Biochimie 92 (2010) 994–1002

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Characterization of the lipid and protein organization in HBsAg viral particles by steady-state and time-resolved fluorescence spectroscopy☆

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ARTICLE INFO

Article history:
Received 22 January 2010
Accepted 19 April 2010
Available online 24 April 2010

Keywords:
HBsAg viral particles
Low density lipoprotein
Lipid membranes
Fluorescence spectroscopy
Fluorescent probes

ABSTRACT

Hepatitis B surface antigen (HBsAg) particles, produced in the yeast *Hansenula polymorpha*, are 20 nm particles, composed of S surface viral proteins and host-derived lipids. Since the detailed structure of these particles is still missing, we further characterized them by fluorescence techniques. Fluorescence correlation spectroscopy indicated that the particles are mainly monomeric, with about 70 S proteins per particle. The S proteins were characterized through the intrinsic fluorescence of their thirteen Trp residues. Fluorescence quenching and time-resolved fluorescence experiments suggest the presence of both low emissive embedded Trp residues and more emissive Trp residues at the surface of the HBsAg particles. The low emission of the embedded Trp residues is consistent with their close proximity in alpha-helices. Furthermore, S proteins exhibit restricted movement, as expected from their tight association with lipids. The lipid organization of the particles was studied using viscosity-sensitive DPH-based probes and environment sensitive 3-hydroxyflavone probes, and compared to lipid vesicles and low density lipoproteins (LDLs), taken as models. Like LDLs, the HBsAg particles were found to be composed of an ordered rigid lipid interface, probably organized as a phospholipid monolayer, and a more hydrophobic and fluid inner core, likely composed of triglycerides and free fatty acids. However, the lipid core of HBsAg particles was substantially more polar than the LDL one, probably due to its larger content in proteins and its lower content in sterols. Based on our data, we propose a structural model for HBsAg particles where the S proteins deeply penetrate into the lipid core.

1. Introduction

Hepatitis B virus (HBV) infection is one of the major causes of both acute and chronic liver hepatitis, liver cirrhosis and primary hepatocellular carcinomas [1,2]. Three types of particles are released from HBV infected cells: tubular lipoproteic structures with a 20–22 nm diameter, 22-nm spherical lipoproteic particles, and 40–44 nm infectious spherical virions, known as Dane particles [3–7]. The 22-nm particles, called HB surface antigen (HBsAg) particles, correspond to the lipoproteic envelop of the mature infectious Dane particles. Moreover, the HBsAg particles found in the serum of patients with HBV infection outnumber mature virus particles, and are strongly immunogenic [8,9] so that they have been used to develop an effective vaccine against HBV [10]. Similar recombinant HBsAg particles are produced in different hosts, such as chinese hamster ovary cells [11], mouse fibroblast cells [12,13] or yeast cells [14], and have been shown to be immunogenically nearly identical to those purified from human serum [7].

HBsAg particles are composed of host-derived lipids and virus-encoded glycoproteins [12], with a protein to lipid weight ratio of about 60:40 [14]. The HBsAg glycoprotein exists in three forms designated L (large), M (medium) and S (small). The S protein is the major form, accounting for 80–90% of the total proteins [7,15]. Whereas HBsAg particles derived from human plasma contain two S proteins, p-25, the major protein, and gp-30, the glycosylated form of p-25 [16]; the *Hansenula polymorpha*-derived HBsAg particles used in this study contains only the nonglycosylated form

Abbreviations: AFM, atomic force microscopy; ApoB100, apolipoprotein B-100; DOPC, dioleoylphosphatidylcholine; DPH, diphenylhexatriene; EM, electron microscopy; ESIPT, excited state-intramolecular proton transfer; FCS, fluorescence correlation spectroscopy; FRET, fluorescence resonance energy transfer; HBV, Hepatitis B virus; HBsAg, Hepatitis B surface antigen; LDL, low density lipoprotein; NATA, *N*-acetyl-L-tryptophanamide; PC, phosphatidylcholine; TMR, carboxytetramethylrhodamine; Trp, tryptophane.

☆ This work was supported by a grant from Sanofi Pasteur.

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doi:10.1016/j.biochi.2010.04.014

of the S protein (24-kDa). About fifty dimers of protein S are found in the mice-derived HBsAg particles [13]. Alpha-helix is the most abundant secondary structure [17], accounting for 50% of the structure of HBsAg S proteins [7]. These proteins are highly hydrophobic, with only two relatively hydrophilic regions [17]. A model for the HBsAg protein organization has been proposed whereby regions within both the NH_2- and COOH-terminal domains of p-25 and gp-30 are buried, while the antigenic 122–150 region is protruding out of the HBsAg particles, being thus exposed to the aqueous environment [16].

Together with the proteins, the lipids are also thought to be involved in the antigenic activity of HBsAg particles [7], likely by stabilizing the proper helical structure of the S proteins and the conformation of their hydrophilic region which contains the antigenic site [14]. The human and yeast HBsAg particles have the same composition including mainly phospholipids, with phosphatidylcholine (PC) being the main phospholipid [14,16], together with sterols, sterol esters and triacylglycerols. The majority of PC is accessible at the surface of the HBsAg particles, where they remain in a highly immobilized state in tight association with S proteins [12]. In addition, cryo-EM reconstitutions lead to the conclusion that unlike in mature virus particles, the HBsAg particles may contain the lipids in an unusual arrangement, being closely intercalated with the proteins [13]. Thus, it has been proposed that lipids in HBsAg particles are not aligned in a typical lipid bilayer configuration [12,14].

A powerful tool for characterization of lipid-based structures is fluorescent probes. For instance, information about the lipid order in the hydrophobic and the interfacial regions of lipid-based structures can be obtained respectively, by diphenylhexatriene (DPH) and its charged derivative (TMA-DPH), which are well-established probes of membrane fluidity [18,19]. Moreover, Nile Red shows high specificity to apolar lipid regions [20,21] and strong sensitivity to environment, providing information on the lipid organization. In addition, recently introduced membrane probes based on 3-hydroxyflavone provide the possibility for multiparametric probing of membrane environment [22]. Due to excited state-intramolecular proton transfer (ESIPT) reaction, they exhibit in the excited state, both a normal (N*) and a tautomer (T*) form, which are differently sensitive to their environment. In membranes, decomposition of their fluorescence spectra into N*, T* and additional hydrated H–N* bands allows simultaneous probing of polarity and hydration [23]. Of particular interest for the present studies are the highly hydrophobic FN4 [24] and the charged derivative F2N8 [23,25] bearing an anchor group for membrane interface, which should allow specific probing of the hydrophobic and interfacial regions of the HBsAg lipid part, respectively. In this study, we used the intrinsic fluorescence of the Trp residues of the S proteins as well as fluorescent membrane probes to characterize the protein and lipid organization of HBsAg particles produced in the yeast *H. polymorpha*.

2. Materials and methods

2.1. Materials

HBsAg particles were obtained from Sanofi Pasteur. HBsAg was produced in the recombinant yeast *H. polymorpha* and obtained in a highly purified form by successive steps of fermentation, extraction and purification. Low density lipoproteins (LDL), Dioleoylphosphatidylcholine (DOPC), Dipalmitoylphosphatidylcholine (DPPC) and Nile Red were from Sigma–Aldrich. DPH and TMA-DPH were from Molecular Probes. 3-Hydroxyflavone dyes F2N8, FN4 and PPZ8 were synthesized as described elsewhere [24–26].

2.2. General procedure

All experiments were performed in 8 mM phosphate buffer saline (PBS) (NaCl 150 mM, pH 7.4), at 20 °C. Concentration of protein for HBsAg particles was 0.4 μM, except for quantum yield measurements, where it was 2 μM. For characterization of the lipid organization, the lipid concentration of HBsAg, LDLs and lipid vesicles was 6 μM. TMA-DPH and DPH were used at a 1 μM concentration. Staining of the particles and vesicles was performed by addition of an aliquot of a stock solution of the DPH or 3-hydroxyflavone dyes in DMSO.

2.3. Steady-state spectroscopy measurements

Absorption spectra were recorded on a Cary 400 spectrophotometer. Extinction coefficient of 81,650 M^{-1} cm^{-1} was used to determine the concentrations of S proteins in HBsAg particles at 280 nm. Fluorescence emission spectra were recorded at 20 °C on a FluoroMax spectrofluorometer (Jobin-Yvon) equipped with a thermostated cell compartment. Excitation wavelength was set at 295 nm to excite selectively the Trp residues. Spectra were corrected for the wavelength dependence of the emission monochromator and photomultiplier. Quantum yields were determined by taking *N*-acetyl-L-tryptophanamide (NATA) in water as a reference [27]. Quenching by acrylamide and KI was carried out by adding aliquots from stock solutions to the samples. The fluorescence intensity changes corrected for the dilution were recorded at the maximum emission wavelength. The data were analyzed by using Stern–Volmer equations. In the case of KI, quenching of fluorescence was described by:

$$F_0/F = 1 + K_{SV}[Q] \quad (1)$$

In the case of quenching by acrylamide, we used a modified equation:

$$F_0/F = (1 + K_{SV}[Q])\exp(V[Q]) \quad (2)$$

In these equations, F_0 and F are the fluorescence intensities in the absence and the presence of quencher, respectively. $[Q]$ is the concentration of quencher, K_{SV} is the Stern–Volmer quenching constant, and V is the static quenching constant. With both quenchers, the bimolecular quenching constant k_q was obtained by $k_q = K_{SV}/\langle\tau\rangle$ where $\langle\tau\rangle$ is the mean fluorescence lifetime in the absence of quencher.

Steady-state fluorescence anisotropy measurements were performed with a T-format SLM 8000 spectrofluorometer at 20 °C. The emitted light was monitored through interferential filters (373 ± 4 nm) (Schott, Mainz). A home-built device ensured the automatic rotation of the excitation polarizer.

2.4. Time-resolved fluorescence measurements

Time-resolved fluorescence measurements were performed with the time-correlated, single-photon counting technique, as previously described [28,29]. Excitation wavelength for Trp residues was at 295 nm. For lifetime measurements, the emission was collected through a polarizer set at magic angle (54.7°) and an 8 nm band-pass monochromator (Jobin-Yvon H10) at 320 nm, 340 nm, 360 nm and 380 nm. For time-resolved anisotropy measurements, this polarizer was set at the vertical position. $I_\perp(t)$ and $I_\parallel(t)$ were recorded alternatively every 5 s, by using the vertical polarization of the excitation beam with and without the interposition of a quartz crystal that rotates the beam polarization by 90°. Time-resolved data analysis was performed by the maximum entropy method using the Pulse5 software [30]. For the analysis of the fluorescence