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Potentiometric determination of formation constants of copper(II)/bile acid/peptide in aqueous solution*

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Abstract: A potentiometric method was used to study the equilibria in aqueous solution for the systems copper(II)/bile acid salt/peptide [bile acid salt–sodium cholate or glycocholate; and peptide–glutamic acid (glutamate), glutamine or glutamylglutamic acid], specifically to assess cooperative binding between small peptides and bile acids in the presence of copper(II). The results obtained suggest large cooperativity in formation of ternary complexes with amino acids when they are coordinated to copper(II) as bidentate ligands.

Keywords: *Bile acids; copper(II); amino acid; peptide; complex formation; cooperativity.*

Introduction

Bile salts are the most important physiological detergents and they have a multifaceted role in the gastrointestinal system [1]. Interactions of bile salts with metal ions, forming soluble complexes of moderate stability may play a very significant effect in their diverse physiological functions [2]. Knowledge of the interactions between bile salts and metal ions can be of importance to understand absorption and dissolution of lipo-soluble compounds, and also in assessing their possible role in hepatobiliary bile salts transport. In the enterohepatic circulation, hepatocytes play a key role in removing bile salts from blood and in secreting them into bile, and both the uptake by the sinusoidal membrane and the secretion by the canalicular membrane are mediated by proteic carriers [3]. Cooperative effects in metal bound proteins can provide insight in the transport mechanism and also in the use of metal ions to promote bile salt transport across biological membranes, and copper(II) due to its presence in the liver is an obvious candidate to foster this cooperativity.

In the present study a very simple model was chosen to assess cooperative binding between small peptides and bile acids in the presence of copper(II) in aqueous solution and at the ionic strength of biological fluids, and at bile acid concentration were well below their critical micelle concentration. The results obtained suggest that in aqueous solution and in the absence of micelle formation, binding of bile acids to small peptides is induced by the presence of copper(II).

Experimental

Reagents and solutions

The bile salts (the Sigma Chemical Company), glutamic acid and its sodium salt (Merck), glutamine (BDH) and glutamylglutamic (Sigma) were used without further purification. All other chemicals were from Merck (grade *pro analysi*); all solutions were prepared with CO_2 free, double deionized water (conductivity less than 0.1 $\mu S\ cm^{-1}$). The concentration of stock solutions of bile salts were established by conductimetric titrations with 0.1 M HCl (Merck; Titrisol) in a Crison Micro CM 2202 conductivity meter, and that of those of copper(II) by titrations with EDTA (Merck; Titrisol).

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Potentiometric determination of acidity and stability constants

All potentiometric measurements were carried out with a Crison 2002 pH meter and 2031 buret controlled by a Philips TC100 microcomputer coupled to a Unisys PW 300 for data manipulation. The electrode assembly was made up of a Metrohm 6.0726.100 double-junction AgCl/Ag reference electrode with a Russel SWL glass electrode. System calibration was performed by Gran method [4] in terms of hydrogen ion concentrations, using strong acid/strong base titrations [HCl (0.001 M)/NaOH (0.01 M)] with solutions whose ionic strength was adjusted to 0.15 M with NaCl. Titrations were always carried out under a nitrogen atmosphere at 25°C in a double-walled glass cell.

Acidity constants. For the bile acids, they have been published elsewhere [5]; for the peptides, they were determined by titration of 20.00 ml of aqueous solutions 1 mM HCl (I = 0.15 M NaCl; 25°C) and 1 and 2 mM in the corresponding peptide with ≈0.01 M NaOH (in some studies sodium glutamate was used instead of glutamic acid). Calculations were performed using the program Superquad [6] with titration data obtained in the pH range 3.0–9.0 for glutamic; 2.6–8.6 for glutamylglutamic acid; and 7.0–9.0 for glutamine.

Bile salt/peptide interactions. Possible interactions between any of the bile acids (or its sodium salts) with any of the peptides was assessed by titration of 20.00 ml of aqueous solutions of bile salt (1–2 mM), peptide (1–2 mM) and HCl (0.2–0.4 mM; for cholate/glutamate no HCl was used) with ≈0.01 M NaOH, under a nitrogen atmosphere. Calculations were performed using program Superquad [6] and BEST [7] with data obtained from 10 independent titrations, each with at least 20 points.

Stability constants of copper (II) complexes. (i) Those with bile salts (cholate and glycocholate) were determined by titration of 20.00 ml of aqueous solutions of bile salt (1–2 mM), copper nitrate (II) (0.5–2 mM) and HCl (0.2 mM) with ≈0.01 M NaOH, under a nitrogen atmosphere. The calculations were performed with data from at least six independent titrations, each with more than 20 data points. The protolysis of ligands and cations were taken in account in all models. (ii) Those with peptides were determined by titration of 20.00 ml of aqueous solutions of the corresponding peptide (1–2 mM), copper nitrate (II) (0.5–2 mM) and HCl (0–0.4 mM) with ≈0.01 M NaOH. (iii) Those of mixed bile acid/peptide were determined by titration of 20.00 ml of an aqueous solutions of peptide (1–2 mM), bile salt (1–2 mM), copper nitrate (II) (0.5–2 mM) and HCl (0–0.4 mM) with ≈0.01 M NaOH. In (ii) and (iii) calculations were performed with data from at least six independent titrations, each with more than 30 data points. All experimental titration data for metal complex formation were analysed using the computer programs Superquad [6] and Best [7], and in all models protolysis of ligands and cations were taken in account.

The errors reported in this work were calculated by the method of Albert and Serjeant [8] in which the errors are calculated as the maximum difference between the logarithm of the average of the antilogarithms of the calculated pK_a values and their individual values. All determinations with bile acids or with their salts were performed at concentrations well below the critical micelle concentration [9].

Results and Discussion

Acidity constants of glutamic acid, glutamine and glutamylglutamic acid

The acidity constants of glutamic acid and glutamine have been extensively studied in the past [10–14] and here are reported values determined for them and for glutamylglutamic acid at I = 0.15 M NaCl, the ionic strength found in most biological fluids, as they will impinge in the results of the latter part of this study. The three peptides show the unusual combination of an amine group bound to a terminal carboxylic acid, and the resonance structures that can take place make the pK_a of this terminal acid much lower than the values typically observed for monocarboxylic acids [15, 16], and a concomitant reduction in the pK_a of the amino group when compared with those of aliphatic amines [17, 18]. No attempts were made to evaluate the acidity constant of these very acidic groups, except for glutamylglutamic acid, as the main objective of this work lies in assessing the behaviour of these peptides at pH values not too far from that observed under physiological conditions. The

Table 1
Acidity constants of the bile acids and peptides used in the present study. Unless stated otherwise the values were obtained in this work

Compound	Cholic acid Literature	Glycocholic acid Literature	Glutamic acid		Glutamine		Glutamylglutamic acid	
			This work	Literature	This work	Literature	This work	Literature
pK_{a_1}	4.66 ± 0.01*	3.64 ± 0.03*	†	2.18 ± 0.01‡	†	2.16 ± 0.01‡	3.09 ± 0.05	—
pK_{a_2}	—	—	4.07 ± 0.01	4.20 ± 0.08‡	8.96 ± 0.02	8.96 ± 0.05‡	3.90 ± 0.05	—
pK_{a_3}	—	—	9.43 ± 0.02	9.59 ± 0.09‡	—	—	4.61 ± 0.05	—
pK_{a_4}	—	—	—	—	—	—	7.98 ± 0.04	—

*From [5].
†Value not obtained in the present work. See text for details.
‡From [19].

Table 2
Equilibrium constants (log β) calculated for copper(II)/bile salt and copper(II)/peptide in aqueous solution*

Ligand	*p*	*q*	*r*	Possible species	pH range	Superquad	Best	Literature†
Cholate	1	1	0	$[CuB]^+$	4.8–5.8	2.24 ± 0.05	2.25 ± 0.05	—
(B^-)	1	1	−1	[Cu(OH)B]	4.8–6.2	−4.24 ± 0.05	−4.23 ± 0.05	—
Glycocholate	1	1	0	$[CuB]^+$	4.0–5.1	2.95 ± 0.06	2.95 ± 0.05	—
(B^-)	1	1	−1	[Cu(OH)B]	4.0–6.2	−3.68 ± 0.05	−3.68 ± 0.04	—
Glutamic	1	1	−2	[CuL]	3.2–5.9	−5.50 ± 0.03	−5.50 ± 0.03	−5.46‡
(H_2L)	2	1	−2	$[Cu(HL)_2]$	3.2–5.9	−2.49 ± 0.05	−2.54 ± 0.05	—
	2	1	−3	$[Cu(HL)L]^-$	3.2–5.9	−7.18 ± 0.05	−6.99 ± 0.04	—
	2	1	−4	$[CuL_2]^{2-}$	3.2–6.7	−12.19 ± 0.04	−12.14 ± 0.04	−12.76‡
	1	1	−3	$[Cu(OH)L]^-$	8.0–9.5	−12.80 ± 0.08	−12.85 ± 0.08	—
Glutamine	1	1	−1	$[CuL]^+$	3.4–6.5	−1.34 ± 0.03	−1.36 ± 0.03	−1.20‡
(HL)	1	1	−1	[Cu(OH)L]	6.5–7.5	−8.54 ± 0.05	−8.57 ± 0.05	—
	2	1	−2	$[CuL_2]$	3.4–6.5	−3.95 ± 0.04	−3.89 ± 0.04	−3.69‡
Glutamylglutamic	1	1	−2	[CuHL]	3.5–6.7	−5.61 ± 0.06	−5.60 ± 0.05	—
(H_3L)	1	1	−3	$[CuL]^-$	3.5–6.7	−9.85 ± 0.06	−9.90 ± 0.06	—
	1	1	−4	$[Cu(OH)L]^{2-}$	3.5–6.7	−14.83 ± 0.04	−14.85 ± 0.05	—
	2	1	−4	$[Cu(HL)_2]^{2-}$	3.5–6.7	−10.80 ± 0.05	−10.76 ± 0.05	—
	2	1	−6	$[CuL_2]^{4-}$	3.5–6.7	−20.56 ± 0.05	−20.50 ± 0.05	—

*All constants were calculated with the programs SUPERQUAD [6] and BEST [7] from data obtained potentiometrically at 25°C and I = 0.15 M NaCl. The symbols p, q and r are used in the programs to indicate the stoichiometric coefficients associated with the possible equilibria in solution: p — coefficient for ligand; q — for copper(II); and r — for protons (note that OH^- binding in this convention contributes −1 to the global r value).
†For equilibria without reported values, none could be found in the literature.
‡From [19].

results obtained are presented in Table 1 and some literature results are also presented for comparison; the acidity constants of cholic and glycocholic acids determined previously [5] are also included.

Copper(II) complexes with cholic and glycocholic acids and with glutamic acid, glutamine and glutamylglutamic acid

The formation constants obtained with copper(II) are presented in Table 2, which includes also the pH range used in data acquisition and some literature values. A more specific discussion of the results is presented with respect to each ligand.

Bile acids. Under the condition used two types of complexes were detected $[CuB]^+$ and [Cu(OH)B], where B^- stands for the conjugate anion of bile acid HB; in all cases B^- acts as monodentate ligand.

Glutamic acid (sodium glutamate). Studies were undertaken with glutamic acid and with sodium glutamate and the data yielded the same results. The best overall fitting of the data assumed the presence in solution of the following copper(II) species: $[Cu(HL)_2]$; $[Cu(HL)L]^-$; [CuL] and $[CuL_2]^{2-}$. In these formulas H_2L represents the neutral acid (zwitterion); in all subsequent discussions L represents the fully deprotonated form of an aminoacid/peptide. It must be pointed out that for a mole ratio copper(II):sodium glutamate of 1:1, formation of $[Cu(OH)L]^-$ was also observed with $\beta = 12.80 \pm 0.08$ but only in the pH range 8.0–9.5; on the other hand detection of $[Cu(HL)]^+$ was not possible, despite earlier claims in the literature [19].

In these complexes, L^{2-} acts probably as a bidentate ligand through the terminal amino/carboxylic moiety forming a six-membered chelate ring, as have been observed for aspartic acid and asparagine [20]. In $[Cu(HL)_2]$ the ion is probably bound to the oxygen atom of the deprotonated C-5 carboxylate, an observation that stems from the fact that the appearance of these species occur at the same pH as formation of HL by loss of the proton at C-5. The distribution diagram also shows that for a mole ratio of 1:1, [CuL] is the most abundant form is solution at pH values smaller than 7; whereas for solutions with ligand excess $[CuL_2]^{2-}$ becomes the main form, probably a distorted six-coordinate complex with two glutamate anions bound equatorially and with the axial positions occupied by weakly coordinated water molecules. Application of the $\Delta \log K$ method [21], with $\Delta \log K = \log K^{CuL}_{CuL_2} - \log K^{Cu}_{CuL}$, yielded for glutamic acid a value of -1.19, thus providing further support for its behavior as a bidentate ligand when fully deprotonated, as the reference values for bidentate ligands lie in the range -1 to -2 log units [21].

Glutamine. The present data support the existence in solution of [Cu(OH)L]; $[CuL]^+$ and $[CuL_2]$; in these complexes and in the pH range used, the amide group shows no coordinating ability and L^- must act also as bidentate ligand, as the value of -1.27 calculated for $\Delta \log K$ also supports. The distribution diagram shows the same pattern for $[CuL]^+$ and $[CuL_2]$ as observed for glutamic acid.

Glutamylglutamic acid. From Table 2 it is clear that five equilibria were detected, but assumptions on the species formed is less straightforward; however, these equilibria must be associated with formation of the following complexes: [CuHL], $[CuL]^-$, $[Cu(OH)L]^{2-}$, $[Cu(HL)_2]^{2-}$ and $[CuL_2]^{4-}$. Support for formation of complexes with HL^{2-} can be gained by noting in the pH range studied the concentration of H_3L can be neglected, and that their concentration is maximal at pH values similar but smaller than that for which concentration of free HL^{2-} is highest, thus suggesting further that Cu^{2+} coordination induces deprotonation. The data also suggest that complexes with L^{3-} are predominant in solution at pH higher than 5; but the role of glutamylglutamate as a bidentate ligand is not so clear cut as the calculated value of $\Delta \log K$ (-0.86) is smaller than the reference values for bidentate ligands and just slightly outside the range for monodentate ligands, -0.5 to -0.8 log units [21]; probably the long peptide chain hinders chelation and the observed value reflects these opposite forces.

Interactions between bile acids and peptides

No interactions between glutamic acid, sodium glutamate and glutamine and any of the bile acids could be detected in the pH range 4–7. For the binary system glutamylglutamic acid/glycocholate (H_3L/B^-) three association

constants were determined (values from SUPERQUAD/BEST) in the pH range 4–8: (i) $p = q = 1$ and $r = 0$, with $\log \beta = 2.95 \pm 0.05$/$\log \beta = 2.89 \pm 0.05$; (ii) $p = q = 1$ and $r = -1$, with $\log \beta = -1.32 \pm 0.05$/$\log \beta = 1.28 \pm 0.05$; and (iii) $p = q = 1$ and $r = -2$, with $\log \beta = -6.03 \pm 0.4$/$\log \beta = -6.00 \pm 0.04$. These data support the existence in solution of the following 1:1 species (i) $[B^- \ldots H_3L]$; (ii) $[B^- \ldots H_2L^-]$; and (iii) $[B^- \ldots HL^{2-}]$. In all cases the interaction must take place between the carboxylate group of the bile salt and the amino group of the peptide, as the formation constant k_n for the reaction $B^- + H_nL^{n-3} \rightleftharpoons [B^- \ldots H_nL^{n-3}]$ $(n = 1–3)$ is approximately constant: $\log k_3 = 2.89 \pm 0.05$; $\log k_2 = 2.62 \pm 0.05$; and $\log k_1 = 2.51 \pm 0.04$ (all data from BEST).

Ternary complexes copper(II)/deprotonated bile acid/peptide

Cooperative effects in the binding of these metal ions to bile salts when coordinated to peptide, can be judged by the application of the $\Delta \log K$ method [21] that compares the difference in stability for reactions between CuL and $Cu(aq)^{2+}$, where $\Delta \log K = \log K^{Cul}_{CuB(L)} - \log K^{Cu}_{CuB}$ can be interpreted as a stability constant that corresponds to the equation $CuL + CuB \rightleftharpoons CuLB + Cu$. The experimentally determined values of $\Delta \log K$ can then be compared with statistical values calculated for the same quantities and thus providing a mean to assess cooperative effects in the formation of ternary complexes. Assuming a coordination number of six for copper(II), as observed in several complexes with amino acids, the relevant statistics parameters have values ≈ -0.30 for a distorted octahedral geometry (assuming one peptide acting as a bidentate and one deprotonated bile acid as a monodentate ligand); for coordination number four the value is -0.48 for tetrahedral geometry and with the same assumptions as above. Values for the calculated equilibrium constants for formation of ternary complexes are presented in Table 3, and Table 4 gives the values of $\Delta \log K$ calculated from data in Tables 2 and 3.

For glutamic acid (sodium glutamate) or glutamine, the main species in solution for both bile acids are $[CuB(H_2L)]^+$ {or$[CuB(HL)]^+$ for glutamine} and mainly $[CuB(L)]^-$ {or $[CuB(L)]$ for glutamine},

Table 3
Equilibrium constants ($\log \beta$) calculated for mixed complexes copper(II)/bile acid salt/peptide in aqueous solution*

Ligand 1	Ligand 2	m	p	q	r	Possible species	pH range	Superquad	Best
Cholate†	Glutamate	1	1	1	0	$[CuB(HL)]$	4.7–6.5	6.65 ± 0.02	6.60 ± 0.04
(B^-)	(HL^-)	1	1	1	1	$[CuB(H_2L)]^+$	4.7–6.5	11.17 ± 0.04	10.99 ± 0.04
		1	1	1	−1	$[CuB(L)]^-$	4.7–6.5	1.67 ± 0.05	1.67 ± 0.05
	Glutamine	1	1	1	−1	$[CuB(L)]$	4.7–6.5	1.59 ± 0.05	1.59 ± 0.05
	(HL)	1	1	1	0	$[CuB(HL)]^+$	4.7–6.5	6.69 ± 0.04	6.65 ± 0.04
Glycocholate	Glutamic acid	1	1	1	0	$[CuB(H_2L)]^+$	3.7–6.5	5.66 ± 0.04	5.69 ± 0.05
(B^-)	(H_2L)	1	1	1	−2	$[CuB(L)]^-$	3.7–6.5	−2.71 ± 0.03	−2.70 ± 0.04
	Glutamine	1	1	1	−1	$[CuB(L)]$	3.7–6.4	2.10 ± 0.03	2.12 ± 0.04
	(HL)	1	1	1	0	$[CuB(HL)]^+$	3.7–6.4	6.54 ± 0.04	6.58 ± 0.04
	Glutamylglutamic acid	1	1	1	−3	$[CuB(L)]^{2-}$	4.1–6.5	−6.85 ± 0.05	−6.82 ± 0.05
	(H_3L)	1	1	1	−4	$[Cu(OH)B(L)]^{3-}$	4.1–6.5	−11.60 ± 0.05	−11.62 ± 0.05

* All constants were calculated with the programs SUPERQUAD [6] and BEST [7] from data obtained potentiometrically at 25°C and $I = 0.15$ M NaCl. The symbols q and r have the meaning described in Table 2; m is the coefficient of ligand 1 and p that of ligand 2.
† In solutions of glutamylglutamic acid/cholate at pH <5 a precipitate is formed.

Table 4
Values of Δ log K for the mixed complexes Cu^{2+}/bile acid salt/peptide

Ligand 1	Ligand 2	Possible species	log $K^{CuL}_{CuB(L)}$	log β*	Δ log K†
Cholate	Glutamate	$[CuB(L)]^-$	3.10 ± 0.10	11.10 ± 0.07	+0.85 ± 0.15
	Glutamine	$[CuB(L)]$	2.93 ± 0.10	10.55 ± 0.07	+0.68 ± 0.15
Glycocholate	Glutamic acid	$[CuB(L)]^-$	2.79 ± 0.08	10.79 ± 0.05	−0.16 ± 0.13
	Glutamine	$[CuB(L)]$	3.44 ± 0.08	11.06 ± 0.05	+0.49 ± 0.13
	Glutamylglutamic acid	$[CuB(L)]^{2-}$	3.00 ± 0.15	9.64 ± 0.09	+0.05 ± 0.20

*$\log \beta = \log K^{Cu}_{CuL} + \log K^{CuL}_{CuB(L)}$. See Table 2 for the values of $\log K^{Cu}_{CuL}$.
†$\Delta \log K = \log K^{CuL}_{CuB(L)} - \log K^{Cu}_{CuB}$. See Table 2 for the values of $\log K^{Cu}_{CuB}$.

although for cholate the complex [CuB(HL)] with glutamate was also detected but in much smaller concentration; under no circumstance could the experimental data be described by any model with equilibria for formation of $[CuB(L)_2]$ (charges omitted). The pH range at which the observed complexes do form is very similar to that of the corresponding bindary complexes metal ion/glutamic acid (glutamate) or metal ion/glutamine. For cholate complexes, the values of Δ log K are positive and larger than 0.5 log units, a value also observed for Cu^{2+}/glycocholate/glutamine: in all cases suggesting a strong cooperativity in formation of these complexes.

The value of Δ log K for Cu^{2+}/glycocholate/glutamate is negative, slightly larger than the reference value of ≈ -0.30, and that of Cu^{2+}/glycocholate/glutamylglutamic acid shows a value of Δ log K near zero, thus in both complexes the case for cooperativity is less strong.

The cooperativity found in binding bile acids to metal ions bound to peptides takes place for small peptides, actually for amino acids, that coordinate through the amino/acarboxylic moiety; furthermore, for the dipeptide the dynamics of the longer chain makes chelation less favourable and also affects possible interactions with bile acids thus reducing cooperativity.

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