that the direction and the relative sizes of the upfield shifts in these binary systems further confirm the presence of stacking between the aromatic heterocycle and the purine ring in all of the binary systems studied.

^{1}H NMR Studies of the Mg^{2+}/phen/ATP System. Protonation, or coordination of a diamagnetic metal ion, usually causes a downfield shift of the resonances of nearby groups, whereas in aromatic systems the ring current shifts those protons lying above an aromatic ring upfield.[38] Thus, the upfield shift of the ATP resonances in a mixture of 2,2'-bipyridyl and ATP on addition of Zn^{2+} confirmed[24] the presence of stacking in the ternary complex Zn(bpy)(ATP)$^{2-}$.

We therefore carried out a similar experiment using Mg^{2+}. On addition of small amounts (0.0025 to 0.005 M) of Mg^{2+} to a phen/ATP^{4-} mixture (each 0.01 M), the resonances of H(1') of the ribose and H(2) and H(8) of the purine part shifted upfield (Figure 2). However, the resonance of H(8) was broadened considerably and at higher [Mg^{2+}] (0.01 M) broadened so much that it was barely visible: H(1') was also broader at higher [Mg^{2+}] ($\Delta\nu_{1/2}$ = ~4 Hz at [Mg^{2+}] = 0.01

Table III. Overall Stability Constants β Used in the Computer Calculation of the Concentrations of the Species Present in the $Zn^{2+}/phen/ATP^{4-}$ and $Zn^{2+}/bpy/ATP^{4-}$ Systems (Figure 5)

Complex	Log β	Ref	Complex	Log β	Ref
$Zn(phen)^{2+}$	6.55	27	$Zn(bpy)^{2+}$	5.30	27
$Zn(phen)_2^{2+}$	12.35	27	$Zn(bpy)_2^{2+}$	9.83	27
$Zn(phen)_3^{2+}$	17.55	27	$Zn(bpy)_3^{2+}$	13.63	27
$Zn(ATP)^{2-}$	5.10	a			
$Zn(ATP)_2^{6-}$	6.1	b			
$Zn_2(ATP)$	8.1	43			
$Zn(phen)(ATP)^{2-}$	11.87	a	$Zn(bpy)(ATP)^{2-}$	10.56	35
$Zn(phen)_2(ATP)^{2-}$	16.97	c	$Zn(bpy)_2(ATP)^{2-}$	14.39	c
$(phen)(ATP)^{4-}$	1.19	a	$(bpy)(ATP)^{4-}$	0.91	22
$(phen)_2(ATP)^{4-}$	1.78	d	$(bpy)_2(ATP)^{4-}$	1.22	d

a This work. *b* Estimated assuming that log $K^{Zn(ATP)}_{Zn(ATP)_2} = 1.0$; this value is probably the upper limit, by comparison with results obtained[42] for the Ni^{2+}/ATP system. *c* Estimated assuming a statistical effect of -0.7 log unit.[6] *d* Estimated assuming a statistical effect of -0.6 log unit.
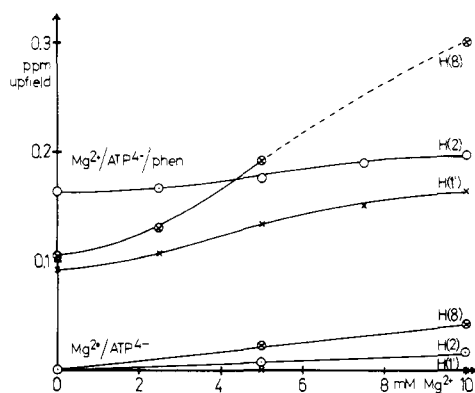
Figure 4. Upper curves: upfield shift of the resonances of $H(1')$ ($\times$), $H(2)$ ($\odot$), and $H(8)$ ($\otimes$) of ATP^{4-} in $phen/ATP^{4-}$ (each 0.01 M) compared with those of the protons in ATP^{4-} itself, with increasing $[Mg^{2+}]$; the broken line (- - -) indicates excessive broadening of the resonance of $H(8)$ and makes its exact position uncertain. Lower curves: shift of the resonances of $H(1')$ ($\times$), $H(2)$ ($\odot$), and $H(8)$ ($\otimes$) of ATP^{4-} (0.01 M) with increasing $[Mg^{2+}]$ (90.025 MHz; 27 °C; $I = 0.1$; $NaNO_3$; pD = 7.4).

M), but was easily measurable, and $H(2)$ remained sharp. The upfield shifts of the resonances of ATP as $[Mg^{2+}]$ is increased from zero to 0.01 M (i.e., to a $[phen]:[ATP^{4-}]:[Mg^{2+}]$ ratio of 1:1:1) are shown in Figure 4.

No broadening was observed in the binary Mg^{2+}/ATP system although earlier workers[39] reported broadening of $H(8)$ of ATP in $Mg(ATP)^{2-}$ and $Zn(ATP)^{2-}$ at lower pH. However, we also observed that at $[Zn^{2+}] > 0.008$ M the resonance of $H(8)$ in ATP (0.01 M) broadens considerably; this is presumably due to relatively slow exchange between free ATP and ATP bound to zinc, as at $[Zn^{2+}] = 0.02$ M the line sharpens again. This variation of line width was not mentioned by Glassman et al.[40]

Although the line broadening in the $Mg^{2+}/phen/ATP$ system hinders precise assessment of the extent of stacking in the mixed-ligand complex and of the mutual orientation of the two aromatic systems, the shifts of $H(1')$, $H(2)$, and $H(8)$, 0.166, 0.196, and ca. 0.28 ppm, respectively, confirm the stacking in $Mg(phen)(ATP)^{2-}$ and imply that $H(8)$ is located more centrally above the phen ring system than is $H(2)$.

^1H NMR Studies of the $Zn^{2+}/phen/ATP$ System. It was known[24] that when Zn^{2+} was added to a solution of equimolar amounts of ATP and bpy both $H(2)$ and $H(8)$ shift upfield to an increasing extent as $[Zn^{2+}]$ is increased. In the binary complex $Zn(ATP)^{2-}$ the signal of $H(8)$ is shifted downfield and that of $H(2)$ is shifted slightly upfield.[24,40]

Thus, we also investigated the effect of Zn^{2+} on phen/ATP^{4-}: the resonances of $H(1')$, $H(2)$, and $H(8)$ of ATP shift upfield from those in free ATP^{4-} by 0.388, 0.404, and 0.535

ppm, respectively, in $Zn^{2+}/ATP^{4-}/phen$ (each 0.01 M), confirming stacking in this ternary complex. Compared with resonances of the binary $Zn^{2+}/phen$ system the stacking in the ternary complex $Zn(phen)(ATP)^{2-}$ causes upfield shifts in the ternary system of 0.151, 0.408, 0.578, and 0.661 ppm for H_α, H_β, H_γ, and H_δ of phenanthroline.

However, the shifts of the resonances of ATP (0.01 M) as $[Zn^{2+}]$ is increased from zero to 0.01 M (Figure 5a) are much more complicated than those observed earlier[24] for the $Zn^{2+}/ATP/bpy$ system. Although the resonance of $H(1')$ shifts fairly uniformly with increasing $[Zn^{2+}]$, $H(2)$ and $H(8)$ change little until the $[Zn^{2+}]$ exceeds 0.005 and 0.002 M, respectively, then change suddenly, but level off in both cases above 0.008 M. Using a 60-MHz instrument a similar shape to that shown in Figure 5a was obtained.

Computer[41] calculation and plotting of the concentrations of all the species likely to be present, i.e., Zn^{2+}, $Zn(phen)^{2+}$, $Zn(phen)_2^{2+}$, $Zn(phen)_3^{2+}$, $Zn(ATP)^{2-}$, $Zn_2(ATP)$, $Zn(ATP)_2^{6-}$, $Zn(phen)(ATP)^{2-}$, $(phen)(ATP)^{4-}$, and $(phen)_2(ATP)^{4-}$, using the stability constants given in Table III, failed to explain the observed dependence of the shift of the resonance with $[Zn^{2+}]$. However, it seemed probable that $Zn(phen)_2(ATP)^{2-}$ would also be formed. As log $K^{Zn(phen)}_{Zn(phen)(ATP)} = 5.32$, on statistical grounds[6] one would predict that log $K^{Zn(phen)_2}_{Zn(phen)_2(ATP)} \simeq \log K^{Zn(phen)}_{Zn(phen)(ATP)} - 0.7$, i.e. 4.62.

From this follows the overall stability constant $\beta^{Zn}_{Zn(phen)_2(ATP)}$, listed in Table III, used in the calculation of the distribution curves shown in Figure 5b. The sum of the concentrations of all the species in which stacking can occur, $(phen)(ATP)^{2-}$, $(phen)_2(ATP)^{2-}$, $Zn(phen)(ATP)^{2-}$, and $Zn(phen)_2-(ATP)^{2-}$, is shown as curve 2 in Figure 5c.[44] This resembles the variation in the shift of $H(8)$ of ATP with increasing $[Zn^{2+}]$ quite well. An exact fit is not expected, as the upfield shift due to stacking is unlikely to be the same in all four species; however, it seems that this shift must at least be similar for these stacked complexes. Similarly, the sum of the concentrations of $(phen)(ATP)^{4-}$, $(phen)_2(ATP)^{4-}$, and $Zn(phen)(ATP)^{2-}$ (curve 3 in Figure 5c) somewhat resembles the variation of the shift of $H(2)$ with increasing $[Zn^{2+}]$. This implies that the shielding effect of the two phenanthrolines in $Zn(phen)_2(ATP)^{2-}$ on $H(2)$ of ATP is smaller than for the other three adducts.[45,46] The change in the shift of the resonance of $H(1')$ as the $[Zn^{2+}]$ is increased resembles the sum of the concentrations of all the species containing coordinated ATP (curve 1 in Figure 5c). However, the coordination of Zn^{2+} to the phosphate chain of ATP is not the only influence, as the resonance of $H(1')$ is considerably shifted by phenanthroline alone (i.e., by the presence of stacking) and as, although $H(1')$ is shifted upfield by Zn^{2+} alone, the upfield shift of $H(1')$ in the binary system (0.056 ppm) is much smaller than in the ternary system (0.388 ppm). Thus, $H(1')$ must also be in the
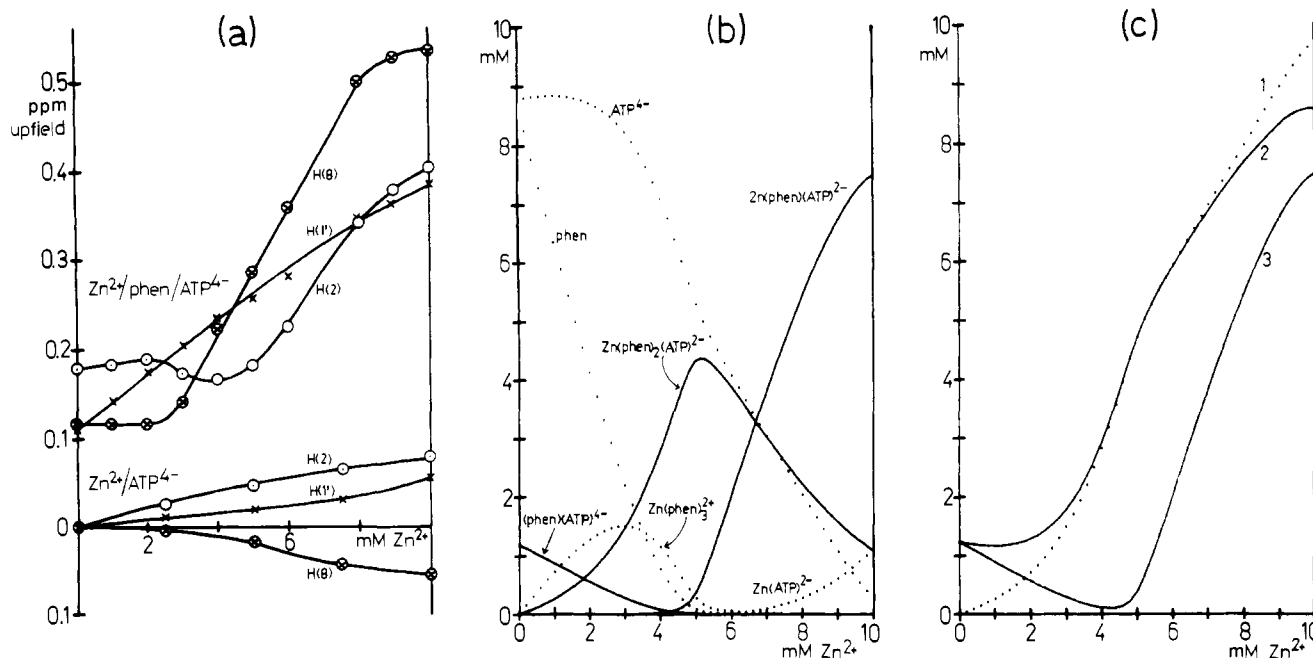
Figure 5. ^1H NMR of Zn^{2+}/phen/ATP^{4-}; experimental upfield shifts and corresponding calculated concentrations of stacked complexes. (a) Upper curves: upfield shift of the resonances of H(1') (×), H(2) (○), and H(8) (⊗) of ATP^{4-} in phen/ATP^{4-} (each 0.01 M) compared with the resonance positions of the protons in ATP^{4-} itself, with increasing $[Zn^{2+}]$. Lower curves: shift of the resonances of H(1') (×), H(2) (○), and H(8) (⊗), of ATP^{4-} (0.01 M) with increasing $[Zn^{2+}]$ (90.025 MHz; 27 °C; $I = 0.1$; $NaNO_3$; pD = 7.4). (b) Concentrations of the species present in phen/ATP^{4-} (each 10^{-2} M) as Zn^{2+} is added up to a maximum concentration of 10^{-2} M (calculated with the constants listed in Table III). The concentrations of free Zn^{2+} (max 3 × 10^{-5} M), $Zn(phen)^{2+}$ (max 1.2 × 10^{-4} M), $Zn(phen)_2^{2+}$ (max 9 × 10^{-5} M), $Zn(ATP)_2^{6-}$ (max 3 × 10^{-6} M), Zn_2ATP (max 3 × 10^{-5} M), and (phen)$_2(ATP)^{4-}$ (max 4 × 10^{-5} M) were too small to show in the figure. (c) Concentrations of the species present in phen/ATP^{4-} (each 10^{-2} M) as $[Zn^{2+}]$ is increased up to 10^{-2} M. (1) The total concentration of ATP^{4-} complexed to Zn^{2+} (i.e., $[Zn(ATP)^{2-}] + 2[Zn(ATP)_2^{6-}] + [Zn_2(ATP)] + [Zn(phen)(ATP)^{2-}] + [Zn(phen)_2(ATP)^{2-}]$). (2) The total concentration of "stacked" complexes (i.e., $[Zn(phen)(ATP)^{2-}] + [Zn(phen)_2(ATP)^{2-}] + [(phen)(ATP)^{4-}] + [(phen)_2(ATP)^{4-}]$). (3) The total concentration of "stacked" complexes, excluding $[Zn(phen)_2-(ATP)^{2-}]$.

vicinity of the phenanthroline. A possible structure for the stacked complex $Zn(phen)(ATP)^{2-}$ is shown in Figure 6.

Conclusions

The values of $\Delta \log K_M$ (Table I) clearly indicate an enhanced stability of the ternary complexes of the type $M(phen)(ATP)^{2-}$. This is especially evident for Mg^{2+} and Ca^{2+}. The enhanced stability of the ternary complexes with Mn^{2+}, Cu^{2+}, and Zn^{2+} may be partly due to stacking, and this has been discussed in detail recently;[2,6,22,24] however, this is not necessarily so, as with these metal ions the combination of a heterocyclic aromatic N ligand and an O-donor ligand such as a phosphate leads to increased stability of the mixed-ligand complexes, owing to electron transfer from the metal ion d orbitals to the π-electron accepting heterocyclic aromatic ligand.[47]

However, such effects cannot exist for the alkaline earth ions, and as the NMR measurements clearly confirm intramolecular stacking within these ternary complexes, this must be the cause of the enhanced stability. This stacking is mainly between the middle ring of phenanthroline and the five-membered imidazole ring of the purine moiety of ATP (cf. Figure 6). The NMR experiment clearly shows that stacking of the aromatic ligands occurs at least as much in the ternary Zn^{2+} complex as in that with Mg^{2+}, although the mode of phosphate coordination to zinc ions[39] may not be the same as to magnesium ions.[48] These cooperative effects are reflected in the formation degree of the complexes. For example, in a solution at pH 7.0 which contains $[Mg^{2+}] = 0.01$ M and $[phen] = 0.001$ M, 22% of the phenanthroline is bound to Mg^{2+}; if the solution also contains 0.01 M ATP^{4-} about 48% of the phenanthroline is bound in the ternary $Mg(phen)-(ATP)^{2-}$ complex.

One could take 1,10-phenanthroline as a simple model of
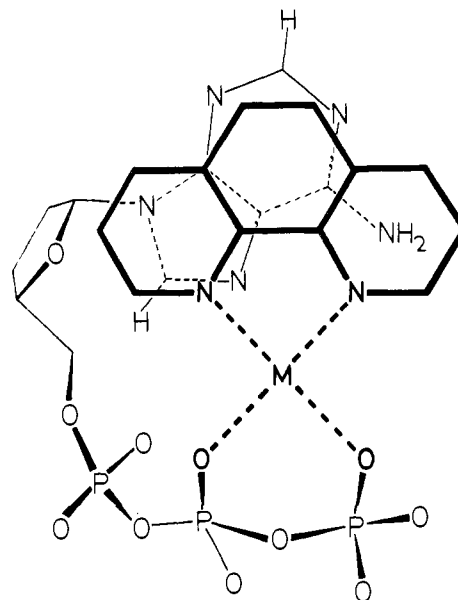
Figure 6. A possible structure for the ternary stacked complex $Zn(phen)-(ATP)^{2-}$.

an enzyme which binds more tightly to the substrate $Mg(ATP)^{2-}$ than to ATP^{4-} itself; the cooperation resulting from the ligand–ligand interaction resembles that observed for enzyme/substrate/metal ion systems and indicates that stacking may be the cause of some of the cooperative effects so often seen in natural systems. For example, arginine kinase binds $Mn(ADP)^-$ and $Mn(ATP)^{2-}$ more tightly than either Mn^{2+} or the nucleotides alone;[49] this increased stability of the

ternary complexes may be due to stacking[43] or to electronic effects.[2,6] Myosin only hydrolyzes ATP in the presence of Mg^{2+} or Ca^{2+}, and the ultraviolet difference spectrum of ATP with a fragment of myosin, heavy meromyosin, has a weak absorbance at about 290 nm which is enhanced[50] by Mg^{2+}; the native enzyme shows[51] a similar difference spectrum in the presence of Mg^{2+} and ATP. Modification of a tryptophan residue in heavy meromyosin indicates[52] that this difference spectrum is due to stacking between the purine residue of ATP and a tryptophanyl indole group in the active center of the enzyme. Indeed, intramolecular stacking occurs not only in the binary ATP/trp complex[53] but also in the ternary complexes M^{2+}/ATP/trp ($M = Mn^{2+}$, Cu^{2+}, or Zn^{2+});[23] it thus seems certain that Mg^{2+} and Ca^{2+} can also stabilize stacked adducts formed by more natural combinations than phenanthroline and ATP.

References and Notes

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(45) Further evidence for the existence of $Zn(phen)_2(ATP)^{2-}$ is hard to obtain either by potentiometric titration or by spectrophotometric measurements as the properties of $Zn(phen)_2(ATP)^{2-}$ and $Zn(phen)_2(ATP)^{2-}$ would be expected to be very similar.
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