Biophysical Chemistry 28 (1987) 183–190
Elsevier

BPC 01192

A spectrophotometric study of the binding of Cu(II) ions to ATP

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Received 3 March 1987
Revised manuscript received 11 August 1987
Accepted 12 August 1987

Optical absorption; Copper ion; Adenosine triphosphate

The interaction of copper(II) with adenosine triphosphate (ATP) has been studied as a function of pH in the range pH 3–12. Our approach is the study of the effect of binding both on the ATP ultraviolet absorption spectrum and on the optical d-d transition of copper ions. The results show that Cu(II)-ATP complexes exist in a variety of forms in equilibrium, the percentage of each species varying according to the state of ionization of the intrinsic adenine, phosphate and ribose groups. These results also show a close correlation between the rate of dephosphorylation of ATP in the presence of Cu(II) ions and Cu(II) bonding to the adenine of ATP, thus supporting the hypothesis that the metal-ion/nucleic-base interactions are crucial for the observation of a metal-ion promoted dephosphorylation of ATP (D.H. Buisson and H. Sigel, Biochim. Biophys. Acta 343 (1974) 45).

1. Introduction

A wide variety of physical methods (pH titration, ultraviolet and infrared spectrophotometry, NMR, ESR, etc.) has been applied to the study of metal-nucleoside and metal-nucleotide interaction [1–4]. The principal importance of these metal binding studies is to provide an understanding of the participation of metal ions in the biological functions of nucleotides and nucleic acids [4]. Because of the biological importance of ATP, adenosine and its derivatives are among the most extensively studied ligands.

Studies on this argument are generally complex, due to the fact that the metal may bind at a number of places, e.g., phosphate groups, base groups and ribose hydroxyl groups. Moreover, the character of the binding depends on the environmental conditions (ionic strength, pH, etc.) and on the metal ion considered.

Despite the numerous studies on binding of divalent metal ions to adenosine and adenine nucleotides there is still controversy about the exact structure of these complexes [2,3]. One of the questions to be resolved regards the binding sites of metals on the bases of nucleosides and nucleotides and the extent to which the phosphate competes with these base sites for the metal ions.

It may be noted that most of these studies look at the effect which metal ion binding produces on some properties of nucleotides and thus, the observations are limited to those binding sites which influence changes in the macromolecular parameters subject to measurements. Another kind of complementary approach may be used by studying the effect that the ligand field produces on the electronic properties of the metal ion. In particular, a study of optical d-d transitions may provide a useful means of investigating the characteristics of binding with nucleic acid ligands, the central ion being a transition metal [5,6]. The splitting of

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the d subshell in the ligand's field leads to low-energy light absorption, the number of bands and their position in the spectrum being dependent on the symmetry and strength of the ligand field. In previous papers we have shown how these circumstances may profitably be used to study Cu(II)-adenine nucleotide complexes [7,8].

Only very few studies have been carried out on the formation of these complexes as a function of pH [7–9,11,12,19], while the pH of the solutions may be an important factor in determining the nature of interaction sites in metal-adenine nucleotide complexes. In fact, in the binding of metal ions to nucleotides there may be competition between these ions and protons for the ionizable groups which serve as ligands. The metal-adenine nucleotide interaction may be expected to depend on the state of ionization of the adenine ring ($pK \approx 4$), of the terminal phosphate group ($pK \approx 7$) and of the ribose hydroxyl group ($pK \approx 12.5$) [8].

In the present work we have considered the binding of Cu(II) ions with ATP over the pH range 3–12 by studying the effect of binding both on the adenine ultraviolet absorption spectrum and on the optical d-d transition of copper ions. The intensity of perturbation of the adenine ultraviolet absorption spectrum was taken as a measure of the extent to which Cu(II) ions bind to nucleotide base [13]. Our purpose was mainly to stress the role of phosphate and ribose groups in the formation of Cu(II)-ATP complexes and the extent to which these groups compete with base sites for the metal ions.

2. Materials and methods

The copper used was $CuCl_2$ reagent grade. The disodium salts of ATP, ADP and AMP were purchased from Boehringer-Mannheim. No buffers were used, in order to avoid interaction of Cu(II) with other ligands. Solutions for optical absorption measurements were prepared by slowly adding concentrated NaOH (2 N) to aqueous solutions initially at pH 3. Absorption spectra were measured with a Shimadzu model 360 spectrophotometer, the cell housing compartment of which was kept at 20.0 ± 0.1°C with water circulating from a thermostatted bath, 3-ml quartz cells of 1 cm length being used.

Spectral ultraviolet absorption changes following the addition of Cu^{2+} to nucleotide solutions were detected by reading the difference in absorbance between a Cu(II)-nucleotide solution and a solution of the nucleotide at the same concentration and pH.

For studies measuring the effect of pH on the change in absorbance, solutions were adjusted to pH 3 with HCl and titrated with NaOH.

The amount of Cu^{2+} added to the nucleotide

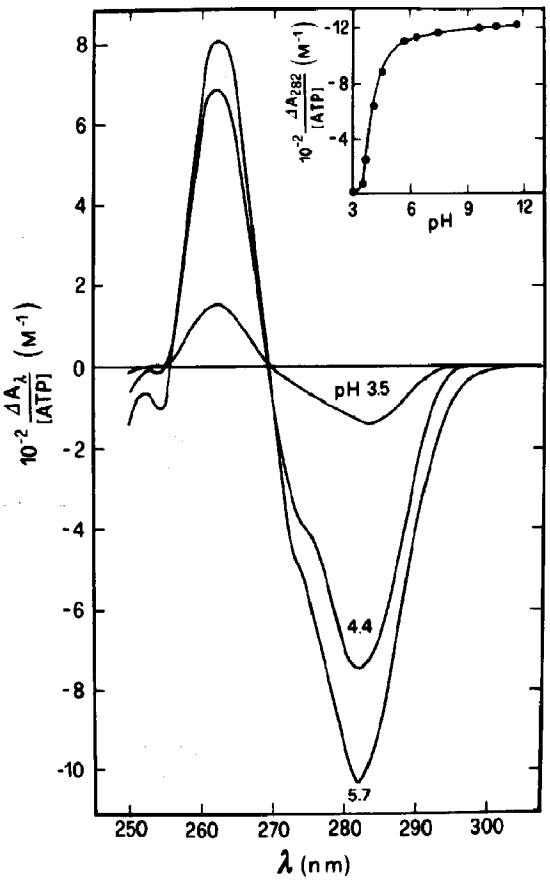

Fig. 1. Absorbance change, ΔA_λ, as a function of pH, for an ATP solution initially at pH 3. The absorption values are divided by the concentration of ATP. These differential spectra show a minimum at 282 nm. The inset shows the variation in ΔA_{282} intensity as a function of pH. Cells with a 1 cm path length were used. The concentration of ATP was 10^{-4} M.

solutions was expressed as r, the molar ratio of ion concentration to nucleotide concentration.

All spectra were recorded immediately after preparation of the solutions.

3. Results and discussion

3.1. Effect of pH change on ATP absorbance

Fig. 1 shows the ultraviolet absorbance change ΔA_λ, as a function of pH, for an ATP solution initially at pH 3. These differential spectra show a maximum at 262 nm and a minimum at 282 nm. The occurrence of an isosbestic point at 269 nm indicates the presence in solution of two different molecular species (one of which is protonated and the other not) and the mutual conversion of one into the other on increasing the pH. The plot of ΔA_{282} intensity at 282 nm vs. pH (inset to fig. 1) shows that the changes in absorbance are mainly in the range pH 3–5.5, an inflection point being located at about pH 4, i.e., in the pH range of adenine deprotonation [3].

These variations in absorbance, although differing in shape, are of the same order of magnitude and occur in the same spectral range as those due to the interaction of the nucleotide base with Cu^{2+} (see fig. 3). This fact imposes a rigorous control on the pH, especially in the range of base ionization ($pK \approx 4$), for measuring the absorbance variations due to the formation of Cu(II)-ATP complexes.

3.2. Optical properties of the Cu(II)-ATP complexes

3.2.1. pH 3

It has been found that ultraviolet spectra of adenosine and adenine nucleotides change on adding metal ions of the 3d transition series [13]. This has been interpreted as an indication of nitrogen chelation on the N7 of the adenine ring. This fact makes ultraviolet differential spectroscopy useful in the study of transition metal complexes with nucleic acids, in particular, by allowing one to estimate the degree of interaction of the nucleotide base with these metal ions.

No absorption changes occur in the ultraviolet

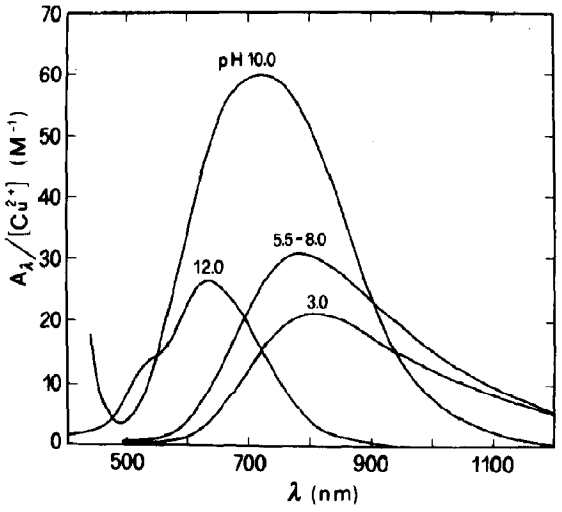

Fig. 2. Absorption spectra of Cu^{2+} in the presence of ATP at various pH values. The absorption values are divided by the concentration of Cu^{2+} present in solution. Cells with a 1 cm path length were used. Overall concentrations: 0.5×10^{-2} M Cu^{2+}, 5×10^{-2} M ATP.

region (250–310 nm range) after addition of Cu^{2+} to an ATP solution at pH 3. Hence, we have no evidence of a metal-ring interaction at this pH value. On the other hand, the Cu(II)-ATP spectrum in the region of the optical d-d transitions (500–1200 nm range) is slightly different from that of the hexaqueo cupric ion $[Cu(H_2O)_6]^{2+}$ [14] this spectrum shows a maximum at 800 nm, similar to the spectrum of the hexaqueo cupric ion, but the molar extinction coefficient is somewhat greater ($\epsilon_{800} = 23$) (fig. 2). The same spectrum was found at pH 3 for the Cu(II)-tripolyphosphate anions ($\epsilon_{800} = 21$), suggesting that at pH 3 the copper binds to the phosphate portion of ATP. At pH 3 the adenine ring is protonated and this seems to hinder the interaction of copper ions with the base; consistent with these conclusions are the results from Raman [15] and EPR [12] studies and from X-ray spectroscopy studies of CuK absorption edges in Cu(II)-ATP complexes, performed recently in our laboratory [16].

3.2.2. pH 3–8

In this pH range the metal-ATP interaction may be expected to depend on the state of ionization of the adenine ring ($pK \approx 4$) and on the state

of ionization of the terminal phosphate group ($pK \approx 7$) [3].

When the pH increases from 3 to 8 we have evidence of some significant changes in the d-d and ultraviolet absorption spectra of the Cu(II)-ATP system. As regards the d-d transition zone (fig. 2), we note a small increase in ϵ ($\Delta\epsilon_{800} \approx 7$) and a small blue shift in the maximum ($\Delta\lambda \approx 25$ nm) when the pH increases from 3 to 5.5, while a subsequent increase in pH from 5.5 to 8 does not produce any change in the Cu(II)-ATP spectrum. For the Cu(II)-tripolyphosphate system, an increase in pH from 3 to 5.5 causes only a very small increase in ϵ ($\Delta\epsilon_{800} \approx 2$), without any change in the band shape.

The model commonly accepted for the Cu(II)-ATP complex at neutral pH values is that of a macrochelate resulting from the coordination of Cu^{2+} to the β- and γ-phosphate group and to the N7 atom of the adenine ring [17]. This question has been the subject of much debate in the literature, particularly with regard to the nature of the metal bond with the N7 of the adenine ring as an inner [18,19] or outer [20,21] sphere binding and the distribution of complex species between exclusive phosphate binding and macrochelation.

It is known that the binding of Cu^{2+} to nitrogen ligands such as ammonia by displacement of a water molecule is accompanied by a blue shift and an increase in the molar extinction coefficient for the copper band [14]. The d-d spectra, recorded for the Cu(II)-ATP system, when the pH increases from 3 to 5.5, show this behaviour, thus suggesting the direct binding of copper ions to the nitrogen atoms of the adenine ring at neutral pH values; however, the spectral changes observed are too small to suggest the direct coordination of all the Cu^{2+} present in solution to the adenine ring.

Another frequently debated question in the literature concerns the occurrence of 1:1, 1:2 or 2:1 metal-ATP complexes under specified conditions. In particular, at metal/ATP ratios greater than unity the existence of a complex containing two bound metal ions per ATP has been suggested [22]. This also arises from the optical absorption measurements as a function of the Cu^{2+}/ATP ratio.

As a matter of fact, if we denote the molar

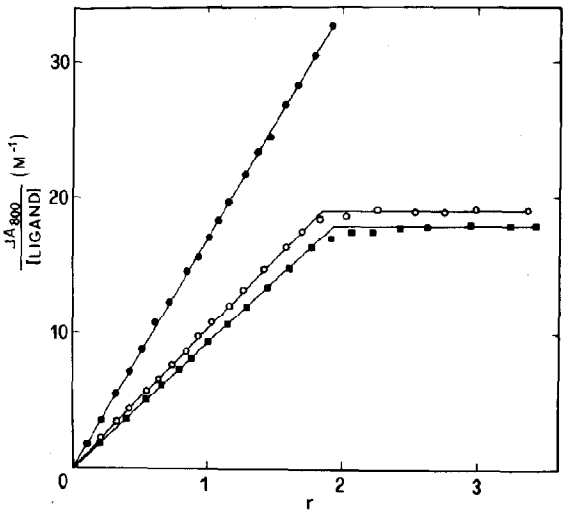

Fig. 3. Differential absorption values at 800 nm, ΔA_{800} (see eq. 2), for a Cu(II)-ATP solution at pH 3 (○———○) and 6.5 (●———●) and for a Cu(II)-tripolyphosphate solution at pH 3 (■———■). ΔA_{800} values only slightly higher than those at pH 3 were observed at pH 6.5 for the Cu(II)-tripolyphosphate system (data not shown). ΔA_{800} was normalized with respect to the ATP or tripolyphosphate concentration.

extinction coefficient of the bound and free copper by ϵ_b and ϵ_f, respectively, and the optical path by l, we can write for the absorption spectrum of the Cu(II)-ATP (or Cu(II)-tripolyphosphate) system

$$A_\lambda = [\text{Cu(II)}]_b \epsilon_b l + [\text{Cu(II)}]_f \epsilon_f l \qquad (1)$$

If from this spectrum we subtract that of a copper solution having a concentration equal to $[\text{Cu(II)}]_b + [\text{Cu(II)}]_f$ (or, in other words, all the copper added) we obtain the quantity:

$$\Delta A_\lambda = [\text{Cu(II)}]_b (\epsilon_b - \epsilon_f) l \qquad (2)$$

which turns out to be proportional to the concentration of only the bound copper.

Fig. 3 shows the value of this quantity (at 800 nm and normalized with respect to the ligand concentration) as a function of r for the Cu(II)-ATP and Cu(II)-tripolyphosphate systems. At pH 3 this quantity increases linearly as r increases up to a plateau value at $r \approx 2$, showing similar values for ATP and tripolyphosphate. At pH 6.5, this quantity again increases linearly with r, but around $r = 2$ a certain amount of scattered light due to the

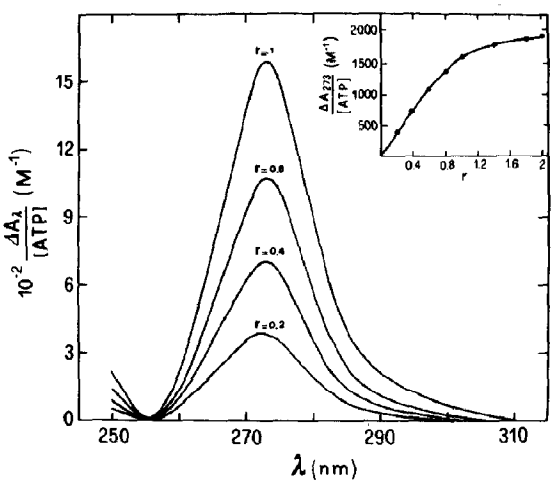

Fig. 4. Differential ultraviolet absorption spectra of 10^{-4} M ATP as a function of the Cu(II)/ATP ratio r at pH 6.5. These differential spectra were detected by reading the difference in absorbance between a Cu(II)-ATP solution and a solution of ATP at the same concentration and pH. These spectra have a maximum at 273 nm whose value, indicated by ΔA_{273}, increases with added copper concentration (see inset).

presence of aggregates is observed for both systems.

These results clearly indicate the formation of a complex, binuclear in metal, for both ATP and tripolyphosphate. The data suggest that in $(Cu(II))_2$-ATP both metal ions may be bound to the phosphate chain and that a neutral pH values they interact to a certain degree with the adenine ring.

The same suggestions arose from the differential spectra in the ultraviolet region. In fact, for pH > 3, we have evidence of some modification in the ATP absorption spectrum upon addition of Cu^{2+} (fig. 4). The observed differential spectrum indicates the appearance of an absorption band with a maximum at 273 nm. We note that such a differential spectrum is different from that due to base protonation (fig. 1); this excludes the possibility that it may be due to a variation in the pK of ring protonation and strongly suggest binding to a ring position. On the other hand, it is unlikely that the Cu^{2+} binds only to a ring position because measurements made with a mixture of Cu^{2+} and adenosine showed no evidence of change in absorbance.

The band intensity at 273 nm, ΔA_{273}, depends on the pH value and metal concentration. The plot of ΔA_{273} at fixed pH as a function of increasing amount of Cu^{2+} appears biphasic (see inset to fig. 4): the curve shows an initial sharp increase in absorbance as the metal ion is added, but, for values greater than unity, we observe a slower increase. At $r > 2$ a certain amount of scattered light is observed due to the presence of aggregates.

The question arises as to the relationship between the 800 nm titration (fig. 3) and the analogous ultraviolet titration at 273 nm (inset to fig. 4). The optical absorption in the d-d zone depends on the ligand field characteristics: the linear increase in ΔA_{800} vs. r and the band shape which does not change for fixed pH on r variation suggest the formation of complexes with a high binding constant and with no substantial modification of the ligand field in the range 0–2 of copper/ATP ratios. This fact supports the idea that the biphasic nature of the ΔA_{273} vs. r curve (inset to fig. 4) is not due to a weak binding regime but to the formation, for $r > 1$, of a new complex, possibly binuclear in metal, characterized by a molar extinction coefficient at 273 nm lower than that observed for low r values.

What is the origin of the 273 nm band? Our data show that (see inset to fig. 3):

$$\left(\mathrm{d}\Delta A_{273}/\mathrm{d}[Cu^{2+}]\right)_{r=0} = 1600\ M^{-1}\,cm^{-1} \qquad (3)$$

By taking into account that, presumably, only a fraction $\alpha < 1$ of the added metal binds directly to the base, it may be inferred that such an interaction is accompanied by a variation $\Delta\epsilon_{273} > 1600$ $M^{-1}\,cm^{-1}$ in the molar extinction coefficient of ATP at 273 nm.

This large change in molar extinction coefficient suggests the attribution of this band to a charge-transfer transition from a ligand to the metal ion. An analogous hypothesis has been adopted for the band occurring in the same spectral range in the absorption spectrum of the Cu(II)-tRNA complex [23] and for that occurring for the Cu(II)-DNA complex [24].

Although the data presented for the Cu(II)-ATP complex at neutral pH values are consistent with an intramolecular chelate structure, they do not

eliminate the possibility of an intermolecular complex in which the Cu^{2+} binds to the phosphate chain and interacts with an adenine ring of another molecule. To clarify this point, we have performed some measurements on equimolar adenosine and tripolyphosphate solutions in the presence of increasing copper content. In the d-d zone, at constant pH (6) value, spectral variations like those for the Cu(II)-tripolyphosphate system were observed. On the other hand, no absorption changes were observed in the ultraviolet region. Thus, we were unable to obtain any evidence for a significant interaction between Cu(II)-tripolyphosphate and adenosine itself under our experimental conditions.

3.2.3. pH 8–12

It is known that on increasing solution pH above 7, hydroxy complexes begin to form, in which the N7 interaction is inhibited [25]. The formation of such complexes is accompanied by the occurrence of a large absorption band with a maximum at about 225 nm (figs. 5 and 6). The tail of this band extends to almost 400 nm and partially overlaps the band at 273 nm (fig. 5). This

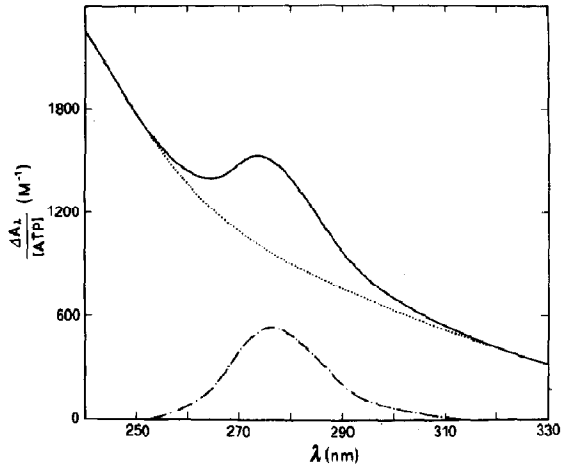

Fig. 5. Differential ultraviolet absorption spectrum at pH 10.3 for Cu^{2+} with ATP. This spectrum was detected by reading the difference in absorbance between a Cu(II)-ATP solution and a solution of ATP at the same concentration and pH. This difference spectrum (———) may be expressed as a linear combination of the corresponding spectra recorded at pH 6.5 (·–·–·) and pH 12 (·····). Concentration of Cu^{2+} and ATP was 10^{-4} M.

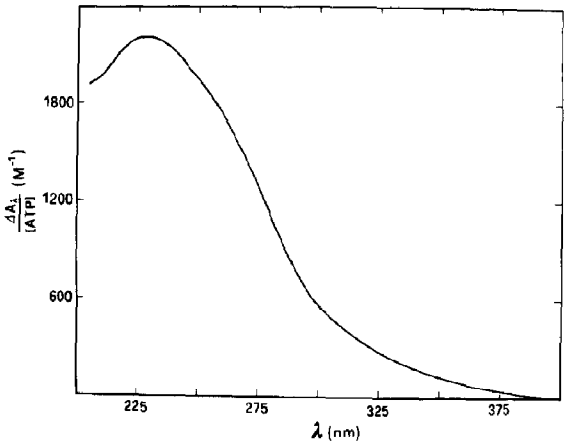

Fig. 6. Differential ultraviolet absorption spectrum at pH 11.5 for Cu^{2+} with ATP. Concentration of Cu^{2+} and ATP was 10^{-4} M.

latter band decreases in intensity on increasing the pH and dies out completely at pH ≈ 11.5 (fig. 6).

The spectra recorded in the range pH 7–12 may be expressed as a linear combination of those recorded at the extremum pH values of the interval considered. In this way it becomes possible to single out from each spectrum the contribution due to the interaction with the adenine ring.

Fig. 7 shows a plot of ΔA_{273} intensity of the Cu(II)-ATP solution ($r = 1$) vs. pH over the full range of pH explored; ΔA_{273} progressively appears in the range pH 3–5.5, remains constant in the range pH 5.5–8 and progressively disappears above pH 8.

As regards the d-d transition zone (fig. 2), we observe that on increasing the pH of the Cu(II)-ATP system from 8 to 10, the absorption maximum is gradually shifted to lower wavelengths (from the initial 800 nm down to 720 nm) and the molar absorption coefficient is enhanced (ϵ_{720} reaches 60 at pH 10). The series of spectra recorded in the range pH 8–10 shows an isosbestic point at $\lambda = 910$ nm.

When the pH is further increased from 10 to 12, the absorption peak is gradually shifted to 640 nm, while ϵ diminishes (fig. 2). Isosbestic points at 470 and 560 nm suggest the presence of two different complexes and the mutual conversion of one into the other in this pH range. The absorp-

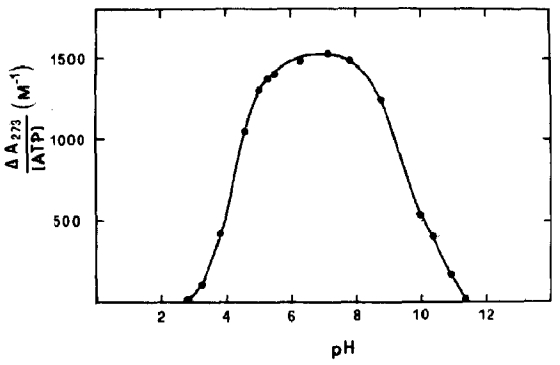

Fig. 7. ATP absorption difference at 273 nm plotted vs. pH: concentration of Cu^{2+} and ATP was 10^{-4} M.

tion spectrum at pH 12 can be resolved into two bands with peaks of about 640 and 520 nm.

The position of the bands in the absorption spectrum depends on the strength of the ligand field, i.e., on the size of the dipole moment of the ligands and on their distance from the absorbing central ion [14]. The observed shift to smaller λ when the pH increases thus indicates the formation of complexes characterized by a stronger ligand field.

The spectra in fig. 2 refer to a 1:10 ratio between copper and ATP concentrations. At pH 10 and 12 the Cu(II)-ATP system shows spectra independent of the copper/ATP ratio over the range 0–1 for this ratio. For greater values of this ratio a certain amount of scattered light is observed due to the presence of aggregates. This fact may indicate that the Cu(II)-ATP complexes which form in this pH range have 1 : 1 stoichiometry.

At high pH values the same characteristic evolution of d-d optical absorption spectra is observed for AMP, ADP and ATP [7,8] and for these nucleotides the absorption spectra at pH 12 (fig. 2) are identical to that of Cu(II)-adenosine and Cu(II)-D-ribose. The observed behaviour revealed at pH > 8 an interaction between the copper ions and hydroxyl groups of ribose, while this effect is not observed in the case of adenine deoxynucleotides, where the copper coordination with the phosphate groups predominates throughout the entire pH range considered [8].

Similar conclusions are to be found in the literature [9,11] and have recently been confirmed by X-ray spectroscopic studies of the CuK absorption edge in Cu(II)-ATP complexes [16]. Thus, the appearance of a differential spectrum in the ultraviolet spectral range is related to the deprotonation of the adenine ring while its subsequent disappearance at pH > 8 is due to the formation of complexes with the ribose group.

The intensity ΔA_{273} (fig. 7) may be taken as a measure of the extent to which Cu^{2+} binds to the nucleotide base. The data show a strict correlation between the degree of interaction of Cu^{2+} with the adenine group of ATP and the observed rate of hydrolysis of ATP to ADP and PO_4^{3-} in the presence of Cu^{2+} as a function of pH [25,26]. This result supports the hypothesis that the metal-ion/nucleic-base interaction is crucial for the observation of Cu^{2+}-facilitated dephosphorylation of ATP [25,27].

4. Conclusions

ATP molecules have potential binding sites in the base, sugar and phosphate groups. From an inspection of the data in the literature it appears that at one time or another all these binding sites have been proposed [3]. In fact, our measurements show that Cu(II)-ATP complexes exist in a variety of forms which are in equilibrium, the percentage of each species varying with pH values and metal/ATP ratios. In some cases, only one copper is bound to a single molecule, but under appropriate experimental conditions, two coppers can be simultaneously bound to a single molecule.

Much of the controversy existing in the literature about the 1 : 1 metal-ATP complex has been about whether the phosphate-bound metal ion interacts with the adenine ring and the extent of this interaction. Our spectrophotometric data show that in the Cu(II)-ATP complex the phosphate-bound metal interacts with the adenine ring and that the extent of this interaction is very sensitive to pH. To be precise, at pH 3, it appears that Cu^{2+} interacts with the triphosphate moiety, but not with the protonated adenine ring. By increasing the pH in the range over which ring deprotonation occurs (pH 3–5.5), we have evidence of an

increasing percentage of coordination of Cu^{2+} to the adenine ring. The measurements in the pH range 5.5–8, in line with NMR studies [10], indicate no significant change in metal-ring interaction; thus, it may be said that the secondary phosphate ionization does not affect metal binding to the adenine ring. At higher pH values an interaction between copper ions and ribose groups is shown. Thus, this chelate site becomes competitive with the nucleic base and phosphate groups as ligands in basic solutions. When the pH is strongly basic, neither the N7 of the base nor the phosphate groups are bound and the ribose residue seems to be the only coordination site for Cu^{2+}. Consistent with our spectrophotometric data are the results from a ^{31}P-NMR study carried out by Gabriel and co-workers [9] which demonstrates that interaction with the ribose hydroxyls (at pH 11.5) excludes the binding with the phosphate groups.

The interaction with ribose does not occur in the case of adenine deoxynucleotides. This result, in line with the previous suggestion by Eichorn et al. [28], shows that there are conditions under which copper might be able to differentiate between ribo- and deoxynucleosides.

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