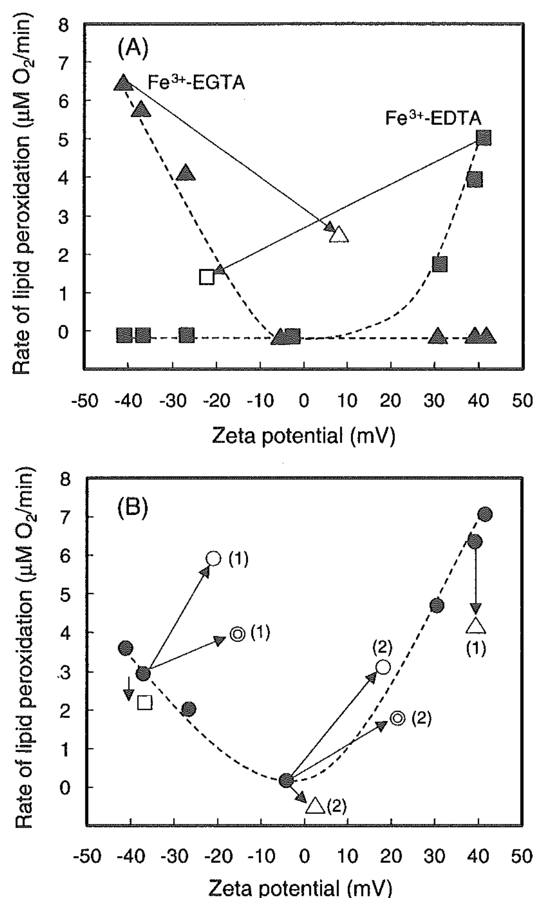

Fig. 8. The effects of polypeptides on the rate of Fe^{3+}-chelate/X-XO-dependent lipid peroxidation and the zeta potentials of EYPC liposomes with DCP or SA. Panel A: Fe^{3+}-EGTA/X-XO-system in the presence (△) or absence (▲) of poly-Lys; Fe^{3+}-EDTA/X-XO-system in the presence (□) or absence (■) of poly-Glu. Concentrations of poly-Lys and poly-Glu were 645 μg/ml (5 mM) and 38.7 μg/ml (0.3 mM), respectively. Panel B: Fe^{3+}-NTA/X-XO-system. (●) EYPC liposomes with different concentrations of SA or DCP in the absence of polypeptides. (○) Poly (Lys-Phe) incorporated into the membrane of the DCP-EYPC(0.1 mM/1.0 mM) or EYPC (1 mM) liposomes. (◎) Poly (Lys-Phe) added to the solution of DCP-EYPC or EYPC liposomes, (△) poly-Lys added to the solution of SA-EYPC(0.1 mM/1.0 mM) or EYPC (1 mM) liposomes, and (□) poly-Glu added to the solution of DCP-EYPC (0.1 mM/1.0 mM) liposomes. Concentrations of poly (Lys-Phe) and poly-Glu were 17.8 μg/ml and 645 μg/ml, respectively. Concentrations of poly-Lys were 645 μg/ml and 6.45 μg/ml in SA-EYPC (0.1 mM/1.0 mM) liposomes and EYPC (1 mM) liposomes, respectively. Other experimental conditions were as shown in Fig. 3.

increased the lipid peroxidation and neutralized the zeta potential of DCP-EYPC liposomes (○ (1), ◎ (1)). In EYPC liposomes, poly (Lys-Phe) also increased the lipid peroxidation and zeta potential (○ (2), ◎ (2)), but the homopolypeptide poly-Lys decreased the lipid peroxidation and slightly increased the zeta potential (△ (2)). On the other hand, poly-Glu (□) and poly-Lys (△ (1)) inhibited Fe^{3+}-NTA-dependent lipid peroxidation without changing membrane charges in DCP-EYPC and SA-EYPC liposomes, respectively, which have the same type of charges as these polypeptides have.

As mentioned above, both BSAs and polypeptides inhibited the lipid peroxidation with and without changing the membrane charge. In liposomes whose zeta potential was neutralized by them, their inhibitory effect was caused by their direct interaction with membranes, whereas in liposomes whose zeta potentials were not affected by them, their inhibitory effect was caused by their binding to Fe^{3+}-chelate and keeping it away from membranes, but not by binding to membranes. In order to prove the binding of the additives to Fe^{3+}-NTA, therefore, we chose systems in which zeta potentials of liposomes were not affected by BSAs and polypeptides, and the binding of BSAs or polypeotides to Fe^{3+}-NTA was estimated by the equilibrium dialysis experiment (see Materials and methods). The percentages binding of Fe^{3+}-NTA to the BSAs were

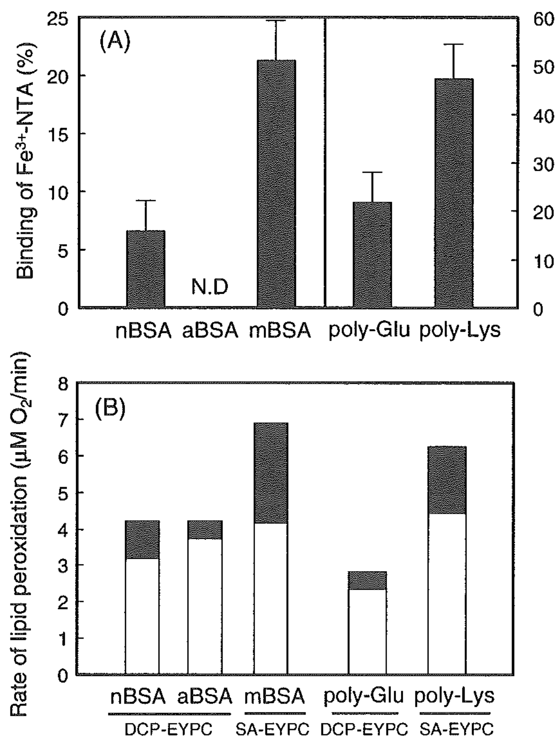

Fig. 9. Correlations between the abilities of BSAs and polypeptides to bind to Fe^{3+}-NTA and to inhibit Fe^{3+}-NTA/X-XO-induced lipid peroxidation without change of the membrane charge. Panel A: concentrations of reagents were: 3 mg BSA/ml and 90 μM Fe^{3+}-NTA for the binding of BSAs to Fe^{3+}-NTA, and poly-Lys (645 μg/ml) or poly-Glu (645 μg/ml) and 60 μM Fe^{3+}-NTA for the binding of polypeptides to Fe^{3+}-NTA. Binding percents are means±S.D. for three experiments. Panel B: (□) BSAs or polypeptides were present; (■+□) BSAs or polypeptides were absent. Values are means for two experiments. Concentrations of BSAs and polypeptides were 1.0 mg/ml and 645 μg/ml, respectively. Concentrations of EYPC, DCP, and SA were 1 mM, 0.2 mM, and 0.2 mM, respectively, in the experiments with BSAs. Concentrations of EYPC, DCP, and SA were 1 mM, 0.1 mM, and 0.1 mM, respectively, in the experiments with polypeptides. Other experimental conditions were as shown in Fig. 3.

6.6±2.4% for nBSA, 0% for aBSA, and 21.2±3.5% for mBSA, and those to poly-Glu and poly-Lys were 21.8±5.9% and 47.0±7.3%, respectively. The results are compared with their inhibiting abilities on Fe^{3+}-NTA-induced lipid peroxidation (Fig. 9). As was expected, there was a good correlation between their relative abilities to bind to Fe^{3+}-NTA (in Fig. 9(A)) and to inhibit lipid peroxidation (in Fig. 9(B)).

4. Discussion

Recently, we proposed a mechanism of peroxidation of membrane lipids exposed to $O_2^{\cdot -}$: an initiation reaction that first requires the binding of iron-chelate to the membrane and is followed by the Haber-Weiss-like reaction of iron reduction by $O_2^{\cdot -}$ with the hydroperoxyl group of preformed lipid peroxides at the membrane surface to form alkoxyl radicals, which penetrate into the hydrophobic inner region and trigger the initiation reaction [15,16].

This hypothesis is supported by the results in Fig. 1 showing that the rate of $O_2^{\cdot -}$ driven lipid peroxidation induced by Fe^{3+}-NTA, which, charged either positively and negatively, can interact with membranes of either charge, was increased with the increase of positive and negative zeta potentials of liposomes, possibly by an increase in coulombic attraction between the charged membrane and Fe^{3+}-NTA. The rates of $O_2^{\cdot -}$-driven lipid peroxidation induced by Fe^{3+}-EGTA and Fe^{3+}-EDTA, which interact with negatively and positively charged membranes, respectively, also increased with an increase of negative and positive zeta potentials, respectively. These results further support the proposal that the binding of iron chelate to the membrane surface triggered the initiation of the lipid peroxidation. In this study, we used this most-likely initiation system for membrane lipid peroxidation in biological circulation because it is most suitable to investigate the antioxidant effect of BSA from the point of view of preventing the occurrence of the Fenton-like reaction at the membrane surface.

The orders of the inhibitions by BSAs of Fe^{3+}-NTA-dependent lipid peroxidation were: (−) charged aBSA>weakly (−) charged nBSA>(+) charged mBSA in positively charged SA-EYPC liposomes, and mBSA>nBSA->aBSA in negatively charged DCP-EYPC liposomes (Fig. 1). These results indicate that BSAs inhibit lipid peroxidation more strongly in oppositely charged membranes than in those with the same charge, suggesting that the inhibition effect of BSA is due to a decrease in the availability of Fe^{3+}-NTA by lowering the membrane charge, followed by a decrease in the amount of Fe^{3+}-NTA bound to membranes. This suggestion was supported by the findings shown in Fig. 4 that BSA-concentration dependencies of the neutralization of the membrane charges, which are opposite to charges of BSAs, were well correlated to the dependencies of the decrease of the rates of Fe^{3+}-NTA-induced lipid peroxidation. The similarly good correlations observed in Fe^{3+}-EDTA- and Fe^{3+}-EGTA-induced lipid peroxidation systems (Figs. 6(A) and 7(A)) further support this suggestion.

To further investigate the inhibiting effect of BSA on lipid peroxidation from its changing effect on the zeta potentials, we compared the effect of BSAs on the rates of lipid peroxidation in two types of liposomes with the same zeta potentials: one was liposomes whose zeta potential was neutralized by the addition of opposite charged BSA, and the other is the liposomes, zeta potential of which was neutralized by the subtraction of charged substances from the membrane. At the same zeta potential, the rates of lipid peroxidation induced by Fe^{3+}-EGTA and Fe^{3+}-EDTA were always higher in the system to which BSAs were added than in the system in which membranous charge substances were reduced (Figs. 6(B) and 7(B)). Similar results were observed when polypeptides such as poly-Glu and poly-Lys were used instead of BSAs; Fig. 8 (A) shows that the rates of lipid peroxidation were always higher in the presence of poly-Glu in SA-EYPC liposomes and poly-Lys in DCP-EYPC liposomes than in their absence in liposomes with the same zeta potential. These results would be explained as follows: that Fe^{3+}-chelate is more available in systems to which BSAs and polypeptides were added than in systems in which membranous charge substances were reduced, possibly because the number of membranous charge molecules available for binding with Fe^{3+}-chelate was larger in the former system, even though the zeta potential of both systems was the same. In cases that the membrane charge was reversed by the addition of BSA, inhibition of lipid peroxidation by BSA as shown in Fig. 6(B) (○ (3) and ○ (4)), as examples, can be explained as follows. The decreased rates of Fe^{3+}-EGTA-dependent lipid peroxidation due to the addition of mBSA at a high concentration to the system of DCP-EYPC liposomes, charges of which became net positive by binding a large amount of positively charged mBSA, might be due to decreased availability of Fe^{3+}-EGTA, because only a small amount of residual positively charged Fe^{3+}-EGTA interacted with the negatively charged membranous DCP left from interaction with mBSA. A similar mechanism would also be applicable to the Fe^{3+}-EDTA-dependent lipid peroxidation decreased by the addition of aBSA in SA-EYPC liposomes (□ (3), □ (4) in Fig. 7(B)). Based on this proposal, the results shown in Fig. 5(C) (○ (2) and ○ (3)) that the rates of Fe^{3+}-NTA-dependent lipid peroxidation by the addition of mBSA at high concentrations to the system of DCP-EYPC liposomes, the apparent charge of which becomes positive, were lower than the rates in the positively charged SA-EYPC liposome system without mBSA (dotted line in Fig. 5(C)) at same zeta potential can be explained as follows. In the mBSA-added system, a large amount of negatively charged DCP is occupied by positively charged mBSA, resulting in a large increase of the amount of silent Fe^{3+}-NTA, which is unable to bind with DCP, for the initiation of lipid peroxidation. Accord-

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.

Acknowledgement

We thank Dr. M. Anraku (Kumamoto University) for technical assistance for measurement of the net charge of the alubumins by determining their migration times in capillary electrophoresis.

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

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Received 30 April 2004; received in revised form 23 September 2004; accepted 27 September 2004
Available online 12 October 2004

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.
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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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doi:10.1016/j.bbapap.2004.09.025

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, [θ], 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

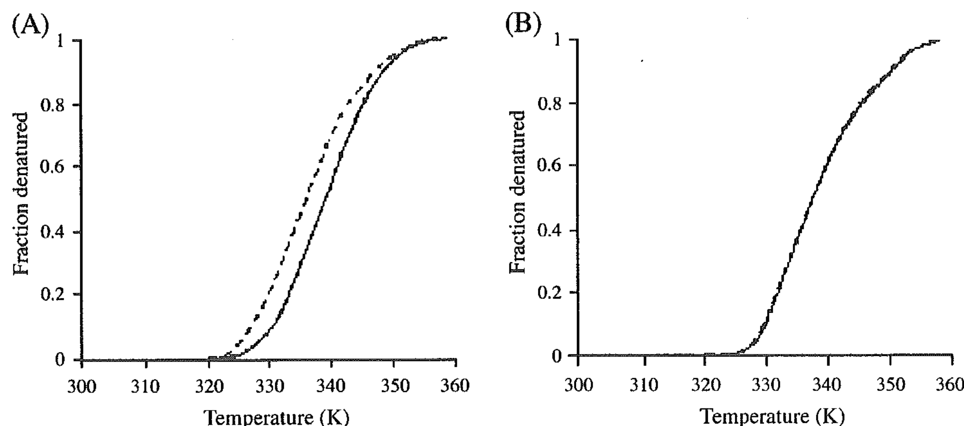

Fig. 1. Thermal denaturation of alloalbumins and their normal, endogenous counterparts. (A) Alb Herborn and (B) Alb Brest; the broken and full curves represent variant and corresponding Alb A, respectively. The curves are averages for three experiments.

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

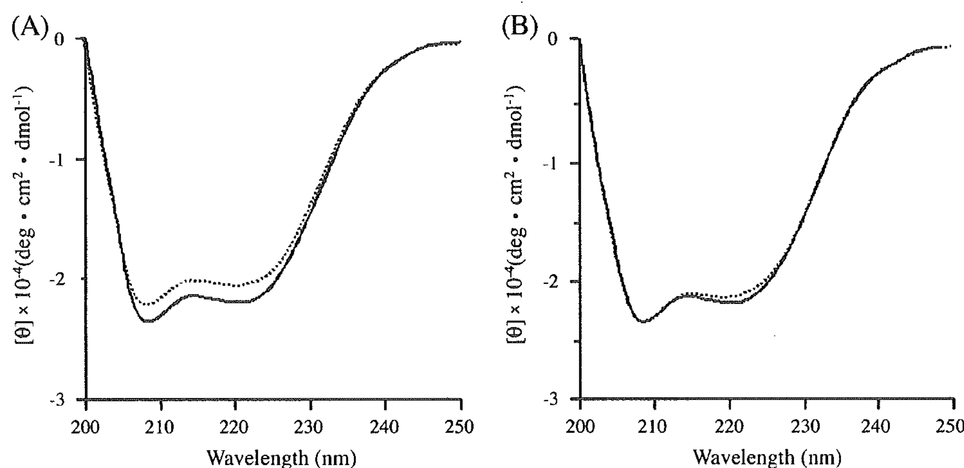

Fig. 2. Far-UV CD spectra of alloalbumins and their normal, endogenous counterparts. (A) Alb Herborn and (B) Alb Brest; the dotted and full curves represent variant and corresponding Alb A, respectively. The curves are averages for three experiments.

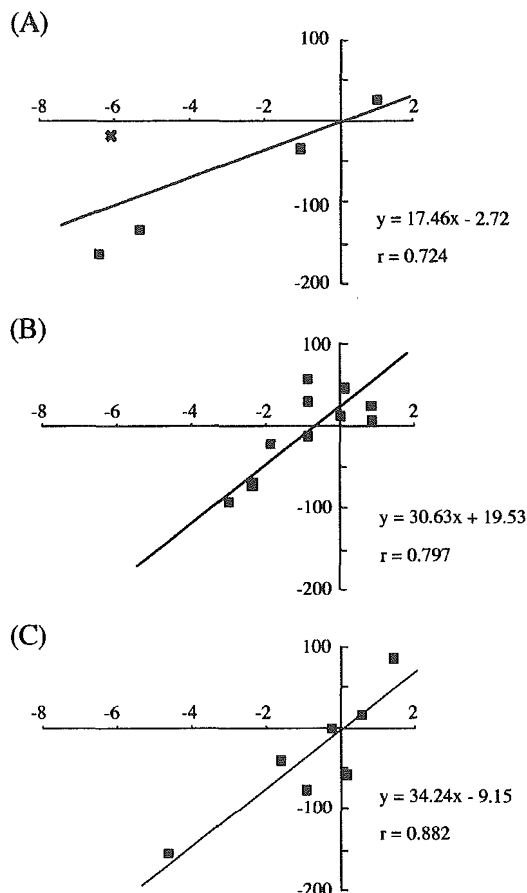

Fig. 3. Abscissas: Changes in percentages of the α-helical content of HSA caused by single amino acid substitutions. Ordinates: Corresponding $\Delta(\Delta H_v)$ given as kJ/mol, taken from Table 1. Results are shown for domain I (A), domain II (B) and domain III (C). In all three cases, the relation 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.

Acknowledgements

We are grateful to the persons mentioned in Materials and methods for the generous gifts of genetic variants of human serum albumin and proalbumin. This work was supported in part by Aarhus University Research Foundation and by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (14370759).

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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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論文受付 2004年12月28日 論文受理 2005年2月7日

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．

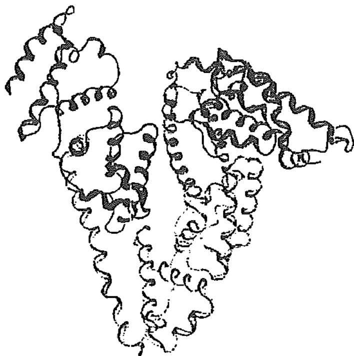

Fig. 1. Crystal structure of human serum albumin.（HSA）（from Ref. 1）

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），シスチ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]．

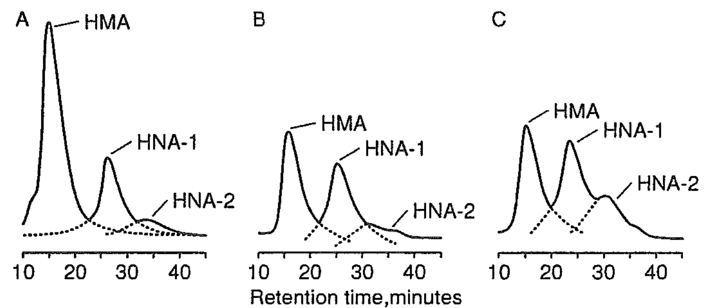

Fig. 2. High-performance liquid chromatography (HPLC) profile of serum albumin from hemodialysis patients (HD) patients with or without intravenous iron administration (IVIR). 5 μL aliquots of serum from HD patients with or without IVIR, and healthy subjects were subjected to HPLC analysis using a Shodex 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

るMetがグルタミン合成酵素の活性サイトの入り口に位置していることに注目し，活性サイトにおいて機能発現や構造維持に重要なアミノ酸残基をラジカルによるダメージから守るスカベンジャーとしてMet残基が機能している可能性を示している[11]．好都合なことには，Metの酸化体であるメチオニンスルフォキシド（MSOX）は細胞内に存在するMSOX還元酵素により元のMetへ変換される[12]．したがって，Metは細胞内ではこのサイクルにより半永久的に抗酸化剤として働くことも可能である．

③サイトⅠに位置する残基（[199]Lys，[214]Trp，[218]Arg，[242]His）

サイトⅠへは，ワルファリン，フェニトイン，フロセミド及びグリベンクラミドをはじめとする多くの薬物が結合する．このサイトⅠは一次配列における[199]Lys～[292]Gluに存在しており，[214]Trpなどの疎水性アミノ酸残基からなる疎水領域に[242]Hisおよび[199]Lysといった陽電荷が局在している．事実，作製したHSA変異体を用いた検討結果から，[199]Lys，[214]Trp，[242]Hisのいずれもが薬物結合に深く係りわり合っていることを確認している[13]．また，[214]Trpは，HSA分子上，中心に位置することから，構造維持に必須なアミノ酸残基として機能している．加えて，Bhagavanらは，[214]Trp→Ala変異体を作製し，抗酸化能が約70%に低下したことから，[214]Trpを抗酸化アミノ酸残基として定義している[5]．最近，Trpの結合特性を活用してHSA製剤に大量に添加されているN-Acetyl-Trpが，抗酸化剤として機能していることが報告された[14]．すなわち，[214]Trpは，HSAの構造と機能を担う必須なアミノ酸残基として役割を果たしていると考えられる．また，家族性高チロキシン血症（FDH）はチロキシンの血中濃度が通常よりも著しく高い疾患であるが，この原因として[218]ArgのHisへの変異が明らかにされている．事実，Bhagavanらは[218]ArgのHisへの変異体を用いてチロキシンの結合性が約65倍増加することを見出している[15]．

④サイトⅡに位置する残基（[410]Arg，[411]Tyr）

著者らはケトプロフェンを用いた光親和性ラベル化法に加えて，部位特異的変異法を用いた実験結果から，サイトⅡに対する薬物結合には[410]Argのグアニジノ基に加え，[411]Tyrの水酸基とその芳香環も重要な役割を果たしていることを明らかにしている[16]．さらに，このサイトへは経腸栄養剤や脂肪乳剤に含まれる中鎖脂肪酸も強く結合しHSAの構造安定性を向上させることが知られている．この特性を活用してHSA製剤にはC_8のカプリル酸が安定化剤として大量に添加されている[14]．サイトⅡは上述した薬物結合能に加え，エステラーゼ様作用を有する．著者らは[410]Arg→Ala変異体，[411]Tyr→Ala変異体あるいは両者の二重変異体を用いた実験結果から，この酵素反応の活性中心が[411]Tyrの水酸基であることとともに，[410]Argがその反応性を高めていることを見出している[17]．また，菊川らは，*in vitro*においてウシ血清アルブミン（BSA）を過酸化水素により処理後，血中セリンプロテアーゼである酸化蛋白質分解酵素により，この酸化BSAを処理したところ，BSAの[409]Arg-[410]Tyr-[411]Thr近傍を特異的に切断することを報告している[18]．この結果を受けて，著者らは，BSAの切断部位に相当するHSAの[410]Arg→Ala，[411]Tyr→Ala変異体を用い，その体内動態をwild-typeと比較検討した結果，[410]Argの変異体においてのみ素早い消失を確認している．すなわち，変異による[410]Arg近傍のコンフォメーション変化が血中の酵素により切断された結果，消失の促進を引き起こしている可能性が考えられる．

2．遺伝子組換え型HSAを利用した医薬への応用

従来，HSAは生体分解性や血中滞留性に富んでいるため，動態特性上問題がある薬物のDDSにおいて好ましい担体として用いられてきた．高分子医薬に対し，アルブミンを担体として付与する場合，アルブミンが有するリガンド結合能を利用した非共有結合，あるいは両者を化学修飾によるいずれかの方法が用いられてきた．しかしながら，化学結合の場合，均一な結合体の調製が難しい上，両成分の生理活性や特性を保持させるためには，適切な架橋条件を見出す必要があるなどの問題点が指摘されている．最近，このような問題点を克服するため，アルブミンと蛋白性医薬品の遺伝子を融合させたfusion-proteinの開発が盛んに行われている．さらに，臓器指向性や，主薬の安定化向上を狙ったHSA自身のサイズ調節をはじめ，新規蛋白医薬品としてのアルブミンの医療への応用も試みられている．

1）チオール基（[34]Cys）を利用した医薬への応用

①一酸化窒素との相互作用

一酸化窒素（NO）やカプトプリル，ブシラミンといったSH含有薬物は[34]Cysに共有結合する．特に，血管弛緩因子であるNOは血液中でS-ニトロソ蛋白として運搬され，その8割がS-ニトロソHSAとして存在している[19,20]．S-ニトロソHSAになると，血管平滑筋を弛緩させたり，血小板凝集を抑制したり，アデニール・サイクレースを活性化させたりする．一酸化窒素（NO）が新しい生理活性物質として認識され，生体内NO結合メディエーター蛋白として，ヘモグロビン（Hb）やHSAがその対象として研究されている．虚血性疾患や臓器移植時などの病態時では，内因性のNO産生低下に伴い外因的なNOの補充が必要不可欠であることから，比較的長寿命で安定な新規NO補充療法の開発が望まれている．著者らは，NO導入効率改善のため，Cysを付加した[410]Arg→Cys変異体を作製し，これにNOを付加した新規S-ニトロソタンパクを作製した．そのNO導入効率は，wild-type-HSAの約4倍まで上昇し，その物性，生物活性及び体内動態特性において，いずれもwile-typeに比べて，S-ニトロソタンパクとして，より優れていることを明らかにしている[21]．今後，Cysを付加した組換え型S-NO-アルブミンの新規NO抱合運搬タンパク（NOトラフィックタンパク）としての応用開発の可能性が大いに期待される．

②[34]Cys架橋による酸素輸液への応用

血液型のない，感染の危険もない輸血に代わる新技術として，人工酸素運搬体の開発が盛んに行われている．土田らは，高濃度酸素輸液の調製を目指して，rHSAに疎水性の合成ヘム

“lipidheme”（ポルフィリン誘導体：Lh）を効率よく包摂させたアルブミン-ヘム複合体（rHSA-Lh）を作製している．このアルブミン-ヘムは自然界に存在しない全く新しい合成ヘム-蛋白質であるが，Hbと同様，酸素分圧に応じて酸素分子を結合解離することが可能であり，HSA1分子あたり８分子のLhを結合できることから，高濃度酸素輸液としての臨床応用が進められている．さらに，最近，rHSA二量体（$rHSA_2$）を^{34}Cysの架橋形成により作製し，二量体による最大16分子のLhが結合可能となっている．また，この$rHSA_2$におけるラットの体内動態特性を評価したところ，rHSAの約1.5倍の半減期を示し（Fig. 3.），更なる高濃度酸素輸液として開発が進められている[22]．

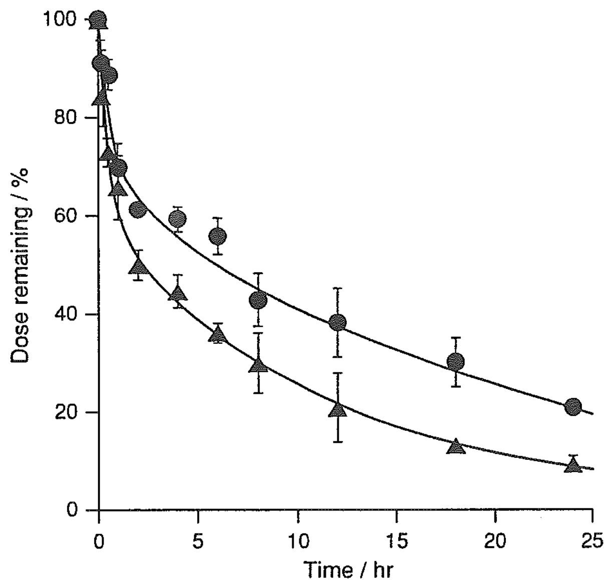

Fig. 3. Plasma levels of ^{125}I-rHSA monomer (△) and dimmer (○) (1.0× 10^{7} cpm, 1.0 mg kg^{-1}) after intracardial administration into Wistar rats. All values are mean ± S.D. (n=3). (From Ref. 22)

③^{34}Cys架橋による効率的薬物輸送

麻薬性鎮痛薬であるDynorphin Aは，その早い消失のため，頻回投与による副作用が問題となっている．しかし，最近，Holmesらは，^{34}CysとDynorphin AをDrug Affinity Complex（DAC）システムにより１対１の割合で化学的に結合させ，血中滞留性の向上とともに，肝臓，腎臓や脳などへの組織移行を制限に伴う副作用の軽減に成功している[23]．現在，ConjuChem社において，第一相臨床試験が行われている．

２）HSAの血中滞留性を利用した薬物動態制御システム

①長期作用型インスリン

インスリンは糖尿病治療薬として汎用されているが，その糸球体濾過に伴う消失の促進による頻回投与が問題となっている．Markussenらは，インスリンの^{29}Lysを長鎖脂肪酸の一つであるミリスチン酸で修飾し，血液中でHSAと結合可能なアシル化インスリンを設計している[24]．HSAとの結合はアシル化インスリンの腎臓や肝臓からの消失を抑制し，血中滞留性を改善することから長時間型インスリンへの応用が期待されている．

②長期作用型新規リウマチ性治療薬

Fiehnらは，リウマチ様関節炎の新しい治療薬へのアプローチとしてアルブミンのLys残基をN, N'-dicyclohexyl-carbodiimide（DCC）及びN-hydroxysuccinimide（HSI）により架橋したメトトレキサート（ＭＴＸ）-アルブミン複合体を設計した[25]．従来，MTXは，その低分子量により，糸球体濾過に伴うすばやい消失のため，頻回投与による副作用の発現が問題となっていた．しかし，MTX-HSA複合体の開発に伴い，糸球体濾過の抑制に伴う半減期は，約15倍に上昇すると共に，浮腫部位への特異的蓄積が認められ，副作用の軽減にもつながることから，リウマチ様関節炎の新しい治療薬として臨床試験がスタートしている．

３）アルブミン融合技術によるコントロールドリリース

①生理活性ペプチドへの応用

米国ヒューマンゲノムサイエンス社はアルブミン融合技術をサイトカインに応用することに成功している．インターフェロン-HSA接合体；Albuferonは，血中におけるインターフェロンの貯蔵庫として機能するため，C型肝炎の治療に際し，副作用の軽減や投与回数の減少により，患者のQOL向上が期待されている[26]．さらに同社では，インターロイキンⅡにおいても同様にインターロイキンⅡ-HSA接合体；Albuleukinが開発されている．Albuleukinは，現在，第一相臨床試験が実施されている．Albuleukinの半減期は，インターロイキンⅡの約40倍長く，従来の投与設計を考えると，Albuleukinは，より簡易な投与設計を提供し，患者の負担軽減に貢献できる可能性を秘めている[27]．

②新規抗癌剤への応用

現在，新規抗癌剤として，血管新生阻害剤アンギオスタチンが注目されている．通常，固形癌は，その伸展・転移にあたり新生毛細血管を必要とするため，種々の血管新生促進因子を産生しているので，その抑制は癌を“兵糧攻め”することになる．また血管新生は正常細胞ではほとんど認められないので，血管新生阻害剤では重篤な副作用が起こりにくいと期待される．Bouquetらは，遺伝子融合技術を用いて，アンギオスタチンとHSAの接合体（Adk3-HSA）の開発を行い，Adk3-HSAの著明な半減期の延長と有意な腫瘍増殖阻害効果を得ている（Fig. 4.）[28]．

４）高機能性アルブミンの設計と評価

①ドメインⅠ，Ⅱ，Ⅲ

前述したようにHSAの基本構造は相同性の高い３つのドメインから構成されているが，蛋白質の機能解析を行う上でドメイン間の相互作用及び各ドメイン自身の機能解析は必要不可欠である．Carterらは，最近，各ドメインを単独に発現させる系を構築・精製し，相同性の高い３つのドメインの構造を明らかにすることにより，ドメイン毎の高い保存性を証明した．著者らは最近，各ドメインを単独に発現させる系を改良し，ドメイン

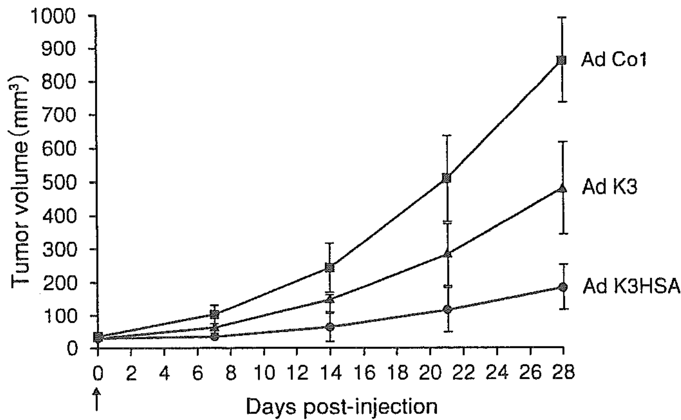

Fig. 4. Systemic treatment of MDA-MB-231 mammary tumors by AdK3-HSA. MDA-MB-231 carcinoma cells were sc implanted into athymic nude mice. 5 × 10^9 pfu of adenovirus AdK3-HSA (n = 12), AdK3 (n = 10), or AdCO1 (n = 8) was iv injected when the tumors had reached a mean volume of 30 ± 8 mm^3 (day 0). Data represent the tumor volume (mean ± SD) for each group. A Student t test was performed for statistical analysis ($P < 0.005$). (From Ref. 28)

毎の構造の安定化を立証するとともに，薬物結合サイトに関して，サイトⅠの構造及びミクロ環境保持には，局在しているドメインⅡだけでなく，隣接している他のドメインとの相互作用の重要性を明らかにした．また，サイトⅡは大部分がドメインⅢに位置し，ドメインⅢに保持されていることを明らかにした．加えて，ドメインⅠはドメインⅡ，Ⅲと比べ，抗酸化活性をよく保持していることが示された（Fig. 5.）[29]．前述したように，ドメインⅠの抗酸化能には34位のfreeのCys残基に加え，Met残基が影響を与えている可能性が考えられている．

②アルブミンダイマー（$rHSA_2$）

従来，アルブミンのダイマー化では，架橋剤を利用した化学的結合のため，均一な結合体の調製の難しさなど多くの問題を抱えていた．最近，W.P.Sheffieldらにより，遺伝子技術を用いた均一なラビット二量体化アルブミンが精製された．しかし，その半減期は，予想に反しwild-typeに比べ短縮していた[30]．この原因として，均一な二量体は発現しているものの，発現・精製過程におけるアルブミン自身の構造変化が惹起され，消失を促進したものと推察される．事実，著者らも同様に二量体化HSAの発現・精製を行った結果，構造及び機能特性においてHSA-monomerと同様であることを確認した．またその体内動態は，HSA-monomerに比べ，有意に延長していた．これらの知見から，二量体化HSAの生体内挙動は，分子サイズに加えて，その構造安定性により大きく左右されるかもしれない．

5）高機能性アルブミンを利用した血液透析への応用

HSAの高い結合率を利用した医薬への応用の一つにアルブミン透析（ECAD; Extra Corporeal Albumin Dialysis）がある．肝不全は種々の毒素の体内蓄積により他の臓器を障害し，しば

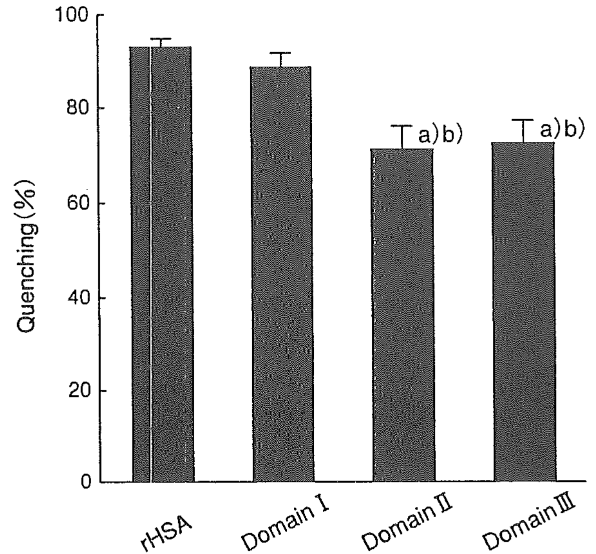

Fig. 5. Quenching of H_2O_2 oxidation of dihydrorhodamine (DRD) by rHSA and individual domains. The sample solutions contained 7.5 μM rHSA or individual domains in 67 mM sodium phosphate buffer (pH 7.4 and 25° C), 5 μM DRD, and 25 mM H_2O_2. Each bar represents the mean ± SD (n = 3). a) $p < 0.01$ vs. rHSA; b) $p < 0.01$ vs. domain I. (From Ref. 29)

しば多臓器不全に進展する．治療として血液濾過透析などの人工肝機能補助療法が行われているが，アルブミンに結合した毒素は除去できず血漿交換との併用が必要である．そこで，ECAD療法により，水溶性毒素とともにアルブミン結合毒素を選択的に除去するシステムである．6時間の治療で血清胆汁酸，ビリルビンが有意に低下することに加え，血漿Nox濃度も有意に低下することから，毒素の除去に伴う血管系，脳，腎及び肝機能の改善に伴う余命の延長が報告されている（Fig. 6.）[31,32]．

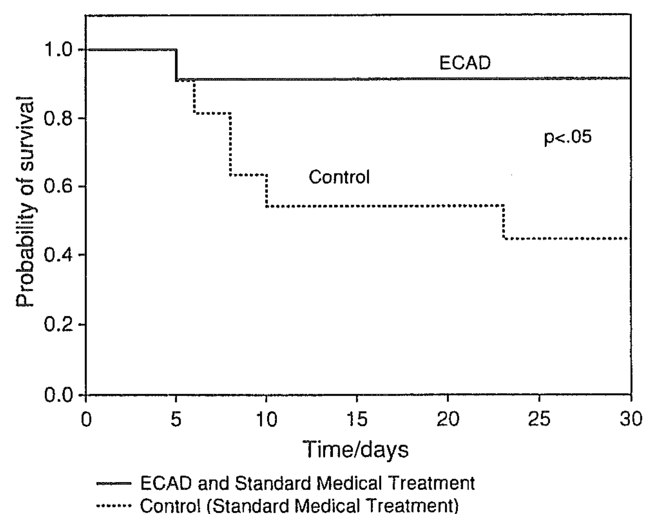

Fig. 6. Kaplan-Meier plot showing 30-day mortality. There was a significant difference between the extracorporeal albumin dialysis and control group ($P < .05$). (From Ref. 32)

このECAD療法に際し，遺伝子組換え型アルブミン，特に高結合能アルブミンの開発は，毒素を結合させるECAD療法において，有用な変異体として利用されうるかもしれない．

おわりに

遺伝子組換え技術により，作製されたHSA変異体により機能発現に必須なアミノ酸残基及びその発現部位が明らかにされてきた．また，機能によって，その発現部位の範囲やドメイン間相互作用の関与に大きな違いが認められた．今後，遺伝子組換え技術により，各ドメインを組み合わせたり，HSAの単量体や多量体分子と接合させることにより，医薬への応用を目指した高機能性組換え型HSAの設計が可能になるかもしれない．

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bjh research paper

Platelet glycoprotein Ib alpha polymorphisms affect the interaction with von Willebrand factor under flow conditions

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Received 22 October 2004; accepted for publication 29 November 2004
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Summary

Interaction of platelet glycoprotein (GP) Ibα with von Willebrand factor (VWF) is essential for thrombus formation, particularly under high shear conditions. Previous case–control studies indicated that two GPIbα polymorphisms, ^{145}Thr/Met and/or variable number (1–4) tandem repeats of 13 amino-acid sequences, are associated with arterial thrombosis. The ^{145}Met-allele and the 3R- or 4R-allele is associated with increased risk. However, there is little clear experimental data to support this association. To elucidate the functional effects of these polymorphisms, we prepared recombinant GPIbα fragments and tested them *in vitro*. The dissociation constants of ristocetin-induced ^{125}I-labelled VWF binding to two forms of soluble recombinant GPIbα [^{1}His–^{302}Ala, either ^{145}Thr (145T) or ^{145}Met (145M)] were not different. Four types of Chinese hamster ovary cells expressing full-length GPIbαβ/IX, 145T with one repeat (T1R), 145M with one repeat (M1R), 145T with four repeats (T4R), and 145M with four repeats (M4R), were prepared, and cell interactions with immobilized-VWF were examined under various shear conditions. The cell rolling velocity of M4R under a shear condition of 114/s was significantly slower than that of T1R. Intermediate values were obtained with M1R and T4R. The results suggest that M4R interacts more strongly with VWF under flow conditions.

Keywords: polymorphisms, mutagenesis, platelets, glycoprotein Ib alpha, von Willebrand factor.

Glycoprotein (GP) Ib-IX-V complex is a platelet membrane receptor for von Willebrand factor (VWF) (Lopez, 1994; Clemetson, 1997; Ware, 1998; Andrews *et al*, 2003). This receptor consists of four subunits, GP Ibα, Ibβ, IX, and V. The largest subunit of the complex, GPIbα, has a VWF-binding site within the N-terminal 45-kDa extracytoplasmic domain of approximately 300 amino acids (Titani *et al*, 1987; Huizinga *et al*, 2002; Uff *et al*, 2002). Interaction of GPIbα with VWF mediates high shear-stress-dependent platelet activation, which is a critical step for thrombus formation (Ikeda *et al*, 1997; Dopheide *et al*, 2001; Ruggeri, 2003). The VWF/GPIbα interaction is not observed under static conditions *in vitro*, but only under shear conditions. Assessment under static conditions requires the presence of non-physiologic inducers, such as ristocetin or botrocetin.

In previous case–control studies, two genetic polymorphisms within the coding region of GPIbα were reportedly associated with arterial thrombosis, such as coronary artery disease and stroke (Hato *et al*, 1997; Murata *et al*, 1997; Sonoda *et al*, 2000; Simmonds *et al*, 2001; Yamada *et al*, 2002; Afshar-Kharghan *et al*, 2004). The first polymorphism is an amino acid dimorphism, Thr/Met, at residue 145 (Murata *et al*, 1992). The second polymorphism is a variable number tandem repeat [1–4 repeats (1R–4R)] of the 13-amino acid sequence, residues 399–411 (VNTR polymorphism) (Moroi *et al*, 1984; Ishida *et al*, 1991; Simsek *et al*, 1994). These two polymorphisms are in linkage disequilibrium (Ishida *et al*, 1991; Simsek *et al*, 1994). The ^{145}Met-allele, which is tightly linked to the 3R- or 4R-allele, is associated with increased risk. There is a race difference in the genotype distribution of the VNTR polymorphism; although 3R is observed in Caucasians, African-Americans (Afshar-Kharghan *et al*, 2004), Japanese, and Koreans (Ishida *et al*, 1996), 4R is observed in Japanese and Koreans (Ishida *et al*, 1996). Epidemiologic data indicate