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ORIGINAL CONTRIBUTION

A comparative study on specific and nonspecific interactions in bovine serum albumin: thermal and volume effects of halothane and palmitic acid

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Abstract Interaction modes of halothane and palmitic acid with bovine serum albumin (BSA) were studied from the thermal and volumetric viewpoints. The thermal stability of BSA was increased by increasing both ligand concentrations. However, the stronger effect of palmitic acid than halothane on BSA was observed at lower concentrations irrespective of the pH-dependent BSA structure. On the other hand, the volume of BSA in the solution shrunk by adding halothane independent of its structure while it expanded by adding palmitic acid. The molar ratio of halothane to BSA at the effective concentration was not consistent with the binding numbers on human serum albumin determined from the X-ray analysis, whereas that of palmitic acid was in good agreement with the numbers. We judged from these facts that halothane is a nonspecific binder to BSA; by contrast, palmitic acid is a specific binder. The stabilization mechanisms of the BSA structure were also revealed.

Keywords Bovine serum albumin · Differential scanning calorimetry · Inhalation anesthetic · Long-chain fatty acid · Specific and nonspecific interactions · Viscosity

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Introduction

Anesthetics affect biological membranes and induce the action. Various kinds of anesthetic theories have been proposed up to now. Nevertheless, there is no unified anesthetic theory that can explain the anesthetic action completely. In recent anesthetic mechanisms, there is a repeated controversy between two theories. One theory is a protein receptor theory, which explains that anesthesia is caused by the specific binding of anesthetic molecules to several signal receptor proteins in the membrane such as a sodium channel [1, 2]. The other one is a lipid membrane theory, which insists that nonspecific binding of the anesthetic molecules to nerve membranes with high content of lipids changes the membrane properties as fluidity and state of phase, and then the secondary effect on the membrane proteins causes anesthesia [3–5]. The main concern for recent anesthetic studies is whether the anesthetic action is based on specific or nonspecific interaction.

The effect of an anesthetic on membrane-constituting substances such as proteins and lipid membranes has been

examined by use of their model systems. An idea obtained from these studies that anesthetics reduce the stability of their structures, namely the promotion of unfolding of proteins and fluidization of membranes, found general acceptance. However, in a recent report by Tanner et al. [6, 7], an opposite effect by an inhalation anesthetic halothane on a model protein, bovine serum albumin (BSA), has been found and attracted a great deal of attention. They showed that the thermal denaturation temperature of BSA increased by adding halothane. This finding corresponds to the enhancement of thermal stability by the folding of BSA and is not consistent with the previous point of view. They also pointed out that BSA has binding sites for the halothane molecules, and the binding of them on BSA is responsible for the thermal enhancement of the structure. On the other hand, Ueda and Suzuki [8, 9] have reported at the same period that an interaction mode of long-chain fatty acid like myristic or palmitic acid with another model protein, firefly luciferase, is significantly different from that of inhalation anesthetic with luciferase. The anesthetic does not compete with a substrate luciferin, destabilizes the structure of luciferase, and increases the volume of luciferase [10], whereas the fatty acid affects luciferase in the opposite direction. Nishimoto et al. [11] have found that there exists a similar difference in interaction modes between long-chain fatty acid and inhalation anesthetic in dipalmitoylphosphatidylcholine bilayer membranes. The above observations suggest that interaction modes between a ligand and bio-macromolecules are different depending on the kinds of both molecules, and there seems to appear either specific or nonspecific interaction, or both interactions. In order to characterize the anesthetic action more obviously, it is advantageous to show essential difference in both specific and nonspecific interactions from a comparative study of interaction modes between a ligand and bio-macromolecules. For this purpose, we chose BSA as a model protein for anesthetic and two kinds of small ligands, an inhalation anesthetic halothane and a palmitic acid in long-chain fatty acids.

Serum albumin is a protein contained greatly in blood plasma. BSA and human serum albumin (HSA) have been used as model proteins for anesthesia because the reagents with high purity are abundantly available in low price, and the binding data of anesthetics to serum albumin by physico-chemical methods, UV spectroscopy [12, 13], fluorescence spectroscopy [14–16, 20], photoaffinity labeling [17–20], hydrogen exchange [19, 20, 24, 26], ^{19}F-NMR [21–23], zonal elution chromatography [24–26], differential scanning calorimetry [6, 7], isothermal titration calorimetry [26–28], densitometry [29, 30], and viscometry [31], have been accumulated. Regarding HSA, the detailed structure and the binding sites of several small ligands have been determined from the X-ray structural analysis [32, 33]. On the other hand, it is well known that in BSA takes place structural changes depending on the solution pH. Foster and Aoki have clarified from the method of electrophoresis in the 1960s [34–36] that BSA has different forms at various pH, which are classified into expanded (E) and fast (F) forms in the acidic pH region, a normal (N) form in the neutral pH region, and basic (B) and aged (A) forms in the alkaline pH region. The physical quantities of BSA definitely change between two forms at certain pH, which is called the structural transition of BSA. The F and B forms are different in α-helix and β-sheet contents, etc., compared with the N form which is the native form of BSA [37]. Hence, we can investigate these states of the different structure in one protein by using BSA.

The solution properties of aqueous protein solutions reflect the structure of proteins in the solution, and the structural changes by adding a ligand produce sensitive change in the solution properties. Because the thermal behavior of protein solutions has a close connection with the volume behavior, it is expected that the examination of both the behavior complementarily gives us useful information regarding the interaction modes between a ligand and bio-macromolecules. In the present study, we investigate the effects of halothane and palmitic acid on BSA by differential scanning calorimetry (DSC) and viscosity measurements of aqueous BSA solutions in the absence and presence of the ligands. The difference in interaction modes of both ligands for BSA is discussed from the thermal and volume changes of BSA induced by the ligands.

Experimental sections

Materials and sample preparation

Essentially globulin and fatty acid free bovine serum albumin (product A0281, lot# 053K7476) and palmitic acid (1-hexadecanoic acid, C15:0-COOH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Halothane (2-bromo-2-chloro-1,1,1-trifuoroethane, $CF_3CHClBr$) was obtained from Takeda Pharmaceutical Co. (Tokyo, Japan). They were directly used without further purification. Methanol was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Phosphate buffer solutions were used as a solvent in the neutral pH region (5.0–8.3) and citrate buffer solutions were used in the acidic pH region (2.5–5.0). Water was distilled twice after deionized treatment, where the second step was done from dilute alkaline permanganate solution.

Powder of BSA was dissolved in 0.1 mol kg^{-1} buffer solutions at various pH. Palmitic acid dissolved in a minimum amount of methanol (ca. 160 mmol kg^{-1}

(0.51%) for DSC measurements and ca. 110 mmol kg^{-1} (0.35%) for viscosity measurements) or liquid halothane was added in the solution in a test tube, and the solution was gently stirred by a vortex mixer until no droplet of halothane was visible or the solution was completely homogeneous. The prepared solutions were further stirred slowly in the tube under no dead volume condition on a magnetic stirrer for an hour at room temperature and were kept at about 5 °C for more than an hour in a refrigerator.

Differential scanning calorimetry

DSC measurements were carried out by a Microcal MCS high-sensitivity calorimeter (Northampton, MA, USA). After sample and reference cells were filled up with respective solutions of 1.5 ml and degassed for 10–15 min, they were pressurized by about 1.2 atm of nitrogen gas. And then, the measurements started from 20 to 95 °C with a constant heating rate. Since the denaturation temperatures of proteins are known to depend on the protein concentration and heating rate in the measurements [9], we adopted the BSA concentration of 2.0 mg ml^{-1} and the rate of 0.5 °C min^{-1} for all DSC measurements. We confirmed in the control measurement that the addition of the same portion of methanol in the absence of a palmitic acid does not affect the thermal phase transition of BSA on the DSC thermogram at all. The obtained thermograms were analyzed by the software Origin 7.0J (Lightstone Co., Tokyo, Japan), and thermal quantities such as transition temperatures and enthalpies were determined.

Viscosity measurements

The viscosities of aqueous BSA solutions were measured by a viscosity measurement apparatus by use of an Ostwald viscometer, which was developed in our laboratory to measure the accurate viscosity values of aqueous solutions rapidly and automatically [38]. The viscometers with efflux time of around 120 and 360 s for pure water were used. The measurements were performed after sample solutions of 10 ml were injected into the viscometer and allowed to stand for 20 min. The viscosities of a buffer solution and BSA solutions in the absence and presence of ligands were measured consecutively in each experiment. Temperature of the measurements was kept constant at 25 °C by a thermostated water bath controlled to ±0.01 K by a thermistor digital controller (Yamashita Giken Co., Ltd., Tokushima, Japan). Viscosity values of the solutions were averaged at least six times in each experiment.

Various viscometric quantities were calculated from the measurement data. Relative viscosity (η_{rel}), which is defined as the ratio of a viscosity of sample solution to that of solvent solution, can be obtained from the following equation

$$\eta_{rel} = \eta/\eta_0 = \rho t/\rho_0 t_0 \approx t/t_0 \quad (1)$$

where η, ρ, and t are the viscosity, density, and efflux time in the viscometer of a sample solution, respectively, and the subscript 0 refers to the value of a reference (i.e., buffer) solution. Here, we assumed that the density values of sample solutions were approximately equal to that of a reference solution because of low concentrations of BSA and ligands. Specific viscosity (η_{sp}) and reduced viscosity (η_{red}) are, respectively, defined as

$$\eta_{sp} = (\eta - \eta_0)/\eta_0 = \eta_{rel} - 1 \quad (2)$$

$$\eta_{red} = \eta_{sp}/C \quad (3)$$

where C is the concentration of BSA in the unit of g dl^{-1}. Further, the extrapolation of the η_{red} value to the zero concentration gives that of inherent viscosity ($[\eta]$),

$$[\eta] = \lim\left(\eta_{sp}/C\right)(C \text{ approaches 0 concentration}) \quad (4)$$

Results and discussion

Effects of ligands on the thermal denaturation temperature of BSA

The DSC thermograms of the BSA solutions in the absence and presence of halothane at pH 7.0 are demonstrated in Fig. 1a. The thermogram of the BSA solution in the absence of halothane exhibited one endothermic peak at about 59 °C, which corresponds to the thermal denaturation of BSA and was in good agreement with the temperatures reported previously [39, 40]. In the presence of halothane, the effect appeared by adding halothane of millimolal concentration range, and the peak shifted to the higher temperature region and grew in a dose-dependent manner. This thermal behavior of BSA induced by halothane was well consistent with that reported by Tanner et al. [6, 7]. The corresponding DSC thermograms for palmitic acid are presented in Fig. 1b. In the case of palmitic acid, a similar peak shift to the higher temperature region was also observed; however, the interaction mode was significantly different from that for halothane. The temperature elevation occurred by adding palmitic acid of micromolal concentration range. Addition of 10 μmol kg^{-1} palmitic acid made the peak shift slightly. With increasing palmitic acid concentration, bimodal thermal behavior was observed: the peak observed in the vicinity of 60 °C decreased and another peak appeared in the higher temperature region. At

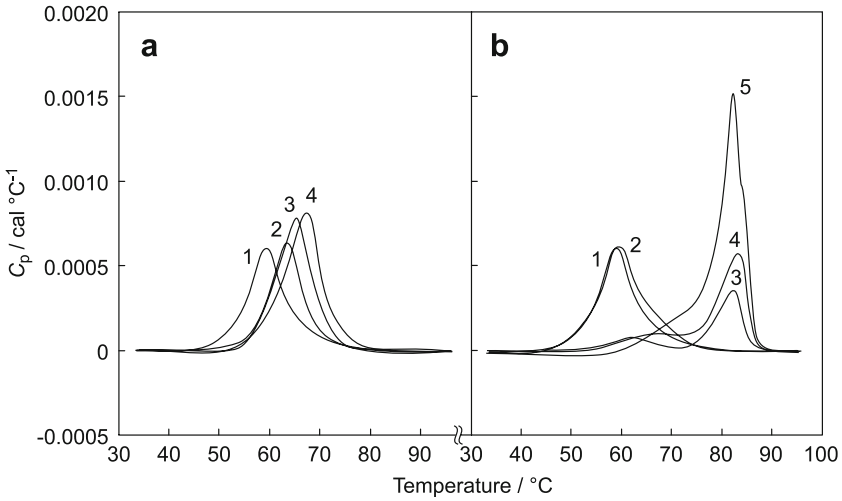

Fig. 1 **a** DSC thermograms of the BSA solution at pH 7.0 in the absence and presence of halothane: *1* 0 mmol kg^{-1}, *2* 5, *3* 7.5, *4* 10. **b** DSC thermograms of the BSA solution at pH 7.0 in the absence and presence of palmitic acid: *1* 0 μmol kg^{-1}, *2* 10, *3* 50, *4* 100, *5* 200

100 μmol kg^{-1} palmitic acid concentration, the former peak became considerably small while the latter peak grew as large as the former peak in the absence of palmitic acid at a higher temperature more than 20 °C. Further, an increase in concentration brought about a complete disappearance of the former peak and a remarkable growth of the latter peak. We observed a small shoulder peak behind the grown peak in the high concentration range. A similar thermal behavior was observed for the system of HSA and palmitate [41].

We determined the peak temperature of DSC thermograms as the denaturation temperatures of BSA (T_D) and calculated enthalpy changes of the denaturation (ΔH_D) from the area of the peak. The values of T_D and ΔH_D of BSA at pH7.0 are plotted against halothane and palmitic acid concentrations in Figs. 2 and 3, respectively. Here, in the case of palmitic acid, two values of T_D (a lower denaturation temperature (T_{DL}) and a higher one (T_{DH})) obtained from two peaks are shown in the figures while the corresponding enthalpy values are shown as the total enthalpy values because two peaks could not be deconvoluted completely. As is seen from Figs. 2a and 3a, the T_D value was raised and the ΔH_D value increased with increasing halothane concentration. The elevation of T_D and the increase in ΔH_D imply that the binding of halothane to BSA increases the thermal stability of the BSA molecule. On the other hand, the palmitic acid also provided the elevation of T_{DL} and T_{DH} and the increase in ΔH_D; however, the greater changes in both quantities occurred at a lower concentration as compared with the

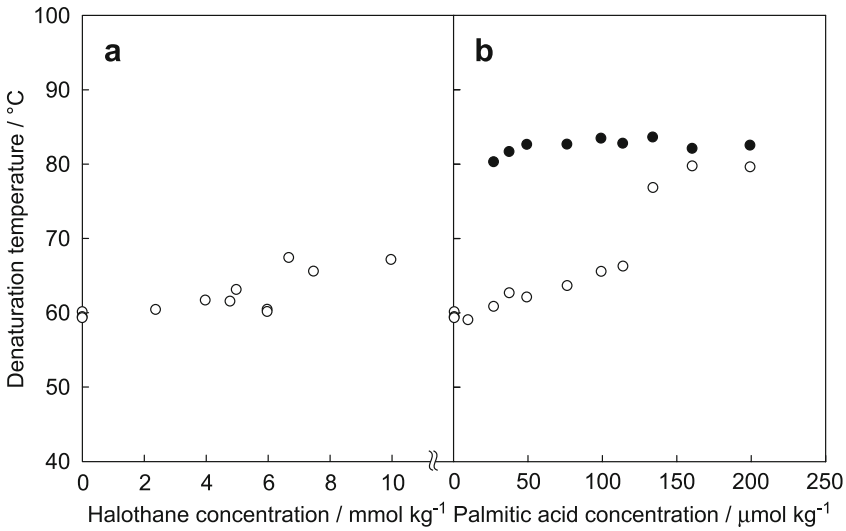

Fig. 2 Thermal denaturation temperatures of BSA at pH 7.0 as a function of ligand concentrations: **a** halothane, **b** palmitic acid. The *open* and *closed circles* in the case of palmitic acid correspond to the temperatures of low- and high-temperature denaturation, respectively

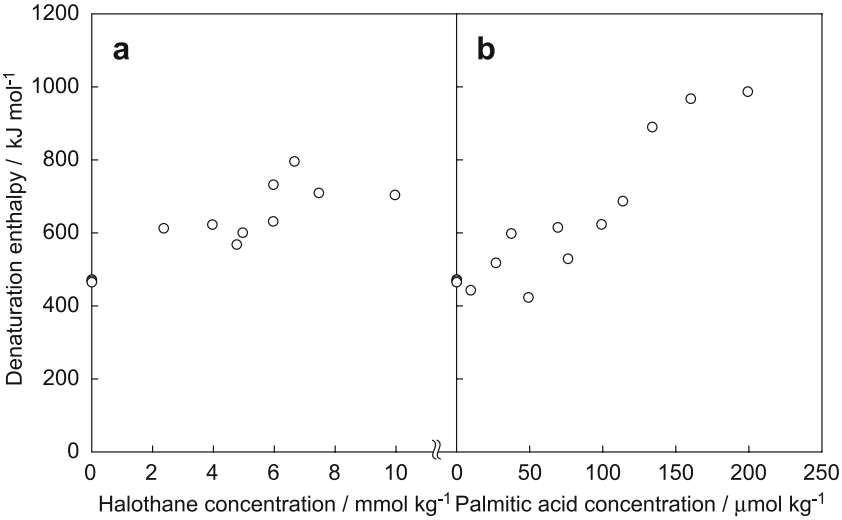

Fig. 3 Thermal denaturation enthalpies of BSA at pH 7.0 as a function of ligand concentrations: **a** halothane, **b** palmitic acid. Total enthalpies of denaturation are given in the case of palmitic acid

case of halothane. For example, the T_D and ΔH_D values were enhanced by ca. 7°C and ca. 300 kJ mol^{-1} at a halothane concentration of 7.5 mmol kg^{-1} while the T_{DH} and ΔH_D values by ca. 23°C and ca. 530 kJ mol^{-1} at a palmitic acid concentration of 200 μmol kg^{-1}. It should be noted in Figs. 2b and 3b that the T_{DL} increased abruptly at concentrations around 120 μmol kg^{-1}and the ΔH_D value nearly saturated at concentrations around 200 μmol kg^{-1}. As revealed later, the concentrations of palmitic acid correspond to the specific concentrations at which the molar ratio of palmitic acid to BSA in the overall sample becomes ca. 4 and 7, respectively. The above findings suggest that there exists a specific interaction between the BSA and palmitic acid molecules. We can say that although the binding of palmitic acid to BSA also increases the thermal stability of the BSA molecule, palmitic acid is incomparably superior in the stabilization ability to halothane.

Since BSA changes its own structure depending on the solution pH as mentioned above, we performed the DSC measurements on the BSA solutions in the absence of ligands at various pH. At acidic pH lower than 5.0 and alkaline pH higher than 10.0, the exact denaturation peak of BSA solutions could not be determined because of the great peak broadening and the poor reproducibility. This fact means that the thermal stability of the F and B forms is considerably low. Yamasaki et al. [42] have reported similar results of the pH dependence of the thermal behavior of BSA in saline solutions. The effect of pH on BSA structure was examined under the condition that the denaturation peak was detected with high reproducibility.

Figure 4 shows the DSC thermograms of the BSA solutions in the absence and presence of halothane and palmitic acid at pH of 6.0, 8.0, and 8.3, respectively. In the neutral pH region (pH6.0 and 7.0), BSA exists as the N form. Since BSA shows the greatest denaturation peak at pH7.0 as shown in Fig. 1, we can judge that its thermal stability is largest at the pH. The peak of BSA at pH6.0 became smaller than that at pH7.0 and there exists a small shoulder peak behind the main peak (Fig. 4a). One reason for the decrease in thermal stability of BSA results from the charge balance of amino acid residues at surface of the BSA molecule. The isoelectric point of BSA is about pH4.8, the solution pH approaches this pH, presumably the structure of BSA fluctuates and BSA is thermally unstabilized. Regarding the appearance of shoulder peak, since it was reported [43] that the denaturation of BSA in the neutral pH region is a three-state transition corresponding to a three-domain structural model for this protein, it may arise from the overlapping of the denaturation of the respective domains. At pH6.0, both halothane and palmitic acid stabilized the structure of BSA but their interaction modes were quite different from each other although the structure of BSA itself was slightly unstabilized by the solution pH. We especially observed that halothane accelerated the denaturations among domains at the pH. On the other hand, in the alkaline pH region (pH8.0 and pH8.3) where the N and B forms coexist in the pH region, the denaturation peak became smaller and shifted to the low temperature region compared with those observed in the neutral pH region. Further, a clear split of the peak into two or three peaks was found for all the peaks at both pH. This drastic change may be attributable to the lower thermal stability of the B form originated from the change in charge balance of surface residues of the BSA molecule and to the different thermal stability change among three domains. However, by adding both ligands, they also enhanced the thermal stability of

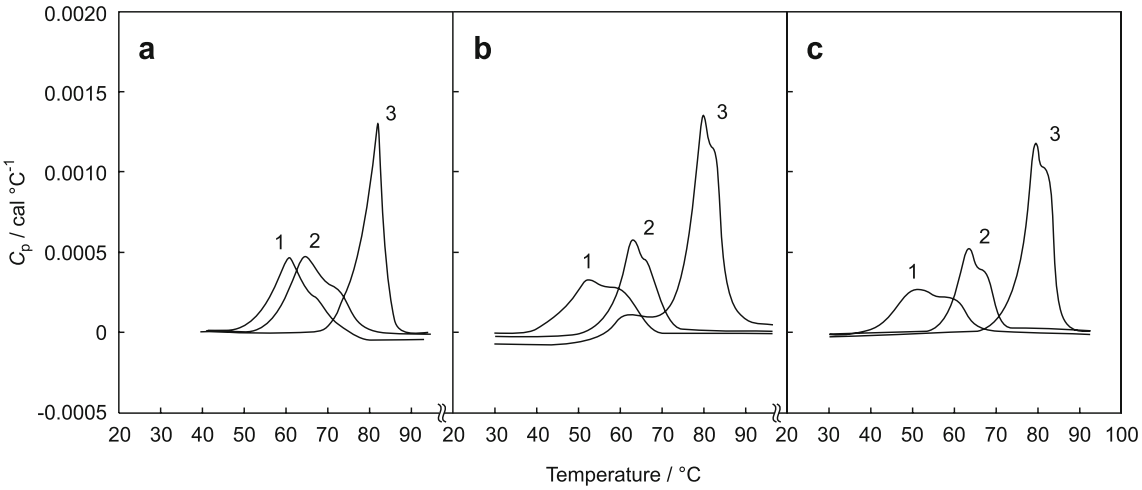

Fig. 4 DSC thermograms of the BSA solutions in the absence and presence of halothane and palmitic acid at various pH: **a** pH 6.0, **b** pH 8.0, **c** pH 8.3. Ligand concentrations: *1* 0 mmol kg^{-1}, *2* 7.5 mmol kg^{-1} halothane, *3* 200 μmol kg^{-1} palmitic acid

BSA and restored the structure with different interaction modes even in the alkaline pH region.

Effects of ligands on the volume behavior of BSA

Ligands affect not only the thermal behavior of BSA but also the volume behavior. Because the enthalpy and volume changes of a protein by the ligands are closely related to each other from the thermodynamic point of view, and a characteristic phenomenon of anesthesia, that is, the pressure reversal of anesthetic action, is also known to be caused by the volume change of bio-macromolecules such as lipid membranes and proteins, we next examined the ligand effect on the volume behavior of BSA. The viscosity measurements of the BSA solutions in the presence of ligand were adopted for this purpose. The values of η_{red} of BSA solutions in the absence of ligands at acidic and neutral pH are plotted against BSA concentration in the inset in Fig. 5. The η_{red} values at a neutral pH increased linearly with increasing BSA concentration, so we could obtain the $[\eta]$ value from the extrapolation of the η_{red} value to zero concentration. The obtained value of 0.037 dl g^{-1} was in good agreement with the values reported by Yang and Foster [44] and Tanford et al. [45, 46], respectively. Contrary to this, the concentration dependence of the η_{red} value at an acidic pH exhibited a shape of hyperbolic curve, and the $[\eta]$ values could not be obtained exactly in the acidic pH region. Consequently, we adopted the η_{red} value as the low concentration of 0.5 g dl^{-1} instead of the $[\eta]$ value for viscosity measurements in this study taking into account that the definite difference of ligand effects is expected to be enhanced at a low BSA concentration.

The pH dependence on the η_{red} value in the absence and presence of halothane is shown in Fig. 5. Here, the halothane concentration of 5.0 mmol kg^{-1} was selected in the measurements. The η_{red} values in the absence of halothane became constant from the weak acidic pH regions to the weak alkaline pH regions, whereas they increased steeply with decreasing pH at acidic pH less than pH4.0. The great increase in the η_{red} value is attributed to the structural transition of BSA as mentioned before: E and F forms of BSA exist in more expanded states in the acidic pH region. In the pH region, we observed two discontinuous changes, which correspond to the E–F and F–N

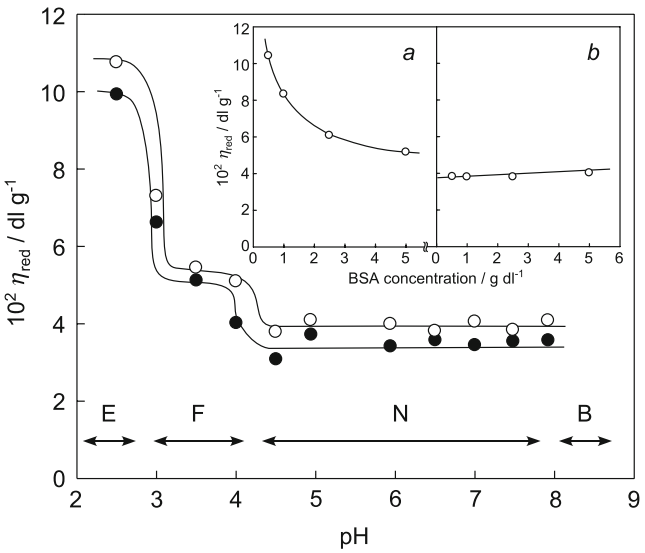

Fig. 5 Reduced viscosity of the BSA solutions at 0.5 g dl^{-1} as a function of pH in the absence and presence of halothane. The *open* and *closed circles* correspond to the results for the BSA solutions in the absence of halothane and those in the presence of 5 mM halothane, respectively. The pH regions of four forms of BSA, *E* (expanded), *F* (fast), *N* (normal), and *B* (basic) are indicated in the figure. The *inset* in the figure shows reduced viscosity of the BSA solutions as a function of BSA concentration at constant pH: *a* pH 2.5, *b* pH 6.0

transitions, in the η_{red} vs. pH curve at pH2.7 and pH4.3, respectively. In the neighborhood of pH8.0 where the N–B transition is anticipated to occur, there was no change on the η_{red} vs. pH curve. Therefore, we can say that the structural transition of BSA at the N–B transition does not induce the volume change enough to affect the solution viscosity. The η_{red} values in the presence of halothane became smaller than those in the absence of halothane at all pH measured. Similar results have been obtained by Ogli [31] for the viscosity of the BSA solution saturated with gaseous halothane by a surface-flow method. The E–F and F–N transitions at acidic pH were also found in BSA solutions even in the presence of halothane. The decrease in η_{red} by halothane suggests that the volume of BSA in the solution shrinks by an addition of halothane, and the volume shrinkage occurs at all BSA structures irrespective of kinds of the structure. This phenomenon is closely related to the nonspecific interaction between BSA and halothane as mentioned later.

Similarly, the effect of palmitic acid on the volume of BSA in the solution was also investigated. In this case, we used the BSA solutions containing a small amount of methanol, which was used as a solvent for palmitic acid. The viscosity of the solution somewhat increased compared with that of the BSA solution in the absence of methanol [47]. This is because the cluster formation of methanol molecules surrounded with clustrated water molecules due to the network of hydrogen bonding found in the low concentration range of methanol [48, 49]. We performed the viscosity measurements in the presence of palmitic acid by using the above solution as a control solution. Figure 6 presents the pH dependence of the η_{red} value in the absence and presence of palmitic acid in the pH range from 6.0 to 8.0. Here, the palmitic acid concentration of 150 μmol kg^{-1} was selected taking account of the results from the DSC measurements. In the whole pH range measured, the η_{red} values in the presence of palmitic acid became larger than those in the absence of palmitic acid. This increase in the η_{red} value means that the volume of BSA in the solution expands by an addition of palmitic acid, and the volume change occurs irrespective of kinds of the structure (here, N and B forms) as found in the case of halothane.

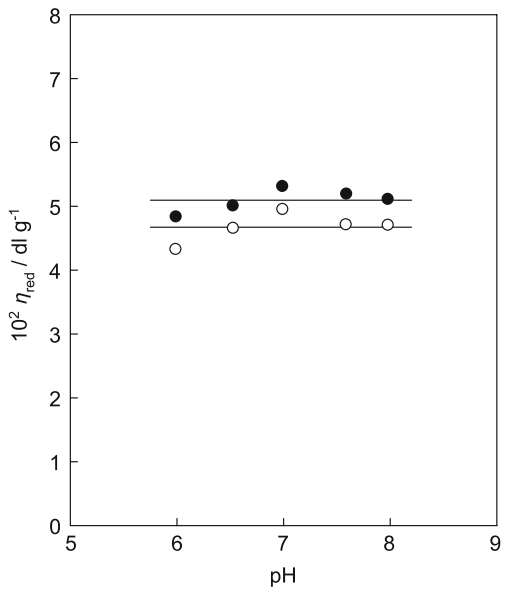

Fig. 6 Reduced viscosity of the BSA solutions as a function of pH in the absence and presence of palmitic acid. The *open* and *closed circles* correspond to the results for the BSA solutions in the absence of palmitic acid and those in the presence of 150 μM palmitic acid, respectively

The viscosity for hard-sphere particles in homogeneous medium is written by Einstein's equation as

$$\eta_{sp} = 2.5(v/V) \tag{5}$$

where v and V are the volume of a hard-sphere particle and total volume of the system, respectively. For nonspherical particles, the numerical coefficient of v/V is larger than 2.5 but should be constant for any given shape provided the rates of shear are sufficiently low to avoid preferential orientation of the particles. Since BSA is one of globular proteins, we roughly calculated the volume of BSA in the absence and presence of ligand assuming that BSA can be regarded as a hard sphere, and estimated the volume change percentage of BSA by adding ligands. The percentage was obtained by the following equations using Eq. (5)

$$v(\mathrm{BSA} + \mathrm{Ligand})/v(\mathrm{BSA}) - 1 = \eta_{sp}(\mathrm{BSA} + \mathrm{Ligand})/\eta_{sp}(\mathrm{BSA}) - 1 \tag{6}$$

where v(BSA+Ligand) and v(BSA) are the volumes of BSA in the presence and absence of ligands, η_{sp}(BSA+Ligand) and η_{sp}(BSA) are the specific viscosity of BSA in the same situation, respectively. We obtained the percentage of ca. −14.7% for halothane of 5 mmol kg^{-1} at pH7.0 and that of ca. 10.4% for palmitic acid of 150 μmol kg^{-1} at pH 7.0. It is evident that halothane and palmitic acid have different effect of the volume behavior of BSA: BSA shrinks by halothane while it expands by palmitic acid. The results for the volume behavior contrasted strikingly with those for the thermal behavior.

Correlation of thermal behavior of BSA with the ligand binding sites

Recently, the X-ray structural analysis for HSA has determined the binding sites of halothane [50] and palmitic acid [51] in HSA, respectively. Since BSA has a very similar primary structure to HSA and form almost the same tertiary structure as HSA [35], it is conceivable that the binding sites of halothane and palmitic acid in BSA are virtually the same as those in HSA. HSA has 585 amino

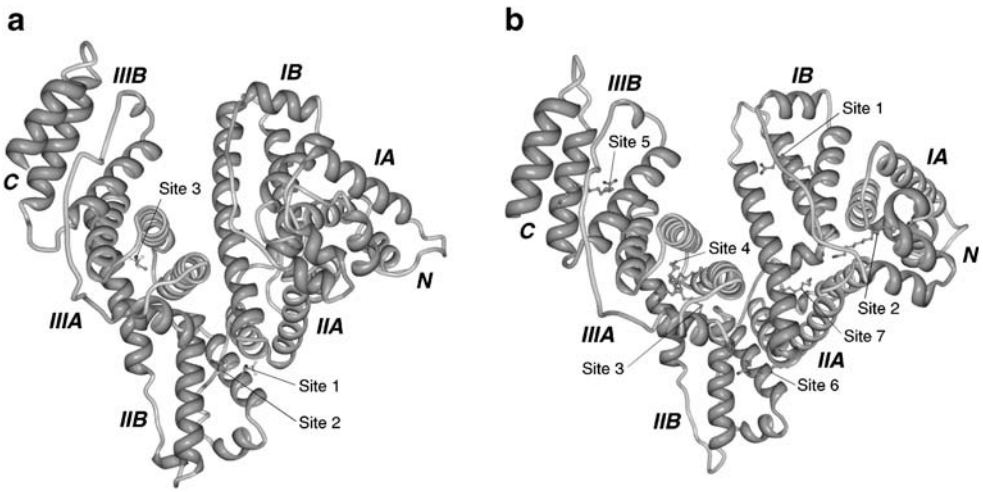

Fig. 7 Structures of HSA complexed with ligands: **a** halothane, **b** palmitic acid. The secondary structure of the protein, three domains I, II, III and sub-domains A and B in each domain, is shown schematically. The ligand in each site is represented by ball and stick models. These figures were prepared from PDB data (1E7B and 1E7H) by using MBT Protein Workshop [69]

acid residues (BSA=583 residues) and consists of three homologous α-helical domains (I, II, and III). Each domain contains ten helices and is divided into six-helix and four-helix sub-domains (A and B). Figure 7 demonstrates the structures of HSA complexed with halothane or palmitic acid, respectively, which are constructed by use of PDB data (1E7B and 1E7H). Halothane binds at three discrete sites on HSA in the clinically relevant concentration range: two sites at the interface between sub-domains IIA and IIB (sites 1 and 2) and one site in sub-domain IIIA (site 3). At high halothane concentrations and in the presence of myristic acid, there exist seven halothane-binding sites on HSA (cf. PDB ID; 1E7C). These binding sites are amphiphilic pockets and clefts pre-formed on HSA, and all reside at the location of fatty acid binding sites. The binding of halothane to HSA causes only very minor changes in local structure. On the other hand, palmitic acid binds at seven discrete sites on HSA: one site in sub-domain IB (site 1), one at the interface between sub-domains IA and IIA (site 2), two in IIIA (sites 3 and 4), one in IIIB (site 5), one at the interface between IIA and IIB (site 6), and one in IIA (site 7). Unlike the case of halothane, the binding of palmitic acid to HSA causes the conformational changes among three domains.

Let us now consider the above structural data with the thermal results. The effective concentration of halothane on BSA is significantly different from that of palmitic acid. Since we used the overall concentrations to express the concentration of ligands in this study, the binding and free concentrations between BSA and ligand at equilibrium could not be determined. However, it is useful to compare the molar ratio of ligand to BSA by using the overall concentration. The molar ratios of effective halothane concentrations of 5 and 10 mmol kg^{-1} correspond to 165 and 330 mol per 1 mol of BSA while that of effective palmitic acid concentrations of 100 and 200 μmol kg^{-1} correspond to 3.3 and 6.6 mol, respectively. Table 1 summarizes the binding numbers of halothane [14, 18, 22, 23, 25–27, 50] or palmitic acid [36, 51–53] to serum albumin determined by various physico-chemical methods. The experimentally determined binding numbers of halothane and palmitic acid are not so different from each other. However, in the molar ratio of both ligands, 50–100 times greater ratio of halothane compared with that of palmitic acid is necessary for the appearance of ligand actions; in other words, the greater numbers of halothane molecules are required for the appearance of the ligand action on BSA whereas palmitic acid is enough for several molecules. Comparing the molar ratio of both ligands at the effective concentrations with the structural data of HSA complexed with ligands, the ratio of halothane was not consistent with the binding numbers on HSA, whereas that of palmitic acid was in good agreement with the numbers. This fact means that there is hardly an observable anesthetic effect on BSA even if the halothane concentration corresponding to the binding numbers determined from X-ray analysis or that reported in the literatures as the overall concentration is added to the BSA solution. Judging from the fact that the molar ratio of halothane at the effective concentration is much larger than the concentration of binding number, there are undoubtedly a lot of free halothane molecules in the BSA solution at the concentration. Therefore, we can conclude that halothane is an essentially nonspecific binder to BSA and affects BSA by the nonspecific solvent effect in the higher concentration range. In contrast with the case of halothane, the binding mode of palmitic acid is quite different. The consistency of molar ratio and structural data exhibited that the overall concentration of palmitic acid added can be substantially regarded as the binding concentration, and then there is almost no free palmitic acid in the BSA solution. Further, it should be noted that the molar ratio of BSA to palmitic acid at optimal stabilization is very close to the maximum binding number seven determined from X-ray analysis. We can say from the

Table 1 Binding number of serum albumin to halothane or palmitic acid

Ligand	Binding number	Method	Reference
Halothane	≥2	Fluorescence	[14]
	≥2	Photoaffinity labeling	[18]
	2.1[a]	Chromatography	[25]
	3	X-ray analysis	[50]
	3	ITC	[27]
	4.2	^{19}F-NMR	[22]
	7.6	ITC	[26]
	19.3[b]	^{19}F-NMR	[23]
Palmitic acid	6	N. A.	[36]
	6.9	Affinity labeling	[52]
	7	X-ray analysis	[51]
	7	^{13}C-NMR	[53]

[a] High affinity site
[b] At 10°C

fact that palmitic acid is a specific binder to BSA and stabilizes the BSA structure at the transition state in the lower concentration range.

Stabilization mechanism of the BSA structure by ligands

Halothane and palmitic acid enhance the stability of the BSA structure. We next reveal the difference in stabilization mechanism of the BSA structure by both ligands. BSA is probably one of the most hydrophilic proteins and can be easily dissolved in large quantities in water. This is confirmed from the nature of BSA that it has a large negative hydropathy index of total amino acid residues (−1,235) [54] and relatively large polarity parameter of Bigelow (1.23) [55], which is defined as the volume ratio of polar amino acids to nonpolar ones. Halothane and palmitic acid are both amphiphilic molecules, but the hydrophobicity of palmitic acid is much larger than that of halothane as is seen in their solubility in water (ca. 19.0 mM at 25°C for halothane [56] and ca. 28 μM at 20°C for palmitic acid [57]). The difference in stabilization of BSA between ligands is closely related to that in interaction mode of a hydrophilic protein with amphiphilic ligands. We speculate that the stabilization of BSA by halothane is attributable to two factors, the predominantly hydrophilic residues on the surface and soft (less rigid) structure of BSA while the stabilization by palmitic acid is responsible for the conformational change of BSA by specific bindings of palmitic acid.

One reason for the stabilization by halothane is a great quantity of hydrophilic residues on the BSA surface. Proteins keep their structural integrity by the balance between hydrophilic and hydrophobic interactions with water. The behavior of a protein is essentially originates from the extensive hydrogen bonding capability of water. Anesthetics act at the protein/water interface and interrupt the interaction between proteins and water. For a protein with predominantly hydrophobic residues on the surface, anesthetics decrease the restrictive force of water (hydrophobic hydration) and loosen the structure. On the other hand, for a protein with predominantly hydrophilic residues at the surface, anesthetics decrease the attractive force of water (electrostriction) and tighten the structure. The above situation is similar to a poor or a good solvent in polymer chemistry. Water is repulsive to hydrophobic solutes and becomes a poor solvent; however, water is strongly attractive to hydrophilic solutes and becomes a good solvent. In a good solvent, a polymer chain is expanded, while in a poor solvent, the chain is contracted. By adding the high concentrations of anesthetics, a solvent changes from water to mixed solvent of water and anesthetic. If the mixed solvent shifts to a good solvent for a hydrophilic protein, the protein tends to unfold while if the solvent does to a poor solvent, the protein conversely tends to fold. It was reported that the biphasic effect of a hydrophilic homopolymer occurs in water by adding a short-chain alcohol [58]. The thermal and volume behavior of BSA in the presence of halothane suggests that the latter case holds for BSA. Furthermore, when the hydrophobic part of anesthetic molecules interact with the hydrophobic residues of protein surface, the volume decreases because the volume of the hydrophobic interaction in water is negative [59]. This effect also contributes to the volume decrease of BSA in addition to the nonspecific solvent effect.

Another reason is the rigidity of the BSA molecule. The rigidity of a protein can be estimated from its compressibility in the aqueous solution. Gekko et al. [60–62] have examined the adiabatic compressibilities of dozens of globular proteins in water by a method of sound velocity measurements and classified the proteins into hard and soft categories. They also revealed the correlation between the compressibility and the structural factors affecting the

compressibility such as the molecular size, hydrophobicity, secondary structure, etc. of a protein. It was found in their studies that BSA has a considerably high compressibility value; its adiabatic and isothermal compressibilities are 10.5×10^{-12} and 14.6×10^{-12} cm^2 dyn^{-1} at 25 °C [61], respectively. Considering that typical globular proteins such as ribonuclease A and lysozyme have less than 5.0×10^{-12} cm^2 dyn^{-1} at 25 °C for their adiabatic compressibilities [61], this means that BSA is a very soft protein. The normal form of BSA contains a high percentage of α-helix structure (about 70% [37]). The α-helix-rich proteins are known to be highly compressible [61]. The elements of α-helix have strong hydrophobicities, and the hydrophobic interaction between α-helices contributes to the stabilization of tertiary structure of a protein. Although the compressibility of α-helix itself is negligibly small [63, 64], the packing of α-helix to surrounding hydrophobic region becomes insufficient due to the bulky structure, and the insufficient packing may make the compressibility of a protein increase. This indicates that it is possible for a part of α-helix in a protein to act as one of dynamic domains, and the structure of α-helix tends to soften the protein structure. Therefore, the shrinkage of BSA by nonspecific solvent effect of halothane may result from the soft structure due to the large α-helix content. Recently, Yamato et al. [65] have reported on the compressibility of myoglobin by a method of molecular simulation that the inside of α-helix is difficult to compress; however, the cavity between α-helices is markedly compressed.

In the case of palmitic acid, it was found from the X-ray structural analysis for HSA [51] that, when binding fatty acids to HSA, the conformational change, that is, the rotations of domains I and III relative to the center domain II, takes place from the native form. This change may be mainly caused by the binding to the site located at the juncture of sub-domains IA and IIA. The structural change of BSA due to the specific binding of palmitic acid is confirmed from the behavior of T_D and ΔH_D in Figs. 2b and 3b. Recent studies of ^{13}C-NMR [66] and molecular dynamic simulation [67] on HSA complexed with palmitic acid exhibited that fatty acid binding sites 2, 4, and 5 are identified as high affinity sites, while sites 1, 3, 6, and 7 as low affinity sites. When a few molecules of palmitic acid occupy the binding sites of BSA, probably high affinity sites including at the interface of sub-domains IA and IIA (site 2), the conformational change of BSA from a native state to a transition state occurs, and the structure of BSA becomes thermally stable (increase in T_{DL} and T_{DH}). Abrupt change in T_{DL} in the vicinity of the molar ratio of 4 in the overall sample shown in Fig. 2b may closely agree with the above behavior. Further increase in palmitic acid concentration brings about the greater stabilization at the transition state and a decrease in number of native forms (increase in ΔH_D). When seven molecules of palmitic acid occupy all the binding sites, the maximum stabilization effect appears and the effect saturates as seen in Fig. 3b. From the volume behavior of BSA in the presence of palmitic acid, we may say simultaneously that the conformational change due to the binding of palmitic acid produces an increase in volume of BSA although the BSA structure becomes more rigid as expected from the great thermal stabilization. This behavior is in accord with the fact revealed from X-ray structural analysis of HSA complexed with a number of drugs [68] that the binding of fatty acids increases the volume of drug site 1 (a preformed binding pocket within the core of sub-domain IIA). We speculate that the native BSA with large structural fluctuation due to the soft structure as mentioned above may be transformed into the complexed BSA of the hard structure with small structural fluctuation by the specific binding of palmitic acid, and the transformation may be accompanied with a volume increase. This stabilization mechanism of palmitic acid forms a striking contrast to that of halothane and confirms that palmitic acid is a specific binder to BSA.

Conclusions

The interaction between BSA and a small ligand, halothane, or palmitic acid was clarified by examining the change in the thermal and volume behavior of BSA by adding each ligand. Although both ligands thermally stabilized the structure of BSA, the large difference in the interaction modes of BSA and the ligands was found. The effective concentration of halothane was much larger than that of palmitic acid, and the effect was not involved with the pH-dependent structural change of BSA. The notable feature of the response of BSA to halothane is its volume shrinkage. Taking into account that the volume change of BSA by halothane is thermodynamically related to the pressure dependence of the association constant between BSA and halothane, the volume shrinkage means that pressure promotes the binding of halothane to BSA, that is, pressure synergizes the anesthetic action, not antagonize the action. Therefore, we may say that BSA does not seem to be relevant to the molecular mechanism of anesthesia. On the other hand, good consistency between the molar ratio from effective concentrations in thermodynamic data and the binding numbers of palmitic acid to HSA from structural data confirms that palmitic acid is a specific binder to serum albumin. The mechanism of BSA response to ligands depends on the nature as a protein and interaction modes of ligands, specific or nonspecific. We showed in the present study that the characteristic of ligand action on a model protein is revealed by comparing the effects of ligands

having different interaction modes on the protein. Such comparative study is very helpful in elucidating the intrinsic interaction between a protein and anesthetics. In the near future, we will report ligand effects on other model proteins to elucidate the interaction modes of anesthetics and the proteins.

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