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Bilirubin, model membranes and serum albumin interaction: The influence of fatty acids

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ABSTRACT

Electronic circular dichroism (ECD), absorption and fluorescence spectroscopy were used to study the enantioselective interactions which involved bilirubin (BR), liposomes, human serum albumin of two different purities, pure (HSA) and non-purified of fatty acids (FA-HSA), and individual fatty acids.
The application of the ECD technique to such a complex problem provided a new perspective on the BR binding to liposomes. Our results demonstrated that in the presence of pure HSA, BR preferred to bind to the protein over the liposomes. However, in the presence of FA-HSA, BR significantly bound to the liposomes composed either of DMPC or of sphingomyelin and bound only moderately to the primary and secondary binding sites of FA-HSA even at high BR concentrations. For the DMPC liposomes, even a change of BR conformation upon binding to the primary binding site was observed. The individual saturated fatty acids influenced the BR binding to HSA and liposomes in a similar way as fatty acids from FA-HSA. The unsaturated fatty acids interacted with BR alone and prevented it from interacting with either 99-HSA or the liposomes. In the presence of arachidonic acid, BR interacted enantioselectively with the liposomes and only moderately with 99-HSA.
Hence, our results show a substantial impact of the liposomes on the BR binding to HSA. As a consequence of the existence of fatty acids in the blood plasma and in the natural structure of HSA, BR may possibly bind to the cell membranes even though it is normally bound to HSA.

1. Introduction

Every day a certain amount of "old" blood cells is replaced by newly produced ones. During this process, an orange-colored pigment bilirubin (BR) is formed as a product of the decomposition of the blood pigment heme. BR is afterwards converted to a more water-soluble form and is excluded from the body [1]. BR belongs to the group of non-planar tetrapyrrolic pigments which form a helical spatial structure, with either a P- or M-sense of helicity [2–5] (Fig. 1). The racemization barrier between these two forms is low, and because of this, the P- and M-forms interconvert rapidly. Therefore, BR exists as a racemate in a homogenous solution and also when it is unbound in the blood plasma. Although there are several polar groups in the BR structure, it behaves mostly as a nonpolar compound, because the polar groups are intramolecularly connected with hydrogen bonds [2–5].

Although it is a waste product, BR fulfills a wide range of biological activities in the human body [1]. In the pathologic state, BR may accumulate and at higher concentrations its negative effects may appear [6–17]. Among the most discussed effects of BR are its deposition in tissues and its influence on nerve cell membranes which may result in cell disturbance. Moreover, BR binding to brain nerve cells is supposed to be one of the main causes of BR encephalopathy in jaundiced newborns. The interaction of BR with cells was previously studied both for model situations and in physiological conditions [13–16,18–28]. It was found that BR preferentially interacts enantioselectively with different liposomic models of cell membranes [18–20,29]. This means that one of the two enantiomeric forms (P- or M-form) of BR binds more to the liposomes and because the two forms interconvert very rapidly, the non-bound BR remains racemic. The enantiodiscrimination of BR on the membranes is supposed to be one of the primary causes of the described neurotoxic effects of BR. Because of the chiral nature of this interaction, chiroptical techniques, electronic and vibrational circular dichroism (ECD, VCD) were advantageously used to study bound BR and its effects [18,29].

Under the normal conditions in the human body, BR should not be dangerous to our well-being because it is mainly transported in the

Abbreviations: 99-HSA, 99% pure HSA; AA, arachidonic acid; BR, bilirubin; DLS, dynamic light scattering; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; ECD, electronic circular dichroism; FA, fatty acid; FA-HSA, human serum albumin with 4% of fatty acids; HSA, human serum albumin; IR, infrared; LA, linoleic acid; LUV, large unilamellar vesicle; MA, myristic acid; OA, oleic acid; PA, palmitic acid; SA, stearic acid; UV–Vis, ultraviolet–visible

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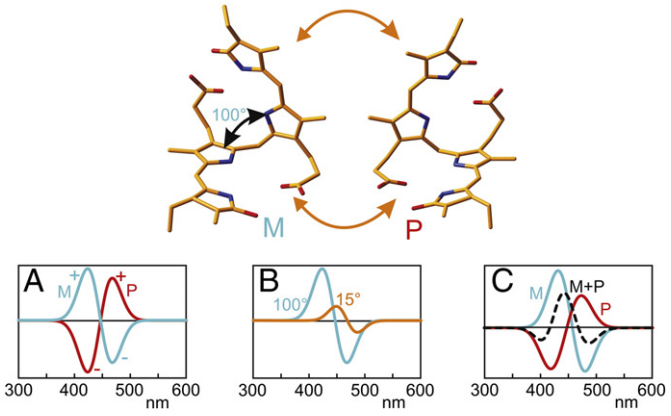

Fig. 1. Spatial structure of the M- and P-forms of BR with the dihedral angle estimated for BR in aqueous solution. (A) The negative couplet (spectral shape composed of two neighboring bands of opposite signs, negative band being at a higher wavelength than the positive) and positive couplet observed typically in the ECD spectra for the M- and P-forms of BR, respectively. (B) A simulation of the influence of the dihedral angle in the BR structure on the ECD spectra estimated with the exciton coupling method (ref. [26]), note a shift of the couplet position and the decrease of its intensity. (C) A simulated spectral overlap possible for the ECD spectra of the M- and P-forms of BR with different dihedral angles in their structure.

blood plasma by human serum albumin (HSA), one of the most abundant carrier proteins. The HSA structure is comprised of three domains, each of which consists of subdomains. The protein has extensive binding possibilities for different drugs, drug-analogs, hormones, steroids and small molecules, and also works as a main transporter of fatty acids (FAs) in the blood plasma. FAs are in fact primary ligands for HSA and at least seven different binding sites of FAs were described in the HSA structure [30–33] with very diverse binding affinities [34]. BR has three potential binding sites in the structure of HSA: one primary site located in subdomain IIA of HSA with a considerably high binding constant ($K_a \sim 10^8\ M^{-1}$) and two secondary sites located in subdomains IB and IIIA with lower and similar binding constants ($K_a \sim 10^6\ M^{-1}$) [35–39]. Remarkably, BR preferentially binds in its P-form to all three binding sites of HSA [35,36,39–43]. It should be noted here that due to the difference in the binding constants, the binding to the secondary sites occurs mostly after the primary site has been saturated. So we cannot observe the binding to the secondary sites alone unless the primary site is blocked by another compound.

Our study is aimed at the situations that may arise on the molecular level in the human body both during standard and pathologic states. As the enantioselective interaction of BR with liposomic models of membranes was confirmed [18,29], our present study focuses on the ternary systems of BR with liposomes and HSA. The object of our study is, first, whether BR will bind to the liposomes even in the presence of HSA, for which it has a high binding constant, and, second, what would occur at increased concentrations of BR when some of the HSA binding sites would be occupied by BR or by other compounds. Previous studies [44,45] showed that the tendency of BR to bind to HSA is mostly higher than to liposomes, but they proved a strong dependence on the ambient conditions and liposome composition.

Hence, we studied the influence of HSA on the BR interactions with liposomes composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, a model of ordinary cells) and of sphingomyelin (a model of nerve cells). These two types of liposomes were chosen, because they serve as an appropriate model of natural membranes [29]. Our previous study [29] also showed a negligible effect of cholesterol on the interactions of the selected liposomes with BR and, therefore, cholesterol was not included in this study. Only a few tests were run for each studied system to confirm that its presence did not influence the systems with the FAs and serum albumins used.

As it is mainly unbound free BR, the effects of which are neurotoxic, and because its concentration is strongly influenced by the binding capacity of HSA, two different HSAs were tested: one pure and one non-purified with bound FAs, which is supposed to have different binding affinities for BR. HSA does not only work as an FA transporter in the blood plasma [32,34,46,47] but FAs bound to HSA were found to play a significant role during many tasks of this protein especially in the case of drug treatment [32,34,48,49] and renal tubulointerstitial injury [50]. Additionally, even in the presence of liposomes or FA-incubated liposomes a preferential binding of FAs to HSA was observed [51,52]. Hence it is truly important to study the equilibrium in the BR/HSA/liposome system not only with pure HSA but also with albumin which was isolated from the blood plasma and which was not purified of FAs.

FAs are well-known to influence the binding capacity of HSA being its natural components and they can also transport from HSA to liposomes and vice-versa [39,51–57]. The effect of six different FAs and their mixtures was studied separately as an addition to the pure HSA solution. Since two thirds of FAs bound to HSA in the blood plasma are unsaturated under normal circumstances, the most common being oleic (OA), linoleic (LA) and arachidonic acid (AA), these three acids were selected as representatives for unsaturated FAs. For saturated FAs, the three most common FAs in the blood plasma were chosen: myristic (MA), palmitic (PA), and stearic acid (SA).

As the major method to study the described systems, the ECD spectroscopy was chosen. It enables the study of different chiral forms of bound BR (Fig. 1) and of changes in the secondary and tertiary structures of HSA as the spectral bands of HSA are in different positions from BR. The spectroscopy also enables a differentiation between BR bound to the liposomes and HSA manifested as different values of molar ellipticity and shifts in couplet positions. The spectra are also very sensitive to the changes of the dihedral angle in the BR structure (Fig. 1B). The ECD method was complemented by the UV–Vis absorption spectroscopy and by the fluorescence emission.

2. Materials and methods

2.1. Materials

The lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and sphingomyelin were purchased from Avanti Polar Lipids (Alabaster, AL). Bilirubin-IXa (BR) was purchased from Frontier Scientific (USA). Human serum albumin (99% A3782 and 96% A1653), palmitic acid (PA), myristic acid (MA), stearic acid (SA), linoleic acid (LA), oleic acid (OA) and arachidonic acid (AA) were purchased from Sigma Aldrich. All of the chemicals were used without further purification.

2.2. Preparation of large unilamellar vesicles

The liposomes were prepared using the standard procedures [58]. The appropriate amount of dried lipid was weighed and dissolved in a chloroform/methanol mixture (2:1, v/v) and vortexed for 10 min. The sample was dried under low pressure to form a thin film on the vial wall, after which it was left under high vacuum for 5 h. The film was then hydrated via the addition of a 2×10^{-2} M phosphate buffer (pH = 7.4) and vortexed extensively for 15 min. The resulting multilamellar liposome suspension was then reduced to uniform large unilamellar vesicles (LUVs) by passing twenty-three times through a polycarbonate membrane with a mean pore diameter of 100 nm using a Mini-Extruder (Avanti Polar Lipids). After extrusion, the LUVs were allowed to equilibrate for at least 2 h before use. The final lipid concentration was calculated based on the weight of the dried lipid [59–63]. The dynamic light scattering (DLS) method confirmed the narrow size distribution of the LUVs with the maximum at approximately (110 ± 3) nm for both the DMPC and sphingomyelin liposomes.

For the testing of the scattering effects, larger liposomes (212 ± 11) nm and (415 ± 12) nm were prepared by extrusion and smaller liposomes