Complexation of thyrotropin-releasing hormone and its related compounds with copper(II) and nickel(II) ions

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Abstract

The complex formation of L-pyroglutamyl-L-histidyl-L-prolinamide (TRH, HA) with Cu(II) and Ni(II) ions was studied by pH-metry, UV spectroscopy and circular dichroism, for aqueous solutions at an ionic strength $I=0.10$ mol dm^{-3} ($NaClO_4$) and temperature of 25 ± 0.1 °C. *N*-Acetyl-L-histidine and L-pyroglutamic acid were chosen as reference materials for discussing the results obtained. Analysis of potentiometric data led to the composition and the formation constant of the metal complexes of these ligands. Thus we have found CuA (3.41), $CuH_{-1}A$ (−3.1), $CuH_{-2}A$ (−7.68), $CuH_{-1}A_2$ (0.9), $CuH_{-2}A_2$ (−5.75), $CuH_{-3}A$ (−18.3); NiA(2.7), $NiH_{-2}A$ (−14.62) and $NiH_{-3}A$ (−25.2) as TRH complexes (logarithmic overall formation constant). Judging from these results, the predominant species at pH 7–8 should be $CuH_{-2}A$ and NiA, respectively, when intraventricular injection was made with the respective metal complexes into a biological body. The effect of the Ni complex is superior to that of TRH on locomotor activity, which may be interpreted in terms of a weak interaction of Ni(II) ion with TRH, and the apparent indifference of copper(II) ion may be explained in terms of the reduction in potency by stronger bonding with the ligand.

Introduction

Biological activity of the thyrotropin-releasing hormone (TRH) has been the subject of intensive studies [1–5], and intracranial injection of this tripeptide produces a number of interesting behavioral responses. Tonoue *et al.* have studied the effect of metal complexes of TRH on the locomotor hyperactivity of the neonatal chicken, and have found that the nickel(II)–TRH complex is more efficacious than the ligand itself on intraventricular injection [5a]. The copper(II)–TRH complex, however, gives a potency comparable to free TRH, but the palladium(II) complex is inert in producing the locomotor activity. In the present study we investigate the behavior of different TRH complexes from the viewpoint of bioinorganic chemistry.

Experimental

Reagents

L-Pyroglutamyl-L-histidyl-L-prolinamide (TRH, abbreviated as HA) from Peptide Institute, Inc. (Osaka) was checked for purity by pH titrations and used without further purification. *N*-Acetyl-L-histidine (Tokyo Kasei Kogyo Co., anal. grade) was purified from 1:1 aqueous ethanol, and L-pyroglutamic acid (Tokyo Kasei Kogyo Co., anal. grade) was used without further purification. Sodium(I), copper(II) and nickel(II) perchlorates were prepared and purified as described elsewhere [6]. Water used as a solvent was double distilled with a Kokubo Model-II sub-boiling distillator.

Equipment

Measurements were carried out at 25 ± 0.1 °C and at an ionic strength $I=0.10$ M**($NaClO_4$). Electrochemical measurements were made by using a Corning 130 pH-meter, which was connected to a Metrohm EA 109T glass electrode and a Metrohm EA 404 calomel electrode. Sodium hydroxide solution was delivered with a Metrohm E274 microburet to a 40 cm^3 titration cell, thermostated with a Neslab RTE-8 refrigerated circulating bath.

Electronic spectra were measured on a Union-Giken SM-401, a Jasco UVIDEC-610BS, or a Carl Zeiss PMQ-II UV–Vis spectrophotometer. Circular dichroism was measured with a Jasco J-500 CD

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**Non-SI unit: 1M = 1 mol dm^{-3}.

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spectrophotometer. Thermogravimetry was done with a Shimadzu DT-30 thermogravimetric apparatus. All data were processed on a FACOM M382 computer at the Computation Center, Nagoya University.

Results

The complex formation of a metal ion M^{2+} with a ligand A may be expressed in general as follows (the charge of A is omitted here)

$$pM^{2+} + qH^{+} + rA \rightleftharpoons M_pH_qA_r \quad (1a)$$

The overall formation constant β_{pqr} corresponding to this reaction is defined as follows

$$\beta_{pqr} = [M_pH_qA_r]/m^p h^q a^r \quad (1b)$$

where m, h and a represent the concentrations of M^{2+}, H^+ and A in free forms, respectively [6, 7]. Hydrogen ion concentration has been obtained from pH-metry and the following notation will be used:

$$\mathrm{ph} = -\log[H^+] \quad (2)$$

The average number of protons, $\bar{q}_H$, bound to a free ligand A was determined experimentally and is defined as follows:

$$\begin{aligned}\bar{q}_H &= (C_H - h + [OH^-])/C_A \\ &= \Sigma q[M_pH_qA_r]/C_A \\ &= \Sigma q\beta_{pqr}m^p h^q a^r / \Sigma r\beta_{pqr}m^p h^q a^r \end{aligned} \quad (3)$$

where C_H and C_A denote the total analytical concentrations of hydrogen ion and the ligand (Fig. S1).

Preliminary experiments with a glass-calomel (saturated NaCl) electrode pair resulted in a Nernstian slope of 58.90 mV/pH, and a 0.01 M perchloric acid solution (0.09 M in $NaClO_4$) was chosen as a pH standard on the concentration scale. Liquid-junction potentials E_j were measured as a function of ph and were in fair agreement, within 0.003 pH unit, with a calculation based on the Henderson equation [8].

Protonation of ligands

Potentiometric titrations of ligands with sodium hydroxide were carried out (Table S1 for the compositions of aqueous solutions), and the resulting stepwise protonation constants are summarized in Table 1, together with values from the literature. The protonation constant of TRH obtained in this work is in fair agreement with those obtained by other groups [9–11].

Deprotonation from the pyrrole ring in TRH occurs at too high a pH to decide the equilibrium constant precisely from potentiometric data alone. Accordingly, the pK_a value was determined by measuring the change in absorbance at 250 nm (Fig. S2), in combination with the acidity function as calculated from analytical concentrations of sodium hydroxide ($C_A = 5.08 \times 10^{-3}$ M) [12].

$$\log K = \log([A]/[H_{-1}A][H^+]) = 13.99 \pm 0.02 \quad (4)$$

This value is similar to that for imidazole, $pK_a = 14.44$ ($I = 0$ M) [13], and this fact substantiates deprotonation at the pyrrole nitrogen (N1) of TRH.

Complex formation as studied by potentiometry

It was possible to determine directly the compositions of metal complexes present in solution and the corresponding formation constants in the cases of *N*-acetyl-L-histidine and L-pyroglutamic acid, since the solution equilibria are simple. However this was not the case for the complexation of TRH with copper(II) ion due to the formation of a number of species, although the predominance of $CuH_{-2}A_i$ (i may be 1 or 2) in the alkaline region could easily be deduced from Gran's graphical presentation of the potentiometric data.

The difficulty could be overcome by a graphical application of Lefebvre's method (la méthode de la surface potentiométrique [16, 17]) to these systems. This method permits evaluating the concentrations of a metal ion and a ligand in free form from the titration curves (Fig. S3–S5). Similar methods have been proposed independently by Sillén [18] and Sarkar and Kruck [19], though they are quite equivalent [17b] to Lefebvre's method. Knowledge of concentrations of species in free form, m and a, leads to semiquantitative prediction of the composition and the formation constant of the metal complexes.

In a region of lower ph for example, the total metal ion concentration, C_M, may be approximated as follows:

$$\begin{aligned}C_M &= [M] + [MA] + [MH_{-2}A] \\ &= m + \beta_{101}ma + \beta_{1\text{-}21}mh^{-2}a \end{aligned} \quad (5)$$

Rearrangement of this equation results in:

$$(C_M - m)m^{-1}a^{-1} = \beta_{101} + \beta_{1\text{-}21}h^{-2} \quad (6)$$

A straight line was observed when the numerical values of the left-hand side were plotted against h^{-2}, and β_{101} was determined as 1.8×10^3 from the intercept and $\beta_{1\text{-}21}$ as 1.6×10^{-8} from the slope for the Cu(II)–TRH system (Fig. 1).

Similar approximations were done successively for possible species and the results are summarized in Table 1. They were successfully adopted as the initial values for the iterative refinement in data processing on the computer.

TABLE 1. Protonation and formation constants

Stepwise protonation constants for ligands	log K		
	[A]/[H_{-1}A][H]	[HA]/[A][H]	[H_2A]/[HA][H]
TRH	13.99	6.297	
		6.79	
Ref. 9[a]		6.25	(I = 1.0 M, $NaClO_4$)
Ref. 10[b]		6.38	
Ref. 11[c]		6.30	
N-Acetyl-L-histidine		7.023	2.823
Imidazole	14.44[d]	7.12[e]	
L-Pyroglutamic acid		3.104	

Formation constants of Cu(II) complexes

	log β_{pqr}						
	MA	MA_2	$MH_{-1}A$	$MH_{-2}A$	$MH_{-1}A_2$	$MH_{-2}A_2$	$MH_{-3}A$
Cu(II)TRH							
(Lefebvre method)	3.3		−2.1	−7.8	1.2	−5.1	
(Computer)	3.41		−3.1	−7.68	0.9	−5.75	−18.3
Ref. 10[b]	3.85			−7.67			
Ref. 11[c]	3.64			−7.67			
N-Acetyl-L-histidine	4.31	7.8	−2.61				
Imidazole[e]	4.15	7.67					
L-Pyroglutamic acid	1.57		−4.43				

Formation constants of Ni(II) complexes

	log β_{pqr}				
	MA	MA_2	$MH_{-1}A$	$MH_{-2}A$	$MH_{-3}A$
Ni(II)TRH	2.7			−14.62	−25.2
Ref. 10[b]	3.17			−14.65	
N-Acetyl-L-histidine	2.96	5.19	−5.78		
Imidazole[e]	3.01				
Ref. 14[f]	3.27	5.95			
L-Pyroglutamic acid	1.5		−5.3		

[a]25 °C. [b]I = 0.1 M, 22 °C. [c]I = 0.2 M, 22 °C. [d]I = 0 M, 25 °C (ref. 13). [e]I = 0.2 M (KNO_3), 25 °C (ref. 15). [f]I = 0.15 M, 25 °C (ref. 14). The other values in the present work were determined at I = 0.1 M and 25 °C, unless otherwise noted.

Use of the Lefebvre method seems to be very sound in such a complicated system, because the concentration of each species in free form can be evaluated directly from the experimental results without assuming any kinds of species present in the solution. Change in [A] at pH > 8.5 could no longer be traced because of various experimental errors in the Cu(II)–TRH system. Accordingly, the formation constant of $CuH_{-3}H$ was determined by the spectroscopic method, which will be described later.

Distribution of the copper(II) species is illustrated in Fig. 2(a) (and Fig S6), which shows that $CuH_{-2}A_i$ (i = 1 or 2) is always predominating, but the formation of bis complexes depends drastically on the total concentrations of copper(II) and TRH, respectively.

The bis complex is less important for the nickel(II)–TRH system, because Ni(II) ion binds to a ligand more weakly than Cu(II) in general. Therefore, the calculation becomes much simpler in the former case, and formation constants could be obtained without applying the Lefebvre method to the Ni(II)–TRH system.

Electronic transition and circular dichroism of copper(II)–TRH complexes

Electronic and CD spectra were measured with a cell designed for simultaneous measurement of ph. The absorption maximum shifted to a shorter

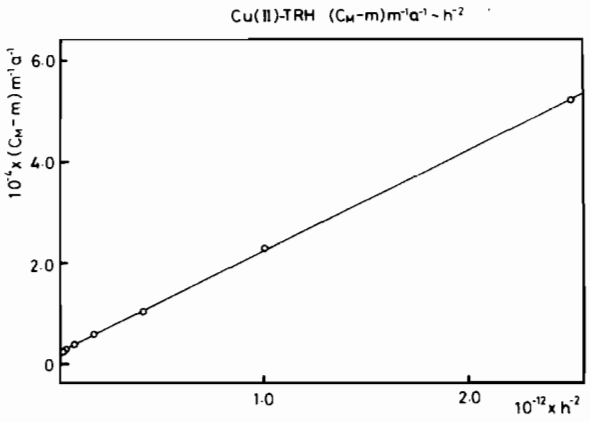

Fig. 1. Graphic determination of formation constants by the Lefebvre method for the Cu(II)–TRH system.

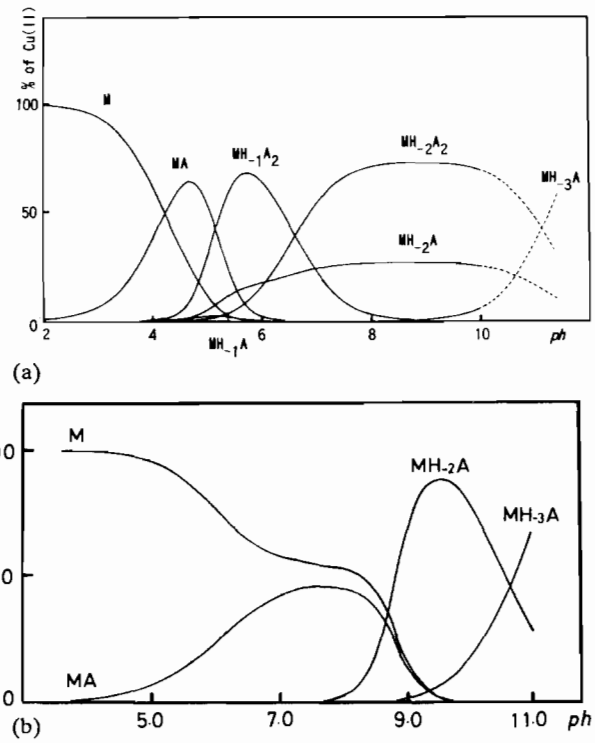

Fig. 2. Variation of composition of metal(II)–TRH solutions with pH; (a) for $C_{Cu}=10^{-2}$ M and $C_A=5\times10^{-2}$ M, (b) for $C_{Ni}=5\times10^{-4}$ M and $C_A=2\times10^{-3}$ M.

wavelength and was strengthened in intensity in accordance with the formation of CuA when ph is increased in the acid region (Fig. 3 and Fig. S7). It is rather difficult to make a spectral assignment due to the appearance of numerous species in a weakly acid solution. Comparison of spectra in alkaline regions (ph = 7.5–9.5) permits us to assign the peak at 590 nm to $MH_{-2}A$ (ph = 8.97, Fig. 3) and the peak at 585 nm to $MH_{-2}A_2$ (ph = 8.23, $C_A=5\times C_M=0.05$ M). It has been pointed out that

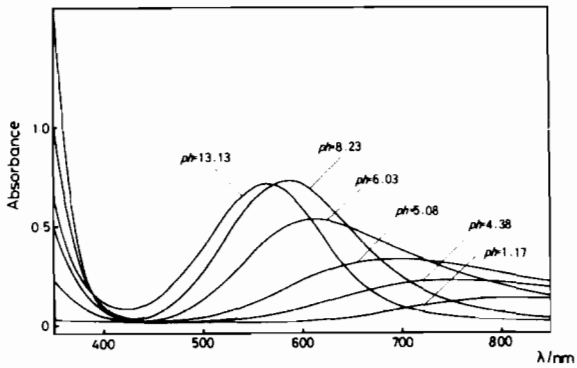

Fig. 3. Change in electronic spectra of Cu(II)-TRH solutions with pH; $C_{Cu}=10^{-2}$ M and $C_A=5\times10^{-2}$ M.

the d–d absorption maximum shifts to a shorter wavelength on substitution of donor atoms with nitrogen atoms at the copper(II) ion [7]. Hence the aforementioned fact indicates that the central metal ion is coordinated by three nitrogen atoms in $MH_{-2}A$ and by four atoms in $MH_{-2}A_2$. Formation of a common species of $MH_{-3}A$ is substantiated by the identical spectra at ph higher than 11.

Although circular dichroism was clearly seen for $CuH_{-2}A$ (ph 7.10) and $CuH_{-3}A$ (ph 12.30), it was not observed appreciably in a ph lower than 5.1. This latter fact seems to be puzzling, in view of the formation of a complex MA as confirmed by the potentiometric and electronic transition evidence between ph 4–5. It may, however, be interpreted in terms of monodentate binding of TRH through imidazole N3 alone to copper(II) ion, which will result in a smaller restriction on free rotation of the ligand. Large dependence on pH of the CD spectrum at 330 nm (Fig. S8) permitted us to determine the protonation constant of the following reaction in the strong alkaline region

$$CuH_{-2}A \rightleftharpoons CuH_{-3}A + H^+ \qquad (7)$$

by plotting the change in spectral intensity against ph. A least-squares technique gave a value of log $K=10.893\pm0.003$, which agrees fairly well with the value of 10.6 from the potentiometric titration.

Electronic transition and circular dichroism of nickel(II)–TRH complexes

A distribution curve of the nickel(II)–TRH system ($C_A=4$ $C_{Ni}=0.002$ M, Fig. 2(b)) is simply characterized by the exclusive presence of aqua ion at ph lower than 5.5, the predominance of NiA at ph = 6.5–8 with abundance nearly equal to aqua ion and of $NiH_{-2}A$ at ph 9–10 (*c.* 80%), and by the gradual increase in $NiH_{-3}A$ beginning at about ph 10. Electronic spectra remained identical for an acid solution

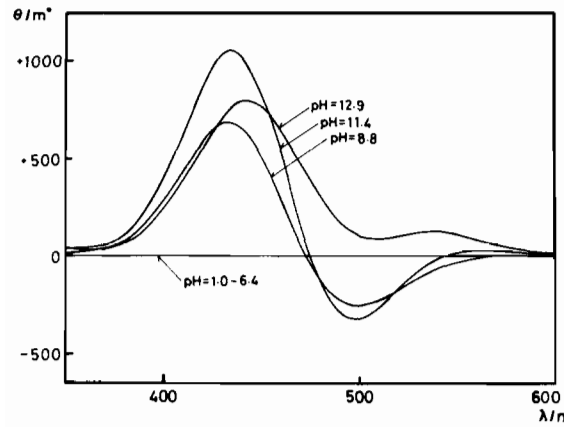

Fig. 4. Circular dichroic spectra of Ni(II)–TRH solutions at different acidity; $C_{Ni}=10^{-2}$ M and $C_A=5\times10^{-2}$ M.

of ph lower than 4.2, but further addition of the alkali solution led to the appearance of absorption maxima at about 390 and 650 nm in accordance with the formation of NiA (Fig. S9). As was the case for CuA, circular dichroism was not observable in this ph region, which suggests again monodentate coordination through imidazole N3 (Fig. 4).

Further increase in ph was accompanied by the appearance of a new peak at *c.* 440 nm in the electronic spectra at ph 8.3 where the formation of $NiH_{-2}A$ commenced, and its intensity increased up to ph 9.6 where this species was sufficiently formed. The peak at 630 nm disappeared gradually in accordance with a decrease in the concentrations of aqua ion and NiA. The position of the absorption maximum shifted to 450 nm at ph 11.3, which may correspond to the formation of $NiH_{-3}A$. Further increase in alkalinity resulted in its shift to a longer wavelength of *c.* 460 nm.

Discussion

Coordination structure of Cu(II)–TRH complexes in solution

The formation constants obtained here can be compared favorably with those obtained by Formicka-Kozłowska *et al.* [10]. They used potentiometry at $I=0.1$ M in KNO_3 and at 22 °C, which are nearly the same as the present experimental conditions. The present formation constant for $CuH_{-2}A$ agrees well with their study. They, however, neglected the formation of higher complexes, which was disclosed by the potentiometric and spectroscopic data in the present study.

The three formation constants of CuA for TRH, N-acetyl-L-histidine, and imidazole may be considered almost the same, if allowance and correction based on the linear free energy relationship (LFER) are made for some differences in pK_a among the ligands. The similarity suggests that copper(II) ion is coordinated with TRH through imidazole N3 alone in MA. This assumption is substantiated by electronic and CD spectral changes, and has been confirmed by the selective ^1H NMR peptide line-broadening [20].

It may be interesting to consider the structures of $CuH_{-1}A$ and $CuH_{-2}A$ based on the potentiometric results. When the following deprotonation process is taken into consideration for the *N*-acetyl-L-histidine complex

$$CuA \rightleftharpoons CuH_{-1}A + H^+;\ pK_a = 6.92 \qquad (8)$$

only the peptide N is a possible candidate for the site of deprotonation. On the other hand, pyroglutamyl N is a candidate in the case of Cu-L-pyroglutamate ($pK_a=6.00$). For the Cu–TRH system, the following deprotonation constants are to be calculated

$$CuA \rightleftharpoons CuH_{-1}A + H^+;\ pK_a = 6.5 \qquad (9)$$

and

$$CuH_{-1}A \rightleftharpoons CuH_{-2}A + H^+;\ pK_a = 4.6 \qquad (10)$$

Since the protons at peptide N and pyroglutamyl N only are to be dissociated, the higher value (6.5) may be attributed to the dissociation at the peptide N to form $CuH_{-1}A$, and the lower one (4.6) to that at the pyroglutamyl N to form $CuH_{-2}A$, respectively. Formicka-Kozlowska *et al.* observed a five-line superhyperfine structure in the ESR spectra at pH = 9.40 for Cu–TRH, and thus proposed a structure of $CuH_{-2}A$ coordinated by two nitrogen donor atoms [20]. On the basis of potentiometric data, however, they suggested coordination with three nitrogen atoms [10] without giving any explanation about the contradiction with the ESR data. According to an ESR study on copper(II) complexes with histidine-containing dipeptides, the coupling constant A_N of imidazole N is smaller than that of alkyl N [21]. Hence the five-line s.h.f.s may be observable, when the coupling constant A_N of the imidazole N3 is too small to give a perceptible superhyperfine structure, compared with those of peptide N and pyroglutamyl N.

Further deprotonation should give $CuH_{-3}A$ at ph higher than 9.5. The deprotonation is thought to occur on pyrrole N, because it has been reported that the deprotonation on pyrrole N becomes easier on coordination with a metal ion [22] and $pK_a=11.7$ for pyrrole N in the Cu(II)–tetrakisimidazole complex [23]. Thus the logarithmic deprotonation constant of 10.6 for Cu–TRH may be acceptable as a value for pyrrole deprotonation, if allowance is made for

some difference in pK_a of the ligands and in coordination structure. In fact deprotonation constants for imidazole N are different between Co(II)–tetrakis(imidazole) ($pK_a = 12.5$) and Co(II)–*N*-acetyl-L-histidine ($pK_a = 10.9$) [23]. Formicka-Kozłowska *et al.* have measured ESR spectra of Cu–TRH at pH 11.40 where $CuH_{-3}A$ predominates [20], and attributed the seven-line s.h.f.s to the coordination of three nitrogen donor atoms. Appearance of the seven-line s.h.f.s may be interpreted in terms of an increase in the coupling constant A_N of imidazole N3 due to enhanced bonding of Cu–N resulting from the deprotonation at pyrrole N.

Bis complexes could be detected in the pH region were $CuH_{-1}A$ and $CuH_{-2}A$ are predominant, according to an elemental analysis [5a] of Cu(II)–TRH crystals. Although Formicka-Kozłowska *et al.* have neglected the formation of bis complexes [10], their presence seems to be reasonable from a structural point of view also. The formation constant of CuA_2 could be determined for *N*-acetyl-L-histidine, but not for TRH due to its poor formation.

The stepwise formation constant for the reaction

$$CuH_{-1}A + A \rightleftharpoons CuH_{-1}A_2 \qquad (11)$$

can be calculated as

$$\log \beta_{1\text{-}12} - \log \beta_{1\text{-}11} = 4.0 \qquad (12)$$

This value may be compared to the formation constant of CuA ($\log \beta_{101} = 3.4$), with allowance made for some errors arising from insufficient concentration of the species. Hence, $CuH_{-1}A_2$ may be formed by coordination of a second A to $CuH_{-1}A$ through imidazole N3.

Deprotonation from $CuH_{-1}A_2$ produces $CuH_{-2}A_2$ and has a logarithmic constant of 6.6(5), which is in good agreement with that of CuA leading to $CuH_{-1}A$ ($pK_a = 6.5$). Accordingly, this process is supposed to be deprotonation of the peptide N. Comparison of the spectra of $CuH_{-2}a$ and $CuH_{-2}A_2$ shows that the latter has an absorption maximum at a shorter wavelength. Since coordination of three nitrogen atoms is evident in the former, the latter bis complex is supposed to be coordinated by four nitrogen atoms [7]. Possible structures of TRH complexes are illustrated in Fig. 5.

Coordination structure of nickel(II)–TRH complexes in solution

Formicka-Kozłowska *et al.* have compared the reactivity of TRH with that of L-pyroglutamyl-L-histidine by potentiometry, CD spectra, ^1H NMR etc., and concluded that the prolinamide moiety is not involved in complexation of the nickel(II) ion [24]. As is evident from Table 1, agreement of the

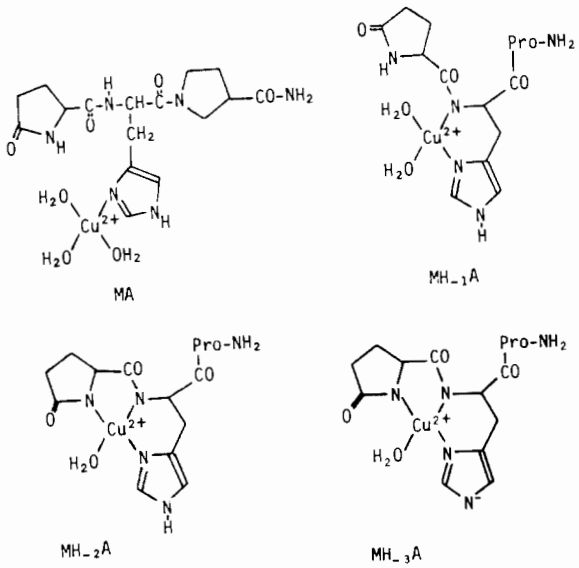

Fig. 5. Possible structures of copper(II)–TRH species in aqueous solutions.

formation constant for the predominant species $NiH_{-2}A$ is remarkable between the two groups, although this is not always the case for minor species. Because the LFER holds in general between the formation constant of MA and the pK_a value of HA [25], it seems to be reasonable that Ni–TRH has a value ($\log \beta_{11} = 2.7$) slightly smaller than that of Ni–imidazole ($\log \beta_{11} = 3.02$). In this work the formation of $NiH_{-3}A$ was taken into consideration for the first time.

As mentioned above, small differences in formation constants of NiA between TRH, *N*-acetyl-L-histidine and imidazole may be interpreted in terms of the difference in pK_a, and thus we can safely conclude that TRH binds to the nickel(II) ion through imidazole N3 in the 1:1 complex. This conclusion is supported by electronic and circular dichroic spectral evidence, and the complex NiA has an octahedral structure judging from the d–d transition spectra [26].

On the other hand, the yellow complex $NiH_{-2}A$ shows electronic spectra that are characteristic of the square planar structure [26]. The following deprotonation of Ni–TRH

$$NiA \rightleftharpoons NiH_{-2}A + 2H^+ \qquad (13)$$

is related to an overall pK_a value of 17.3. One step deprotonations ($NiA \rightleftharpoons NiH_{-1}A + H^+$) of Ni(II)–*N*-acetyl-L-histidine ($pK_a = 8.74$) and of Ni(II)–L-pyroglutamate ($pK_a = 6.8$) give a sum total of 15.5, if similar types of deprotonation process are considered to be responsible for the two-step deprotonation. Since it is not far from the experimental value of

17.3, then the complex $NiH_{-2}A$ is supposed to have three nitrogen donor atoms from imidazole N3, peptide N and pyroglutamyl N.

Further deprotonation from $NiH_{-2}A$ will give rise to $NiH_{-3}A$, and the corresponding pK_a is calculated as 10.6 from the equilibrium constants summarized in Table 1. The pyrrole N has pK_a values of 10.6 for Cu(II)–TRH, 11.7 for Cu(II)–tetrakis(imidazole), 10.9 for Co(II)–bis(*N*-acetyl-L-histidine), and 12.5 for Co(II)–tetrakis(imidazole) [23], which are not very different for different metal ions and ligands. Accordingly, the value mentioned above for Ni(II)–TRH must be related again to the deprotonation at pyrrole N. Formicka-Kozłowska *et al.* have measured the ^1H NMR spectra of a nickel(II) complex with L-pyroglutamyl-L-histidine, a TRH-related peptide, at pH 11.5 where $NiH_{-3}A$ predominates [24]. They have found large upfield chemical shifts of imidazole H(2), histidine αCH, and pyroglutamyl αCH, and thus proposed coordination of Ni(II) ion with the three nitrogen atoms. Moreover they have suggested deprotonation of pyrrole N on Ni(II) coordination, based on the large upfield chemical shifts of H(2) and H(5) of the dipeptide imidazole compared to the metal-free dipeptide. Judging from these data, we may propose a possible structure of $NiH_{-3}A$ as illustrated in Fig. 5.

Further increase of pH over 11.3 resulted in a red shift of the peak of electronic spectra (Fig. S9). Although alkalinity was too high to apply potentiometry to straightforward analysis of the spectral change, it is highly probable that hydrolysis of $NiH_{-3}A$ should give rise to $NiH_{-4}A$.

Biological activity of TRH in reference to the structure of its complexes

Tonoue *et al.* found that intraventricular injection of the Ni(II)–TRH complex into the neonatal chicken resulted in more prominent hyperactivity at pH 7.4 than the ligand itself with respect to locomotion [5a]. The Cu(II)–TRH complex, however, showed a potency only comparable to TRH, and the palladium(II) complex was inert in producing hyperactivity. They suggested that the effectivity of metal complexes does not come from higher resistibility to the enzymatic degradation but from conformational change caused by chelation.

As can be seen from Fig. 2 (and S6), solution equilibria of Ni(II)– and Cu(II)–TRH are rather simple at pH 7–8, and NiA and $CuH_{-2}A$ should be predominant species also in a living body. The nickel monomer has a conformation where weak monodentate coordination is operating, but the copper deprotonated species is characterized by strong coordination through the three nitrogen atoms, as has been discussed above.

An interesting example is given by a study on the copper(II) complexes of dipeptides containing histidine, which may be model compounds for the active site of superoxide dismutase (SOD) [27]. According to this report, only weak biological activity is shown by histidyl peptide complexes with strong coordination with three nitrogen donor atoms, although a prominent biological effect is seen in complexes of histidine-containing peptides with weak coordination through imidazole N3.

In any organism, TRH is supposed to be weakly bonded to the Ni(II) ion and accordingly interact with it by retaining a relatively free conformation. Weak interaction through imidazole N3 alone may give stronger biological activity, but it will be reduced by tight coordination to the Cu(II) ion.

Supplementary material

The following materials are available from author N.N. upon request. Table S1: solution compositions for pH titrations. Fig. S1: plot of $\bar{q}_H$ against ph for *N*-acetyl-L-histidine. Figs. S2–S4: graphical presentations of the Lefebvre method for the Cu(II)–TRH system. Fig. S5: deprotonation of the pyrrole ring in TRH as seen from the change in spectral intensity at 250 nm. Fig. S6: distribution curve of copper(II)–TRH; $C_{Cu}=5\times10^{-3}$ M and $C_A=6\times10^{-3}$ M. Fig. S7: electronic spectra of Cu(II)–TRH; $C_{Cu}=5\times10^{-3}$ M and $C_A=6\times10^{-3}$ M. Fig. S8: deprotonation of $CuH_{-2}A$ giving $CuH_{-3}A$ as seen from CD spectra ($C_{Cu}=2\times10^{-3}$ M and $C_A=10^{-2}$ M). Fig. S9: electronic spectra of Ni(II)–TRH in (a) acid and (b) alkaline regions; $C_{Cu}=10^{-2}$ M and $C_A=5\times10^{-2}$ M.

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