Complex Formation of Cu(II) with ATP§ and Aliphatic Dipeptides in Aqueous Solution

ERNST R. WERNER and BERND M. RODE

Institute of Inorganic and Analytical Chemistry, University of Innsbruck, Innrain 52a, A-6020 Innsbruck, Austria

Received June 14, 1983

The formation of binary and ternary complexes was investigated by ESR§ spectroscopy in aqueous solutions of Cu(2+), ATP and the dipeptides glygly§ and gly-L-pro§ at room temperature. Spectra and stability constants of two ternary complexes for each peptide. (GG)Cu(ATP)(3–)§, (GG)Cu(ATP)(4–), (GP)Cu(ATP)(3–)§ and (GP(Cu(ATP)(5–) were determined. Assuming that complexes of similar structure show similar spectra, some conclusions could be drawn about the structure of the complexes. The characteristic difference between gly-L-pro and glygly is attributed to the lack of the peptide proton in gly-L-pro. At acidic pH Cu(2+) is bound in binary ATP complexes, at neutral to basic pH in binary peptide or in ternary peptide–Cu–ATP complexes.

Introduction

Interactions between proteins, metals and ATP play an important role in nature. The selectivity of these interactions is achieved by the structure of the macromolecules in solution and may also be influenced by the metal ion [1]. Dipeptides are the simplest 'model compounds' as they contain all binding sites of interest [2] including the peptide bond. The results derived from such a model study give only limited information concerning the complex formation of the macromolecules [3]. A knowledge of the complex formation of the model system, however, allows us to decide whether a specific behaviour of a macromolecule is to be assigned to a special property of this particular molecule or if it reflects only the behaviour of the basic functional groups. Another aim of this work was to test the utility of the ESR method [4], [5] to investigate complex formation in aqueous Cu(2+) solutions with more than one ligand, before applying this method to more complex systems such as macromolecules.

Copper was chosen as the metal ion, due to its excellent properties for ESR measurements. Recently a specific biological action of ATP–Cu solutions has been reported: the stimulation of the release of luteinizing hormone releasing factor from hypothalamic granules [6].

In former papers we have reported ATP–Cu(2+) [5] and dipeptide–Cu(2+) [4, 7] complex formation in aqueous solution (see these papers for further references). Ternary ATP–Cu complexes in solution have been investigated by various methods: potentiometric titrations [8–10], spectrophotometric titrations [11, 12, 15, 17], and calorimetric measurements [14], and the compounds forming ternary complexes with ATP–Cu in these investigations were: catechols and ethanolamines [8], 2,2′ bipyridyl [9–11, 15], ethylenediamine and pyrocatecholate [10], biogenic amines [12], 1,10 phenanthroline [13], L-tyrosine and L-phenylalanine [14], imidazole and ammonia [16].

The ESR method used in this work proved to be especially useful to investigate ternary systems, as complex formation can be followed by observation of the metal and not simply by the study of the influence of the metal on ligand properties as in most other methods.

Experimental

Materials

Copper was used as $CuCl_2 \cdot 2H_2O$ analytical grade (Mallinckrodt), ATP was obtained from Serva as $Na_2H_2ATP \cdot 3H_2O$ 'puriss.', the peptides gly-L-pro and glygly were Sigma analytical grade products. All solutions were prepared using CO_2 free distilled water and contained 0.1 *M* $NaNO_3$. The acid and base used for titration were Titrisol products (Merck).

Potentiometric Titrations and ESR Experiments

Stock solutions of ATP(4–) were always freshly prepared by rapidly titrating the dissolved $Na_2H_2ATP \cdot 3H_2O$ to the equivalence point. After addition of the copper solution, the peptide and an appropriate amount of HCl the titration was carried out immediately to prevent errors due to dephosphorylation [15]. 3 cm^3 solution were titrated at 20 ± 0.2 °C using a 0.2 cm^3 burette (Gilmont). The copper concentration was always 0.00490 *M*, the ATP and/or

§ Abbreviations: ATP adenosine 5′ triphosphate, glygly = GG glycylglycine, gly-L-pro = GP glycyl-L-proline, ESR electron spin resonance.

0020-1693/84/$3.00

peptide concentrations were varied independently from a ratio of 1:1 to 5:1 to Cu(2+). For the ESR measurements 8 to 15 samples of 100 mm^3 per titration were taken from the titration vessel by micropipette, immediately frozen in liquid nitrogen and rapidly defrosted just before the ESR measurement. The spectra were recorded at 20 °C within two minutes.

Apparatus

For the pH measurements a Schott pH-meter CG 803 and an Ingold electrode 104051393 calibrated with standard buffer solutions (Merck) were used. The ESR spectra were recorded on a Varian E 104 spectrometer (calibrated microwave frequency = 9.097 GHz) in tubes with a much smaller diameter (1 mm, Wilmad Cat. Nr. 800) than standard ESR tubes in order to reduce the dielectric losses caused by water.

Calculations

All calculations were carried out using the CDC Cyber 74 computer of the University of Innsbruck. 24 to 35 points per potentiometric titration curve and 400 points per ESR spectrum (digitized with a Summagraphics ID 2000, resolution 0.1 mm) were included.

Method

The ESR titration method was used as described in ref. [5]. The main differences from most other ESR investigations in this field are that the spectrum is recorded at room temperature, and that the evaluation is focused on quantitative data (peak heights, stability constants calculated therefrom). The basic assumption used in the interpretation is, that the amplitude of the ESR signal is proportional to the concentration of a complex in solution. The spectrum recorded for a metal ligand solution is assumed to be the direct sum of the spectra of all species present. A twofold integration of the signals gives a rough measure of the amount of ESR detectable copper. Small complex molecules containing two copper atoms show a zero ESR spectrum under the present experimental conditions.

The calculation of single species spectra and pK-values of these species starts with an arbitrary set of species and pK-values. (The deprotonation constants of the ligands without metal are determined by potentiometric titration). In a stepwise manner, beginning with few species and only a section of the spectra (*e.g.* specific pH range and metal ligand ratio), more and more species and spectra are taken into account and their stability constants iteratively varied. A good description of the complex formation is obtained, when the difference between measured spectra and calculated spectra (which are the sum of the spectra of the occurring species) is as small as the experimental error. In this work, only one combination out of many assumed combinations led to this fit and is presented as a result of this work.

Results and Discussion

Summary of the Complex Formation of the Binary Systems ATP–Cu, GG–CU, GP–Cu

Equilibria and pK-values of the binary systems are shown in Table I. Compared to the peptides, the complex formation of Cu(2+) with ATP starts at a more acidic pH due to the already negatively charged phosphate groups. In the case of ATP, above pH = 9 all copper is bound in complexes showing no ESR spectrum. They are assumed, therefore, to contain two copper atoms (species 5, 6, 10, 11). A strong ESR signal at more basic pH (>10.5) appears only at a ATP/Cu ratio > 1:1 (species 9). Further, a comparatively weak $(ATP)_2Cu$ complex (species 7) is formed.

Glygly forms much stronger complexes than gly-L-pro because it can lose the peptide proton upon complex formation. Species 16 (GG)Cu(OH)(–) is particularly stable, so that at pH = 11.5 this 1:1 complex is formed by 80% of the Cu(2+) even for a GG:Cu ratio of 3:1. In contrast to this, gly-L-pro forms a large amount of the 2:1 $(GP)_2Cu$ species 22 and 23 at GP:Cu ratios >1:1 and neutral to basic pH. A $(peptide)_2Cu_2$ complex is only detected for glygly (species 18). Figs. 1a, 1b, 1c show the spectra of the binary species.

Ternary Complexes (Peptide–Cu–ATP)

Two ternary complexes for each peptide were detected (Table II).

The stability of the two ternary species of glygly is comparatively low, they form only up to 40% in the 3:3:1 case (Fig. 2). Both complexes, however, are essential to describe the ESR spectra and are detected unequivocally. At pH = 11.5, as in the binary system, species 16 (GG)Cu(OH)(–) is formed by 80% of the Cu(2+).

Gly-L-pro forms ternary complexes with ATP and Cu to a much greater extent (Fig. 3) than glygly. As a precipitate is formed at a gly-L-pro/Cu ratio of 1:1 (without ATP) and as ATP–Cu solutions show no ESR spectrum at this pH, the occurrence of a clear solution showing an ESR spectrum of all copper (2 × integral) for GP:Cu:ATP = 1:1:1, pH = 9 gives direct evidence for the formation of a ternary complex. Quantitative calculation shows that this species is (GP)Cu(ATP)(3–). At pH > 10 a sharp new signal occurs in the ternary system which is species 27 (GP)Cu(ATP)(5–), a (4–) species as for glygly is not formed. The spectrum of species 27 is very similar to the spectrum of the binary $(ATP)_2Cu$ species 9 (Fig. 4). For this $(ATP)_2Cu$ species it is proposed that complex formation occurs via ribose hydroxyls

TABLE I. Equilibria of Binary Systems (see ref. 4, 5 for details)

pK	Nr.		
−6.51	1	ATP(4−) + H(+)	→ HATP(3−)
−4.12	2	HATP(3−) + H(+)	→ $H_2ATP(2-)$
−3.45	3	Cu(2+) + HATP(3−)	→ Cu(HATP)(−)
−6.10	4	Cu(2+) + ATP(4−)	→ Cu(ATP)(2−)
−6.85	5	2Cu(2+) + 2ATP(4−) + H_2O	→ $Cu_2(ATP)_2(OH)(5-)$ + H(+)
1.19	6	2Cu(2+) + 2ATP(4−) + $2H_2O$	→ $Cu_2(ATP)_2(OH)_2(6-)$ + 2H(+)
−7.96	7	Cu(2+) + 2ATP(4−)	→ $Cu(ATP)_2(6-)$
12.5	8	ATP(4−)	→ ATP(5−) + H(+)
20.85[β]	9a	Cu(2+) + 2ATP(4−) + H_2O	→ $Cu(ATP)_2(OH)(9-)$ + H(+)
31.85[β]	9b	Cu(2+) + 2ATP(4−)	→ $Cu(ATP)_2(10-)$ + 2H(+)
9.90[β]	10	2Cu(2+) + 2ATP(4−) + nH_2O[α]	→ $Cu_2(ATP)_2(OH)_n(7-)$ + 3H(+)
19.45[β]	11	2Cu(2+) + 2ATP(4−) + nH_2O[α]	→ $Cu_2(ATP)_2(OH)_n(8-)$ + 4H(+)
−3.18	12	GG(0) + H(+)	→ GGH(+)
8.25	13	GG(0)	→ GG(−) + H(+)
−5.91	14	GG(−) + Cu(2+)	→ (GG)Cu(+)
−1.57	15	GG(−) + Cu(2+)	→ (GG)Cu(O) + H(+)
8.24	16	GG(−) + Cu(2+) + H_2O	→ (GG)Cu(OH)(−) + 2H(+)
−4.50	17	2GG(−) + Cu(2+)	→ $(GG)_2Cu(-)$ + H(+)
4.08	18	2GG(−) + 2Cu(2+) + H_2O	→ $(GG)_2Cu_2(OH)(-)$ + 3H(+)
−2.85	19	GP(0) + H(+)	→ GPH(+)
8.66	20	GP(0)	→ GP(−) + H(+)
−6.75	21	GP(−) + Cu(2+)	→ (GP)Cu(+)
−11.27	22	2GP(−) + Cu(2+)	→ $(GP)_2Cu(0)$
−1.66	23	2GP(−) + Cu(2+) + H_2O	→ $(GP)_2Cu(OH)(-)$ + H(+)

[α]'n' denotes that it was not distinguished whether H(+) comes from metal bound water or from deprotonation of the ATP(4−) molecule. [β]Different to ref. (5) (results of potentiometric titrations (not shown) up to pH = 11 included).

TABLE II. Stability of Ternary Complexes.

pK	ΔpK	Nr.		
−10.58	±0.4	24	GG(−) + Cu(2+) + ATP(4−)	→ (GG)Cu(ATP)(3−)
−3.59	±0.4	25	GG(−) + Cu(2+) + ATP(4−)	→ (GG)Cu(ATP)(4−) + H(+)
−11.44	±0.3	26	GP(−) + Cu(2+) + ATP(4−)	→ (GP)Cu(ATP)(3−)
3.61	±0.3	27	GP(−) + Cu(2+) + ATP(4−)	→ (GP)Cu(ATP)(5−) + 2H(+)

(see ref. 5). If it is assumed that the (GP)Cu(ATP)-(5−) complex has a similar structure, it can be understood why only a (5−) and not a (4−) (GP)Cu(ATP) species is formed: the ribose hydroxyl groups are in a vicinal position and emerge at the same side of the ribose ring. Therefore it seems plausible that, when complex formation occurs at this site, both ribose hydroxyls are involved at once in complex formation, thus explaining the lack of a (4−) species (this argument also favours 9b as a description of the ATP–Cu binary system rather than 9a; both 9a and 9b have been proposed as a possible description [5]).

If the assumption, that complexes showing similar ESR spectra have similar structures, is applied to the other complexes, some conclusions about the structure of the ternary complexes can be drawn. In the (GG)Cu(ATP)(3−) complex two possibilities can be distinguished:

a) glygly has (1−) charge and ATP (4−) or

b) glygly has (2−) charge (peptide proton lost) and ATP is HATP(3−).

In gly-L-pro, no peptide proton can be lost and therefore in (GP)Cu(ATP)(3−) gly-L-pro can be assumed to be (1−), and ATP (4−) as in possibility a). In species 17 $(GG)_2Cu(-)$ one peptide has lost its peptide proton as in possibility b). Comparison of the spectra of species 24 (GG)Cu(ATP)(3−) to species 26 (GP)Cu(ATP)(3−) (Fig. 5) clearly favours possibility a). The spectrum of species 25 (GG)Cu-(ATP)(4−) (Fig. 5), however, is similar to the spectrum of species 17 $(GG)_2Cu(-)$ (Fig. 1a), indicating that in species 25 the peptide proton has dissociated.

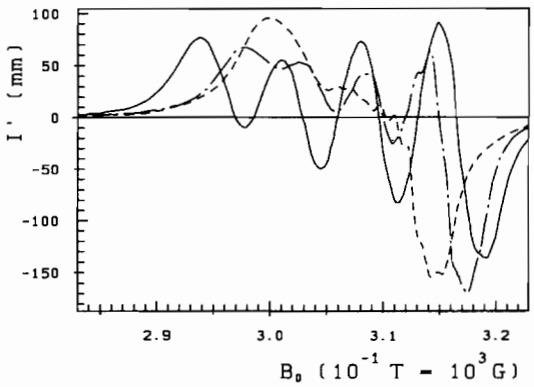

Fig. 1a. Concentration of all species = 0.0049 *M*. —— species 15 (GG)Cu(O); – – – species 16 (GG)Cu(OH)(–); – · – species 17 $(GG)_2Cu(-)$.

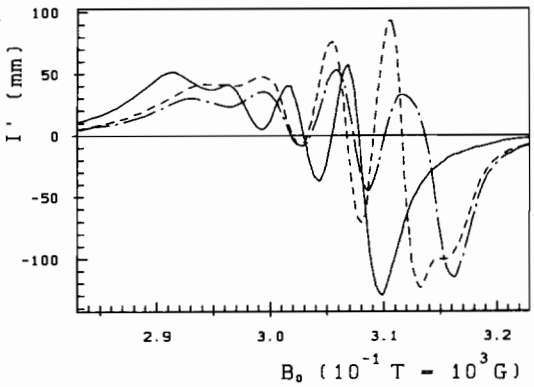

Fig. 1b. Concentration of all species = 0.0049 *M*. —— species 21 (GP)Cu(+); – – – species 22 $(GP)_2Cu(O)$; – · – species 23 $(GP)_2Cu(OH)(-)$.

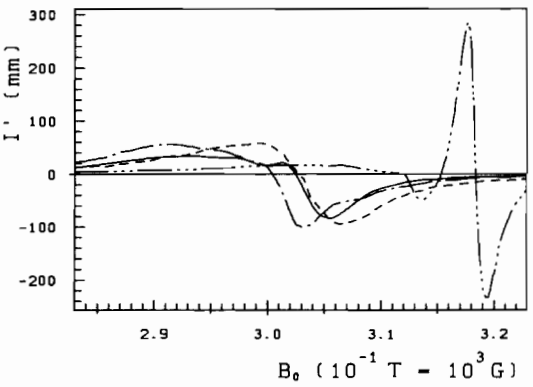

Fig. 1c. Concentration of all species = 0.0049 *M*. —— species 4 Cu(ATP)(2–); – · – species 3 Cu(HATP)(–); – – – species 7 $Cu(ATP)_2(6-)$; – ··· – species 9 $Cu(ATP)_2(10-)$ or $Cu(ATP)_2(OH)(9-)$. Although the signal of species 9 is much larger than the other signals, it gives the same twofold integral as the other signals within the error of the integration procedure.

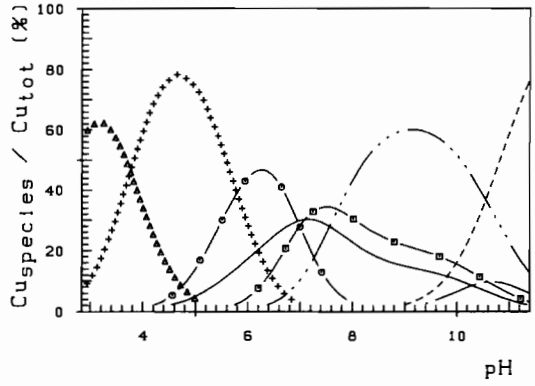

Fig. 2. (glygly) = 0.015 *M*, (Cu) = 0.005 *M*, (ATP) = 0.015 *M*. Peptide species: —— 15 (GG)Cu(O); – – – 16 (GG)Cu(OH)(–); – ··· – 17 $(GG)_2Cu(-)$; – · – 18 $(GG)_2Cu_2(OH)(-)$. ATP species: △ 3 Cu(HATP)(–); + 4 Cu(ATP)(2–). Ternary species: –○– 24 (GG)Cu(ATP)(3–); –□– 25 (GG)Cu(ATP)(4–).

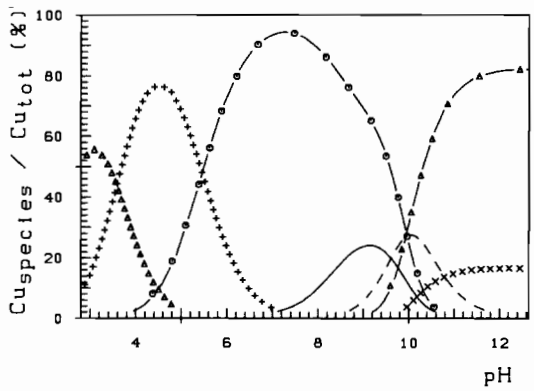

Fig. 3. (gly-L-pro) = 0.015 *M*, (Cu) = 0.005 *M*, (ATP) = 0.015 *M*. Peptide species: —— 22 $(GP)_2Cu(O)$; – – – 23 $(GP)_2Cu(OH)(-)$. ATP species: △ 3 Cu(HATP)(–); + 4 Cu(ATP)(2–); × 11 $Cu_2(ATP)_2(8-)$. Ternary species: –○– 26 (GP)Cu(ATP)(3–); –△– 27 (GP)Cu(ATP)(5–).

The lack of a (GG)Cu(ATP)(5–) species analogous to species 27 for gly-L-pro can be explained by the particularly high stability of species 16 (GG)Cu(OH)(–). As gly-L-pro cannot lose a peptide proton, a similar species cannot be formed, which on the other hand enables the formation of species 27 (GP)Cu(ATP)(5–). Due to the lack of the peptide proton a species (GP)Cu(ATP)(4–) analogous to species 25 of glygly cannot be formed by gly-L-pro.

At acidic pH Cu(2+) is bound in all cases in ATP–Cu complexes (Figs. 2, 3) because the ATP molecule is already charged (2–) or (3–), whereas the peptide molecules are (1+) or not charged. From neutral to basic pH, Cu(2+) is bound in either binary Cu-peptide or in ternary peptide–Cu–ATP complexes (Figs. 2, 3).

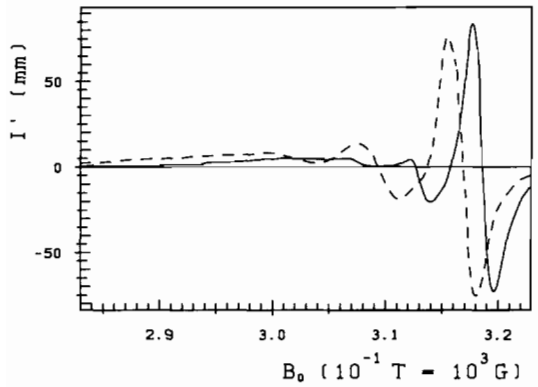

Fig. 4. Two experimental spectra are shown. In both cases ca. 30% of the copper forms the species that shows the ESR spectrum, the balance of the copper is bound in $Cu_2(ATP)_2$ species (10 and/or 11) showing no spectrum. —— (Cu) = 0.00439 *M*, (ATP) = 0.0236 *M*, species 9 $Cu(ATP)_2(10-)$. – – – (Cu) = 0.00438 *M*, (ATP) = 0.00445 *M*, (gly-L-pro) = 0.00438 *M*, species 27 (GP)Cu(ATP)(5–).

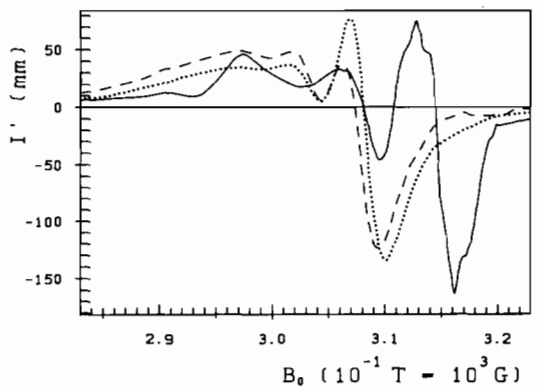

Fig. 5. Concentration of all species = 0.0049 *M*. —— species 25 (GG)Cu(ATP)(4–); – – – species 24 (GG)Cu(ATP)-(3–); · · · species 26 (GP)Cu(ATP)(3–).

Figure 6 shows one example of a spectrum of a ternary system, how the spectrum is composed of the spectra of the species and how this calculated spectrum agrees with the experimental one. Due to the possible error combination the fit of the experimental to the calculated spectra is not as good as is found in binary systems (compare ref. 4, 5).

Complex Formation in the Biological pH-Range (6–8)

Glygly

In a 3:1 GG:Cu solution at pH = 6.2 the greatest part of the copper is contained as species 15 (GG)Cu-(O) (95%) where glygly carries no peptide proton. Addition of ATP (ATP:GG:Cu = 3:3:1) results in the formation of the ternary species 24 (GG)Cu(ATP)-

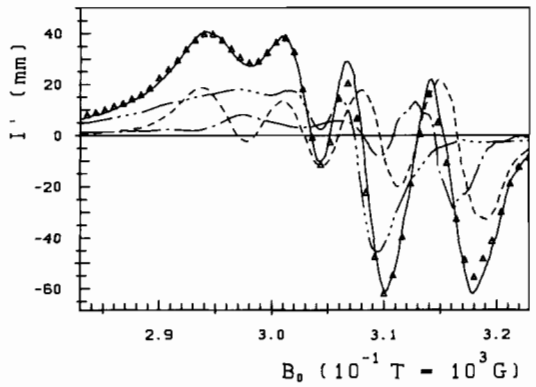

Fig. 6. (Cu) = 0.00423 *M*, (ATP) = 0.0138 *M*, (glygly) = 0.0129 *M*. —— experimental spectrum; ▵ calculated spectrum (every 8th point); – – – species 15 (GG)Cu(O) 27.9%; – · – species 25 (GG)Cu(ATP)(4–) 18.7%; – · · · – species 24 (GG)Cu(ATP)(3–) 39.1%; balance species <5% not shown.

(3–) (45%) where glygly carries a peptide proton, the remaining copper is bound as ATP complex (20% species 4 Cu(ATP)(2–)) or in species 15 (GG)Cu(O) (20%). Changing the pH to 8.0 leads to a complete deprotonation of the peptide nitrogen in the ternary complex to form species 25 (GG)Cu(ATP)(4–) (30%), the remaining copper being bound in the two peptide species (GG)Cu(O) (24%) and $(GG)_2Cu(-)$ (40%) only. At pH = 7 Cu(2+) is present either as peptide + ATP or as pure peptide complex.

Gly-L-Pro

In a 3:1 GP:Cu solution of pH = 8 most Cu is bound in species 22 $(GP)_2Cu(O)$ (94%). Addition of ATP (ATP:GP:Cu = 3:3:1) results in the formation of the ternary species 26 (GP)Cu(ATP)(3–) (90%, balance 10% $(GP)_2Cu(O)$), which remains the dominating species also upon acidification up to pH = 6.6 (90% (GP)Cu(ATP)(3–), 10% species 4 Cu(ATP)-(2–)).

ATP

At pH = 8 (ATP:Cu = 1:1) 70% of the copper is bound in the dimeric species 5 and 6 $Cu_2(ATP)_2$-$(OH)(5-)$ and $Cu_2(ATP)_2(OH)_2(6-)$ (35% each), 25% in species 4 Cu(ATP)(2–) [5]. In solutions containing glygly or gly-L-pro in at least equimolar amounts almost all Cu–ATP complexes are converted into ternary complexes (this is valid for pH = 7 to 9).

Acknowledgement

Financial support by the Austrian Federal Ministry of Science and Research (Erl. Zl. 18 854/6-10/81) is gratefully acknowledged.

References

1 H. Sigel, B. E. Fischer and B. Prijs, *J. Am. Chem. Soc., 99* (13), 4489 (1977).
2 H. Sigel, *Inorg. Chem., 14* (7), 1535.
3 H. C. Freeman in 'Bioinorganic Chemistry', Vols. 1 and 2, G. L. Eichhorn, pp. 159–162, Elsevier, Amsterdam, 1973.
4 W. S. Kittl and B. M. Rode, *J. Chem. Soc. Dalton Trans., 3*, 409 (1983).
5 E. R. Werner and B. M. Rode, *Inorg. Chim. Acta, 80*, 39 (1983).
6 G. H. Burrows and A. Barnea, *Endocrinology, 110* (4), 1456 (1982).
7 W. S. Kittl and B. M. Rode, *Inorg. Chim. Acta, 55*, 21 (1981).
8 R. W. Colburn and J. W. Maas, *Nature, 208* (5005), 37 (1966).
9 H. Sigel, W. Becker and D. B. McCormick, *Biochim. Biophys. Acta, 148*, 655 (1967).
10 H. Sigel, *J. Inorg. Nucl. Chem., 39*, 1903 (1977).
11 P. Chaudhudri and H. Sigel, *J. Am. Chem. Soc., 99*, 3142 (1977).
12 K. S. Rajan, S. Mainer and J. M. Davis, *Bioinorg. Chem., 9*, 187 (1978).
13 M. S. Mohan and M. M. T. Khan, *J. Coord. Chem., 8*, 207 (1979).
14 G. Arena, R. Call, V. Cucinotta, S. Musumeci, E. Rizzarelli and S. Sammartano, *Congr. Naz. Chim. Inorg. (Atti) 13th*, 288 (1980).
15 D. H. Bouisson and H. Sigel, *Biochim. Biophys. Acta, 343*, 43 (1974).
16 N. Saha and H. Sigel, *J. Am. Chem. Soc., 104* (15), 4100 (1982).
17 P. W. Schneider and P. E. Amsler, *J. Am. Chem. Soc., 98*, 7390 (1976).