Investigation of Slow Dynamics of the Sulfhydryl in the Solution and Gel States of Bovine Serum Albumin: A Vector Electron Paramagnetic Resonance Study

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Abstract: Serum albumin has one reactive sulfhydryl (Cys-34) that is one of the important binding sites. Cys-34 is located in the crevice on the surface of the albumin molecule and is therefore restricted in its motion. Bovine serum albumin (BSA) Fraction V forms a transparent gel at pD 4.0 (F-form) in D₂O at protein concentrations above 7% (BSA*-gel). We studied the molecular motion of Cys-34 on BSA in the solution and gel states by the vector electron paramagnetic resonance (EPR) method using a maleimide spin label. The rotational correlation times of the spin label bound to Cys-34 in the BSA solution and BSA*-gel were in the order of 10⁻⁶ and 10⁻⁵ s,

respectively. A longer rotational correlation time of the Cys-34 spin label in the BSA*-gel suggested that the gel network formed in BSA may drastically slow the motion of Cys-34. The integrated value obtained from the vector EPR spectra also showed an extremely dramatic slowing of the Cys-34 spin label during the gel formation. On the other hand, the values for order parameter and the inclination of the principal axis (z) of the Cys-34 spin label to the rotational axis (μ) were the same in the BSA solution and BSA*-gel. [Japanese Journal of Physiology, 49, 27–33, 1999]

Key words: bovine serum albumin, gel, maleimide spin label, sulfhydryl environment, vector electron paramagnetic resonance.

Serum albumin is the principal protein in blood and is clearly an extraordinary molecule with many different functions and applications. One of the most important characteristic features of the albumin molecule is the presence of a free sulfhydryl residue at position 34 (Cys-34) [1]. This free sulfhydryl is known to bind with cysteine, glutathione and various metals in the bloodstream [1]. There are only a few previously reported electron paramagnetic resonance (EPR) studies on Cys-34 of bovine serum albumin (BSA), which include the use of the spin label to investigate the mobility of Cys-34 and its microenvironment [2–5]. According to these reports, Cys-34 is considered to be located in the crevice on the surface of the

albumin molecule and the motion of the spin label bound to Cys-34 might be immobilized; hence, reporting on the overall motion of the BSA molecule. However, controversy remains about the detailed microenvironment for Cys-34 in the crevice.

Wilson and Foster [6] reported that BSA Fraction V contains small amounts of a proteolytic enzyme which catalyzes a very limited and specific cleavage of BSA in the F-form near pH 3.8, resulting in the formation of partially hydrolyzed BSA (BSA*). BSA* has a tendency to form a transparent gel at pD 4.0 in D₂O at protein concentrations above 7% and it is tentatively called BSA*-gel [7, 8].

The vector EPR method [9–13] is an extended satu-

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Abbreviations: BSA, bovine serum albumin; EPR, electron paramagnetic resonance; γ , inclination of the principal axis (z) of the spin label to the rotational axis (μ); ¹H-NMR, proton nuclear magnetic resonance; HPLC, high-performance liquid chromatography; I , integrated value; S , order parameter; τ , rotational correlation time; V_2' spectrum, out-of-phase second harmonic spectrum.

ration transfer EPR method [14–16] to investigate more accurately the molecular motion of the spin label bound to the specific residues and the rotational dynamics of protein molecules. Although the saturation transfer EPR spectrum of the complex of stearic acid spin probe [17] or maleimide spin probe [18] with BSA has been already reported, these reports on BSA-spin probe complex were a reference for studying the rotational motion of the rhodopsin molecule in the membrane. In the present study, however, we were able to demonstrate the power of the vector EPR method for the characterization of the Cys-34 microenvironment in the solution and gel states of BSA, and for the detection of the slow molecular motion range from 10^{-7} to 10^{-5} s, which is outside the limit of measurement by the conventional EPR method.

MATERIALS AND METHODS

Materials. BSA Fraction V was purchased from Sigma Chem. Co. (Lot 122H9318). 3-Maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl was purchased from Aldrich Chemical Co. (Lot 12206kz). BSA in 0.20 M KCl was allowed to react with spin labels (1.0 mol spin label per 1.0 mol BSA) with continual stirring for 48 h at 4°C (pH 6.45). The mixture was dialyzed exhaustively with distilled water at 4°C for 2 weeks and underwent ultrafiltration with Centricut Mini (Kurabo Ind. Ltd.) at 4°C. The solution was then lyophilized and stored in a cold room until use.

For making the 10% spin-labeled BSA solution, a lyophilized sample was dissolved in D₂O and the pD was adjusted to 4.10 (pD range of the F-form) by adding 0.10 M DCl. The solution was filtered through a Millex filter (0.45 µm), sealed into sterilized capillaries (0.5 mm ID) and placed in an EPR tube (5 mm ID) in order to avoid dielectric loss in the EPR cavity. The EPR tubes containing spin-labeled BSA D₂O solutions were kept at $35 \pm 0.3^\circ\text{C}$, resulting in the formation of a transparent spin-labeled BSA*-gel. The experiment for the gel formation was carried out under sterilized conditions.

Conventional and vector EPR measurements. The EPR experiments were performed on a JEOL JES-FE1X EPR spectrometer equipped with a lock-in amplifier (NF Electronic Instr., 5610). The conventional EPR spectra were recorded with the microwave field strength of 0.01 mT and 100 kHz field modulation (0.2 mT), in-phase for the first harmonic mode. Vector EPR spectra were recorded with the microwave field strength of 0.055 mT in the second harmonic mode, that is, 100 kHz/200 kHz of the modulation/demodulation scheme, as recently reported [13].

The microwave field strength was calibrated by the standard method [11]. The phases of vector EPR spectra were measured with both in-phase (V_2) and out-of-phase (V_2') displays. For studying the effect of temperature variation ranging from -20 to 40°C on vector EPR spectra, a variable temperature controller (JEOL, JES-VT-3A2), a temperature adaptor (JEOL, JES-UCT-2AX) and liquid nitrogen were used. All experiments except for temperature variation were performed at $24 \pm 0.5^\circ\text{C}$.

Rotational correlation time (τ) for the slow motional signal was evaluated from the peak-height ratios, that is, low-field (L'/L), central (C'/C), and high-field (H'/H) peak-height ratios in the V_2' spectra [15]. The sensitivity of the detection mode of 100 kHz/200 kHz is inferior to that of 50 kHz/100 kHz, but the order of magnitude of τ does not alter. The ratio between modulation amplitude and modulation frequency has been defined as a modulation parameter [12]. The net strength of field modulation is influenced by modulation frequency. However, the same order of magnitude of τ is obtained even by raising the 50 kHz modulation frequency to 100 kHz, which is a factor of two. The value for τ in the fast motional domain was obtained by the conventional EPR method using the theory for isotropic motion [19].

The integrated value (I) obtained from the V_2' spectra, which is associated with τ , was used in the present study. However, we observed a slight shift of phases in the V_2' spectra and this may be due to an error in I . The corrected value is given in the following equation:

$$I = \left(\int V_2'(H) dH \right) / L, \quad (1)$$

where H is the magnetic field and L is the peak height at the lower magnetic field in the V_2' spectrum.

The order parameter (S) was calculated from the following equation [20]:

$$S = \{ (A_{//} - A_{\perp}) / [A_{zz} - 1/2(A_{xx} + A_{yy})] \} (a_0/a_0'), \quad (2)$$

where $A_{//}$ and $A_{\perp}$ are the hyperfine interactions parallel ($//$) and perpendicular ($\perp$) to the symmetrical axis, respectively, and A_{xx} , A_{yy} and A_{zz} are tensors in the principal axis system. The reported principal values of the hyperfine tensors were employed from those for deuterated maleimide spin label [21]. a_0 is the isotropic component of the hyperfine interaction and a_0' is the hyperfine splitting in the polarity environment of the spin label.

The inclination of the principal axis (z) of the spin label to the rotational axis (μ) (γ (degree)) was calculated by the following equation [20]:

$$S = 1/2(3\cos^2\gamma - 1). \quad (3)$$

Miscellaneous. The pH measurements were carried out with a Hitachi-Horiba F-7SS equipped with an expanded scale, using a Radiometer GK-2401C combined electrode. The pD value is given by $pD = pH(\text{reading}) + 0.407$, assuming the atomic fraction of deuterium in the D_2O solution to be 0.9975 [22]. The protein concentrations were determined with a Hitachi 320S spectrophotometer, assuming E (1%, 1 cm) at 279 nm to be 6.67.

RESULTS AND DISCUSSION

Before starting the main results and discussion, it is appropriate to note briefly the effects of lyophilization of BSA and temperature on vector EPR spectra. In order to confirm whether the lyophilization procedure of BSA affects vector EPR spectra or not, the V_2' spectra of the BSA solution (7.5%, pH 5.24, 0.10 M Cl^-) were measured before and after lyophilization. Spectral features of the V_2' spectra were almost identical, and I values obtained from the V_2' spectra were 241.6 and 237.9, respectively, indicating that lyophilization did not affect the motion of the spin label bound to the Cys-34 of BSA.

The temperature variation of I values for BSA solution (9.7%, pD 4.10, 0.10 M Cl^-) and BSA*-gel (9.7%, pD 4.10 before gelation, 0.10 M Cl^-) was also investigated over a range of temperature from -20 to $40^\circ C$. The I values for BSA solution and BSA*-gel were gradually decreased continuously from -20 to $40^\circ C$ (data not shown), indicating the motion of the spin label bound to Cys-34 became faster with increasing temperature. It follows that, in this study, we have chosen the temperature as $24 \pm 0.5^\circ C$ for the sake of simplicity.

Spectral features of BSA solution and BSA*-gel

In previous reports on BSA using the maleimide spin label [2–5], the conventional EPR spectrum showed a typical powder pattern spectrum, and this type of spectrum indicated that the motional mobility of the spin label went beyond the limit of measurement by the conventional EPR method (so-called “rigid limit”) [23]. The conventional EPR spectrum of BSA had two major components, one corresponded to a strongly immobilized signal and the other to a weakly immobilized one [2–5]. Griffith and McConnell [2] considered that strongly immobilized signal is due to the spin label bound to Cys-34 in the crevice, whereas the weakly immobilized one is due to

binding to the $\epsilon-NH_2$ of lysine residues on the surface of albumin molecules.

The V_2' spectra of the BSA solution (9.7%, pD 4.10, 0.10 M Cl^-) and BSA*-gel (9.7%, pD 4.10 before gelation, 0.10 M Cl^-) are shown in Fig. 1, A and B, respectively. The spectrum shown in Fig. 1A was obtained immediately after the preparation of BSA solution in the F-form (pD 4.10) in D_2O . That shown in Fig. 1B was obtained at 13 d after incubation at $35 \pm 0.3^\circ C$. Both spectra had four slow motional peaks (L , L' , H , and H'), two fast motional peaks (F_L and F_H), and two slow motional peaks contaminated with fast motional peaks (C and C'). As mentioned in the experimental sections, the BSA solution was mixed with the maleimide spin label, and the solution (pH 6.45) was dialyzed exhaustively with distilled water for an extended period of time at $4^\circ C$ in order to remove the free spin label from the solution, as stated by Benga and Strach [3]. Consequently, in Fig. 1, A and B, the four slow motional peaks (L , L' , H , and H') may have originated from the spin label bound to Cys-34, and the two fast motional peaks (F_L and F_H) may have originated from that bound to the $\epsilon-NH_2$ of lysine residues, as pointed out by Griffith and McConnell [2].

As demonstrated in Fig. 1, A and B, the spectral shapes between solution and gel state of BSA were considerably different. The peak heights of L' , C' , and H' in BSA*-gel were larger than those in BSA solution, suggesting that the molecular motion of the spin label bound to Cys-34 in the BSA*-gel was more strongly immobilized than that in the BSA solution. Indeed, the calculated τ values for slow motional signal in the BSA solution and BSA*-gel were 4.0×10^{-6} and 1.3×10^{-5} s for L'/L ratio, and 3.2×10^{-6} and 3.2×10^{-5} s for H'/H ratio, respectively. That for fast motional signal was 1.0×10^{-9} s in both states. Thus, the vector EPR method can be quite useful in simultaneously detecting the molecular motions in both the Cys-34 in the crevice and the $\epsilon-NH_2$ of lysine residues on the surface of BSA, as discussed in the later section.

Gelation process on partially hydrolyzed BSA (BSA*)

Evans demonstrated that there is a correlation of I value obtained from the saturation transfer EPR spectra with τ of a slow motional spin label [24]. He also indicated that such a correlation may be useful in measuring an accurate τ value of a slow motional spin label in the presence of fast one [24]. Since the integration of fast motional signal in the V_2' spectrum tends toward zero, the fast motional signal no longer

Fig. 1. Vector EPR spectra detected at second harmonic mode at $24 \pm 0.5^\circ\text{C}$. The second harmonics displays were recorded by a 100 kHz/200 kHz, modulation/demodulation scheme, with a microwave field strength of 0.055 mT and modulation amplitude of 0.2 mT. The arrows indicate

signal peaks for determining rotational correlation times (τ) of the spin labels. **(A)** Out-of-phase spectrum of BSA solution (9.7%, pD 4.10, 0.10 M Cl^-). **(B)** Out-of-phase spectrum of BSA*-gel (D_2O) (9.7%, pD 4.10 before gelation, 0.10 M Cl^-).

has any influence upon I , obtained from the V_2' spectrum. Thus, I is only related to the amplitude of the slow motional signal and the overall amplitude in the V_2' spectrum correlates with τ . Consequently, it may be worth noting that the I value obtained from the V_2' spectra in the present study reports directly on the molecular motion of the spin label bound to Cys-34 in the crevice of the BSA molecule. The integrated spectra of BSA solution (9.7%, pD 4.10, 0.10 M Cl^-) and BSA*-gel (9.7%, pD 4.10 before gelation, 0.10 M Cl^-) are shown in Fig. 2. The signal intensity for the integrated spectrum of the BSA*-gel was increased considerably as compared with that of the BSA solution. Using Eq. 1, calculated I values for BSA solution and BSA*-gel were 179.4 and 658.7, respectively. These values, together with the results shown in Fig. 1, A and B, indicate that the molecular motion of the spin label bound to the Cys-34 in BSA*-gel is considerably immobilized during the gelation of BSA*.

The partial hydrolysis of the main chain between Gln-392 and Cys-435 in one or two portions of the BSA molecule resulted in the formation of BSA* in the F-form near pH 3.8 [6], and then the BSA* was

Fig. 2. Integrated spectra of BSA solution (9.7%, pD 4.10, 0.10 M Cl^- ; dotted curve) and BSA*-gel (9.7%, pD 4.10 before gelation, 0.10 M Cl^- ; solid curve) obtained from the V_2' spectra at $24 \pm 0.5^\circ\text{C}$. See Fig. 1 for experimental conditions.

readily formed into a transparent gel in D_2O at pD 4.0 at protein concentrations above 7% [7, 8]. During gelation on BSA*, the I value increased steeply for 3 d and then peaked to a plateau from 4 to 13 d after incu-

Fig. 3. Integrated values (I) obtained from the V_2' spectra at $24 \pm 0.5^\circ\text{C}$, as a function of the time (d) of hydrolysis at $35 \pm 0.3^\circ\text{C}$ by protease with BSA (9.7%, pD 4.10, 0.10 M Cl^-). See Fig. 1 for experimental conditions.

bation at $35 \pm 0.3^\circ\text{C}$, as shown in Fig. 3. The time course of I shown in Fig. 3 was almost similar to that for BSA* formation at pH 3.8 and at $35 \pm 0.3^\circ\text{C}$, which was analyzed by ion-exchange high-performance liquid chromatography (HPLC) on TSK SP-5PW column (see fig. 1B in Era *et al.* [8]). Based on these results, the formation of BSA* might be the rate-limiting step for the gelation process. Consequently, the time-dependent variation of I value reflects the gelation process and the immobilization process of the spin label bound to Cys-34.

Sulfhydryl environment in the solution and gel states, and structural characteristics of BSA*-gel

The values for S and γ of the spin label bound to Cys-34 were calculated using the data from conventional and vector EPR spectra. In determining the values for S and γ , both conventional and integrated EPR were utilized. The integrated spectra were obtained from the in-phase (V_2) spectra of vector EPR, because they yielded the same lineshape of conventional EPR spectra. As given in Table 1, the values for S and γ obtained by the two different methods were almost identical. Furthermore, it is noteworthy that there was no difference between the S and γ values in the BSA solution (9.7%, pD 4.10, 0.10 M Cl^-) and BSA*-gel (9.7%, pD 4.10 before gelation, 0.10 M Cl^-). In general, S and γ indicate the degree of wobbling motion of the spin label around the rotational axis. The values given in Table 1 show a markedly anisotropic motion of the spin label bound to Cys-34 in both states, and there was no change in solution $\rightarrow$ gel transition.

Era *et al.* [8] reported that BSA* solution in H_2O at pH 3.80 in 0.10 M Cl^- was a transparent solution,

Table 1. Order parameter (S) and the inclination of the spin label bound to Cys-34 to the rotational axis (γ (degree)) in BSA solution (9.7%, pD 4.10, 0.10 M Cl^-) and BSA*-gel (9.7%, pD 4.10 before gelation, 0.10 M Cl^-) at $24 \pm 0.5^\circ\text{C}$.

	Conventional EPR spectra	Integrated EPR spectra [†]
BSA solution		
S	0.85	0.85
γ	19	19
BSA*-gel		
S	0.85	0.84
γ	19	20

[†] The integrated spectra were obtained from in-phase (V_2) spectra of vector EPR, because they yielded the same lineshape of conventional EPR spectra.

even at a protein concentration of 11.53% and even after 7-d incubation at $35 \pm 0.3^\circ\text{C}$. It has been reported by several groups [25–28] that D_2O might strengthen the intra- and intermolecular hydrophobic and/or hydrogen bonds. Furthermore, Kuwata *et al.* [25] found the suppression of the structural fluctuation of the BSA molecule in D_2O . The strengthening of intermolecular hydrophobic and/or hydrogen bonds in D_2O may favor the network formation between BSA* molecules, that is, the transparent BSA*-gel formation [7, 8, 29]. In the series of ^1H -NMR cross-relaxation investigations on the physical state of water in the BSA solution and BSA*-gel, Sogami *et al.* [7, 29] and Era *et al.* [8] reported that the shortening of cross-relaxation times of water in the solution $\rightarrow$ gel transition of BSA might be attributable to not only a large amount of bound water but also physical differences in the protein-protein interaction, that is, characteristic of large protein aggregates in the gel state.

As already mentioned, τ values for slow motional signal in the BSA solution and BSA*-gel were in the order of 10^{-6} and 10^{-5} s, respectively. Although the value for τ is semi-qualitative because of the limiting of signal-to-noise ratio in the V_2' spectra, the results suggest that the molecular motion of the spin label bound to Cys-34 is strongly immobilized in the solution $\rightarrow$ gel transition. On the other hand, the τ value in the fast motional domain was kept at almost the same value (1.0×10^{-9} s) in both states, indicating that the molecular motion of the spin label bound to the $\epsilon\text{-NH}_2$ of lysine residues on the surface of BSA did not change during gel formation. These are necessary observations for our conclusion that the molecular motion of both the Cys-34 and $\epsilon\text{-NH}_2$ of lysine spin labels observed by vector EPR spectra reports directly on the structural characteristics of the BSA molecule

itself. Furthermore, from the sets of results shown in Figs. 1 and 2 and Table 1, it can be speculated that the suppression of the molecular motion of the spin label bound to Cys-34 might be predominantly caused by the immobilization of BSA main chain during the three-dimensional gel network formation, whereas the microenvironment for the spin label bound to Cys-34, such as crevice structure, might not alter in the solution $\rightarrow$ gel transition.

Based on the present results obtained through the vector EPR method together with previously reported results using $^1\text{H-NMR}$ [7, 8, 29], we can assume that the three-dimensional networks observed in the transparent BSA*-gel might be the cross-linked lattice structure formed by linear polymers. Such structure might be similar to the aggregation of "string of beads"-type polymers, as classified by Tombs [30]. However, the detailed molecular mechanism in the solution $\rightarrow$ gel transition of BSA necessitates more information on protein-protein interaction.

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