Role of Electrostatic Forces in Hydroxy and Keto Bile Salt-Albumin Interactions: Some Experimental Observations

B. FARRUGGIA and G. PICÓ

Departamento de Química-Física and Instituto de Fisiología Experimental (CONICET), Facultad de Ciencias Bioquímicas y Farmaceúticas, Universidad Nacional de Rosario, Suipacha 570, (2000) Rosario, Argentina.

Abstract. Proton binding to bovine serum albumin and effects on hydroxy and keto bile salts-albumin binding were studied within a pH range between 5 and 10. Electrostatic forces contribute to the binding of these ligands to albumin; prototropic groups of albumin such as imidazol are involved in the interaction. Bile salts binding produces a shift in pK of these groups. It is postulated that hydroxy bile salt-albumin binding is linked whith the N⇌B transition of the protein, while for keto bile salts a microarrangement in the protein binding sites is driving the interaction.

Key words: Bile salts — Albumin binding — Acid-base titration — Acid-base equilibrium

Introduction

A study of acid-base equilibrium dependence of ligand-protein macromolecule interaction may help in understanding factors which contribute to the reversible binding. Numerous reports have appeared concerning the influence of pH on enzyme-substrate interaction; nevertheless, little is known about the pH effects on binding of small ligands to albumin (Janssen et al. 1981). A similar study can be expected to contribute to the understanding of molecular mechanisms (electrostatic forces) that play a role in the formation of albumin complexes (Janssen et al. 1985). Bile salts were used in the present experiments for this purpose. This choise was based on the important physiological role these ligands play. In previous reports (Picó and Houssier 1989; Picó 1990) the bile salts binding sites on bovine serum albumin were identified. The present work was focused on the study of the effects of hydroxy and keto bile salts binding on the acid-base equilibrium of bovine serum albumin.

Materials and Methods

Bovine serum albumin (BSA), cholanic 3-one (C3), cholanic 3,6-dione (C3–6), dehydrocholic acid (DHC), cholic acid (C), hydroxycholic acid (HDC) and lithocholic acid (LC) were all purchased from Sigma (USA) and used without further purification. Solutions of bile salts (BS) were prepared from the respective compounds in solid state and neutralized whith an equivalent amount of NaOH. BSA solution was deionized by repeated passing through an ion-exchange column until showing constant electric conductivity. The average pH of the resulting solution was 5.4 (NaCl 0.15 $mol.l^{-1}$, 20 °C, protein concentration $150.10^{-6} mol.l^{-1}$). The BSA concentration was determined from absorbance at 280 nm. BS-BSA binding was determined using the quenching effect of BS on native fluorescence emission of BSA (Picó and Houssier 1989). The effect of the ligands on the acid-base equilibrium of the protein was determined by acid-base titration of albumin according to Janssen and van Wilgenburg (1978) in the presence of different concentrations of BS (in 0.15 $mol.l^{-1}$ NaCl) or in the absence of BS. The fluorescence measurements were performed using a Jasco FP 770 spectrofluorometer, and an Orion 720 pH-meter with an accuracy of 0.001 pH units was used for pH determinations.

Results

The following BS were tested: monohydroxy (LC), dihydroxy (HDC) and trihydroxy derivatives (C) and their keto homologues C3, C3–6 and DHC. BS binding to BSA assayed by the fluorescence quenching method gave linear Scatchard plots for C3 and C (Fig. 1*a*), while the other BS yielded non-linear plots (Fig. 1*b*). DHC quenched BSA fluorescence with a low yield; this prevented as from studying the binding of this BS to BSA. The non-linear plots suggested the presence of several types of binding sites in the protein, or interactions between them, a situation no usually encountered with albumin. The Scatchard plots were interpreted assuming two classess of independent binding sites, as reported previously (Picó and Houssier 1989). Upon testing BS-BSA binding at different pH (ranging-between 5 and 10), a change in the affinity constant was found. The dependence of log K on pH is show in Fig. 2. It can be seen that K value for hydroxy BS increases upon raising pH from 5 to 10, a similar behavior was observed for the keto BS.

To obtain more information about the influence of pH on the BS-BSA complex formation, the protein was titrated in the presence and/or absence of BS. The results were expressed as different acid-base titration curves (ΔZH^+ vs. pH) where ΔZH^+ is the difference between the number of protons bound by the albumin in the presence and absence of BS respectively at the same pH. As judged by the ligand-protein ratio range analyzed (between 1 and 4), only the primary BS binding sites of the albumin are occupied, the ligand fraction bound to the secondary sites being negligible (Picó et al. 1987). Thus, the results obtained from the acid-base titration curves may be considered to reflect BS binding to the primary sites of the protein.

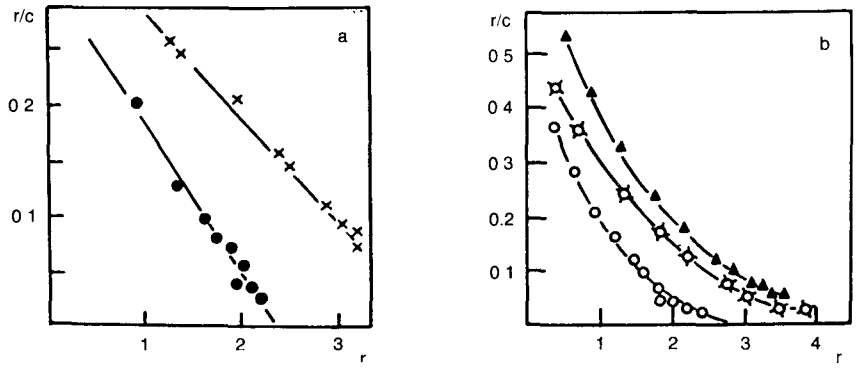

Figure 1. Scatchard plots for the binding of BS to BSA. Albumin concentration: 20.10^{-6}mol.l^{-1}. Temperature 20 °C. Medium phosphate buffer, ionic strength 0.15 mol.l^{-1} (NaCl): c is the free ligand concentration, and r is the ratio between the ligand bound to the protein and the total protein concentration. *a*: C3 (•), C (×); *b*: C3–6 (○), HDC (¤), LC (▲)

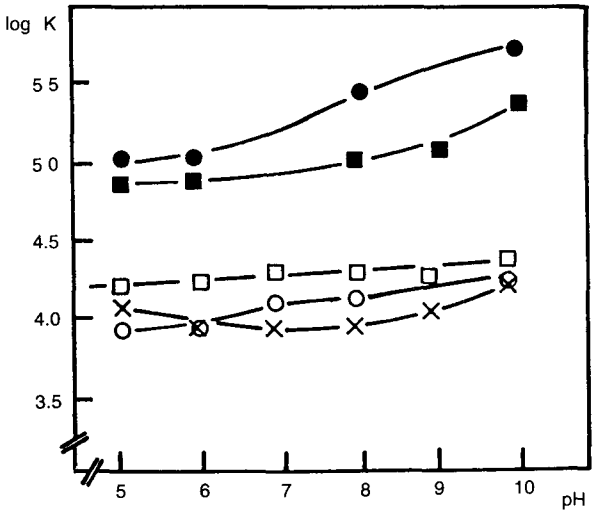

Figure 2. Effect of pH on the affinity constant (log K) of the BS-BSA complexes. For other conditions see legend to Figure 1. C3–6 (○), HDC (□), LC (■), C (×), C3 (•). The K values relate to the primary binding sites of the protein.

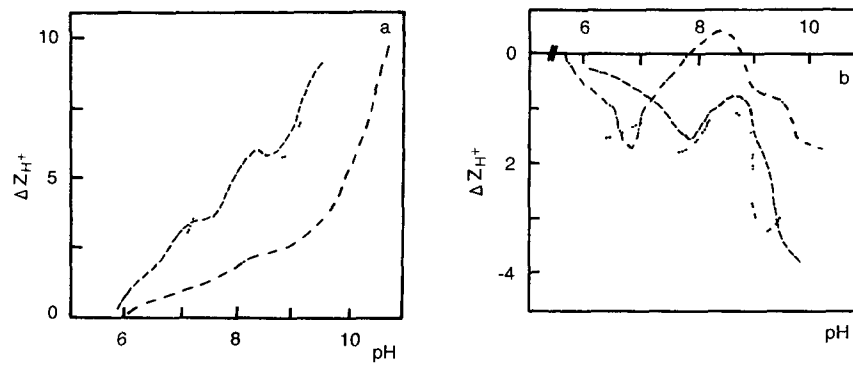

Figure 3. Differences between the number of protons bound by BS-BSA and BSA The ΔZH^+ values are given per mole of protein Temperature 20 °C, ionic strength 0 15 mol l^{-1} (NaCl) Albumin concentration 150 10^{-6} mol l^{-1} The BS-protein ration was 4 a C3 ( ), C3-6 (– – –), DHC (– –), b LC (– –), HDC ( ), C (– – –)

Fig 3 shows the albumin titration curves obtained in the presence of BS for a BSA-BS ratio of 4 There is a clear BS effect on the acid-base equilibrium of the albumin as the ΔZH^+ values in the pH range tested were not zero In the pH range studied, ΔZH^+ for hydroxy BS is negative, which means that at a given pH the albumin has less protons bound in the presence of hydroxy BS In other words, protons are released by the protein upon BS addition In terms of pK values it means that some groups titrable in the neutral region undergo a pK shift downwards upon addition of hydroxy BS Judging by the observed amplitude of ΔZH^+ the number of protons involved in this process is dependent on the hydroxy groups present in the BS molecule

For the keto BS, in the pH range from 6 to 10, a positive ΔZH^+ value was obtained According to the definition of ΔZH^+, this indicates an upward pA change of the same groups of the protein Considering the pH region within which this effect occured, imidazole groups are the most likely candidates for being involved in this binding Hydrogen ion concentration affects the charge of the carboxyl group of the BS molecule, the pK values for all the BS tested range between 6 and 6 5, the effect of pH variation on the BS-BSA interaction, as shown in Fig 3, is thus a consequence of changes in the ionization state of the BS between pH 6 and 7 At high pH values, the charge of the BS molecule is dependent on pH, and the ΔZH^+ value may only be considered a contribution of the prototropic groups in the albumin molecule

C3 and C produced ΔZH^+ peaks at the same pH value (8 2), while DHC did not produce any peak in the ΔZH^+ value Hydroxy BS produced negative a ΔZH^+

Table 1.

Bile salt	ΔZH^+ (mol H^+/mol BSA)	pH
LC	-3.5	7.2
HDC	-4.0	7.8
C	-3.0	8.0
C3	7.3	8.2
C3–6	6.2	8.2
DHC	–	–

peak between pH 7 and 8.2.

Discussion

Several authors have reported that in the neutral region albumin can exist at least in two conformations: conformation B is encountered at pH 8, the N form is the predominat at pH below 6. The B form seems to have a higher affinity for BS than does the N conformation form, as suggested by Fig. 2. This means that upon addition of BS to BSA the conformational equilibrium between the N and B form will shift towards the B state. In B form imidazole groups have lower pK values than in N form (Janssen et al. 1985), resulting in a release of protons upon BS binding (Fig. 3). The effects of hydroxy BS on the shift of N⇌B equilibrium towards the B form increases with the increasing number of polar substituents (hydroxy groups) present in the BS molecule. Similar results have been reported for the anion warfarin binding to albumin (Janssen et al. 1981). Parameter ΔZH^+ also describes the pH dependence of the logarithm of K on the BS-BSA affinity constant: $d \log K/dpH = \Delta ZH^+$ (Steinhard and Reynolds 1969), where ΔZH^+ is the difference in protons bound, associated with the binding process characterized by $\log K$. For hydroxy BS, ΔZH^+ is positive (see Fig. 3 and Table 1); this means that $\log K$ increases with the increasing pH (Fig. 2). Keto BS have pK values similar to those of hydroxy BS: increasing pH produced increases in the K values, resulting in a greater affinity for the B than the N form (Fig. 2). The inverse effect of keto BS on the BSA acid-base equilibrium may be due to a mechanism of binding different from that described for hydroxy BS. The effect of pH on ΔZH^+ (Table I) shows that keto BS produced an increase in the pK value of imidazole groups of albumin. This change was independent of the number of keto groups present in a BS molecule, but there was a close relation between the ΔZH^+ value and the BS structure.

It has been postulated that the increase of pK values of the prototropic groups may be due to the presence of negative charge near the imidazole group (Janssen

et al. 1981). Keto groups have a dense electron charge and are able to disperse the positive charge of the proton groups of imidazole, while the effect of the hydroxy groups is very weak in this respect. Thus, the positive charge density on the hydrogen bound to the amino group of imidazole is reduced, producing an increase in the pK value of this prototropic group.

Acknowledgements. This research was supported by a grant from the Consejo Nacional de Investigaciones Cientificas y Técnicas de la República Argentina (CONICET). G.P. is a Member of CONICET and B.F. is a Member of CIUNR.

References

Janssen L. H. M., van Wilgenburg M. T. (1978): Experimental observation of ionic forces in drug-protein interaction as illustrated by the binding of sulfaethindole by human serum albumin. Mol. Pharmacol. **14,** 884—889

Janssen L. H. M., van Wilgenburg M. T., Wilting J. (1981): Human serum albumin as an allosteric two-state protein. Evidence from effect of calcium and warfarin on proton behaviour. Biochim. Biophys. Acta **669,** 244–250

Janssen L. H. M., Drige J., Durlinger F., Fruytier F. J. (1985): The pH dependence of the thermodynamics of the interaction of diazepam with human serum albumin. Biol. Chem. **260,** 11442—11445

Picó G. (1990): Use of 1-anilino-8-naphthalene sulfonate as a reporter molecule to study the bile salts-bovine serum albumin binding. Stud. Biophys. **136,** 21—26

Picó G., Favazza M., Gatti C. (1987): Thermodynamic study of taurocholate binding to rat serum albumin. Gen. Physiol. Biophys. **6,** 637—643

Picó G., Houssier C. (1989): Bile salts-bovine serum albumin binding: spectroscopic and thermodynamic studies. Biochim. Biophys. Acta **999,** 128—134

Steinhard J., Reynolds J. (1969): Multiple Equilibria in Protein. Academic Press. New York.

Final version accepted December 15, 1991