Participation of Band 3 Protein in Hypotonic Hemolysis of Human Erythrocytes

Yukio SATO,* Hiroshi YAMAKOSE and Yasuo SUZUKI

Pharmaceutical Institute, Tohoku University, Aobayama, Aoba-ku, Sendai 980, Japan. Received July 22, 1992

The study was designed to show whether band 3, anion exchange protein in human erythrocytes, is responsible for hemolysis in hypotonic solution. From ESR spectra of the spin labeled erythrocytes, it was found that the change of the osmotic condition had no effect on membrane fluidity at pH 7.4. At pH 6.0, however, the fluidity at the central part of the lipid bilayer was dependent on osmotic stress. In this case, the fluidity change as a function of KCl concentration was similar to the hemolysis curve. From circular dichroism (CD) spectra of erythrocyte ghosts, it was found that the conformational and/or associative changes of the membrane proteins correlated with the hypotonic hemolysis. In contrast to the osmotic stress, sodium dodecyl sulfate (SDS) changed the fluidity of the membranes but not the conformation of the membrane proteins regardless of the medium pH. In this case, the changes in the fluidity corresponded to the hemolysis curves in SDS solutions. Further, by using anion transport inhibitors, 4,4'-diisothiocyanostilbene-2,2'-disulfonate, eosin 5-isothiocyanate and eosin-5-maleimide, a correlation between the effect on the hypotonic hemolysis and the conformational and/or associative change of band 3 was suggested. These results demonstrate that band 3 is responsible for the hypotonic hemolysis.

Keywords erythrocyte; hypotonic hemolysis; ESR spectrum; CD spectrum; membrane fluidity; band 3

Introduction

Studies on human erythrocyte hemolysis have been promoted by a number of investigators, and it is known that there are many initial reactions which lead ultimately to cell lysis.^{1,2)} On the basis of the initial reactions, the hemolyses of the cells are designated as photo hemolysis,³⁾ thermal hemolysis,⁴⁾ osmotic hemolysis,⁵⁾ immune hemolysis,⁶⁾ pressure-induced hemolysis,⁷⁾ drug-induced hemolysis,⁸⁾ and drug-induced immune hemolysis.⁹⁾ In hypotonic hemolysis, red blood cells are swollen and burst by exposing them to a hypotonic solution, followed by releasing a cellular content, hemoglobin. The hemoglobin is released through membrane defects or holes. There is an idea that the hole formation is dependent on the initial reactions. Further, it is well known that the membrane hole developed during hypotonic hemolysis is transient. That is, after the hemolysis, when intracellular and extracellular osmolalities reach isosmolar, the membrane seals up spontaneously and becomes again impermeable.¹⁰⁾

It was reported that human erythrocytes create a single large hole in each membrane under hypotonic conditions and subsequently contract to a size that is determined only by the permeability of buffers.^{11–14)} However, the mechanisms of the hole formation in the membranes has not been elucidated. The process of sealing can be explained by the abrupt transition from a highly permeable to a highly impermeable state rather than the gradual reduction of hole radius in each ghost.^{14b)} Changes of membrane fluidity and conformation and/or associative changes of membrane proteins seems to be closely correlated with each other in hypotonic hemolysis.

This paper describes the relationship between the changes in membrane fluidity and the conformational and/or associative changes of membrane proteins in hypotonic hemolysis. We propose that band 3 protein, an anion transporter in the human erythrocytes, is responsible for the formation of the hemolytic hole in the erythrocyte membrane.

Experimental

Materials DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonate) was purchased from the Dojindo Laboratory. EITC (eosin-5-isothiocyanate) was prepared by bromination of fluorescein isothiocyanate (from Tokyo Kasei Kogyo Co., Ltd.) according to the procedure of Cherry *et al.*¹⁵⁾ EMI (eosin-5-maleimide) was obtained from Molecular Probe Inc. The fatty acid spin labels, 5-doxyl stearic acid and 16-doxyl stearic acid, were purchased from Aldrich Chem. Co. SDS (sodium dodecyl sulfate) was from Wako Pure Chemical Industries, Ltd.

Good's reagents, Mes (2-(*N*-morpholino)ethanesulfonic acid) and Hepes, were obtained from the Dojindo Laboratory. These reagents were used for buffers of pH 6.0 and 7.4, respectively. The pH values were adjusted to the indicated values by the addition of 0.1 *N* NaOH. Other chemicals were of reagent grade. All aqueous solutions were prepared with deionized glass-distilled water. Samples were prepared in duplicate or triplicate, and the mean of scans was used for analysis.

Preparation of Labeled Erythrocytes and Ghosts Heparinized fresh human blood was obtained from Miyagi Prefectural Red Cross Blood Center. Red blood cells were washed three times with 0.9% NaCl and then three times with 310 ideal milliosmolarity (mosM) sodium phosphate buffer, pH 7.4. Resealed ghosts were prepared by the method of Dodge *et al.*¹⁶⁾ To prepare labeled erythrocytes, a band 3 modification reagent, DIDS, EITC or EMI of 5×10^{-4} M, was added to react with the erythrocytes in 310 mosM phosphate buffer in the dark at 37°C for 3 h. The hematocrit of erythrocyte suspension was of about 45%. Then, the labeled erythrocytes were washed several times with the same buffer to remove unreacted reagents. The modified erythrocytes were washed again with the appropriate pH buffer of 5 mM Mes or Hepes containing 150 mM KCl. These intact and modified erythrocytes were subjected to hemolysis assay and ESR experiments.

To prepare labeled ghosts, the labeled erythrocytes in 310 mosM phosphate buffer were hemolyzed in 20 mosM phosphate buffer, pH 7.4, and washed several times with the same buffer till hemoglobin was free. Then the ghosts were incubated in the isotonic buffer at 37°C for 1 h to reseal them. The resealed ghosts were washed several times with 5 mM Mes or Hepes containing 150 mM KCl. All operations except for the resealing were carried out at about 4°C. The ghosts obtained were used for circular dichroism (CD) experiments.

Hemolysis Assay In the experiment of hypotonic hemolysis, 0.1 ml of packed erythrocyte suspensions in Mes or Hepes buffer containing 150 mM KCl were added to 10 ml of the same buffer containing various amounts of KCl.¹⁷⁾ In the experiment of SDS hemolysis, the packed erythrocyte suspensions in Mes or Hepes were added to the same buffer containing 150 mM KCl and various amounts of SDS. These suspensions were incubated at 37°C for 10 min followed by centrifugation for 10 min at $750 \times g$. From the absorbance of hemoglobin at 415 nm in the supernatant, the percentage of lysis was calculated. The value of 100% hemolysis

was obtained from the supernatant after incubation without KCl as an absorbance reading of *ca.* 2.4 when using 1.0 mm cell.

Preparation of Spin-Labeled Erythrocytes Ten ml of spin labels in chloroform (2×10^{-4} M) were added to a flask and evaporated to form a thin film on the wall of the flask. Then, 5 ml of packed erythrocytes were added to the flask and incubated for 10 min at 37°C. The spin-labeled erythrocytes were separated by centrifugation and were washed several times with 5 mM Mes or Hepes buffer containing 150 mM KCl to remove the unbound spin labels. Finally, the labeled erythrocytes of about 45% hematocrit were used for ESR measurement.

Spectral Measurements The absorption spectra or absorbance were taken by a Hitachi 220 spectrophotometer. The ESR spectra were recorded by a JEOL JES-FE ESR spectrometer with a field intensity of 3290 G. The usual spectrometer settings were X-band; 100 kHz modulation amplitude, 6.3 gauss; microwave power, 4 mW; amplitude, 1×1000 — 2×1000 ; response, 0.03—0.1 s; sweep time, 8 min. At these conditions, no power saturation effect was observed. The spectra were recorded at room temperature. The erythrocyte suspensions prepared and incubated in a similar manner as the hemolysis assay were centrifuged for 10 min at 750 $\times g$, and were packed in a hematocrit capillary tube of 1.1 ml i.d. (Hyland Co.) for measurement. The CD spectra were taken by a Jasco J-400X spectropolarimeter equipped with a data processor. The measurements were carried out at room temperature by the use of a 1 mm cell. The observed CD spectra were expressed in terms of molar ellipticity, $[\theta]$, in $\text{deg} \cdot \text{cm}^2/\text{dmol}$. The protein concentration of erythrocyte ghosts was determined by method of Lowry *et al.*¹⁸⁾ using bovin serum albumin as the standard. The ghost suspension of 0.13 mg proteins/ml corresponded to about 1×10^{-3} M when a mean residue weight of 130 was used to compute the ellipticity of membrane proteins.

Membrane Fluidity When the acyl chain motion of the membranes is isotropic, a rotational correlation time (τ_c) can be estimated from ESR spectra according to

$$\tau_c = 6.5 \times 10^{-10} \Delta H_0 [(h_0/h_{+1})^{1/2} + (h_0/h_{-1})^{1/2} - 2] \quad (1)$$

where ΔH_0 is the peak-to-peak width of the central line, and h_{+1} , h_0 and h_{-1} are the heights of the low-, central- and high-field lines, respectively.^{5c,19,20)}

When the molecular motion is anisotropic, an empirical parameter, the ratio of signal height, h_{+1}/h_0 , has been shown to reflect the fluidity of the lipid matrix. This parameter reflects (i) the rate of axial rotation of the spin-label moiety, (ii) the rate of tumbling motion of the rotational axial and (iii) the angular amplitude of the tumbling motion.²⁰⁾ In the present study, the membrane fluidity near the polar groups was estimated by this parameter for convenience, since it is more sensitive to small changes in fluidity than the conventional order parameter.^{19,21)}

Estimation of Secondary Structure of Proteins in Ghosts The fractions of α -helix, β -sheet, β -turn and random-coil structures in the proteins were estimated by utilizing parameters derived from these four basic secondary structures presented by Brahms and Brahms.²²⁾ The observed CD spectra of erythrocyte ghosts can be expressed as

$$[\theta]_\lambda = f_\alpha [\theta]_{\lambda,\alpha} + f_\beta [\theta]_{\lambda,\beta} + f_{\beta_t} [\theta]_{\lambda,\beta_t} + f_r [\theta]_{\lambda,r} \quad (2)$$

where $[\theta]_\lambda$ is the observed mean residue ellipticity at wavelength λ , the fractions of α -helix, β -sheet, β -turn and random-coil structure are indicated by f_α , f_β , f_{β_t} and f_r , respectively. $[\theta]_{\lambda,\alpha}$, $[\theta]_{\lambda,\beta}$, $[\theta]_{\lambda,\beta_t}$ and $[\theta]_{\lambda,r}$ are values of the basic protein CD intensities for corresponding structures at wavelength λ . The curve fitting analysis for the CD spectra was made in the wavelength region of 200—250 nm at 2 nm intervals under the conditions of $f_\alpha + f_\beta + f_{\beta_t} + f_r = 1$ and all $f > 0$.

The quality of the fit of the reference spectra to the experimental data was evaluated by calculating a fitting parameter, the normalized root mean square deviation (NRMSD).²²⁾ The maximum value of NRMSD was 0.163 in the EMI-modified ghosts at pH 6.0 in the presence of 150 mM KCl. When $0.1 < \text{NRMSD} < 0.2$, the calculated structure is characterized as having secondary structures generally similar to the actual types.

Results

Hypotonic Hemolysis The shape of hemolysis curve reflects the various states of erythrocytes. In the present study, an osmotic gap was performed by various KCl concentrations. The effect of pH on hemolysis was shown in Fig. 1 as a function of KCl concentration. The hypotonic hemolysis seems to be pH-dependent and KCl concentra-

Fig. 1. Hemolysis Percent as a Function of KCl Concentration —●—, pH 6.0; —○—, pH 7.4.

Fig. 2. Effects of Osmotic Gap on ESR Spectra of 16-Doxyl Stearic Acid and 5-Doxyl Stearic Acid in Erythrocyte Membranes

(a) 16-Doxyl stearic acid, pH 6.0: ---, $[\text{KCl}] = 0$; —, $[\text{KCl}] = 150$ mM. (b) 5-Doxyl stearic acid, pH 7.4: ---, $[\text{KCl}] = 0$; —, $[\text{KCl}] = 150$ mM. All are plotted at the same instrument sensitivity so that their spectra intensities can be directly compared. (The spectrum in the presence of 150 mM KCl in (a) was drawn on a scale of half intensity.) The spectra were measured under the spectrometer settings as described in Experimental.

tions of 50% hemolysis, $[\text{KCl}]_{50}$, are about 85 and 75 mM at pH 6.0 and 7.4, respectively. This result indicates that the resistivity of erythrocytes against osmotic change may be higher at the neutral pH region than at the acidic region as previously reported.^{5b,23)}

Effects of Osmotic Gap on the Fluidity of Erythrocyte Membrane To elucidate the change of physicochemical properties of the membrane structure in the process of osmotic hemolysis, ESR spectra were measured under various conditions. Figure 2a depicts the ESR spectra of erythrocytes labeled with 16-doxyl stearic acid at pH 6.0. This spin probe reflects mobility in the internal hydrophobic core of the lipid bilayer. The spectral intensity is increased by the addition of KCl. Since the spectrum corresponds approximately to isotropic motion, an acyl chain motion in the hydrophobic end of the chain is disordered, then the correlation time, τ_c , can be calculated by Eq. 1. The correlation times at pH 6.0 in the presence of 150 mM KCl and in the absence of KCl were calculated as 34.9×10^{-10} and 29.4×10^{-10} s, respectively. In Fig. 3, the correlation times are plotted as a function of KCl concentration.

At pH 7.4, the osmotic stress seems not to induce a change in the correlation time τ_c . In Fig. 3, the changes of the empirical order parameter, h_{+1}/h_0 , plotted as a

Fig. 3. Change of τ_c of 16-Doxyl Stearic Acid and h_{+1}/h_0 of 16-Doxyl Stearic Acid and 5-Doxyl Stearic Acids in Erythrocytes

16-Doxyl stearic acid: τ_c : —▲—, pH 6.0; —△—, pH 7.4; h_{+1}/h_0 : —●—, pH 6.0; —○—, pH 7.4. 5-Doxyl stearic acid: h_{+1}/h_0 : —●—, pH 6.0; —○—, pH 7.4.

function of KCl concentration are also shown. The changes of the parameter for 16-doxyl stearic acid are in fair agreement with those of the correlation times τ_c . These results indicate that at pH 7.4, osmotic change caused by KCl has no influence on the fluidity at the central portion of the membrane bilayer. At pH 6.0, on the other hand, the membrane fluidity depends on the osmotic gap. This reflects a heterogeneity of erythrocyte membrane to pH. However, when liposomes composed of egg phosphatidylcholine (PC) were used instead of erythrocytes, the membrane fluidity was not affected by the osmotic gap (data not shown). Therefore, it can be said that, at pH 6.0, the change in membrane fluidity arises from interactions between lipids and proteins in the membrane and also from the complex formation between the membranous proteins with the cytoplasmic proteins. The fluidity change at the central portion of the bilayer observed at pH 6.0 corresponds to the hemolysis curve at pH 6.0 (Fig. 1).

The ESR spectrum of 5-doxyl stearic acid reflects the fluidity of the surface portion of the membrane (polar end). In Fig. 2b, ESR spectra of erythrocytes labeled with 5-doxyl stearic acid at pH 7.4 are shown. In contrast to the spectrum of 16-doxyl stearic acid, the spectral pattern of 5-doxyl stearic acid shows that molecular motion of the spin label is sufficiently anisotropic. The spectral intensity is slightly increased by the addition of KCl. It is obvious that the parameter h_{+1}/h_0 is not influenced by the osmotic gap both at pH 6.0 and 7.4. The membrane fluidity at the surface portion in the erythrocyte membranes are not influenced by the osmotic stress. Since a reorganization of the membrane components is required for membrane defect or hole formation, it can be said that membrane proteins are responsible for the hypotonic hemolysis.

Conformational Change of Ghost Proteins In order to prove participation of membrane proteins to hypotonic hemolysis, the change in secondary structures of ghost proteins was examined by means of CD under various conditions. The CD spectra of the ghosts in the presence and absence of KCl at pH 6.0 and 7.4 are presented in Fig. 4. A major negative band around 223 nm and another band around 210 nm are observed. By increasing the KCl concentration up to 150 mM, these bands decrease in intensity. The intensity of these bands decreases with

Fig. 4. CD Spectra of Erythrocyte Ghosts under Various Conditions

pH 6.0: —, [KCl]=0; —●—, [KCl]=150 mM; pH 7.4: —, [KCl]=0; —○—, [KCl]=150 mM. Inset: variation of α -helix fraction of membrane proteins as a function of KCl concentration. —●—, pH 6.0; —○—, pH 7.4. The data of percent hemolysis as a function of KCl concentration are from Fig. 1. —●—, pH 6.0; —○—, pH 7.4.

Fig. 5. Hemolysis as a Function of SDS Concentration

—●—, pH 6.0; —○—, pH 7.4.

increasing pH. An isoelliptic point is observed at about 202 nm. The spectral change of the ghosts reflects not only the conformational change of membrane proteins but also the change of associative conditions of the proteins. In this study, an apparent fraction of α -helix content was calculated from the CD data by unconstrained least-square fitting to the reference spectra of Brahms and Brahms.²²⁾ Percent changes of the α -helix content as a function of KCl concentration (f_α -curve) at pH 6.0 and 7.4 are shown in Fig. 4 (inset). The concentrations of KCl responsible for the transition point, $[KCl]_T$, are about 90 mM at pH 6.0 and 64 mM at pH 7.4. As shown in the inset, these values of $[KCl]_T$ are compatible with the values of $[KCl]_{50}$ in the hypotonic hemolysis at a corresponding pH, suggesting that the conformational and/or associative change of membrane proteins should be correlated with the hypotonic hemolysis.

Effect of SDS on the Erythrocyte Membranes It seemed that the hemolysis by detergents presented great contrast to the osmotic hemolysis. Thus, the effect of anionic detergent SDS, a typical detergent, on the erythrocyte membranes was compared with that of hypotonic hemolysis. Figure 5 represents the hemolysis as a function

Fig. 6. Effects of SDS on ESR Spectra of 16-Doxyl Stearic Acid in Erythrocyte Membranes

(a) pH 6.0: —, [SDS] = 0.03 mM; — —, [SDS] = 0.09 mM; — · —, [SDS] = 0.16 mM. (b) pH 7.4: —, [SDS] = 0.01 mM; — —, [SDS] = 0.2 mM. The spectra in the presence of 0.16 mM SDS (a) and 0.2 mM SDS (b) were drawn on a scale of two fold intensity. The spectra were measured under the spectrometer settings as described in Experimental.

Fig. 7. Changes of h_{+1}/h_0 of 16-Doxyl Stearic Acid and 5-Doxyl Stearic Acid in Erythrocyte Membranes as a Function of SDS Concentration

16-Doxyl stearic acid: —●—, pH 6.0; —○—, pH 7.4; 5-doxyl stearic acid: —●—, pH 6.0; —○—, pH 7.4.

of SDS concentration both at pH 6.0 and 7.4. The lysis is also pH-dependent in this system. The values of $[SDS]_{50}$, the concentration that leads to 50% hemolysis, at pH 6.0 and 7.4 are about 0.06 and 0.13 mM, respectively. The rather gradual rise in pH 7.4 may result from the permeability effect corresponding to a prelytic hemoglobin release.²⁴ At pH 6.0, the hemolysis curve is sharp. This is interpreted as a damage of membranes. Such a difference in these two curves should originate from a difference of the interaction of SDS to membrane constituents in these pH regions.

In order to examine a correlation between the hemolysis and the changes in the fluidity of the lipid matrix in the presence of various SDS concentrations, the ESR spectra of 16-doxyl stearic acid were measured at pH 6.0 and 7.4 (Fig. 6). The spectral intensity is decreased by the addition of SDS at both pHs. The change in the parameter h_{+1}/h_0 at pH 6.0 and 7.4 are shown in Fig. 7. As SDS concentration increases, the fluidity increases gradually and reaches maxima in the SDS concentrations of about 0.09 mM at pH 6.0 and 0.16 mM at pH 7.4. These values of SDS con-

Fig. 8. Effects of SDS on ESR Spectra of 5-Doxyl Stearic Acid in Erythrocyte Membranes

(a) pH 6.0: —, [SDS] = 0.03 mM; — —, [SDS] = 0.2 mM; (b) pH 7.4: —, [SDS] = 0.01 mM; — —, [SDS] = 0.2 mM. The spectra in the presence of 0.2 mM SDS were drawn on a scale of two fold intensity. The spectra were measured under the spectrometer settings as described in Experimental.

centration roughly correspond to those of $[SDS]_{50}$ in the hemolysis curve at pH 6.0 and 7.4 (Fig. 5). These results indicate that the hemolysis induced by SDS resulted from disruption of the membranes. However, the fluidity of the membranes decreases with a further increase of SDS concentration (Fig. 7). This may be due to the absorption of SDS on membrane constituents.

The spectral changes of 5-doxyl stearic acid at pH 6.0 and 7.4 are shown in Fig. 8. The spectra show anisotropic motion. The intensity of the spectrum decreased with the concentration of SDS. The values of the parameter h_{+1}/h_0 at pH 6.0 and 7.4 are plotted as a function of SDS concentration (Fig. 7). It should be noticed that the fluidity of membrane surface decreases with increasing SDS concentration, reflecting direct interaction of SDS with phospholipid molecules near the polar head groups. The transitions are observed at the concentrations of SDS about 0.07 mM at pH 6.0 and 0.13 mM at pH 7.4, nearly corresponding to $[SDS]_{50}$. Thus, the hemolysis of erythrocytes induced by SDS parallels the changes of membrane fluidity. These results strongly suggest that the hemolysis by SDS resulted mainly from the physicochemical change of the lipid bilayer.

Further, the treatment of the ghosts with SDS of 0.25 mM at pH 6.0 and 7.4 resulted in little change in CD spectra (data not shown). This suggests that SDS has no effect on the membrane proteins in the present experimental conditions, supporting the view that the disruption of the lipid bilayer is exclusively responsible for the hemolysis by SDS.

Effects of Band 3 Modification on Hypotonic Hemolysis
Band 3 is the most abundant protein of the human erythrocyte membrane, comprising about 30% of the total membrane protein. Rao²⁵ reported a structural change in band 3 with hemolysis. These evidences led us to inquire as to whether an associative or conformational change of band 3 proteins correlates with the hypotonic hemolysis or not. The hemolysis curves of erythrocytes induced by osmotic change at pH 6.0 after modification of band 3 proteins were obtained. By incubation of erythrocyte suspensions with band 3 specific modification reagents, DIDS,^{26,27} EITC,²⁸ and EMI,²⁹ the values of $[KCl]_{50}$ were observed at about 75, 85 and 80 mM in the DIDS-, EITC- and EMI-modified erythrocytes, respectively. The DIDS- and EITC-modified erythrocytes did not show

TABLE I. The Values of $[KCl]_{50}$ and $[KCl]_T$ of Various Erythrocytes and Ghosts

Condition	System	$[KCl]_{50}$ (mM)	$[KCl]_T$ (mM)
pH 6.0	Intact	85	90
	DIDS-modified	75	60
	EITC-modified	85	105
	EMI-modified	80	100
pH 7.4	Intact	75	64
	DIDS-modified	69	80
	EITC-modified	67	70
	EMI-modified	67	80

$[KCl]_{50}$: KCl concentration for 50% hemolysis of erythrocytes. $[KCl]_T$: KCl concentration responsible for the transition of α -helix fraction of ghost proteins.

100% hemolysis in the absence of KCl. Especially, DIDS-modification suppressed maximum lysis until 86%. In this case, a prelytic hemoglobin release was observed at the higher KCl concentration regions (100–120 mM). The lysis of the modified erythrocytes at pH 7.4 was also examined. The value of $[KCl]_{50}$ was shifted to 67 mM for the EITC- and EMI-modified erythrocytes and to 69 mM for the DIDS-modified. The EITC-modified erythrocytes only showed 100% hemolysis. The prelytic hemoglobin release of the DIDS-modified erythrocytes was observed at the KCl concentration region of 80–100 mM. Thus, the modification of band 3 raised the resitivity of erythrocytes against the hypotonic hemolysis in a modification reagent dependent manner. These results are summarized in Table I.

Conformational Change of Modified Ghost Proteins In an attempt to investigate whether the hypotonic hemolysis is related to conformational change of band 3, CD spectra of the DIDS-, EITC- and EMI-modified ghosts were measured. The CD spectra of the modified ghosts in the presence of 150 mM KCl at pH 6.0 and 7.4 are shown in Figs. 9 and 10, respectively. An isoelliptic point can be observed at about 205 nm in both figures, indicating that the influence of the modified reagents themselves on the spectra is negligible. From the results of secondary structure analysis for the CD data of these modified ghosts, the change of α -helix fraction are shown as a function of KCl concentration (f_α -curve) in the inset of Figs. 9 and 10. The values of $[KCl]_T$ of the DIDS-, EITC- and EMI-modified ghosts are about 60, 105 and 100 mM at pH 6.0 and are about 80, 70 and 80 at pH 7.4, respectively (Table I). By comparing f_α -curves in Figs. 9 and 10 with the hemolysis curves of the modified erythrocytes, of which the characteristics are shown in Table I, $[KCl]_T$ of the modified ghosts can be correlated to the respective values of $[KCl]_{50}$. It seems that the values of $[KCl]_{50}$ parallel those of $[KCl]_T$, though their values are not equal to each other. Thus, the spectra of the modified ghosts reflect characteristic change of band 3 protein. The discrepancy between the values of $[KCl]_{50}$ and $[KCl]_T$ may result from the facts that CD spectrum of ghosts is composed also of other membrane proteins and that a conformational change in band 3 is produced during the ghost preparation. From these results, it can be said that band 3 proteins are mainly related to the hypotonic hemolysis.

Fig. 9. CD Spectra of Modified Ghosts at pH 6.0 in the Presence of 150 mM KCl

—, unmodified ghosts; ---, DIDS-modified ghosts; —·—, EITC-modified ghost; ···, EMI-modified ghosts. Inset: variation of α -helix fraction of membrane proteins as a function of KCl concentration. —△—, DIDS modification; —○—, EITC modification; —●—, EMI modification.

Fig. 10. CD Spectra of Modified Ghosts at pH 7.4 in the Presence of 150 mM KCl

—, unmodified ghosts; ---, DIDS-modified ghosts; —·—, EITC-modified ghosts; ···, EMI-modified ghosts. Inset: variation of α -helix fraction of membrane proteins as a function of KCl concentration. —△—, DIDS modification; —○—, EITC modification; —●—, EMI modification.

Discussion

Seeman²⁾ reported that the hypotonic hemolysis proceed in step with the formation of a hole in each erythrocyte membrane. The process of events in hypotonic hemolysis can be described as follows: When erythrocytes were placed directly into hypotonic solution, as a result of rapid entry of water into the erythrocytes, then the erythrocytes from prelytic spherical shapes in seconds or minutes before disrupting and releasing hemoglobin.²⁹⁾ The membrane holes or defects appear about 10 s after the onset of the water entry (rapid hemolysis).^{10,30)} These membrane holes tend to cluster in a region of about 1 μ m in diameter, finally leading to a single wide open hole in each erythrocyte membrane.^{2,10)}

There are at least two possible explanations for the hole formation during hypotonic hemolysis. (i) A change of membrane fluidity or of lipid distribution causes a reorganization of the membrane components, following a formation of membrane hole or defect. (ii) A conformational or associative change of membrane proteins results in a hole formation in the erythrocyte membrane and leads to hemolysis.

In the present study, the hemolysis curves at pH 6.0 and 7.4 are compared with the curves for the membrane fluidity change and f_a -curves to consider which explanation is appropriate for hypotonic hemolysis. From the data obtained in this study, it can be said that the hemolysis induced by detergents is interpreted by (i). A similar example to the detergent case also reported drug-induced hemolysis, which is not due to the denaturation of the membrane proteins.³¹⁾ The drug-induced hemolysis is caused mainly by direct interaction of the drug with erythrocyte membranes. Thus, the drugs disturb the arrangement of phospholipids and the hydrophobic interactions between lipids and proteins as a result of translocation into membranes.

On the other hand, hemolysis in the hypotonic medium is explained by (ii). From the ESR spectra of 5-doxyl stearic acid and 16-doxyl stearic acid, there was little influence of the osmotic gap on membrane fluidity at pH 7.4. At pH 6.0, membrane fluidity at the center of the lipid bilayer is influenced by the osmotic gap (Fig. 3). The change of membrane fluidity near the internal hydrophobic region of the lipid bilayer at pH 6.0 in the hypotonic medium should result from a disturbance in the interaction between lipids and proteins, because the osmotic gap had no influence on the membrane fluidity of egg PC liposomes. The change of membrane fluidity restricted in this region may reflect a change of interactions among the proteins in the cytosol. Therefore, it can be said that the hypotonic hemolysis should be closely related to a change in the property of membrane proteins, corresponding to the case (ii).

The aim of the present study is to clarify the relationship between band 3 protein and hemolysis induced in hypotonic solution. The results shown in Table I revealed that band 3 modification influences hypotonic hemolysis. We can say that the modification exerts two effects on band 3. First, the modification induces an inhibition of water transport by band 3 proteins, resulting in protection against hemolysis (Table I).³²⁾ The second effect is the change of conformational and/or associative properties of band 3 proteins. The fact that the hemolysis curve depends on the modification reagents and pH can be explained by the idea that the binding sites of these inhibitors in band 3 are different from each other. Such changes of band 3 properties should influence the hole formation, leading to the change of the hemolysis curve. The prelytic hemoglobin release or hemolysis less than 86% (pH 6.0) or 95% (pH 7.4) observed in the DIDS-modified erythrocytes should be a typical example of the band 3 modification effect. From these considerations, it can be said that band 3 proteins are responsible for the hypotonic hemolysis. Band 3 proteins seem to play an important role in edge formation, stabilization and expansion during hemolytic hole formation.

The studies concerning the responsibility of band 3 for

several types of hemolysis have been reported.³⁻⁹⁾ In photohemolysis, band 3 itself is a site of photooxidative attack.^{3b,c)} This was supported by the observation that 4,4'-diisothiocyandihydrostilbene-2,2'-disulfonate (H₂DIDS) bound to band 3 inhibited the photohemolysis. In hyperthermia-induced hemolysis, a coupling of band 3 to the critical target was proposed.^{4b)} Beth *et al.*³³⁾ reported a rotational diffusion of band 3 by employing a bifunctional spin-labeling reagent. They showed that hypotonic hemolysis can cause a major increase in the rotational mobility of band 3. On the basis of characterization of pyridoxal 5'-phosphate labeling of band 3 protein, Salhany *et al.*³⁴⁾ proposed that a structural change in band 3 was caused by hypotonic hemolysis and resealing. Very recently, Sauer *et al.*³⁵⁾ suggested that a glycerol-induced hemolysis is also mediated by a process involving band 3. These works present strong evidence in support of band 3 responsibility for hypotonic hemolysis. Further, according to Bakács *et al.*,³⁶⁾ the relative susceptibility of erythrocytes to complement mediated lysis is dependent upon the amount of IgG bound to erythrocytes and is influenced by the distribution of that antibody on the erythrocyte membrane. Hemolysis occurs only when the IgG molecules are arranged in clusters.³⁷⁾ There is other evidence that the surface bound IgG is clustered at band 3 and forms several other membrane protein aggregation sites.³⁸⁾ These aggregation sites are recognized as foreign and antigenic to the immune system. Thus, band 3 is also involved in the immune hemolysis. It seems that the role of band 3 proteins is of fundamental significance also for control of osmotic homeostasis other than cytosolic pH.

The more direct visual evidence of a hole formation with the association of band 3 proteins in the hypotonic medium has been observed by a fluorescence microscopic study. This result and a mechanism of hole formation will be reported in the following paper.

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