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Bovine serum albumin with glycated carboxyl groups shows membrane-perturbing activities

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ABSTRACT

The aim of the present study aimed to investigate whether glycated bovine serum albumin (BSA) showed novel activities on the lipid–water interface. Mannosylated BSA (Man-BSA) was prepared by modification of the carboxyl groups with *p*-aminophenyl α-D-mannopyranoside. In contrast to BSA, Man-BSA notably induced membrane permeability of egg yolk phosphatidylcholine (EYPC)/egg yolk sphingomyelin (EYSM)/cholesterol (Chol) and EYPC/EYSM vesicles. Noticeably, Man-BSA induced the fusion of EYPC/EYSM/Chol vesicles, but not of EYPC/EYSM vesicles. Although BSA and Man-BSA showed similar binding affinity for lipid vesicles, the lipid-bound conformation of Man-BSA was distinct from that of BSA. Moreover, Man-BSA adopted distinct structure upon binding with the EYPC/EYSM/Chol and EYPC/EYSM vesicles. Man-BSA could induce the fusion of EYPC/EYSM/Chol vesicles with K562 and MCF-7 cells, while Man-BSA greatly induced the leakage of Chol-depleted K562 and MCF-7 cells. The modified BSA prepared by conjugating carboxyl groups with *p*-aminophenyl α-D-glucopyranoside also showed membrane-perturbing activities. Collectively, our data indicate that conjugation of carboxyl groups with monosaccharide generates functional BSA with membrane-perturbing activities on the lipid–water interface.

Introduction

Serum albumin is the most abundant protein in the circulatory system, and it plays a key role in the transport of a large number of metabolites, endogenous ligands, fatty acids, bilirubin, hormones, anesthetics, and commonly used drugs [1]. Bovine serum albumin (BSA)[2] BSA demonstrates structural flexibility with changes in the pH, suggesting a mechanism for drug binding and/or distribution of serum albumin [2]. Moreover, Celej et al. [3] found that the binding of BSA with different anilinonaphthalene sulfonate derivatives induces the production of different conformers of BSA. These past results suggested that BSA possesses inherent structural flexibility. Although the structural flexibility of proteins is believed to be favorable for interacting with the binding partners [4,5], the interactions of a serum albumin with biological macromolecules such as proteins or cell membrane *in vivo* remains to be determined. Previous studies revealed that BSA can transfer lipid amphiphile to the lipid bilayer membranes [6], reflecting that BSA could exert its function at the lipid–water interface. Moreover, several studies have suggested that membrane-interface or membrane-mimicking surface induces conformational changes in human serum albumin [7–9]. Taken together, these findings suggest that serum albumin could interact with the lipid bilayers.

Previous studies have shown that mannosylated BSA (Man-BSA) prepared by conjugation its carboxyl groups with *p*-aminophenyl-mannopyranoside is a ligand of hyaluronan-binding proteins [10]. Several lines of evidence have revealed that Man-BSA specifically binds with M-type phospholipase A_2 (PLA_2) receptor, which is a member of the mannose receptor family [11,12]. Hernandez et al. [13] found that Man-BSA competitively blocks the cellular activity of secreted PLA_2. Conversely, Triggiani et al. [14] and Choi et al. [15] reported that Man-BSA mimics or even enhances the activity of secreted PLA_2. Considering that PLA_2 is a phospholipid-hydrolyzing enzyme, these past results suggest that Man-BSA shows a preference to target the lipid–water interface. Thus, it is possible that Man-BSA exhibits its biological activity via binding with the cellular membrane. Noticeably, several studies revealed

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[2] *Abbreviations used:* BSA, bovine serum albumin; Chol, cholesterol; EDC, 1-ethyl-3-(diethylaminopropyl)-carbodiimide hydrochloride; EYPC, egg yolk phosphatidylcholine; EYSM, egg yolk sphingomyelin; FPE, N-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoylphosphatidyl-ethanolamine; Glu-BSA, glucosylated BSA; Man-BSA, mannosylated BSA; MβCD, methyl-β-cyclodextrin; NBD-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycerol-3-phosphoethanolamine; Rh-PE, lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine.

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that carbohydrate show an affinity for binding with lipid [16,17]. Accordingly, it is possible that incorporation of carbohydrate moieties into BSA may enhance the interaction of BSA with phospholipid bilayers. To address this question, the membrane-perturbing activities of Man-BSA and BSA were investigated in this study.

Materials and methods

BSA (Fraction V, fatty acid free, catalog number 775835, purity > 99%) was obtained from Roche Applied Science. Calcein, cholesterol (Chol), egg yolk phosphatidylcholine (EYPC), egg yolk sphingomyelin (EYSM), 1-ethyl-3-(diethylaminopropyl)-carbodiimide hydrochloride (EDC), methyl-β-cyclodextrin (MβCD), *p*-aminophenyl β-D-glucopyranoside and *p*-aminophenyl α-D-mannopyranoside were purchased from Sigma–Aldrich Inc., and 10,12-tricosadiynoic acid was obtained from Fluka. Lissamine rhodamine B 1, 2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Rh-PE), N-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoylphosphatidylethanolamine (FPE) and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1, 2-dihexadecanoyl-*sn*-glycerol-3-phosphoethanolamine (NBD-PE) were the products of Molecular Probes. Sepharose 6B was obtained from Amersham Biosciences. All other reagents were analytical grade.

Preparation of glycated BSA

Man-BSA was prepared by coupling *p*-aminophenyl α-D-mannopyranoside to BSA through water-soluble EDC. Briefly, BSA (6.78 mg) was added to a 6-ml stirring solution of *p*-aminophenyl α-D-mannopyranoside (136 mg) in water (pH 4.75). Then EDC (155 mg/ml) was added dropwise over a period of 30 min at room temperature and allowed to stand for 6 h. Man-BSA was dialyzed against water and then lyophilized. Meanwhile, conjugation of carboxyl groups with *p*-aminophenyl β-D-glucopyranoside was also conducted for preparing glucosylated BSA (Glu-BSA). The molecular weight of Man-BSA and Glu-BSA was determined using MALDI-TOF mass analyses.

Release of entrapped fluorescent markers from liposomes

Membrane-damaging activity was determined by measuring the release of the liposome-entrapped self-quenching fluorescent dye calcein according to the procedure described by Kao et al. [18]. Lipid mixtures with indicated compositions were dissolved in chloroform/methanol (v/v, 2:1) at indicated molar ratio and dried by evaporation. Buffer (10 mM Tris–HCl, 100 mM NaCl, pH 7.5) containing 50 mM calcein was added to the film of lipids, and after hydration the suspension was shaken vigorously. The multilamellar vesicles obtained in this way were extruded 10 times, above the transition temperature, through a 100 nm polycarbonate filter and applied to a Sepharose 6B column (2 × 15 cm) to separate the liposome from the free calcein. Leakage was induced by adding aliquots of glycated BSA to a vesicle suspension directly in the cuvette used for fluorescence determination at 37 °C. The kinetics of membrane damage was monitored by the increase in fluorescence with emission at 520 nm and excitation at 490 nm, and the signal was expressed as percentage of total calcein release after the addition of 0.2% Triton X-100.

Fusion assay of phospholipid vesicles

Glycated BSA-induced liposome fusion was measured by increase in fluorescence resonance energy transfer between two lipid probes (NBD-PE and Rh-PE). NBD-PE and Rh-PE were used as donor and acceptor fluorescent lipid, respectively. NBD-PE and Rh-PE (1 mol% in phospholipid vesicles) were incorporated into phospholipid vesicles. The NBD emission was monitored at 530 nm with the excitation wavelength set at 465 nm. As the two liposome groups interacted, the fluorescence energy emitted from NBD-PE labeled liposomes was transferred to the Rh-PE labeled liposomes resulting in a decreased fluorescence signal of NBD. The percentage of fusion was defined by the following relationship: $\%\text{Fusion} = 100 \times (1 - F/F_0)$, where F and F_0 are the fluorescence intensities in the presence and absence of Rh-PE, respectively. For the inner monolayer phospholipid fusion assay, phospholipid vesicles were treated with sodium dithionite to completely reduce the NBD-labeled phospholipid located at the outer monolayer of the membrane. Final concentration of sodium dithionite was 100 mM and the inner leaflet mixing assay was carried out at the level in which 55–60% fluorescence intensity of NBD were reduced.

Lipid-binding experiments

FPE was incorporated at 2 mol% into indicated lipid compositions, which were prepared by extrusion through 100-nm pore size polycarbonate filters. Excitation was at 490 nm, and emission intensity at 515 nm was monitored. A plot of $1/\Delta F$ versus 1/[Man-BSA] gives lines with a slope corresponding to the dissociation constant of phospholipid-Man-BSA complexes.

Colorimetric phospholipid/polydiacetylene membrane assay

Penetration of glycated into phospholipid/polydiacetylene (PDA) vesicles was detected essentially according to the methods described by Kolusheva et al. [19]. EYPC/EYSM/Chol/10,12-tricosadiynoic acid (14.7/13.3/12/60, mol/mol/mol/mol) or EYPC/EYSM/10,12-tricosadiynoic acid (21/19/60, mol/mol/mol) was dissolved in chloroform/ethanol (1:1, v/v) and dried together *in vacuo* followed by addition of de-ionized water and sonication at 70 °C. The vesicle solution was then cooled to room temperature and kept at 4 °C overnight. The vesicles were then polymerized using irradiation at 254 nm for 30–40 s, with the resulting phospholipid/PDA solution exhibiting an intense blue appearance. Vesicle samples for experiments were prepared at concentration of 0.5 mM (total lipid) in 10 mM Tris–HCl-100 mM NaCl (pH 7.5). Following addition of Man-BSA or BSA, the changes in the absorbance at 500 nm and 640 nm of vesicle solution were measured.

A quantitative value for the extent of the blue-to-red color transitions within the vesicle solutions is given by the colorimetric response (%CR), which is defined as follows: $\%\text{CR} = [(\text{PB}_o - \text{PB}_1)/\text{PB}_0] \times 100$, where $\text{PB} = A_{640}/(A_{640} + A_{500})$. A_{640} and A_{500} are the absorbance measured at 640 nm and 500 nm, respectively. PB_o is the A_{640}/A_{500} ratio of the control sample (before addition of Man-BSA or BSA), and PB_1 is the value obtained for the vesicle solution after addition of Man-BSA or BSA.

Analyses of Fourier transform infrared (FTIR) spectra of phospholipid-Man-BSA complexes

Fourier transform infrared (FTIR) spectra were measured using a 66 v/s FTIR spectrometer (Bruker Optics). Man-BSA (44 μM) was dissolved in D_2O-based buffer containing 10 mM HEPES-100 mM NaCl (pH 7.5) in the absence or presence of 880 μM phospholipid vesicles. Then the samples were transferred to a demountable cell composed of two CaF_2 windows separated by a 25-μm Teflon spacer clamped together in a brass holder. The FTIR spectra were recorded at a spectral resolution of 2 cm^{-1} for 200 scans, and then inverted to second-derivative spectra. Quantification of secondary structure of Man-BSA was performed as described by Goormaghtigh et al. [20].