EFFECT OF DIVALENT METAL ION BINDING ON THE RAMAN SPECTRUM
OF ATP IN AQUEOUS SOLUTION

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SUMMARY

The apparent dissociation constants for the secondary H^+ of $HATP^{-3}$, $CaHATP^{-1}$, $MgHATP^{-1}$ and $ZnHATP^{-1}$ were determined by studying the pH dependent frequency of a particular line in the Raman spectrum of ATP. We obtained the following values for the pK_a: 6.50 for ATP alone and 5.48, 4.43, 4.21 for the Zn, Mg and Ca complexes respectively. The Raman technique yields the above results more directly than methods previously employed. The order of binding strengths for the three cations to the triphosphate moiety inferred from the present results is reversed from earlier published data.

In order to understand the molecular mechanism of biological energy transfer reactions involving ATP hydrolysis, it is necessary to have a microscopic description of the interactions between ATP and the divalent metal ions such as Ca^{++}, Mg^{++}, since a complex of ATP with such ions is associated with enzyme driven hydrolysis.[1,2,3,4] While ATP hydrolysis involved in muscle contraction requires the presence of Mg^{++} or Ca^{++}, a special role for Zn^{++} has been observed in muscle relaxation.[5] The knowledge of relative binding properties of these metal ions to ATP is of great importance for any microscopic model of the ATPase active site. We present here the study of detailed effects of pH variation on a particular Raman line of ATP and its complexes with the divalent cations mentioned. We determine the apparent dissociation constant pK_a of the secondary triphosphate proton. The pK_a is obtained directly from the pH dependence of a line center frequency, rather than from comparison of amplitudes as in earlier IR measurements,[6] or from calculated fits to titration curves.[7,8] We also obtain the frequency shifts for this line caused by the

complexing with the divalent cations. Significantly the present results indi-
cate that the binding strength of the three ions to the triphosphate moiety
increases from Zn^{++} to Ca^{++} in reverse order to that previously obtained by
other methods. We reach this conclusion consistently from the pK_a values and
also from the value of the frequency shifts upon addition of these cations.
Presumably a detailed study of the ^{31}P NMR could also yield a similar direct
determination of the pK_a from a determination of the pH dependence of the
chemical shift. Most such measurements have been performed at a few discrete
values of pH, and mainly for relatively low concentrations of transition metal
ions.[9,10,11] The one NMR experiment reported with the above biologically im-
portant ions[9] did not show such a result, possibly because the pH dependence
was not carried out over a sufficiently fine grained set of pH values.

The Raman spectrum of ATP in aqueous solution has the following features
pertinent to the present work:[12] 1. Below 1700 cm^{-1} there is a large number
of strong sharp lines which can be assigned to the adenine moiety; many of
these undergo changes when the pH of the solution is varied through the pK for
dissociation of the H^+ from the adenine. The group of lines characteristic of
protonated adenine, as the pH increases over the pK_a, is progressively replaced
by a set of lines characterizing the deprotonated base. 2. In this region
there are no strong lines that can be associated with the ribose component.[13]
3. There is one sharp polarized line that can definitely be associated with
the triphosphate.[12] It occurs at 1127 cm^{-1} for pH well below the pK_a of the
secondary phosphate proton, and at 1118 cm^{-1} well above it.[14] More specific
assignment of this line to a definite phosphate eigenmode cannot be made at
this point. The interpretation of our results does not depend on such a pre-
cise assignment considering that there is no detectable change in the adenine
lines when the pH is varied around the pK for the secondary proton. There is
a corresponding polarized phosphate line in the spectra of both ADP and AMP;
however, the difference in its frequency between the protonated and ionized
forms is much larger than in ATP,[12,13] and it is clearly observed that the line

characteristic of the protonated ADP or AMP decreases in intensity and disappears as the pH is increased above the pK_a, and is replaced by the line characteristic of the ionized form. This type of behavior indicates that for the mono- and diphosphates the average proton exchange frequency is, at all times, smaller than the frequency difference between the spectral lines associated with the two configurations. This difference is less in ATP than in the higher phosphates, namely 7 cm^{-1}[12] as compared to 18 in ADP and 102 in AMP.

As a preliminary to the study of the effect of divalent cation complexing on this vibration we have examined in detail its pH dependence in pure ATP solutions. The samples were prepared with ATP disodium purchased from Nutritional Biochemicals Corporation of Cleveland. The pH was adjusted by addition of Na_2O and measured to $\pm.02$ with a Beckman Expandomatic instrument. The samples were filtered into the scattering cell through 100 mμ millipore filters. All measurements were made at $25 \pm 0.5^{\circ}C$. The divalent metal ions were used as chlorides, analytical reagent grade. The data were obtained at a number of ATP concentrations and the divalent metal ion concentration was equimolar to that of ATP. Figure 1 shows samples of the actual recorder traces. The spectra were obtained with argon ion 4880 Å excitation and a Jarrell Ash double monochromator, with photon counting detection and completely digital data handling.[12] The results for the line frequency as a function of pH are plotted in Fig. 2. It is clear that, contrary to the results for the lower phosphates, the line in ATP shifts in frequency continuously as a function of pH. The transition in molecular conformation occurs in a narrow pH range indicated by a sharp but still continuous drop in the line frequency. The fact that in the transition region we see a single line shifting in frequency between the values corresponding to the two configurations, rather than a pair of conjugate lines exchanging intensities, can be understood in the same general terms as the motionally narrowed NMR spectra of liquids.[16] If the exchange frequency is larger than the splitting between the conjugate lines, it can be shown that the spectrum still consists of two lines but only one remains
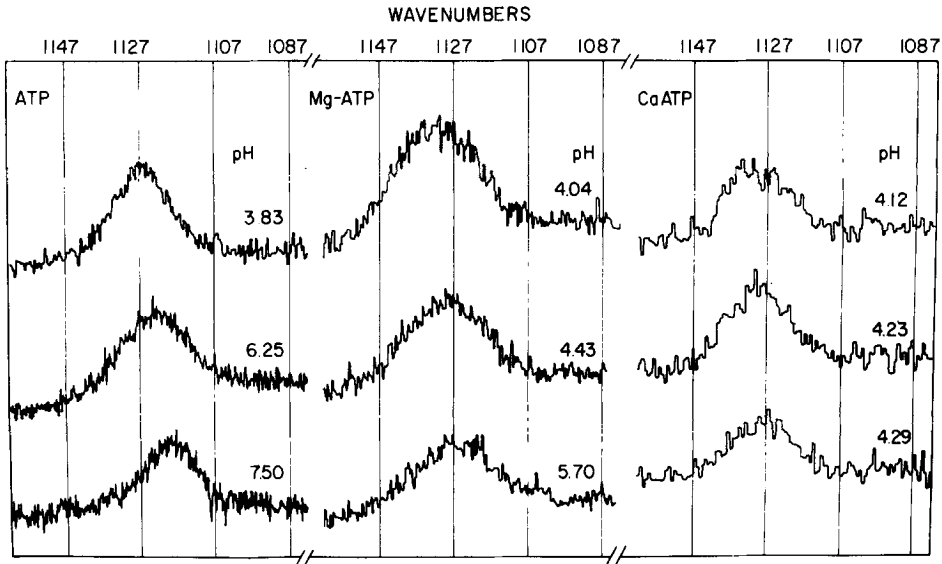
Fig. 1 Recorder traces of the Raman line associated with the triphosphate
        moiety of ATP.   Left: ATP, Center: ATP-Mg, Right: ATP-Ca
        Spectral Slit Width - 3 cm^{-1} Speed - 10 cm^{-1}/min.
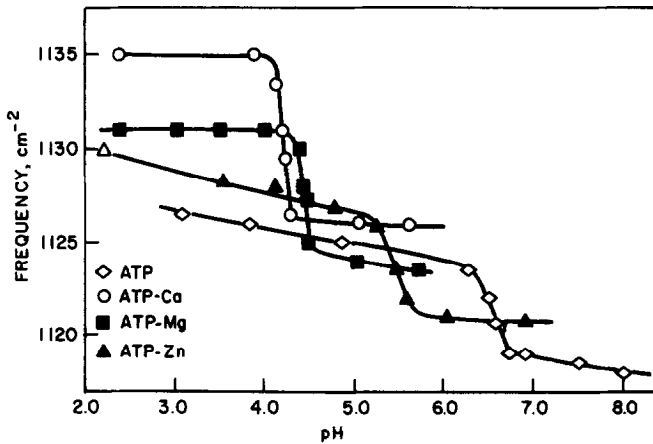

Fig. 2 pH dependence of the center frequency of the triphosphate Raman line
        in various ATP solutions.

sharp; its frequency shifts continuously between the two conjugate values, the

shift being linear in the concentration difference between the two configu-

rations.   Therefore the pK_a will correspond to the midpoint of the transition.

The other line has a much larger width and was not observed under our

experimental conditions. We did observe some broadening even for this sharp

component, indicating that the exchange frequency is not too much larger than

the splitting. Comparison of the results between ATP and ADP put it in the

range 10^{11} to 10^{12} sec^{-1}. Such high values of exchange frequency (above the

diffusion limit) have been reported in the past for acidic protons in water

solution,[17,18,19,20] in particular from Raman line shape analysis.[21] The

proton exchange rate that is relevant in such spectroscopic measurements is the

jumping rate between a site covalently bound to the base oxygen of the terminal

phosphate and a hydrogen bond site associated with the nearby hydration water

molecules, which is still a very short distance away. In Table I we show the

apparent pK_a values obtained directly from the data in Fig. 2.[22] The de-

pendence of the pK_a on ATP concentration for the range of the present measure-

ments was very small. For pure ATP our results are in agreement with those of

earlier investigations,[6,7,8] in particular with those obtained from the

analysis of IR spectra. As shown in Table I, the addition of divalent metal

ions depresses the transition region in qualitative agreement with the earlier

results. However, quantitative discrepancies appear, as indicated in Table I.

In an attempt to clarify these differences we have measured an extensive series

of IR spectra in the range 800 - 1500 cm^{-1} by the same technique of Khalil

et al.[6] We concluded that whereas for pure ATP the conjugate lines employed

to obtain the pK_a are quite clearly resolved, this is not so for the solutions

containing divalent metal ions. For the latter the lines are broader and there

is considerable interference between the various lines. There are also measur-

able pH dependent variations in line frequencies and widths which make the

intensity measurements used as a basis for pK_a determination rather unreliable.

Such complicating factors are absent in the Raman spectra where the transition

is monitored by the center frequency of an isolated sharp spectral line. Our

data also show that the addition of the divalent metal ions increases the

frequency of the line about equally both below and above the pK_a, as seen ex-

plicitly in Table I. If this frequency shift is taken as an indication of the

binding of the ion to the ATP, the conclusion is that we are dealing with a

TABLE I

SAMPLE	CONC.(M)	Apparent ionization constant, pK_a		Frequency shifts related to the frequency in ATP (cm^{-1})	
		Raman pH*	Infrared pD*	$pH \ll pK_a$	$pH \gg pK_a$
ATP	.3	6.50			
	.2	6.50			
	.1	6.50	6.5		
	.05	6.50			
ATP-ZnCl$_2$	.2	5.48		2.5	2.5
	.1		3.9		
ATP-MgCl$_2$	.2	4.43		3.7	4.5
	.1		4.7		
ATP-CaCl$_2$	.1	4.21	5.3	7.5	7.5
		pD*			
ATP-MgCl$_2$(D$_2$O)	.2	4.56			

*pH and pD values refer to H_2O and D_2O solutions, respectively.

metal ion - ATP complex both below and above the pK_a. This fact may by itself account for the difference between the values for the pK_a obtained here as compared to those obtained from analysis of titration curves,[7,8] since the latter depends on assumptions concerning the formation constants of such complexes. The numerical value of these frequency shifts increases mono-tonically with the depression of the pK_a by the corresponding metal ion, indicating that the more strongly bound metal also depresses the pK_a by the larger amount. Such considerations would also indicate that on a time average basis the binding of the metal ion to both the protonated and the ionized form of the ATP is the same, since the frequency shift upon addition of the metal ion is about the same on both sides of the pK_a. It is worth noting that both Mg^{++} and Ca^{++} seem to bind much more strongly to the triphosphate than Zn^{++}, and that a number of previous results indicate that the reverse may be true with respect to binding with the adenine base.[23,9] The possible differences in complex structure for the two kinds of ions that evolve may very well be related to differences observed in their biological activity, as mentioned in

the introduction. In view of the preceding arguments we are led to assign the following order for binding strength of the metal ions to the triphosphate moiety of ATP: Zn < Mg < Ca. It is our conclusion that the variance of this result from those obtained by other methods should be seriously considered, since with the present technique the transition pH is obtained rather directly and unequivocally from the experimental results.

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