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Antioxidant effect of bovine serum albumin on membrane lipid peroxidation induced by iron chelate and superoxide

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Abstract

Albumin is supposed to be the major antioxidant circulating in blood. This study examined the prevention of membrane lipid peroxidation by bovine serum albumin (BSA). Lipid peroxidation was induced by the exposing of enzymatically generated superoxide radicals to egg yolk phosphatidylcholine liposomes incorporating lipids with different charges in the presence of chelated iron catalysts. We used three kinds of Fe^{3+}-chelates, which initiated reactions that were dependent on membrane charge: Fe^{3+}-EDTA and Fe^{3+}-EGTA catalyzed peroxidation in positively and negatively charged liposomes, respectively, and Fe^{3+}-NTA, a renal carcinogen, catalyzed the reaction in liposomes of either charge. Fe^{3+}-chelates initiated more lipid peroxidation in liposomes with increased zeta potentials, followed by an increase of their availability for the initiation of the reaction at the membrane surface. BSA inhibits lipid peroxidation by preventing the interaction of iron chelate with membranes, followed by a decrease of its availability in a charge-dependent manner depending on the iron-chelate concentration: one is accompanied and the other is unaccompanied by a change in the membrane charge. The inhibitory effect of BSA in the former at high concentrations of iron chelate would be attributed to its electrostatic binding with oppositely charged membranes. The inhibitory effect in the latter at low concentrations of iron chelate would be caused by BSA binding with iron chelates and keeping them away from membrane surface where lipid peroxidation is initiated. Although these results warrant further in vivo investigation, it was concluded that BSA inhibits membrane lipid peroxidation by decreasing the availability of iron for the initiation of membrane lipid peroxidation, in addition to trapping active oxygens and free radicals.

Keywords: Serum albumin; Lipid peroxidation; Superoxide; Liposome; Membrane; Antioxidant; Free radical; Xanthine oxidase; Nitrilotriacetate; Iron

1. Introduction

Reactive oxygen species participate in the development of many pathologic events by causing oxidative deterioration of biological macromolecules such as DNA, proteins, and membrane lipids [1,2]. In particular, oxidation of membranes and lipoprotein lipids in circulation has been implicated in the pathogenesis of many vascular disorders, including atherosclerosis, diabetes, and hypertension [3,4], and there have been extensive studies on the initiation and prevention of lipid peroxidation.

Albumin is considered the major circulating antioxidant in the blood [5–7], which is exposed to continuous oxidative

Abbreviations: BSA, bovine serum albumin; aBSA, acetylated BSA; mBSA, methylated BSA; nBSA, native BSA; DCP, dicetylphosphate; EDTA, ethylenediamine tetraacetic acid; EGTA, bis(2-aminoethyl ether)-ethyleneglycol tetraacetic acid; EYPC, egg yolk phosphatidylcholine; HEPES, 2-hydroxyethylpiperazine-2-ethanesulfonic acid; NTA, nitrilotriacetic acid (nitrilotriacetate); PC-OOH, hydroperoxides of egg yolk phosphatidylcholine; poly-Glu, poly-L-glutamic acid; poly-Lys, poly-L-lysine; poly (Lys-Phe), poly-L-lysine-L-phenylalanine; SA, stearylamine; TBA, thiobarbituric acid; X, xanthine; XO, xanthine oxidase

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stress. In vitro experiments have shown that serum albumin protects human low-density lipoproteins from copper-mediated oxidation and red blood cells from hemolysis due to free radical membrane damage [8]. Further, it was reported that serum albumin protects cultured cells, such as renal tubular cells, macrophages, aortic endothelial cells, and lung fibroblasts, from oxygen radical damage [9,10]. Albumin protects cells mainly by directly scavenging reactive oxygen species by trapping them. Dean et al. [11] reported that bovine serum albumin (BSA) inhibited membrane lipid peroxidation of liposomes induced by lipid-soluble radicals less than that induced by water-soluble radicals, an effect that is probably due to lower scavenging efficiency for lipid-soluble radicals than for water-soluble radicals.

The superoxide anion radical ($O_2^{\cdot -}$) produced in aerobic organisms is widely held to be a major initiator of biological damage resulting in pathophysiological events associated with a variety of diseases [12,13]. The biological effectiveness of $O_2^{\cdot -}$ is usually explained by the formation of more-reactive species derived from it. A specific example is the oxidation of unsaturated lipids in membranes, an important event in pathophysiology, which cannot be initiated directly by $O_2^{\cdot -}$. The conversion of $O_2^{\cdot -}$ to more reactive intermediates requires the participation of metal catalysts, of which iron is the most important in biological systems [12–15].

Recently, we investigated the membrane lipid peroxidation in liposomes exposed to $O_2^{\cdot -}$ in the presence of chelated iron, and proposed that a Fenton-like reaction of iron-chelate with preformed lipid peroxides at the membrane surface triggers the initiation of lipid hydroperoxide (LOOH) formation [15,16]:

$$Fe^{3+} - \text{chelate} + O_2^{\cdot -} \rightarrow Fe^{2+} - \text{chelate} + O_2 \quad (1)$$

$$Fe^{2+} - \text{chelate} + L - OOH \rightarrow Fe^{3+} - \text{chelate} + L - O^{\cdot} \quad (2)$$

$$L - O^{\cdot} + LH \rightarrow L - OH + L^{\cdot} \quad (3)$$

$$L^{\cdot} + O_2 \rightarrow L - OO^{\cdot} \quad (4)$$

$$L - OO^{\cdot} + LH \rightarrow L - OOH + L^{\cdot} \quad (5)$$

where LH is the unoxidized lipid and L$^{\cdot}$, L-O$^{\cdot}$, and L-OO$^{\cdot}$; the lipid carbon-centered, alkoxyl, and peroxyl radicals, respectively.

This initiation system seems most likely to occur in blood circulation. Therefore, in this study, we used this lipid peroxidation system and investigated the inhibitory effect of BSA on it from the point of view of obstruction of the Fenton-like reaction that occurs at the membrane surface, and which differs from its well-known antioxidant effect due to trapping active oxygens and free radicals.

2. Materials and methods

2.1. Materials

Egg yolk phosphatidylcholine (EYPC) was obtained from Nippon Oil and Fats (Tokyo, Japan). The fatty acid composition (mol%) of EYPC was 33.1 palmitate, 1.2 palmitoleate, 11.6 stearate, 30.0 oleate, 15.5 linoleate, 3.3 arachidonate, and 5.3 docosahexaenoate. The commercial EYPC sample was contaminated with 0.3–0.4 mol% of hydroperoxide, as determined by the ferric–xylenol orange method [17]. Bovine serum albumin, fatty acid-free (native BSA:nBSA), carboxymethylated BSA (mBSA), xanthine oxidase (XO), stearyl amine (SA), dicetylphosphate (DCP), poly-L-lysine (poly-Lys) (m.w., 48100), poly-L-glutamic acid (poly-Glu) (m.w.,72500), and poly-L-lysine-L-phenylalanine (1:1) (poly (Lys-Phe)) (m.w., 35400) were purchased from Sigma Chemical (St. Louis, MO). Sodium ethylenediamine tetraacetate (EDTA-2Na), bis (2-aminoethyl ether) ethyleneglycol tetraacetic acid (EGTA), 2-hydroxyethylpiperazine-2-ethanesulfonic acid (HEPES), xylenol orange, and $Fe(NO_3)_3$ were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Sodium nitrilotriacetate (NTA-2Na) and $FeSO_4$ were from Nacalai Tesque (Kyoto, Japan). All other reagents were of analytical grade. Acetylated BSA (aBSA) was prepared using a modification of the method of Basu et al. [18].

2.2. Preparation of liposomes

Liposomes were prepared as described previously [15]. Stock solutions of EYPC in chloroform with or without DCP or SA were evaporated under nitrogen. The resulting thin lipid films were dispersed in 10 mM HEPES buffer, pH 7.4, in a vortex mixer and subjected to ultrasonic irradiation in a Bransonic-12 sonic bath (Yamato Tokyo, Japan) at 40 °C for 5 min (charged liposomes) or 10 min (uncharged liposomes). When required, the EYPC was freed from contaminating hydroperoxides of EYPC (PC-OOH) by treatment with triphenylphosphine (TPP) [19] in chloroform just before the preparation of liposomes.

2.3. BSA acetylation and characterization of BSAs

nBSA was acetylated using acetic anhydride [18]. To 3 ml of 0.15 M NaCl solution of nBSA (15 mg/ml), 3 ml of sodium acetate-saturated solution was added, and then 40 μl of acetic anhydride was slowly added (2 μl/3 min) at room temperature. After incubation for 30 min, acetylated BSA was dialyzed using a hydrated dialysis membrane (Wako Chem., USA) at 4 °C in 0.15 M NaCl for 24 h and further in 10 mM HEPES buffer solution (pH 7.4) for 3 h. The protein concentration of the BSA preparation was determined using the Lowry method [20]. The net charge of the albumins was investigated by determining their migration times in capillary electrophoresis [21]. The migration times for

aBSA was 12.71±0.10 min (S.D., n=3), which was slightly but significantly long as compared with that of nBSA (12.21±0.02 min), indicating additional net negative charge. The amounts of carbonyl groups measured according to the dinitrophenylhydrazine method [22] were 119% higher in aBSA (0.166 mol carbonyl/mol BSA) than that in nBSA (0.139 mol carbonyl/mol BSA). Purchased mBSA prepared by Mandel and Hershey [23] was well confirmed as a net positively charged basic protein [24].

2.4. Assays of lipid peroxidation

Lipid peroxidation of EYPC liposomes was measured by following oxygen consumption [16]. The following conditions were used for Fe^{3+}-chelate-dependent lipid peroxidation initiated by $O_2^{\cdot-}$ in liposomes. The test systems consisted of liposomes containing 1 mM EYPC with or without DCP or SA in 3.6 ml of 10 mM HEPES buffer pH 7.4 at 37 °C. The oxidation was started by the addition of 10 μl Fe^{3+}-chelate (final concentrations 30 μM Fe^{3+} and 33 μM chelator), 10 μl xanthine (30 μM), and finally 10 μl XO (1 mU/ml) to 3.6 ml of liposome suspensions in 10 mM HEPES buffer (pH 7.4). The Fe^{3+}-chelates were prepared by mixing $Fe(NO_3)_3$ in H_2O or dilute HCl solution with the chelator (1.0 to 1.1 molar ratio). The rate of oxygen consumption associated with lipid peroxidation was measured with a Clark-type oxygen electrode, assuming an oxygen concentration of 217 nmol/ml in the initial incubation mixture at 37 °C.

The generation of $O_2^{\cdot-}$ in human granulocytes from normal blood samples during phagocytosis was reported to be 5–30 mU XO equivalent/ml [25]. In this study, the XO content (1 mU XO/ml) was decided considering the suitable condition for measurement of lipid peroxidation, under which O_2 consumption due to $O_2^{\cdot-}$ generation is negligible in comparison with that due to lipid peroxidation. The rate of $O_2^{\cdot-}$ generation by 1 mU XO/ml used in this experiment is equivalent to the rate (1 μM $O_2^{\cdot-}$/min) observed in our previous study that $O_2^{\cdot-}$ was responsible for apoptosis in rat vascular smooth muscle cells [26]. The thiobarbituric acid (TBA) method was used as described previously [16] to provide another assay for lipid peroxidation. The amount of TBA-reactive substances (TBARS) formed was expressed as equivalents of malondialdehyde (MDA). Other experimental details are described in figure legends.

2.5. Assay of binding of Fe^{3+}-NTA to BSA

Equilibrium dialysis experiments were performed with an Equilibrium Dialyzer (Sanplatec, Osaka, Japan) using a total volume of 13 ml of cells. A hydrated dialysis membrane was washed in deionized water and dried with N_2 gas. To one side of the membrane was added 7 ml of dialysate solution (50 mM HEPES–Tris in 10 mM NaCl buffer, pH 7.4) containing a certain concentration of Fe^{3+}-NTA solution. On the other side of the membrane, 7 ml of the sample (BSA or polypeptide) solution was dialyzed for 24 h. The volumes of the solutions on either side of the membrane were kept constant during the dialysis procedure. Aliquots from the chamber were removed and then free concentrations of Fe^{3+}-NTA were determined by the ferric–xylenol orange method [27]. The percentage binding of Fe^{3+}-NTA to the sample was calculated using the following equation:

$$B\ (\%) = (Abs_a - Abs_b)/Abs_a \times 100$$

where B (%) is the percentage of the added Fe^{3+}-NTA bound to the sample, and Abs_b and Abs_a are the absorbances at 560 nm of Fe^{3+}-NTA in the dialyzed solution with and without a sample, respectively.

2.6. Measurement of zeta potential of liposome membranes

The zeta potentials of the liposomes (1 mM EYPC with or without 0–0.2 mM DCP or SA) in 10 mM HEPES/10 mM NaCl buffer at pH 7.4 were measured electrophoretically in a NICOMP (model 380 apparatus, Particle Sizing Systems, Santa Barbara, California) at room temperature.

3. Results

Preliminary experiments showed that the complete system, made up of liposomes formed from 1 mM EYPC supplemented with 0.2 mM SA or 0.1 mM DCP, 10 mM HEPES buffer at pH 7.4, 1 mU/ml XO, 30 μM X, and Fe^{3+}-NTA (30 μM Fe^{3+}, 33 μM NTA), rapidly consumed oxygen, indicating the occurrence of lipid peroxidation (solid lines (1) in Fig. 1). Oxygen was not consumed if XO was excluded, but slightly consumed if Fe^{3+}-NTA was excluded, possibly due to the generation of $O_2^{\cdot-}$ from oxygen by X-XO (broken lines in Fig. 1 (left)), indicating that lipid peroxidation was not induced directly by either $O_2^{\cdot-}$ or Fe^{3+}-NTA. The involvement $O_2^{\cdot-}$ in this process was confirmed by the complete inhibition of oxygen uptake by the addition of superoxide dismutase (100 U/ml) (data not shown). Similarly, oxygen consumption was completely inhibited if liposomes were prepared from EYPC pretreated with triphenylphosphine (TPP) (broken line (1) in Fig. 1 (right)), which reduces endogenous EYPC hydroperoxides (PC-OOH) to the corresponding alcohols (PC-OH) [19], indicating that the presence of PC-OOH was a prerequisite for the induction of lipid peroxidation.

Lines (2)–(4) in Fig. 1 show the time course of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation in liposomes with positive and negative charges in the presence of various BSAs. Three types of BSAs, nBSA with a small net negative charge, negatively charged aBSA, and positively charged mBSA, were used. All BSAs inhibited the lipid peroxidation. The orders of their inhibitory effects were: aBSA>nBSA>mBSA in positively charged SA-EYPC liposomes, and mBSA>nBSA>aBSA in negatively

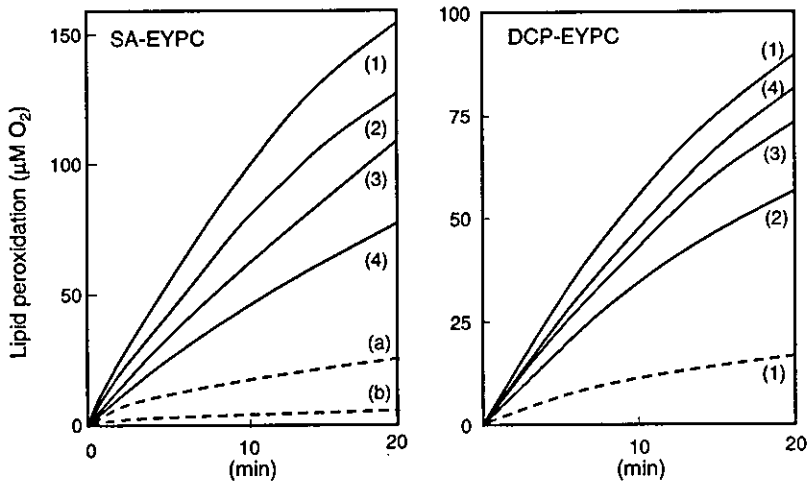

Fig. 1. Inhibition by BSAs of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation in DCP-EYPC and SA-EYPC liposomes. (1) Control; (2) +mBSA; (3) +nBSA; (4) +aBSA. Broken lines in SA-EYPC liposome system indicate the oxygen consumption in the system that excluded only (a) Fe^{3+}-NTA or (b) X-XO from the control system (line (1)). The system of broken line (1) in DCP-EYPC liposome system is all the same to the control system (solid line (1)) but EYPC was pretreated with TPP before the preparation of liposomes. The concentration of BSAs added to the control system was 1.0 mg/ml. Concentrations of reagents in the control system were: 1 mM EYPC, 0.1 mM DCP or 0.2 mM SA, 10 mM HEPES buffer, pH 7.4, 1 mU/ml xanthine oxidase (XO), 30 μM xanthine (X), and iron chelate formed from 30 μM $Fe(NO_3)_3$ and 33 μM NTA. Incubation was at 37 °C, and the reaction started by the addition of XO.

charged DCP-EYPC liposomes. Same orders were observed by the assay of TBARS values (data not shown). Furthermore, nBSA more strongly inhibited TBARS formation in SA-EYPC liposomes (63% inhibition) than in DCP-EYPC liposomes (28% inhibition). These results indicate that the inhibitory effect of BSAs is higher in oppositely charged membranes.

Fe^{3+}-NTA, a renal carcinogen, has been reported to cause apoptosis associated with increase of lipid peroxide and 8-hydroxydeoxyguanosine levels in vivo [28,29] and in vitro [30]. In cultured cells treated with 20–100 μM Fe^{3+}-NTA, oxidative damage [31] and apoptotic [30], cytotoxic [32], and mutagenic [33] effects were observed. Therefore, we used 10–60μM Fe-NTA and examined its concentration dependency for the induction of X-XO-dependent lipid peroxidation in EYPC liposomes containing different amounts of positively charged SA (Fig. 2). The rate of lipid peroxidation increased with an increase in Fe^{3+}-NTA concentration. A decrease of the membranous SA concentration in EYPC liposomes lowered the rate of lipid peroxidation. The addition of nBSA (1 mg/ml) to SA-EYPC (0.2 mM/1 mM EYPC) liposomes slowed down the lipid peroxidation rate. The change in the lipid peroxidation rate in the presence of nBSA was similar to that of the rate depending on the reduction of SA concentration at concentrations of Fe^{3+}-NTA higher than 20 μM. On the contrary, the rate change was quite different from that at concentrations of Fe^{3+}-NTA lower than 10 μM, and the addition of nBSA almost completely inhibited the lipid peroxidation.

Before the antioxidant effect of BSA on the Fe^{3+}-chelate/$O_2^{\cdot-}$-dependent membrane lipid peroxidation at the membrane surface was examined in detail, we investigated the role of Fe^{3+}-chelate at the membrane surface, where a Fenton-like reaction initiates lipid peroxidation. The concentration of Fe^{3+}-chelate chosen was 30 μM, which was suitable for the investigation of inhibiting effect of BSA.

Fig. 3 shows the effect of the membrane charge on the rate of O_2 consumption indicative of lipid peroxidation by Fe^{3+}-chelates (Fe^{3+}-NTA, Fe^{3+}-EGTA, and Fe^{3+}-EDTA). Fe^{3+}-NTA catalyzed the lipid peroxidation both in positively

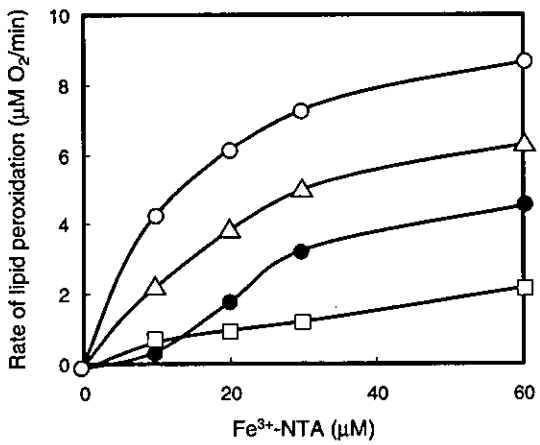

Fig. 2. Inhibitory effect of nBSA on the rate of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation in the presence of various concentrations of Fe^{3+}-NTA in EYPC liposomes containing different amounts of SA. (○) SA-EYPC (0.2 mM/1.0 mM) liposomes; (△) SA-EYPC (0.05 mM/1.0 mM) liposomes; (□) EYPC (1.0 mM) liposomes; (●) SA-EYPC (0.2 mM/1.0 mM) liposomes+nBSA (1.0 mg/ml). The rate in each system was obtained after the subtraction of the value of blank, which was the system that only omitted Fe^{3+}-NTA from the control system.

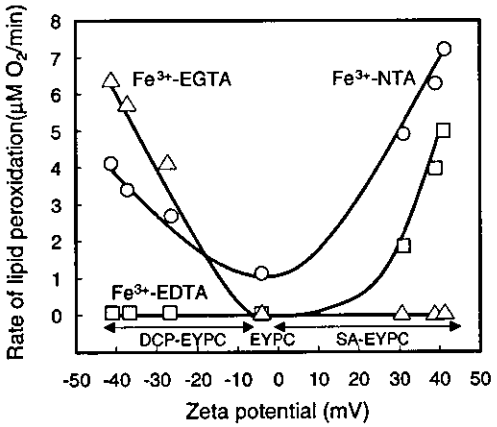

Fig. 3. Rates of Fe^{3+}-chelate/X-XO-dependent oxygen consumption indicative of lipid peroxidation in liposomes with various membrane charges. (○) Fe^{3+}-NTA; (△) Fe^{3+}-EGTA; (□) Fe^{3+}-EDTA. Liposomes were prepared from EYPC containing different amounts of DCP or SA. Concentrations of reagents for lipid peroxidation were: 1 mM EYPC, 0–0.2 mM DCP or SA, 10 mM HEPES buffer, pH 7.4, 1 mU/ml xanthine oxidase (XO), 30 μM xanthine (X), and iron chelate formed from 30 μM $Fe(NO_3)_3$ and 33 μM NTA, EDTA, or EGTA. Incubation was at 37 °C, and the reaction started by the addition of XO.

charged SA-EYPC liposomes and negatively charged DCP-EYPC liposomes, and the rates of lipid peroxidation increased with an increase in the zeta potential of liposomes. Our previous work revealed that the binding of Fe^{3+}-chelates with liposomes is indispensable for the initiation of $O_2^{\cdot-}$ driven lipid peroxidation [16]. Because of the good correlations between the abilities of Fe^{3+}-chelates to induce lipid peroxidation and electrostatic binding to membranes, we supposed that the increase of negative and positive zeta potentials of membranes causes more binding of Fe^{3+}-NTA to membranes, increasing its facilitating effect on lipid peroxidation. The initiation of lipid peroxidation by Fe^{3+}-EGTA and Fe^{3+}-EDTA was also dependent on the zeta potentials of the liposome membranes. The former and the latter catalyzed the lipid peroxidation in negatively charged DCP-EYPC liposomes and positively charged SA-EYPC liposomes, respectively, indicating that membrane charges of DCP-EYPC liposomes and SA-EYPC liposomes are closely associated with the electrostatic binding ability of oppositely charged Fe^{3+}-chelates, such as net positively charged Fe^{3+}-EGTA and net negatively charged Fe^{3+}-EDTA. Increased amounts of Fe^{3+}-chelates bound to the membrane would result in an increase of the availability of the Fe^{3+}-chelates required to initiate lipid peroxidation at the membrane surface.

As shown in Fig. 1, the inhibitory effect of BSA depending on membrane charge was suggested to be due to the neutralization of the membrane charge by electrostatic interaction with the membranes. Thus, we chose systems in which the charges of liposomal membranes and BSAs were opposite, and investigated the correlation between the effects of BSAs on the zeta potentials of liposomes and the rates of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation. The rate of lipid peroxidation was obtained from the initial gradient of the curve in Fig. 1. As shown in Fig. 4, the rates of Fe^{3+}-NTA-dependent lipid peroxidation as a function of BSA concentration were well correlated to the changes in the zeta potentials of liposomes.

To determine the details of the ability of BSA to change the zeta potentials and lipid peroxidation, we compared the rates of lipid peroxidation in liposomes with same zeta potentials, which depend on the concentrations of BSAs added to the membranes and of charged molecules removed from the membranes. The rate of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation in liposomes with different charges was plotted as a function of zeta potential of liposomes in the presence and absence of BSAs (Fig. 5). Under the same zeta potential conditions in systems in which the charge of the liposomes is opposite to that of the BSAs added, the rates of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation were

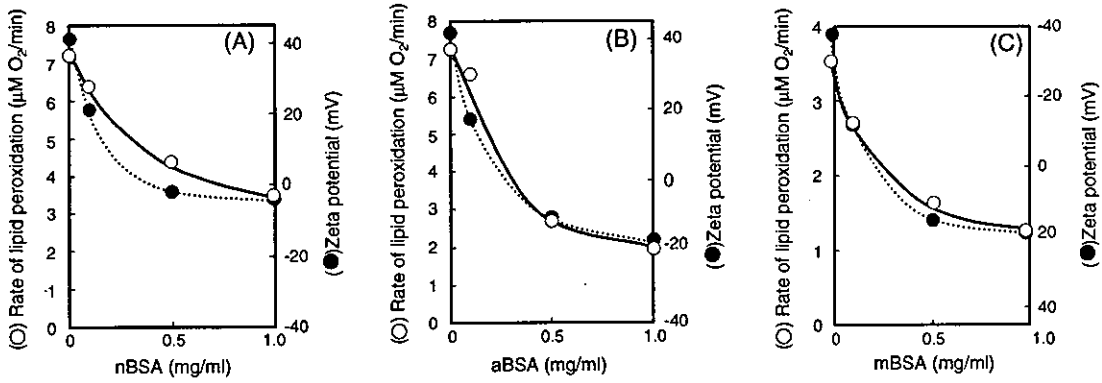

Fig. 4. The effects of the concentration of BSAs on the rate of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation and the zeta potential of EYPC liposomes with DCP or SA. Panel A: nBSA in SA-EYPC liposomes; panel B: aBSA in SA-EYPC liposomes; panel C: mBSA in DCP-EYPC liposomes. (○) Lipid peroxidation; (●) zeta potential. Concentrations of BSAs added to the DCP-EYPC (0.1 mM/1.0 mM) or SA-EYPC (0.2 mM/1.0 mM) liposomes were 0.1 mg/ml–1.0 mg/ml. Other experimental conditions were as shown in Fig. 3.

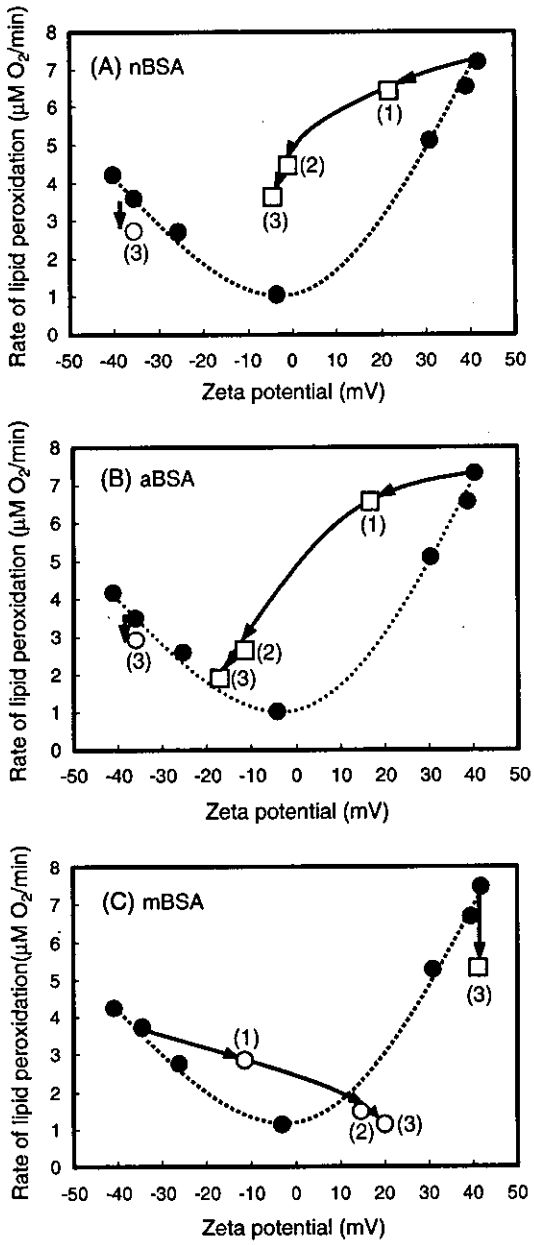

Fig. 5. The rate of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation as a function of zeta potential of liposomes with different charges in the presence or absence of nBSA, aBSA, or mBSA. Panel A: nBSA; panel B: aBSA; panel C: mBSA. (○) DCP-EYPC (0.1 mM/1.0 mM) liposomes in the presence of BSAs; (□) SA-EYPC (0.2 mM/1.0 mM) liposomes in the presence of BSAs; (●) DCP-EYPC (0–0.2 mM/1.0 mM) and SA-EYPC (0–0.2 mM/1.0 mM) liposomes in the absence of BSAs. The concentrations of BSAs added to the liposomes are indicated by numbers: (1) 0.1 mg/ml; (2) 0.5 mg/ml; (3) 1.0 mg/ml. Experimental conditions were as shown in Fig. 3.

always higher in the presence of BSAs (□ in Fig. 5(A) and (B), ○ (1) in Fig. 5(C)) than in their absence (dotted lines in Fig. 5), except for the results shown in Fig. 5(C) (○ (2) and ○ (3)). The reductions of the rate of lipid peroxidation were larger for the addition of aBSA with a high net negative charge (□ in Fig. 5 (B)) than for addition of nBSA with a low net negative charge in SA-EYPC (0.2 mM/1 mM) liposomes (□ in Fig. 5(A)).

On the contrary, in the systems in which BSA was added to identically charged liposomes, Fe^{3+}-NTA/X-XO-dependent lipid peroxidation was inhibited without affecting the zeta potential: nBSA and aBSA with a net negative charge did not affect the zeta potential but weakly inhibited the lipid peroxidation in negatively charged DCP-EYPC (0.1 mM/1 mM) liposomes (○ (3), as shown in Fig. 5(A) and (B)), and mBSA with a net positive charge did not affect the zeta potential but did lower the rate of lipid peroxidation in positively charged SA-EYPC liposomes (□ (3) in Fig. 5(C)).

Fig. 6 shows the effects of mBSA and aBSA on the zeta potentials of liposomes and the rates of Fe^{3+}-EGTA/X-XO-dependent lipid peroxidation. The addition of mBSA lowered both the negative value of the zeta potential and lipid peroxidation in DCP-EYPC liposomes, and their mBSA concentration-dependent decreases correlated well (Fig. 6 (A)). At the same zeta potential, the rates of lipid peroxidation in DCP-EYPC liposomes in the presence of mBSA (○ in Fig. 6 (B)) were always higher than those in their absence (dotted line in Fig. 6 (B)). Furthermore, Fe^{3+}-EGTA/X-XO-dependent lipid peroxidation was not induced in positively charged SA-EYPC liposomes (□ (1) in Fig. 6 (B)) but was induced in DCP-EYPC liposomes with a net positive charge by the addition of a high concentration of mBSA (0.5 mg, 1.0 mg/ml) (○ (3),○ (4) in Fig. 6(B)). On the contrary, Fe^{3+}-EGTA/X-XO-dependent lipid peroxidation was induced in negatively charged DCP-EYPC liposomes (dotted line in Fig. 6(B)) but was not induced in SA-EYPC liposomes with a net negative charge by the addition of a high concentration of aBSA (1.0 mg/ml) (□ (5) in Fig. 6(B)).

Fig. 7 shows the effects of aBSA and mBSA on the zeta potentials of liposomes and the rates of Fe^{3+}-EDTA/X-XO-dependent lipid peroxidation. The addition of aBSA also concentration-dependently neutralized the charge of liposome membranes and lowered the lipid peroxidation rate in SA-EYPC liposomes (Fig. 7 (A)). However, in SA-EYPC liposomes, the rates of lipid peroxidation were always higher in the presence of aBSA (□ in Fig. 7(B)) than in the absence of it (dotted line) at the same zeta potential (Fig. 7 (B)). Fe^{3+}-EDTA/X-XO-dependent lipid peroxidation was not induced in negatively charged DCP-EYPC liposomes (○ (1) in Fig. 7(B)) but was induced in SA-EYPC liposomes with a net negative charge by the addition of a high concentration of aBSA (0.5 mg, 1.0 mg/ml) (□ (3), □ (4) in Fig. 7(B)). On the contrary, Fe^{3+}-EDTA/X-XO-dependent lipid peroxidation was induced in positively charged SA-EYPC liposomes (dotted line in Fig. 7(B)) but was not induced in DCP-EYPC liposomes with a net positive charge by

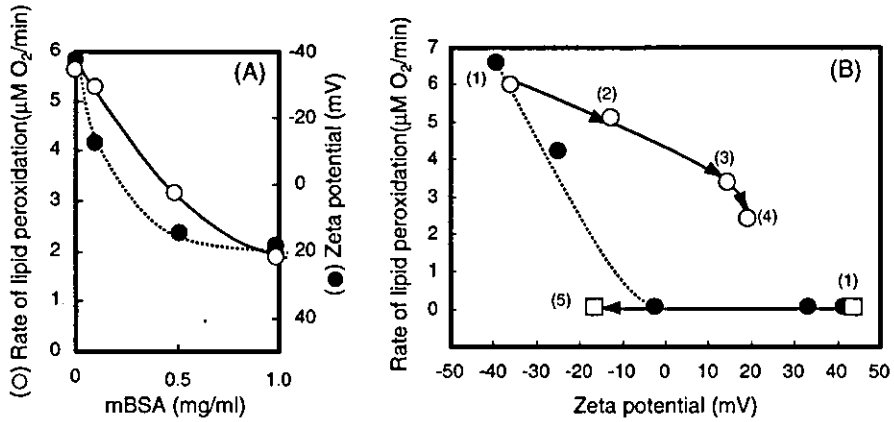

Fig. 6. The effects of the concentration of BSAs on the rate of Fe^{3+}-EGTA/X-XO-dependent lipid peroxidation and the zeta potential of EYPC liposomes with DCP or SA. Panel A: (○) lipid peroxidation; (●) zeta potential. Concentrations of mBSA added to the SA-EYPC (0.2 mM/1.0 mM) liposomes were 0.1–1.0 mg/ml. Panel B: (○) DCP-EYPC (0.1 mM/1.0 mM) liposomes in the presence of BSAs; (□) SA-EYPC (0.2 mM/1.0 mM) liposomes in the presence of BSAs; (●) DCP-EYPC or SA-EYPC liposomes in the absence of BSA. (1) control (no BSA); (2) 0.1 mg mBSA/ml; (3) 0.5 mg mBSA/ml; (4) 1.0 mg mBSA/ml; (5) 1.0 mg aBSA/ml. Other experimental conditions were as shown in Fig. 3.

the addition of a high concentration of mBSA (1.0 mg/ml) (○ (5) in Fig. 7(B)).

The effects of polypeptides, which were used instead of positively charged mBSA and negatively charged aBSA to simplify the experimental system, on the zeta potentials and the rates of lipid peroxidation were investigated further. As shown in Fig. 8(A), the addition of positively charged poly-Lys inhibited Fe^{3+}-EGTA/X-XO-dependent lipid peroxidation in DCP-EYPC liposomes (△), and the addition of negatively charged poly-Glu inhibited Fe^{3+}-EDTA/X-XO-dependent lipid peroxidation in SA-EYPC liposomes (□). Fe^{3+}-EGTA/X-XO-dependent lipid peroxidation was not induced in positively charged SA-EYPC liposomes (dotted line) but was induced in DCP-EYPC liposomes with a net positive charge by the addition of poly-Lys (△). Similarly, Fe^{3+}-EDTA/X-XO-dependent lipid peroxidation was not induced in negatively charged DCP-EYPC liposomes (dotted line) but was induced in SA-EYPC liposomes with a net negative charge by the addition of poly-Glu (□). Thus, the inhibition of lipid peroxidation by polypeptides as well as by BSAs with charges opposite those of the membrane was associated with neutralization of the membrane charge.

We further investigated the effects of positively charged copolypeptides with a hydrophobic residue, poly (Lys-Phe), on the zeta potentials and the rates of Fe^{3+}-NTA/X-XO-dependent lipid peroxidation in EYPC and DCP-EYPC liposomes. As shown in Fig. 8(B), poly (Lys-Phe) behaved similarly to the charged molecule SA in the membranes, and

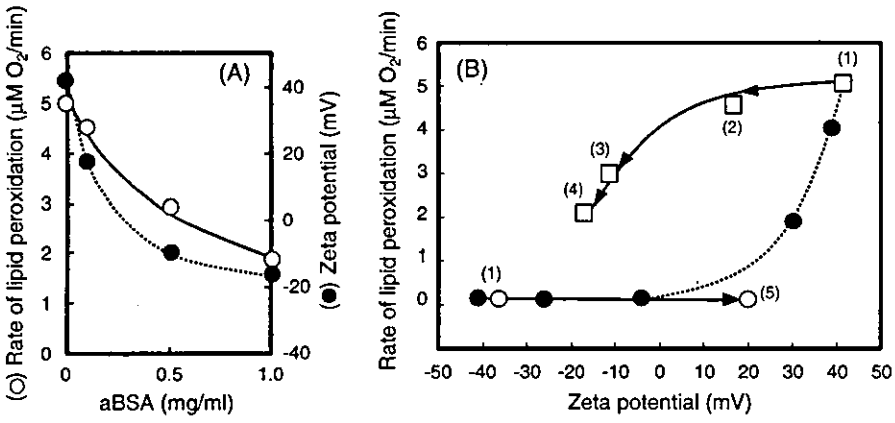

Fig. 7. The effects of the concentration of BSAs on the rate of Fe^{3+}-EDTA/X-XO-dependent lipid peroxidation and the zeta potentials of EYPC liposomes with DCP or SA. Panel A: (○) lipid peroxidation; (●) zeta potential. Concentrations of aBSA added to DCP-EYPC (0.1 mM/1.0 mM) liposomes were 0.1–1.0 mg/ml. Panel B: (○) DCP-EYPC (0.1 mM/1.0 mM) liposomes in the presence of BSAs; (□) SA-EYPC (0.2 mM/1.0 mM) liposomes in the presence of BSAs; (●) DCP-EYPC and SA-EYPC liposomes in the absence of BSAs. (1) control (no BSA); (2) 0.1 mg aBSA/ml; (3) 0.5 mg aBSA/ml; (4) 1.0 mg aBSA/ml; (5) 1.0 mg mBSA/ml. Other experimental conditions were as shown in Fig. 3.

ingly, the amount of DCP bindable with positively charged Fe^{3+}-NTA remains small, and thus only a small amount of residual Fe^{3+}-NTA is available to initiate lipid peroxidation. Under conditions with the same zeta potential, the lower rate of lipid peroxidation in the DCP-liposome system to which mBSA was added than in the SA-liposome system to which no BSA was added, would be due to the lower availability of Fe^{3+}-NTA depending on the amount bound to charge molecules. Similarly, the higher availability of Fe^{3+}-NTA would cause the higher rate of lipid peroxidation in the SA-liposome system to which aBSA was added than in the DCP-liposome system to which no BSA was added under conditions with the same zeta potential (□ (2), □ (3) in Fig. 5(B)).

BSAs and polypeptides inhibited lipid peroxidation without affecting the zeta potentials of the liposomes, which have the same type of charges as BSAs and polypeptides; as shown in Fig. 9(B), nBSA, aBSA, and poly-Glu did not affect the zeta potential but weakly inhibited lipid peroxidation in DCP-EYPC liposomes, while mBSA and poly-Lys also lowered lipid peroxidation in SA-EYPC liposomes without affecting their zeta potential. This indicates that they inhibit lipid peroxidation without direct interaction with membranes. We concluded that their inhibiting effect on lipid peroxidation was due to their binding with Fe^{3+}-NTA and excluding it from membranes. A good correlation between their abilities to bind to Fe^{3+}-NTA and to inhibit lipid peroxidation (Fig. 9(A) and (B)) supports this conclusion.

Surprisingly, an amphiphilic polypeptide, poly (Lys-Phe), increased the rate of Fe^{3+}-NTA-dependent lipid peroxidation and the zeta potential of EYPC liposomes, but a hydrophilic poly-Lys decreased the rate of Fe^{3+}-NTA-dependent lipid peroxidation and increased their zeta potential (○ (2), ⊙ (2), and △ (2) in Fig. 8(B)). In EYPC liposomes, poly (Lys-Phe) binds to the membranes by hydrophobic interaction and increases their membrane charge by acting as a membrane charge molecule like SA, resulting in an increase in the amount of Fe^{3+}-NTA bound to membranous poly (Lys-Phe), and an increase in lipid peroxidation follows. Similar behaviors by poly (Lys-Phe) were observed in negatively charged DCP-EYPC liposomes (○ (1), ⊙ (1) in Fig. 8(B)). In DCP-EYPC liposomes, some parts of membranes charged positively with poly-(Lys-Phe) and other of parts charged negatively with DCP results in an increase of the total number of membrane-charged molecules, that is, an increase of the amount of membranous sites bound with Fe^{3+}-NTA, which can interact with molecules of either charge. Accordingly, the initiation reaction induced by Fe^{3+}-NTA occurs charge-site dependently, and the initiation ability of Fe^{3+}-NTA depends on the total number of sites charged positively and negatively in a membrane rather than the net charge of the membrane.

Recently, Anraku et al. [7] reported that additional net negative charge was increased in oxidized human serum albumin. This finding suggests that the oxidized BSA functions just like an aBSA. It is very interesting to investigate the antioxidant effect of the oxidized BSA from this point.

Finally, we will discuss the antioxidant effect of the native type of BSA based on the above-mentioned considerations. The rate of lipid peroxidation depending on the Fe^{3+}-NTA concentration in SA-EYPC liposomes increased with an increase of SA concentration (Figs. 2 and 3). At concentrations of Fe^{3+}-NTA higher than 20 μM, the decrease of the Fe^{3+}-NTA concentration-dependent lipid peroxidation due to the addition of nBSA (1 mg/ml) to SA-EYPC liposomes (0.2 mM/1.0 mM) with the zeta potential of +41 mV was similar to that of the lipid peroxidation rates decreased by a lower SA concentration in liposomes (Fig. 2), which had about +20 mV of zeta potential as approximated from the rate of lipid peroxidation in SA-EYPC liposomes shown in Fig. 3. These results suggest that net negatively charged nBSA binds to positively charged SA and decreases the amount of membranous SA available for Fe^{3+}-NTA binding, resulting in a decrease in the rate of lipid peroxidation. The zeta potential measured when nBSA (1 mg/ml) was added to SA-EYPC (0.2 mM/1.0 mM) liposomes was about −5 mV, which corresponds to the zeta potential of EYPC liposomes containing no charged substance (Fig. 5(A)). The rate of lipid peroxidation depending on the Fe^{3+}-NTA concentration in the nBSA-added system of SA-EYPC liposomes with −5 mV (● in Fig. 2) was higher than that in nBSA-free system of the EYPC liposome with a charge of −5 mV (□ in Fig. 2), indicating that the membranes of SA-EYPC liposomes that interact with nBSA would be more likely to bind with Fe^{3+}-NTA than with the membranes of EYPC liposomes, even if their membrane zeta potentials were almost the same.

On the contrary, at a concentration of Fe^{3+}-NTA lower than 10 μM, the rate of lipid peroxidation was slower in the system in which nBSA (1 mg/ml) was added to SA-EYPC (0.2 mM/1 mM EYPC) liposomes (● in Fig. 2) than in the nBSA-free system of EYPC liposomes containing no SA (□ in Fig. 2), although the zeta potentials of liposomes in both systems were almost the same (−5 mV). These results suggest that nBSA binds with most of the Fe^{3+}-NTA and prevents it from interacting with membranes by keeping it away from the membrane, resulting in little induction of lipid peroxidation.

In conclusion, the binding of Fe-chelate to membrane surfaces triggered initiation of the Fe-chelate/$O_2^{\cdot-}$-dependent lipid peroxidation of the membrane. BSA inhibits this lipid peroxidation by decreasing the availability of Fe-chelate in two manners, which are dependent on Fe-chelate concentration: ① at high concentrations of Fe-chelate, BSA directly interacts with the membrane and prevents the interaction of Fe-chelate with the membrane surface, where lipid peroxidation is initiated; and ② at low concentrations of Fe-chelate, BSA interacts with Fe-chelate and excludes it from the membrane.

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Effect of genetic variation on the thermal stability of human serum albumin

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Abstract

Reversible thermal denaturation of 33 genetic variants of human serum albumin (HSA) appeared to be a two-state process when studied by circular dichroism (CD). Fourteen single-residue variants have T_m values (midpoint of denaturation) higher than, and nine have T_m values lower than, their endogenous, wild-type counterpart. Nine single-residue variants have ΔH_v values (van't Hoff enthalpy) higher than, and 14 have ΔH_v values lower than, normal albumin. All types of combinations of positive and negative ΔT_m values and $\Delta(\Delta H_v)$ values were found. Good linear correlations between mutation-induced changes of α-helical content and $\Delta(\Delta H_v)$ values, but not ΔT_m values, were found especially for the variants mutated in domains I and III. The effect of altered chain length and glycosylation on T_m and ΔH_v was also studied. For all variants, no clear relationship was found between the changes in the thermodynamic parameters and the type of substitution, changes in protein charge or hydrophobicity. However, the protein changes taking place in domain I have a rather uniform effect (almost all of the nine variants have positive ΔT_m values and negative $\Delta(\Delta H_v)$ values, i.e., they denature more easily than normal albumin but they do so at a higher temperature). The present results can be of both protein chemical relevance and of clinical interest, because they could be useful when designing stable, recombinant HSAs for clinical applications.

Keywords: Human serum albumin; Genetic variant; Thermal stability; Midpoint of denaturation; van't Hoff enthalpy; α-Helical content

1. Introduction

Mutations of surface residues as well as of amino acids in the interior of proteins can effect their stability. Even single-residue substitutions can influence stability as has been observed for, e.g., intracellular fatty acid-binding proteins [1], lysozyme [2], apoflavodoxin [3], a thermophilic cold shock protein [4], crystallins [5] and staphylococcal nuclease [6]. The results referred to have all been obtained by using recombinant mutants. In the present work, we have studied the effect of genetic variation on the thermal stability of human serum albumin (HSA).

HSA is a single-chain protein synthesized in and secreted from liver cells. Normally, it is a simple protein, i.e., it lacks prosthetic groups and covalently bound carbohydrate and lipid. The protein has 585 amino acids and a molecular mass of 66.5 kDa [7]. According to X-ray crystallographic analyses of HSA and its recombinant version, the albumin has about 67% α-helix but no β-sheet. The analyses also showed that the polypeptide chain forms a heart-shaped protein with three homologous domains (I-III), each comprised of two subdomains (A and B) with distinct helical folding patterns that are connected by flexible loops [8,9]. HSA has 35 cysteine residues, and all of these but one, 34 Cys, are involved in the formation of stabilizing disulfide bonds.

Abbreviations: HSA, human serum albumin; Alb, albumin; proAlb, proalbumin; Alb A, normal (wild-type) albumin; CD, circular dichroism; T_m, midpoint of denaturation; ΔH_v, van't Hoff enthalpy

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The temperature behaviour of albumin has been studied by several techniques but mostly by circular dichroism (CD) [10–14] and differential scanning calorimetry [12,15–21]. Among other things, the results showed increased thermal stability of the protein in the presence of aliphatic fatty acid anions of different chain length [13,15–17] or of *N*-acetyl-L-tryptophanate [13,17]. The effect of species differences has also been investigated [21]. Here, the effect of natural mutation of HSA has been examined. The work made use of 33 structurally different genetic variants which represent all kinds of known albumin isoforms, namely single-residue substitutions, proalbumin variants, chain termination mutants and glycosylated albumins. The thermal stability of these alloalbumins, as compared with that of wild-type albumin isolated from the same heterozygous carriers, was monitored by CD at 222 nm. Stability was quantitated in terms of midpoint of the denaturation curve (T_m) and van't Hoff enthalpy (ΔH_v). In addition, in the case of the 23 single-residue variants, the changes in T_m and ΔH_v were related to changes in α-helical content.

2. Materials and methods

2.1. Protein samples

The genetic variants of HSA and their normal (wild-type) counterparts (endogenous Alb A) were isolated from serum from heterozygous carriers by ion-exchange chromatography. After isolation, the albumins were checked by electrophoresis, and no denaturation or significant (no more than 5%) cross-contamination was detected. The proteins were put at our disposal by Drs. M. Galliano and L. Minchiotti, University of Pavia, Pavia, Italy; Dr. S.O. Brennan, Canterbury Health Laboratories, Christchurch, New Zealand; Dr. A.L. Tárnoky, University of Reading, Reading, UK; Dr. F.M. Salzano, Universidade de Federal do Rio Grande do Sul, Porto Alegre, Brazil; Dr. D. Donaldson, East Surrey Hospital, Redhill, UK and Dr. O. Sugita, Niigata University School of Medicine, Niigata, Japan. Before use, the albumins were delipidated by treatment with a hydroxyalkoxypropyl-dextran at pH 3.0 as previously described [22]. After defatting, the albumins were dialysed extensively against deionized water, lyophilized and stored at −20 °C until use. Thus, the albumins from a donor have been exposed to exactly the same conditions from the time the blood samples were taken until the present experiments were performed.

Fraction V HSA (96–99% pure), assumed to be Alb A, was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and delipidated, dialysed and lyophilized in the same way as the other albumin samples.

2.2. Thermal denaturation measurements

The protein concentration was 10 μM, as determined by the method of Bradford [23], and the buffer was 67 mM sodium phosphate, pH 7.4. CD measurements were made with a Jasco J-720 spectropolarimeter (Tokyo, Japan), and CD melting curves were determined by monitoring the changes in the dichroic intensity at 222 nm as a function of temperature. The albumin solutions were heated by a Peltier effect in the CD-cuvette. The thermal denaturation was studied in the range 298–358 K using a heating rate of 1.0 K/min which was applied with a Jasco PTC-348 thermostat (Tokyo, Japan). Similar results (not shown) were obtained with a heating rate of 0.5 K/min. The calorimetric reversibility of the thermally induced transition was checked by reheating protein solutions in the calorimetric cell, flushed with nitrogen, after cooling from the first run. It was observed that heating to or above 358 K caused irreversible denaturation. This finding is in accordance with results of differential scanning calorimetry [20,21] and fluorescence spectroscopy [20].

The denaturation process was characterized by determining the midpoint of denaturation (melting temperature, T_m) and the van't Hoff enthalpy (ΔH_v). T_m is the temperature at which half of the protein is in a denatured state (D) and the other half is in the native state (N). ΔH_v was determined according to the following method, which is essentially the same as that of Budisa et al. [24]. At each temperature an equilibrium constant for the denaturation (K) was calculated from [D]/[N], where the squared brackets represent concentrations. Next, ln$K$ was plotted as a function of temperature (T) according to the van't Hoff equation:

$$\ln K = (-\Delta H_v/R) \times 1/T.$$

In this equation, R is the gas constant. Finally, ΔH_v was determined from the slope of the straight line obtained.

2.3. Far-UV CD spectra

The protein concentration was 1.5 μM, and the buffer was 67 mM sodium phosphate, pH 7.4, 25 °C. Far-UV intrinsic spectra were recorded from 200 to 250 nm using the Jasco J-720 spectropolarimeter. For calculation of the mean residue ellipticity, $[\theta]$, the molecular masses of normal albumin and of the albumins with single amino acid substitutions were assumed to be 66.5 kDa. The α-helical content of these proteins was estimated from the ellipticity values at 222 nm as described by Chen et al. [25].

3. Results and discussion

3.1. The genetic variants

The 33 alloalbumins used in this study have been named after the place from where the first detected carrier originates, and their molecular changes are summarized in Tables 1 and 2.

The majority of the albumin variants results from single-residue substitutions and almost all have net charges which

Table 1
Thermal denaturation of albumin variants with a single amino acid substitution[a]

Variant (mutation[b])	Reference[c]	ΔT_m (K)[d]	$\Delta(\Delta H_v)$ (kJ/mol)[e]
Alb Blenheim (1 Asp→Val)	[26]	1.94	−132.61
Alb Malmö-95 (63 Asp→Asn)[f]	[27]	6.07	−163.15
Alb Vibo Valentia (82 Glu→Lys)	[28]	2.03	−35.48
Alb Tregasio (122 Val→Glu)	[29]	0.57	26.85
Alb Hawkes Bay (177 Cys→Phe)	[30]	−1.59	−17.66
Alb Tradate-2 (225 Lys→Glu)	[31]	−4.86	44.13
Alb Herborn (240 Lys→Glu)	[32]	−2.74	−71.99
Alb Niigata (269 Asp→Gly)	[33]	3.67	−70.90
Alb Caserta (276 Lys→Asn)	[31]	4.87	13.42
Alb Canterbury (313 Lys→Asn)	[34]	−7.16	6.84
Alb Brest (314 Asp→Val)	[35]	−0.38	24.09
Alb Roma (321 Glu→Lys)	[36]	1.42	28.98
Alb Sondrio (333 Glu→Lys)	[37]	−2.56	−21.89
Alb Trieste (359 Lys→Asn)	[38]	−6.56	−13.91
Alb Parklands (365 Asp→His)	[39]	0.89	58.06
Alb Milano Slow (375 Asp→His)	[38]	−0.09	−94.33
Alb Kashmir (501 Glu→Lys)	[40]	0.13	−1.52
Alb Ortonovo (505 Glu→Lys)	[41]	1.87	−83.36
Alb Maku (541 Lys→Glu)	[42]	6.12	−58.32
Alb Church Bay (560 Lys→Glu)	[43]	0.70	15.23
Alb Paris-2 (563 Asp→Asn)	[37]	4.17	−154.35
Alb Verona (570 Glu→Lys)	[44]	−6.53	83.92
Alb Milano Fast (573 Lys→Glu)	[45]	2.08	−41.45

[a] The table gives average values for two to three experiments, which coincided with each other within ±6%.
[b] The positions of Alb A are from 1 to 585.
[c] See the references for more information about, for example, isolation and sequencing.
[d] ΔT_m is T_m for the variant minus T_m for the corresponding Alb A.
[e] $\Delta(\Delta H_v)$ is ΔH_v for the variant minus ΔH_v for the corresponding Alb A.
[f] The unglycosylated form of the variant. The name of this variant, as well as of its glycosylated form (Table 2), was taken from Ref. [31].

differ from Alb A at physiological pH (Table 1): Alb Vibo Valentia, Roma, Sondrio, Kashmir, Ortonovo and Verona are all +2 variants (i.e., they have two positive charges more than Alb A); Alb Blenheim, the unglycosylated form of Malmö-95, Niigata, Brest, Parklands, Milano Slow as well as Paris-2 are +1 variants; Alb Tregasio, Caserta, Canterbury and Trieste are −1 variants; and, finally, Alb Tradate-2, Herborn, Maku, Church Bay and Milano Fast are −2 variants. Thus, all kinds of changes in net charge are represented in this work. The only variant with no change in net charge is Alb Hawkes Bay. This albumin is also special in the sense that it has one disulfide bond less than Alb A but one free sulfhydryl group more than Alb A. Most of the single amino acid substitutions are placed in domain II (residues 200–391), namely 11. Domains I (residues 1–199) and III (residues 392–585) are represented by five and seven examples, respectively.

Proalbumin (proAlb) is an albumin molecule to which the propeptide, Arg-Gly-Val-Phe-Arg-Arg-, is still bound at the N-terminus. Normally, this protein does not occur in detectable amounts in the circulation, because the propeptide is cleaved off by propeptidase within the liver cells. However, substitution of −2 Arg (as in proAlb Lille) or 1 Asp (as in proAlb Blenheim) inhibits the proteolytic cleavage of the propeptide but not the secretion of the protein, and proalbumin variants, in contrast to wild-type proalbumin, can be isolated from the serum. In the periphery, part of proAlb Blenheim (Table 2) becomes converted to Alb Blenheim (Table 1), i.e., the normal propeptide is hydrolysed [26].

Among the C-terminal variants most are truncated albumins (Table 2). Thus, Alb Catania is three amino acids shorter than Alb A, and the three last residues in the new C-terminal end are changed from Gln-Ala-Ala to Lys-Leu-Pro. Alb Venezia has been shortened by seven amino acids, and the new C-terminal end is changed from Gly-Lys-Lys-Leu-Val-Ala-Ala to Pro-Thr-Met-Arg-Ile-Arg-Glu. Alb Bazzano has been shortened by three amino acids, and 14 of the last 16 amino acids in the new C-terminal end have been substituted: from Cys-Phe-Ala-Glu-Glu-Gly-Lys-Lys-Leu-Val-Ala-Ala-Ser-Gln-Ala-Ala to Ala-Leu-Pro-Arg-Arg-Val-Lys-Asn-Leu-Leu-Leu-Gln-Val-Lys-Leu-Pro. Here the 567 Cys→Ala substitution has caused the loss of the C-terminal disulfide bridge. The most pronounced modification, however, is found in Alb Kénitra which is an extended, and not a truncated, variant [49]. The last 11 amino acids of Alb A have been changed from Leu-Val-Ala-Ala-Ser-Gln-Ala-Ala-Leu-Gly-Leu to Thr-Cys-Cys-Cys-Lys-Ser-Ser-Cys-Leu-Arg-Leu. The extension consists of Ile-Thr-Ser-His-Leu-Lys-Ala-Ser-Gln-Pro-Thr(596)-Met-Arg-Ile-Arg-Glu-Arg-Lys; in the circulation the two C-terminally placed Arg-Lys are cleaved off by basic carboxypeptidases. In Alb Kénitra

Table 2
Thermal denaturation of proalbumin variants, albumins modified at the C-terminal end and of glycosylated variants[a]

Variant (mutation[b])	Reference[c]	ΔT_m (K)[d]	$\Delta(\Delta H_v)$ (kJ/mol)[e]
proAlb Lille (−2 Arg→His)	[46]	4.64	−13.82
proAlb Blenheim (1 Asp→Val)	[26]	7.10	−118.49
Arg-Alb (Alb A having −1 Arg)	[47]	0.23	12.65
Alb Catania (580–582 substituted, 583–585 deleted)	[48]	0.13	−57.27
Alb Venezia (572–578 substituted, 579–585 deleted)	[48]	−5.74	99.43
Alb Bazzano (567–582 substituted, 583–585 deleted)	[31]	4.67	−8.54
Alb Kénitra (575–585 substituted, extended with 586–601)	[49]	−5.30	12.72
Alb Malmö-95 (63 Asp→Asn, glycosylated at 63 Asn)	[27]	4.06	−107.41
Alb Redhill (−1 Arg retained, 320 Ala→Thr, glycosylated at 318 Asn)	[50,51]	1.93	−9.52
Alb Casebrook (494 Asp→Asn, glycosylated at 494 Asn)	[52,53]	−1.11	54.36

[a] The table gives average values for two to three experiments, which coincided with each other within ±6%.
[b] The positions of proalbumin are from −6 to −1 (the juxtaposition to albumin itself), and those of Alb A are from 1 to 585.
[c] See the references for more information about, for example, isolation and sequencing.
[d] ΔT_m is T_m for the variant minus T_m for the corresponding Alb A.
[e] $\Delta(\Delta H_v)$ is ΔH_v for the variant minus ΔH_v for the corresponding Alb A.

the four additional cysteine residues form two new S–S bridges, and 596 Thr is partially *O*-glycosylated by a monosialylated oligosaccharide.

It is uncommon for an amino acid substitution to result in the formation of an oligosaccharide attachment sequence. However, that has happened to Alb Malmö-95 (partly), Redhill and Casebrook, which are glycosylated in domain I, II and III, respectively (Table 2). In all three cases, the glycan is a disialylated (mainly or totally) biantennary complex type oligosaccharide *N*-linked to an asparagine residue [51]. Alb Redhill is unique, because it is the only example so far of an albumin with two mutations. One is the 320 Ala→Thr, which leads to glycosylation of 318 Asn; the other is −2 Arg→Cys, which, in this case, results in abnormal hydrolysis of preproalbumin within the liver cells and to the formation of albumin still possessing an Arg at position −1 [50].

All the albumins were isolated from heterozygotes, who, except for two cases, had one proalbumin or albumin variant and Alb A in the circulation. In one exception, a member of a New Zealand family had both proAlb Kaikoura and Arg-Alb [47]. The explanation of this condition is that most of the modified preproalbumin (about 85%) is cleaved after the mutated residue, −2 Cys, giving rise to Arg-Alb (Table 2), with about 15% processed normally, i.e., hydrolysed before −6 Arg by signal peptidase, leading to the formation of proAlb Kaikoura. The liver excretes both alloalbumins, and both can be isolated from the serum. Unfortunately, the proalbumin variant was not available for this study. In the other example, a Swedish one, the person carried a variant (Alb Malmö-95) of which about 50% was glycosylated [27].

According to the literature cited [26–53], none of the mutations seem to affect the oligomeric state of albumin.

3.2. Thermal denaturation

The temperature behaviour of albumin is strongly dependent on the environmental conditions. For example, thermal denaturation has been reported to be dependent on protein concentration [10,16], the presence of ligands [13,17], pH [15,18], type of salt [19] and salt concentration in the medium [15,18]. In the present work, we have used our standard conditions with respect to medium and rate of heating; see Section 2.2. In this way we should be able to compare the results obtained in this work with those in which the effect of recombinant mutation [14] and species differences were examined [21]. Thus, the effect of temperature in the range 298–358 K (25–85 °C) on the reversible denaturation of the albumins was monitored by CD at 222 nm. In this temperature range, the thermal denaturation process can be regarded as a two-state transition, i.e., in these proteins, the probability of all the intermediate states between the native and denatured ones is very low, and the denaturation appears as a single cooperative system [21]. Fig. 1 shows examples of mutations having a moderate effect (Fig. 1A) and a small effect (Fig. 1B) on thermal stability. In all cases, the process was characterized by determining the midpoint of denaturation, T_m, and the van't Hoff enthalpy, ΔH_v, and the results are included in Tables 1 and 2. The average value of T_m for endogenous Alb A was 336.67 K. This value is similar to that determined for commercial HSA (337.02±0.21 K, n=4). The average value of ΔH_v for wild-type HSA was calculated as 262.55 kJ/mol, a value which is somewhat higher than that obtained for the commercial protein (237.49±0.18 kJ/mol, n=4). The differences between the average values determined for endogenous and commercial Alb A are most probably due to differences in isolation procedures.

3.2.1. Single-residue variants

From Table 1 it is seen that 20 of the 23 single-residue mutations resulted in ΔT_m values which are larger than two times the standard deviation determined for commercial Alb A (0.42 K). Thirteen of the ΔT_m values are positive, and seven are negative. Apparently, there is no simple relationship between the ΔT_m values and the domains in which the mutations are placed, or between the ΔT_m values and the

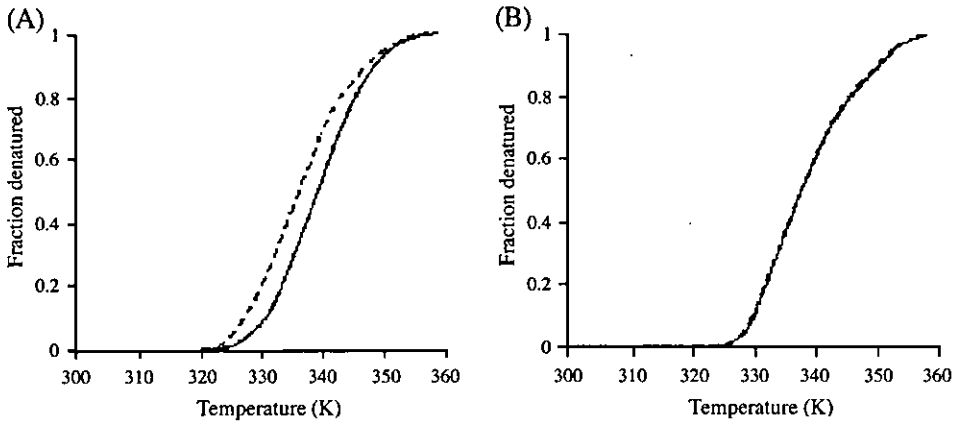

Fig. 1. Thermal denaturation of alloalbumins and their normal, endogenous counterparts. (A) Alb Herborn and (B) Alb Brest; the broken and full curves represent variant and corresponding Alb A, respectively. The curves are averages for three experiments.

change in protein charge (see Section 3.1). However, eight of the 20 mutations giving significant changes in ΔT_m are mutations resulting in more hydrophobic albumins. These include the albumins Blenheim, Malmö-95, Niigata, Caserta, Parklands and Paris-2, having positive changes in T_m, and albumins Canterbury and Trieste, which have very big negative ΔT_m values. For Alb Hawkes Bay, see below.

All of the mutations result in $\Delta(\Delta H_v)$ values larger than 0.36 kJ/mol (2×SD for commercial Alb A) (Table 1). Among these, 14 have ΔH_v values lower than their corresponding Alb A, i.e., the variants are easier to denature, more unstable, than Alb A. Nine of the 14 variants have positive ΔT_m values, i.e., the albumins denature more easily than Alb A but do so at a higher temperature. Although most of the positive $\Delta(\Delta H_v)$ values are placed in domain II, there seems to be no clear relationship between the $\Delta(\Delta H_v)$ values and the domains in which the mutations are placed. Finally, apparently there is no relationship between the $\Delta(\Delta H_v)$ values and the change in protein charge or between the $\Delta(\Delta H_v)$ values and protein hydrophobicity.

All the genetic variants included in this study were originally detected by electrophoresis performed under non-denaturing conditions. Among other things, this fact implies that the single-residue mutations are exposed to the solvent and result in modifications of albumins surface charges, a proposal which is supported by inspection of the crystal structure of HSA [8,29,31,35,38,43]. Alb Hawkes Bay is an exception, because the abnormal electrophoretic mobility of this alloalbumin was due to a gross conformational change caused by the mutation (177 Cys→Phe) [30]. The reason for the conformational changes is that this variant has lost the disulfide bond between 177 Cys and 168 Cys. The new SH-group of 168 Cys seems to bind to the nearby 124 Cys leaving 169 Cys unbound [30]; i.e., the variant has two free cysteine residues, namely the usual 34 Cys and 169 Cys. Surprisingly, the molecular rearrangements do not effect the thermal denaturation much (Table 1).

By using recombinant mutants, Watanabe et al. [14] studied the effect of single-residue mutations placed in subdomain IIA (Sudlow's binding site I) and IIIA (site II) on the thermal stability of HSA. These authors observed that mutations in subdomain IIA have a larger impact on the stability than those placed in subdomain IIIA, which only had marginal effects on the thermodynamic parameters. The mutation with the biggest effect was 214 Trp→Ala, which resulted in a ΔT_m value of −3.0 K and a $\Delta(\Delta H_v)$ value of −178.7 kJ/mol. The first value is within the range of the results found in this study, whereas the numeric value of the latter is higher than the numeric values of those found here (Table 1).

3.3. Relation between thermal stability and α-helical content

The molecular changes of the albumin variants had in themselves an impact on the CD spectrum. Fig. 2A shows that the mutation of Alb Herborn has a fairly large effect, whereas that of Alb Brest (Fig. 2B) has a small effect on the far-UV CD spectrum of albumin. Comparison of these observations with the results illustrated in Fig. 1 suggests that there exists a correlation between the mutation-induced effects on α-helical content and thermal stability of albumin. Therefore, a more detailed study was performed involving all the single-residue variants. Alloalbumins with more extensive molecular changes were not included in these experiments, because the effects on α-helical content were assumed to be more complex. Fig. 3 relates changes in α-helical content (abscissas) and $\Delta(\Delta H_v)$ (ordinates), and it is seen that there exists a good linear correlation between these parameters. Apparently, the correlation is best for mutations in domain III. However, if the results for Alb Hawkes Bay are ignored, an excellent correlation (r=0.998) is obtained for the domain I variants. The results of Fig. 3A also support the information in the literature [30], that the 177 Cys→Phe

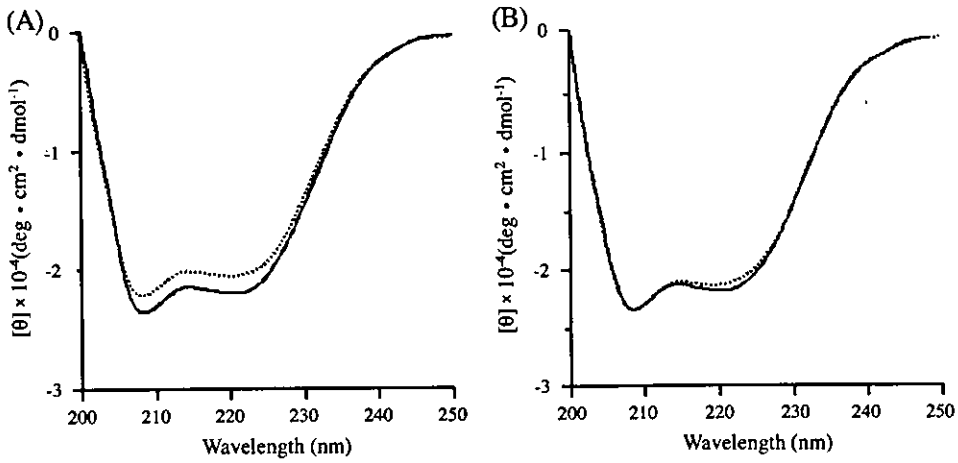

Fig. 2. Far-UV CD spectra of alloalbumins and their normal, endogenous counterparts. (A) Alb Herborn and (B) Alb Brest; the dotted and full curves represent variant and corresponding Alb A, respectively. The curves are averages for three experiments.

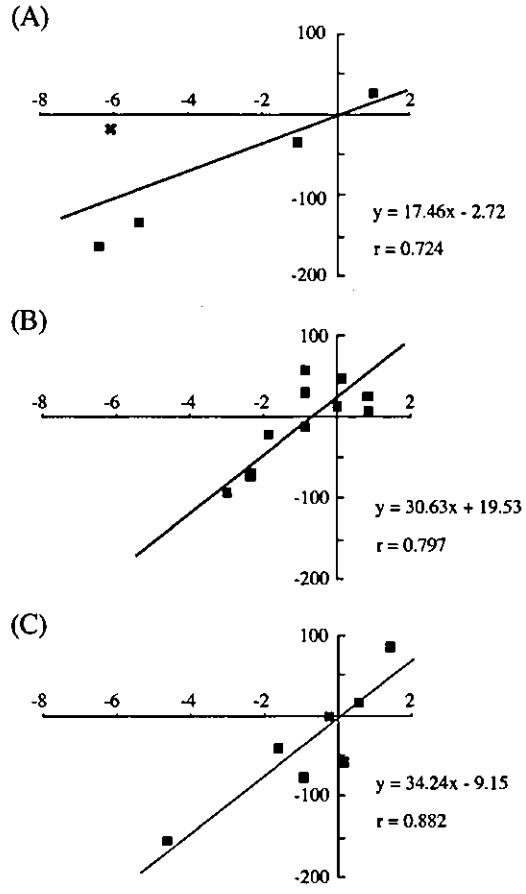

Fig. 3. Abscissas: Changes in percentages of the α-helical content of HSA caused by single amino acid substitutions. Ordinates: Corresponding $\Delta(\Delta H_v)$ given as kJ/mol, taken from Table 1. Results are shown for domain I (A), domain II (B) and domain III (C). In all three cases, the relation between changes in α-helical content and $\Delta(\Delta H_v)$ has been characterized by regression lines, and the expressions of these are given in the panels. The × in (A) represents the single variant in which a cysteine residue has been mutated (177 Cys→Phe). Without this protein, the domain I variants can be described by the following regression line: $y=25.00x-3.16$, $r=0.998$. The figure shows average values for two to three experiments, which coincided with each other within ±2%.

mutation results in a large conformational change, because the α-helical content of Alb Hawkes Bay is diminished by ca. 6%..

The changes in α-helical content were also related to ΔT_m (results not shown). However, this relationship was poor: the regression coefficients for the lines were only 0.242 (domain I), 0.319 (domain II) and 0.548 (domain III).

3.4. Effect of other types of mutation on thermal stability

3.4.1. Effect of altered chain lengths

The thermal stability of albumin is not effected much by an extra arginine residue at the N-terminus (Table 2). By contrast, the presence of a propeptide has a pronounced influence, and proAlb Blenheim is among the alloalbumins mostly effected. For both proalbumins, T_m is increased, and ΔH_v is decreased (Table 2).

A relatively small molecular change of the C-terminus (Alb Catania) has no effect on T_m (Table 2). By contrast, a relatively large decrease in ΔH_v was found. Alb Bazzano has lost its C-terminal disulfide bridge, namely the one between 558 Cys and 567 Cys. Surprisingly, this molecular change, as well as the relative large number of amino acid substitutions (see Section 3.1), has a pronounced increasing effect on T_m (ΔT_m=4.67 K), whereas ΔH_v is decreased somewhat. Alb Kénitra has only one free SH-group (34 Cys) but two new disulfide bridges, both of which are placed in the modified C-terminal end (see Section 3.1). Apparently, the two additional disulfide bonds have only a minor stabilizing effect because T_m is decreased by −5.30 K and ΔH_v is only slightly increased. However, the results are most probably a combined effect of the unusually many changes, which have taken place in this variant [49]. Finally, Alb Venezia has the mostly modified parameters, because T_m is much decreased, and the ΔH_v value is very high (Table 2).

3.4.2. Effect of N-glycosylation

All three variants have disialylated oligosaccharides linked to an asparagine residue (Table 2). Alb Malmö-95 has its glycan in domain I, and the molecular change causes a big increment of T_m and a large decrease of ΔH_v. Alb Redhill is so far the only example of an albumin with two mutations but they have only a relatively small impact on the proteins thermal stability. Finally, attachment of an oligosaccharide to domain III (Alb Casebrook) results in a more stable protein.

4. Concluding remarks

Apparently, there is no clear relation between changes in thermal stability of HSA and the type of substitution, change in protein charge or hydrophobicity. These observations probably reflect the fact that the effects on stability are strongly dependent on the molecular environment of the individual mutations. However, the protein changes taking place in domain I have a fairly uniform effect, because eight of the nine variants have positive ΔT_m values and seven of them have negative $\Delta(\Delta H_v)$ values (Tables 1 and 2); i.e., almost all of the alloalbumins modified in domain I denature more easily than normal albumin but they do so at a higher temperature. Furthermore, four of the five mutations resulting in very big changes in ΔH_v [$\Delta(\Delta H_v)<-100$ kJ/mol] are placed in domain I. This finding suggests that domain I is the most thermally unstable domain.

A good correlation was found between changes in α-helical content and $\Delta(\Delta H_v)$. Ignoring the variant with a modified disulfide bond pattern, the correlations were best for the peripheral domains. For all three domains, the trend is that a diminished α-helical content results in a negative

value of $\Delta(\Delta H_v)$. However, examples of increased α-helical content were also found. These increments were generally associated with positive $\Delta(\Delta H_v)$ values, i.e., more stable albumins.

Kosa et al. [21] have determined the T_m values for five species of serum albumin, namely human, bovine, dog, rabbit and rat. Of these, HSA was the most stable, and rat serum albumin was the least stable, and T_m for the former minus T_m for the latter was 2.0 K. Even though the sequence homologies between the five species of mammalian albumins are greater than 70% [21], many differences exist between them with respect to amino acid sequence. Therefore, it is surprising that half of the genetic variants with single amino acid substitutions have ΔT_m values larger than 2.0 K or lower than −2.0 K (Table 1).

Mutations resulting in change of size or shape of nonpolar residues in the hydrophobic core almost always modify thermal and chemical stability as well as structural properties of a protein [54,55]. However, the presence of cavities in the protein can to some extent neutralize the effect of single site mutations [55]. On the other hand, it has also been argued that filling of cavities leads to better packing and thereby helps stabilize the native state of the protein [56]. In general, it is expected that replacements on the surface of a protein have little or moderate effect on protein stability unless either the original or the introduced residue has specific roles. However, mutations of residues on the protein surface can also affect the proteins stability by more unspecific means. For example, the effect can be brought about by the alteration of charge–charge interactions [57], the packing and thereby the stability of the protein can be modified by changing the number of van der Waals interactions throughout the protein [56]. Thus, the observed changes in albumin stability could in some cases be due to structural alterations localized to the surface of the protein, whereas in other examples interior parts of albumin could be affected as well.

The present results can be of both protein chemical relevance and of clinical interest. HSA is a very widely used therapeutic agent. However, a major problem at present in the usage of the protein for clinical applications is viral contamination by, for example, human immunodeficiency virus, or vira causing herpes or hepatitis. Therefore, solutions of HSA are pasteurized by heating at 60 °C for 10–11 h before use [7]. Such a prolonged treatment can cause both reversible and irreversible denaturation of the protein. In the near future, the supply of recombinant HSA with high stability as well as inexpensive cost will be awaited. The results obtained here will be basically useful for designing these recombinant albumins.

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総　説

血清アルブミンの構造特性と医薬への応用

Structural Properties and Pharmaceutical Application of Human Serum Albumin

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和文抄録

ヒト血清アルブミン（HSA）は，膠質浸透圧の維持，薬物結合能，抗酸化能など生体内の恒常性維持に必要不可欠な機能を数多く有していることから，各種病態時に伴う血中HSAの量的・質的変動は，これらの機能低下を引き起こす．最近，部位特異的変異法によりHSAの機能発現に必須なアミノ酸残基が同定され，特に，薬物結合や抗酸化能に重要な残基が明らかにされつつある．また，HSAは，安全性，生体適合性や血中滞留性に富んでいるため，ドラッグデリバリーシステム（DDS）における担体としての開発も盛んに行われており，遺伝子組換え技術によるアルブミンと蛋白性医薬品の遺伝子を融合させたアルブミン融合医薬品をはじめ，アルブミンの断片化，多量体化によるサイズ調節など，アルブミン自身の改良による医療への応用も試みられている．特に，HSAの高いリガンド結合性を利用したアルブミン循環透析は，肝機能補助療法として利用されている．

Abstract

Human serum albumin (HSA) has many essential functions for homeostasis, such as the maintenance of osmotic pressure, drug binding capacity and antioxidant activity. In some diseases, the qualitative and quantitative variation of HSA in blood induced the depression of these functions. Site-directed mutagenesis studies of HSA, made it possible to examine the participation of various amino acids residues in the functional properties of HSA, such as binding capacity and antioxidant activity. In addition, the development of HSA as carrier in the drug delivery system (DDS) is advanced, such as albumin fusion protein and fragment and polymeric albumin by using recombinant DNA technology. Especially, albumin circulation dialysis using the high ligand binding capacity of HSA will be utilized as liver function adjuvant therapy.

Keywords

Human serum albumin, X-ray crystallographic analysis, site-directed mutagenesis, pharmaceutical application, albumin circulation dialysis

はじめに

HSAは，血漿蛋白質のなかでも最も高濃度（約4g/dL）に存在し，生体内において血漿膠質浸透圧の調節（正常血漿の膠質浸透圧のうち，80%がアルブミンによって維持されている），脂肪酸，ビリルビン，尿毒症物質，一酸化窒素をはじめとする内因性及び薬物などの外因性リガンドの輸送担体，抗酸化能など数多くの機能を有している[1-4]．種々の病態・疾患時では，アルブミンの質的・量的変動が認められ，膠質浸透圧の低下による浮腫の発現，血中薬物濃度増大による副作用の発現，抗酸化能の減少など生体内恒常性の異常を来す．

ところで，このようなマルチな機能を持つHSAは，優れた生体適合性に加えて非常に長い半減期（生物学的半減期：15～19日）を持つため，低分子のみでなく，薬物動態学的特性に問題がある生理活性ポリペプチドにおいても好ましい担体として利

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用されてきた．さらに，最近，遺伝子組換え技術によるHSAを用いたDDSの開発研究は，アルブミンと蛋白性医薬品の遺伝子を融合させたアルブミン融合医薬品をはじめ，アルブミンの断片化，多量体化によるサイズ調節など，アルブミン自身の改良による医療への応用も試みられている．

本稿では，X線結晶構造解析並びに化学修飾等の結果を基に，HSA分子上の薬物結合サイトを始めとした機能発現に必須なアミノ酸残基に着目し，遺伝子組み換え技術を用いたHSAの機能に及ぼすそれらアミノ酸残基の役割について紹介する．加えて，ドラッグデリバリーシステム（DDS）担体として利用されているHSAについて，遺伝子組換え技術を応用したHSAの医療への新たな展開について述べる．

1．HSAの機能発現に係るアミノ酸残基

HSAは585個のアミノ酸残基から成る分子量約66.5kDaの糖鎖を持たない単純蛋白質である．HSAの基本構造はαヘリックスとそれを結ぶ柔軟なhinge領域より形成される3つのドメインである（ドメインⅠ，Ⅱ，Ⅲ）が，水溶液中ではこれら3つのドメインがさらに折り重なって，心臓の様な形状をした特有の立体構造をとっている（Fig. 1.）．また，HSAはその分子中に35個のシステイン残基（Cys）を有しているが，そのうち17対は分子内ジスルフィド結合を形成し，9つのループの構築やHSAの構造安定化に大きく寄与している．一方，一次配列上34番目のCysはSH基が唯一遊離な状態で存在しており，このSH基が抗酸化能やリガンド輸送といったHSAの特有な機能発現において重要な役割を果たしている．

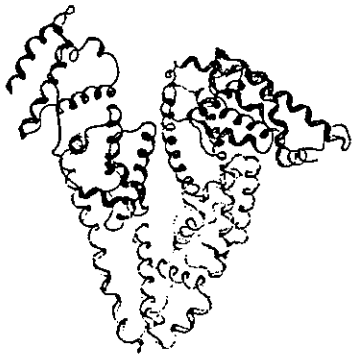

Fig. 1. Crystal structure of human serum albumin. (HSA) (from Ref. 1)

①^{34}Cys（サブドメインIA）

^{34}Cysは遊離の反応性の高いSH基を有しており，これが血中におけるラジカルやある種の薬物のスカベンジャーあるいはリザーバーとして機能している．Bhagavanらは，Cys→Ser変異体を作製し，NO消去能が約60%に低下したことから，^{34}Cysを抗酸化アミノ酸残基として定義している[5]．また，Eraらは，^{34}CysのSH基に着目し，SH基がフリーの状態を還元型アルブミン（メルカプトアルブミン;HMA），システインや酸化型グルタチオンなどの含硫アミノ酸とジスルフィド結合した状態を酸化型アルブミンとし，血中において，これら両者が混合物として存在することを報告している[6]．この^{34}Cysの還元型と酸化型アルブミンの割合はHPLC法により，簡便に定量できることから，これまでに腎疾患，肝疾患など種々の疾患における生体内の酸化還元動態の解析に用いられている[7]．最近，著者らもまた，このカラムにより腎透析患者に貧血を防ぐために併用投与されている鉄剤による生体の酸化亢進を確認している（Fig. 2.）[8]．このように細胞内では多量の還元型グルタチオンの存在によってかなりの還元性が保たれているが，一方細胞外では，血管内外を問わず体内に多量に存在する血清アルブミンがこのSH基の酸化・還元機構によって，非酵素的な抗酸化蛋白質として広く機能している．事実，Evanceらは，急性呼吸窮迫症候群（ARDS）の患者に対して，20%アルブミンを投与した結果，非投与群に比べて生存率が30%上昇するとともに，血中のチオールレベルが回復したことから，^{34}CysのSH基が血清中における抗酸化アミノ酸残基として機能する可能性を示唆している[9]．

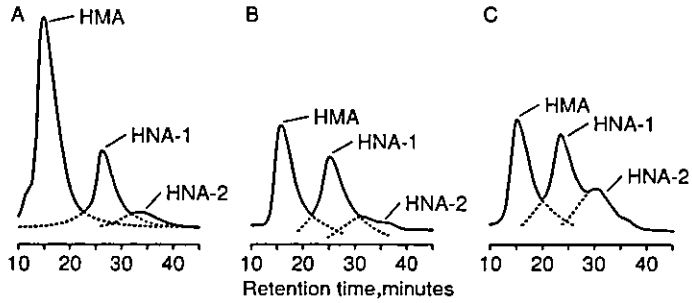

Fig. 2. High-performance liquid chromatography (HPLC) profile of serum albumin from hemodialysis patients (HD) patients with or without intravenous iron administration (IVIR). 5 μL aliquots of serum from HD patients with or without IVIR, and healthy subjects were subjected to HPLC analysis using a Shodex Asahipak ES-502N column. A representative tracing of HPLC profiles of serum albumin from normal subjects (A). A representative tracing of HPLC profiles of serum albumin from HD patients without IVIR (B). A representative tracing of HPLC profile of serum albumin from patients treated with IVIR (C). HMA; mercapto albumin, HNA-1 nonmercapto albumin (S-S), HNA-2; nonmercapto albumin (-SOOH or -SOOOH) (from Ref. 8)

②Met残基（^{123}Met，^{548}Met）

これまでに^{34}Cysの重要性を示してきたが，当然，その他のアミノ酸残基も修飾を受ける．特に，Metの酸化は，^{34}Cysにつぐ酸化ストレスに対する必須残基として位置づけられている．事実，Finchらは，^{34}Cysの抗酸化能には及ばないものの，^{123}Met，^{548}Metが活性酸素である過酸化水素の標的部位であることを明らかにしている[10]．著者らもまた，Met→Ala変異体を用いた検討より，^{123}Met，^{548}MetがHSA自身の酸化に対して抗酸化剤として機能することを見出し，この結果の妥当性を裏付けている．また，アルブミンの事例ではないものの，Metの重要性を示す例として，肺水腫によるα_1-アンチトリプシン欠損症において，Metの酸化が蛋白自身の機能消失を引き起こすことが報告されている．加えて，Stadtmanらは，抗酸化能を有す