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Copper(II) increases bile acid binding to asparagine

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Abstract

Interactions of two bile acids (cholic and glycocholic acids) with asparagine have been studied by potentiometry in aqueous solutions under conditions similar to those observed in biological fluids (37°C and $I = 0.15$ M NaCl), and in the absence and presence of copper(II). To characterize the equilibria for the systems copper(II)/bile acid/asparagine, specifically to assess cooperative binding between bile acids and asparagine, the acidity constant of asparagine and formation constants for copper(II)/bile acid and copper(II)/asparagine were also obtained under the same conditions. The results obtained suggest cooperativity in the binding of bile acid to asparagine in the presence of copper(II). © 1998 Elsevier Science B.V.

Keywords: Asparagine; Bile acids; Complex formation; Cooperativity; Copper(II)

1. Introduction

Bile acids are detergent molecules which are synthesized in the liver from cholesterol and represent the major pathway for cholesterol elimination from the body. Bile acids are stored in the gall bladder prior to secretion, and undergo enterohepatic circulation several times a day via the small intestine and the terminal ileum before being returned in the portal blood. This transport through the enterohepatic system includes transport of bile acids across the sinusoidal membrane, for absorption from the portal blood, and across the canalicular membrane for excretion into the bile [1]. Three different mechanisms, at least, have been proposed to be involved in the basolateral uptake of bile salts into hepatocytes [2]. One of these mechanisms is thought to involve non-ionic diffusion followed by binding to cytosolic protein [3]. These proteins are also believed to be responsible for the intracellular transport of bile salts from basolateral to canalicular membrane sites, hence playing an important role in the hepatic circulation of bile salts. Other transport mechanisms have also been suggested that invoke transport by membrane-bound compartments, e.g., vesicles [2].

Bile acid transport studies have known a recent resurgence, not only to help understand the action, transport and overall function of bile acids in the enterohepatic system, but also mainly to

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evaluate the possible use of bile acids as therapeutic agents [4]. A 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. Cooperative effects in binding to metal ions coordinated to proteins can provide insight into the transport mechanisms and also into the use of metal ions to promote bile salt transport across biological membranes.

In the present study, a very simple model was chosen to assess cooperative binding in aqueous solution between asparagine and bile acids (cholic and glycocholic) mediated by copper(II), due to its presence in the liver, and under conditions similar to those observed in physiological media, namely 37°C and at the ionic strength of biological fluids. The results obtained in this work suggest that in aqueous solution and in the absence of micelle formation, binding of bile acids to amino acids is induced by the presence of copper(II).

2. Experimental

2.1. Reagents and solutions

The bile salts (sodium cholate and sodium glycocholate) and asparagine (all from Sigma) were used without further purification. All other chemicals were from Merck (grade pro analysi); and all solutions were prepared with CO_2-free, double-deionized water (conductivity less than 0.1 μS cm^{-1}). The concentration of stock solutions of bile salts was established by conductimetric titrations with 0.1 M HCl (Merck; Titrisol) in a Crison Micro CM 2202 conductivity meter, and that of stock solutions of copper(II) by potentiometric titrations with EDTA (Merck; Titrisol).

2.2. Potentiometric measurements

All potentiometric measurements were carried out with a Crison 2002 pH meter and 2031 buret controlled by a Philips TC 100 microcomputer coupled to an IBM-compatible personal computer for data manipulation. The electrode assembly was made up of an Orion 900029/4 AgCl/Ag reference electrode with a Russell SWL glass electrode. System calibration was performed by the Gran method [5] in terms of hydrogen ion concentration, 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 37°C in a double-walled glass cell.

2.3. Potentiometric determination of acidity and stability constants

The acidity constants of the bile salts have been published elsewhere [6]; for asparagine, it was reevaluated by titrating 20.00 ml of acidified (1 mM HCl) aqueous solutions of asparagine (both 1 and 2 mM). Interactions between the bile acids salts with the asparagine was assessed by titrating 20.00 ml of aqueous solutions (4 mM HCl) of the corresponding bile salt (1–2 mM) and of asparagine (1–2 mM). Stability constants of copper(II) complexes with bile salts (cholate and glycocholate) and with asparagine were determined by titrating 20.00 ml of aqueous solutions of the appropriate bile salt or of asparagine (1–2 mM), copper nitrate(II) (0.5–2 mM) and HCl (0.2–0.4 mM). The mixed stability constants copper(II)/bile salt/asparagine were evaluated by titrating 20.00 ml of aqueous solution of bile salt (1–2 mM), asparagine (1–2 mM), copper nitrate (II) (0.5–2 mM) and HCl (0.2–0.4 mM). Titrations were performed at 37°C using approximately 0.01 M NaOH; the titrant solutions had the ionic strength adjusted to 0.15 M with NaCl. All determinations with bile acids salts were performed at concentrations well below the critical micelle concentration [7].

Calculations were performed with data obtained from at least six independent titrations, each with more 30 points, and the experimental titration data were analyzed using the computer programs Superquad [8] and Best [9]; 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

Table 1
Equilibrium constants (log β) calculated for copper(II)/bile acid salts and copper(II)/asparagine in aqueous solution[a]

Ligand	p	q	r	Possible species	pH range	Superquad	Best
Asparagine (HL)	1	0	1	[HL]	3.0–5.0	2.16 ± 0.05	2.12 ± 0.03
	1	0	−1	$[L]^-$	7.0–9.0	-8.72 ± 0.04	-8.70 ± 0.04
	1	1	−1	$[CuL]^+$	3.0–5.0	-0.62 ± 0.03	-0.69 ± 0.03
	1	1	−2	[Cu(OH)L]	3.5–7.0	-6.96 ± 0.08	-6.90 ± 0.08
	2	1	−2	$[CuL_2]$	3.0–8.0	-2.78 ± 0.02	-2.81 ± 0.06
Cholate (B^-)	1	0	1	$[B]^-$	4.0–6.0	4.75 ± 0.03	—
	1	1	0	$[CuB]^+$	4.5–5.5	2.48 ± 0.04	2.54 ± 0.03
	1	1	−1	[Cu(OH)B]	4.5–6.0	-4.12 ± 0.08	-4.11 ± 0.08
Glycocholate (B^-)	1	0	1	$[B]^-$	3.5–5.0	3.67 ± 0.01	—
	1	1	0	$[CuB]^+$	4.0–5.5	3.04 ± 0.03	3.03 ± 0.03
	1	1	−1	[Cu(OH)B]	4.0–6.0	-3.69 ± 0.08	-3.62 ± 0.08

[a] All constants were calculated with the programs SUPERQUAD [8] and BEST [9] from data obtained potentiometrically at 37°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).

[10] in which the errors are calculated as the maximum difference between the logarithm of the average of the antilogarithms of the calculated pK values and their individual values.

3. Results and discussion

3.1. Acidity constants

The acidity constants for cholic and glycocholic acids, under conditions identical to those used in the present work, have been obtained previously [6]. For asparagine (HL stands for the zwitterion), experimental data were obtained in the pH ranges 3.0–5.0 and 7.0–9.0, the first associated with deprotonation of the carboxylic and the second with the amino group (Table 1). Acidity constants for asparagine have been extensively reported in the literature and the values recommended by IUPAC [11] ($pK_{a1} = 2.15$ and $pK_{a2} = 8.71$; 0.10 M $< I <$ 0.20 M NaCl and 25°C) are very similar to those reported in this work ($pK_{a1} = 2.16$ and $pK_{a2} = 8.72$).

The unusual combination of a carboxylic and an amino group bound to the same carbon atom in all amino acids decreases the pK_a of the acid by two units and that of the amine by one unit, when compared with typical values of carboxylic acids and amines. Also, for simple amino acids, those without any other functional group attached to the carbon skeleton, the average of their pK_{a1} and pK_{a2} values is almost constant (5.98 ± 0.05), what suggests that these different groups affect both groups in a similar way.

The introduction of side chains with electron-withdrawing groups (hydroxyl, carboxylic or amide groups) decreases even further the pK_a values of the terminal carboxylic acid and amine (thereby reducing their average). More interesting is the observation that these electron-withdrawing substituents are also going to affect the pK_a values of both groups in a manner similar to that of electron-releasing groups. In fact, a plot of the IUPAC recommended values for the lowest (or the highest) pK_a value [11,12] for several amino acids against their difference yields a straight line (Fig. 1). This plot can be used to judge the quality of the acidity constants reported/determined. As an example, we note that for the values reported in the literature for aspartic [13–17] and glutamic acid [13,18–21], only one set for each amino acid set falls near the line.

3.2. Bile acid/asparagine interactions

Direct evidence for formation of a molecular complex bile acid/asparagine in solution in the pH

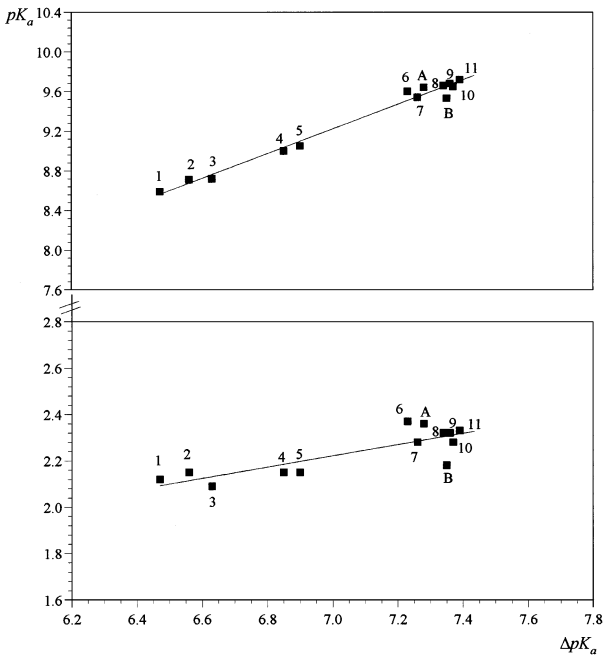

Fig. 1. Plot of IUPAC recommended values for the lowest and highest pK_a values of several amino acids [11,12] against their difference, ΔpK_a: (1) threonine, (2) serine, (3) methionine, (4) asparagine, (5) glutamine, (6) glycine, (7) valine, (8) leucine, (9) alanine, (10) isoleucine and (11) 2-aminohexanoic acid. Literature values for (A) aspartic acid [14] and (B) glutamic acid [19] are also included.

range used, can be gathered by noting that a formation constant was obtained (Table 2) for the equilibrium

$$B^- + HL \rightleftarrows B(HL)$$

where B^- stands for the conjugate anion of a bile acid. This result supports formation of a bond between the deprotonated carboxylic group of the bile acid and the protonated amino group of the zwitterionic form of asparagine.

3.3. Copper(II) complexes

Table 1 also presents the formation constants ($\log \beta$) for copper(II) with cholic and glycocholic acids, and with asparagine, as well as the pH intervals in which data were collected. For bile acids, with the assumption that B^- behaves as a monodentate ligand, the data support the existence of only two complexes with Cu(II): $[CuB]^+$ and [Cu(OH)B]. For the binary system Cu(II)/asparagine, the model that best fits the data assumes the occurrence of three equilibria in solution that correspond to formation of the following species: $[CuL]^-$, $[CuL_2]$ and [Cu(OH)L] (L^- represents fully deprotonated asparagine). The values reported in the literature for the first two stability constants, both at $I = 0.1$ M NaCl and 25°C ($\log K_1 = 7.86$ and $\log K_2 = 6.56$ [11], and $\log K_1 = 7.69$ and $\log K_2 = 6.69$ [22]), and at $I =$ 0.15 M NaCl and 37°C ($\log K_1 = 7.71$ and $\log K_2 = 6.50$ [23]), are similar to, although systematically a little lower than, those obtained in the present work, $\log K_1 = 8.03$ and $\log K_2 = 6.60$.

As reported previously, both asparagine and aspartic acid [22], and glutamine and glutamic acid [24], act probably as a bidentate ligand bound through the amino and carboxylic moi-

Table 2
Equilibrium constants ($\log \beta$) calculated for binary systems bile acid salt asparagine and for mixed complexes copper(II)/bile acid salt/asparagine in aqueous solution[a]

Ligand 1	Ligand 2	m	p	q	r	Possible species	pH range	Superquad	Best
Asparagine(HL)	Cholate(B^-)	1	1	0	0	[(B)HL]	5.0–7.0	2.63 ± 0.03	2.63 ± 0.01
		1	1	1	0	$[Cu(B)HL]^+$	4.0–6.5	8.38 ± 0.08	8.37 ± 0.06
		1	1	1	−1	[Cu(B)L]	4.0–6.5	2.25 ± 0.05	2.25 ± 0.06
	Glycocholate(B^-)	1	1	0	0	[(B)HL]	4.0–7.0	2.96 ± 0.08	2.93 ± 0.01
		1	1	1	0	$[Cu(B)HL]^+$	3.5–4.5	6.51 ± 0.05	6.49 ± 0.08
		1	1	1	−1	[Cu(B)L]	3.5–4.5	3.03 ± 0.04	3.00 ± 0.05

[a] All constants were calculated with the programs SUPERQUAD [8] and BEST [9] from data obtained potentiometrically at 37°C and $I = 0.15$ M NaCl. The symbols q and r have the meaning described in Table 1; m is the coefficient of ligand 1 and p that of ligand 2.

Table 3
Values of $\Delta\log K$ for the mixed complexes copper(II)/bile acid salt asparagine

Ligand 1	Ligand 2	Possible species	$\log K^{CuL}_{Cu(B)L}$	$\log \beta$ [a]	$\Delta\log K$ [b]
Cholate	Asparagine	[Cu(B)L]	2.94 ± 0.10	10.97 ± 0.06	$+0.46 \pm 0.15$
Glycocholate	Asparagine	[Cu(B)L]	3.72 ± 0.08	11.75 ± 0.05	$+0.68 \pm 0.13$

[a] $\text{Log } \beta = \log K^{Cu}_{CuL} + \log K^{CuL}_{Cu(B)L}$. See Table 1 for the values of $\log K^{Cu}_{CuL}$.
[b] $\Delta\log K = \log K^{CuL}_{Cu(B)L} - \log K^{Cu}_{CuB}$. See Table 1 for the values of $\log K^{Cu}_{CuB}$.

eties, thus forming five membered chelate rings. In $[CuL_2]$, both anions are bound to the metal ion as bidentate ligands, thus yielding a square-planar copper(II) moiety, to which two water molecules may weakly coordinate to the axial positions. Further support for the bidentate behavior of fully deprotonated asparagine as a bidentate ligand can be gained by applying the $\Delta\log K$ method [25], where:

$$\Delta\log K = \log K^{CuL}_{CuL_2} - \log^{Cu}_{CuL}$$

to its copper complexes. The calculated value, $\Delta\log K = -1.43$, lies in the range -1 to -2, typical of ligands bound to copper in a bidentate mode, whereas the limits for typical values for monodentate ligands are -0.5 and -0.8.

For solutions of copper(II), asparagine and one bile acid (cholic or glycocholic), the best fit of the experimental data was obtained assuming the formation of two ternary complexes in solution, [Cu(B)L] and $[Cu(B)HL]^-$, and the calculated formation constants are reported in Table 2. Under no circumstance could the experimental data be fitted to any model in which formation of $[CuB(L)_2]$ was included.

To assess cooperativity in the formation of copper/bile acid/asparagine complexes, the value of $\Delta\log K$ was obtained from the formation constants of the binary and ternary complexes determined in this work, and is given by $\Delta\log K = \log K^{Cu}_{Cu(B)L} - \log^{Cu}_{CuB}$. It has been shown, based on statistical considerations, that this value should be about -0.30 for complexes with a distorted octahedral geometry, and equal to about -0.48 for four-coordinate complexes (these values presuppose that one ligand acts as a bidentate) [25]. As the calculated values (Table 3) are positive for both bile acids (0.46 for cholic and 0.68 for glycocholic), a strong cooperativity is expected in the binding of bile acids to copper ions bound to asparagine, as was found with other amino acids [24].

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References

[1] C.J. Sippel, M. Ananthanarayanan, F. Suchy, Am. J. Physiol. 258 (1990) 728–737.
[2] P.B. Hylemon, Adolf Windaus prize lecture: biochemistry and genetics of intestinal bile salt metabolism, in: A. Stiehl, W. Gerok, (Eds.), Falk Symposium 58, Kluwer Academic Publishers, London, 1990, pp. 1–21.
[3] A. Stolz, H. Takikawa, O. Murad, N. Kaplowitz, Annu. Rev. Physiol. 51 (1989) 161.
[4] A. Stiehl, W. Gerok, Bile acids as therapeutic agents. From basic science to clinical practice, in: A. Stiehl, W. Gerok, Falk Symposium 58, Kluwer Academic Publishers, London, 1990.
[5] G. Gran, Analyst 77 (1952) 661–671.
[6] B. Castro, J.L.F.C. Lima, M.S.F.F.H. Reis, Analusis 22 (1994) 281–286.
[7] A. Roda, H. Hoffman, J. Biol. Chem. 258 (1983) 6362–6370.
[8] P. Gans, A. Sabatini, A. Vacca, J. Chem. Soc., Dalton Trans. (1985) 1195–1200.
[9] A.E. Martell, R.J. Motekaitis, Determination and Use of Stability Constants, VCH, Weinheim, 1988.
[10] A. Albert, E.P. Serjeant, The Determination of Ionization Constants, 2nd ed., Chapman and Hall, London, 1971, pp. 20–22.
[11] G. Berthon, Pure Appl. Chem. 67 (1995) 1117–1240.
[12] I. Sovago, T. Kiss, A. Gergely, Pure Appl. Chem. 65 (1993) 1029–1080.
[13] A.E. Martell, R.M. Smith, Critical Stability Constants, vol. 5, Plenum Press, New York, 1982, p. 12–13.

[14] G. Barnard, T. Boddington, J. Gregor, L. Pettit, Talanta 37 (1990) 219–224.
[15] J. Pessoa, R. Marques, Polyhedron 9 (1990) 81–87.
[16] M. Khaledi, A. Rodgers, Anal. Chim. Acta 239 (1990) 121–125.
[17] M. Maeda, K. Okada, Y. Tsukamoto, J. Chem. Soc., Dalton Trans. (1990) 2337–2341.
[18] J. Pessoa, R. Marques, L. Boas, Polyhedron 11 (1992) 1449–1454.
[19] K. Burger, P. Sipos, M. Veber, I. Horvath, Inorg. Chim. Acta 152 (1988) 233–238.
[20] D. Kovala, M. Dermetzis, Bull. Soc. Chim. Fr. 2 (1988) 793–795.
[21] K. Prasad, A. Rao, M. Mohan, J. Coord. Chem. 16 (1987) 251–256.
[22] L. Lomozik, A. Wojciechowska, Polyhedron 8 (1989) 1–6.
[23] G. Berthon, B. Hacht, M.-J. Blais, P. May, Inorg. Chim. Acta 125 (1986) 219–227.
[24] B. Castro, J.L.F.C. Lima, M.S. Reis, J. Pharm. Biom. Anal. 13 (1995) 465–470.
[25] H. Sigel, Angew. Chem., Int. Ed. Engl. 14 (1975) 394–401.