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Induced circular dichroism of eosin-5-maleimide bound to band 3 of human erythrocytes

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The inhibition of anion exchange in human erythrocyte membrane by eosin-5-maleimide (EMI) was examined at various pH values. At the pH region between pH 6.0 and 8.0, EMI inhibited the sulfate efflux by about 90%. Further, the interaction of EMI molecules with erythrocyte ghosts was studied by induced circular dichroism (CD). At acidic pH, the EMI-ghost system showed a positive band at about 552 nm and negative bands at about 523 and 505 nm. When the ghosts had been preincubated with *N*-ethylmaleimide, which is a modifying reagent for cysteine residues, the intensity of the CD bands was decreased. On the other hand, when the ghosts had been preincubated with 4,4′-diisothiocyanostilbene-2,2′-disulfonate or eosin-5-isothiocyanate, which inhibit the anion exchange by binding to membrane from outside of the cell, EMI CD was not influenced. These results and the experiment of trypsin digestion, suggested that the induced CD originated from the complexation of EMI molecules with SH groups on band 3 protein. A conventional Gaussian analysis of the CD spectrum at pH 6.0 revealed that the CD spectrum was composed of three components; one of them may be from EMI monomers bound to a cryptic SH group on the 17K fragment and two of them were coupling-type CD bands originating from EMI dimer and/or trimer. The EMI dimer and trimer, which should be located predominantly on the cytoplasmic SH groups on the 43K fragment, were considered as 'stacking' and/or 'head to tail' arrangements. At pH 7.4, the CD spectrum originating from EMI monomers, which showed a negative band at about 560 nm and a positive band at about 535 nm, could be observed.

Introduction

Band 3 protein in erythrocyte membrane is the major integral protein, which catalyzes HCO_3^--Cl^- exchange [1–5]. Kopito and Lodish [6] determined the primary structure of murine band 3 using a complementary DNA clone. Very recently, the complete sequence of human band 3 has been reported by Tanner et al. [7]. The molecular mechanism of the anion transport and the structural organization of this protein, however, have remained enigmatic. Some amino acid residues which participate in the anion transport system have been reported. Histidine [8–10], arginine [11–14], lysine [15], and amino acids that have a carboxyl side chain [16] have been proposed to participate in the anion transport and recognition. Compounds which bind to SH groups have long been known to alter the permeability characteristics of cell membranes. Lukacovic et al. [17] supposed that the SH groups are not responsible for the anion exchange, because both *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzenesulfonate (pCMBS) do not inhibit the anion exchange. However, eosin-5-iodoacetamide which covalently binds to the SH group in the 17K fragment is highly inhibitory to the anion exchange [18,19]. Thus, the role of SH groups in the anion transport is not yet ascertained.

The present paper deals with the inhibitory effect of eosin-5-maleimide (EMI) on sulfate exchange and the characteristics of the binding sites of EMI based on CD experiments.

Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); EITC, eosin-5-isothiocyanate; EMI, eosin-5-maleimide; NEM, *N*-ethylmaleimide; pCMB, *p*-chloromercuribenzoate; pCMBS, *p*-chloromercuribenzenesulfonate.

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Materials and Methods

Materials

EMI was purchased from Molecular Probe Inc. EITC was prepared by the method of Cherry et al. [20].

4,4′-Diisothiocyanostilbene-2,2′-disulfonate (DIDS) was from Dojindo Laboratory. Trypsin was from Sigma Chemical Co. Other chemicals were of analytical reagent grade.

Preparation of ghosts

Fresh human red blood cells (treated with sodium citrate anticoaglant) were obtained from Miyagi Prefectural Red Cross Blood Center. Human erythrocyte ghosts were prepared according to the procedure of Dodge et al. [21]. All operations were carried out at 0–4°C. Leaky ghosts were resealed at various pH by incubating for 30 min at 37°C in isotonic KCl solution buffered with 5 mM sodium citrate (pH 4.0 and 4.5), Mes (pH 5.0–6.75), Hepes (pH 7.0 and 7.5), Taps (pH 8.0 and 8.5), or Caps (pH 9.0 and 9.5) [10].

In this study, two types of EMI-ghost systems were examined. In the first system (the EMI-ghost system), the leaky ghosts were resealed at various pH values, and then incubated with EMI solution of the corresponding pH. In the second system (the EMI-prelabeled ghost system), ghosts were prelabeled with EMI at pH 6.0 by the following methods: The packed erythrocytes (10 ml) were washed twice with 310 mosM phosphate buffer (pH 7.4) and then with the isotonic KCl solution buffered with 5 mM Mes (pH 6.0) (buffer A). The washed erythrocytes were incubated with EMI solution of $2 \cdot 10^{-4}$ M (pH 6.0) for 2 h at 37°C. The erythrocytes were then washed twice with the same buffer to remove unreacted EMI molecules. These EMI-modified erythrocytes were hemolyzed and washed several times with 20 mosM phosphate buffer (pH 7.4). Thus, the EMI-prelabeled leaky ghosts were obtained, and then resealed in the buffer A.

Inside-out ghosts. Inside-out ghosts were prepared by the method of Kondo et al. [22]. The pelleted ghosts obtained by the method of Dodge et al. [21] were suspended in 40 vols. of ice-cold 0.5 mM Tris-HCl (pH 8.0) for 2 h, and washed twice with the same buffer, followed by centrifugation at $22000 \times g$ for 30 min. The pellet was then vesiculated by passing through a 27 gauge needle five times. The vesicles were used after incubation in buffer A for 30 min at 37°C.

Chemical modification of ghosts

The leaky ghosts were resealed in buffer A, and then several SH-modifying reagents, NEM, pCMB, pCMBS and DTNB, were added to react with the ghosts in the same buffer, in the dark at 37°C for 2 h. After this incubation of the ghost suspension containing each reagent, an EMI solution of pH 6.0 was added to the reaction mixture and the mixture was incubated for another 2 h. Initinal concentrations of EMI and ghosts were $2 \cdot 10^{-5}$ M and 0.13 mg protein/ml, respectively. When a mean residue weight of 130 was used to compute the ellipticity of the membrane proteins, the ghost suspension of 0.13 mg proteins/ml corresponds to about $1 \cdot 10^{-3}$ M.

Sulfate efflux

The sulfate efflux was measured as reported previously [9]. Intact erythrocytes were washed twice with isotonic phosphate-buffered KCl (pH 7.4) and once with the appropriate buffers above mentioned. After preincubation of the erythrocytes in the same medium for 2 h at 37°C, the erythrocytes (10% hematocrit) were incubated with [^{35}S]Na_2SO_4 (1 μCi/ml) for 2 h. ^{35}S-labeled erythrocytes were allowed to react with EMI of various concentrations for 2 h at 37°C, and then washed three times with the corresponding ice-cold buffers to remove extracellular isotope and unreacted EMI. Sulfate efflux was started by resuspending the erythrocytes to 2.5% hematocrit in the buffer at 37°C. After 2 h incubation, the isotope content in supernatant was measured after deproteinization with 2% trichloroacetic acid.

EDTA- or NaOH-extraction and trypsin digestion

Ghosts (0.13 mg protein/ml) were reacted with EMI ($2 \cdot 10^{-5}$ M) in the dark at 37°C for 2 h in the buffer A. The reaction mixture was centrifuged and the pellet was washed three times using buffer A containing 0.1% bovine serum albumin. The EMI-modified ghosts were resuspended in 5 mM Taps, 150 mM KCl and 1 mM EDTA (pH 8.0), and incubated for 2 h at 4°C. The ghosts were washed three times with buffer A and adjusted to the first volume.

NaOH extraction. The EMI-modified ghosts were suspended in 10 volumes of ice-cold 0.1 M NaOH and immediately centrifuged at $20000 \times g$ for 30 min to collect the membranes. After washing with the buffer A, the ghost suspension was adjusted to the first volume.

Trypsin digestion. EDTA-extracted ghosts were treated with trypsin according to the procedure of Steck [23]. The pellet of the ghosts was diluted with 1 vol. of phosphate buffer (pH 8.0) and digested with 1 μg/ml of trypsin at 0°C for 1 h. After stopping the trypsin digestion by the addition of 1 mM diisopropylfluorophosphate, the mixture was centrifuged at $22000 \times g$ for 10 min to remove water-soluble fragments. The sediment was washed three times with the buffer A and resuspended in the buffer A to the first volume.

The extent of the trypsin digestion and spectrin depletion were confirmed by the SDS-PAGE (polyacrylamide gel electrophoresis) using Laemmli's system [24].

Spectral measurements

The absorption spectra were taken by a Hitachi 220 spectrophotometer. The CD spectra were measured by a Jasco J-400X spectropolarimeter equipped with a data processor. The measurements were carried out at room temperature by the use of 1 cm cell. All the observed CD and absorption spectra were expressed in terms of

molar ellipticity, $[\theta]$, in $deg \cdot cm^2 \cdot dmol^{-1}$, and molar absorption coefficient, ε, calculated on basis of the initial concentration of EMI.

Results

Inhibition of sulfate efflux by EMI

Previously, we reported the pH-dependence of the inhibitory effect of EITC on the sulfate efflux in erythrocytes [10]. Above pH 8.0, EITC inhibited the sulfate efflux by about 83%. As the pH value decreased, the inhibitory effect became weaker. Below pH 6.5, the sulfate efflux was not apparently inhibited by EITC. In the present study, we first examined the inhibitory effect of EMI on the sulfate efflux at several pH values in order to compare it with that of EITC. Fig. 1 shows the inhibitory effect of EMI. The data reveal that the concentration giving 50% inhibition of the sulfate efflux seems to span from about $2.5 \cdot 10^{-5}$ to $10 \cdot 10^{-5}$ M EMI at the pH values studied. It seems that the maximum inhibition of sulfate exchange by EMI occurs around pH 7. Such pH dependence of the inhibitory effect is very different from that of EITC. These results reflect that the EMI binding site or the environmental character around the binding site is different from that of EITC binding site.

Induced circular dichroism of the EMI-ghost system

Next, the absorption and CD spectra of the EMI-ghost system were examined. The absorption spectrum of EMI shows a band at about 519 nm and a shoulder at about 480 nm at pH 6.0 (Fig. 2A (dotted line)). When EMI was bound to ghost proteins at pH 6.3, the absorption maximum of EMI shifted to 521 nm with decreasing its intensity, and a weak shoulder appeared at about 536 nm (Fig. 2A (solid line)).

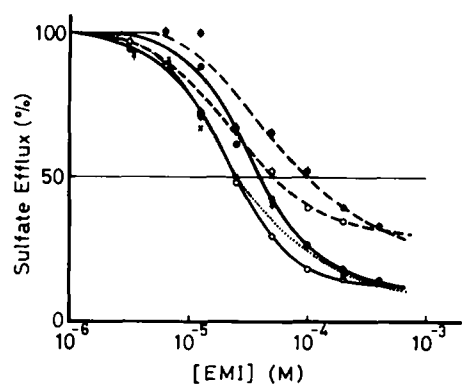

Fig. 1. Inhibitory effect of EMI on sulfate efflux at various pH values (2.5% hematocrit). o---o, pH 6; o——o, pH 6.5; ●——●, pH 7; ×····×, pH 7.5; ●---●, pH 8. Sulfate efflux = (the count in supernatant of EMI-labeled erythrocytes/the count in supernatant of intact erythrocytes) × 100. Each point represents the mean of three samples ± S.D.

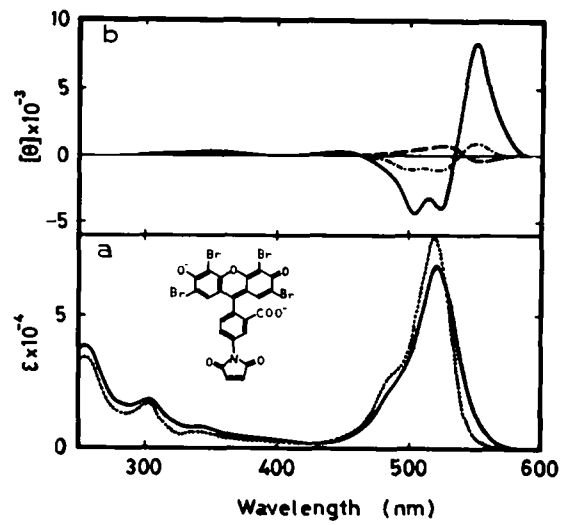

Fig. 2. Circular dichroism and absorption spectra of the EMI – ghost system. Samples were prepared by incubating the EMI-ghost system for 2 h at 37 °C in the MES-KCl buffer, at pH indicated. [ghost] = 0.13 mg proteins/ml (corresponding to $1 \cdot 10^{-3}$ M), [EMI] = $2 \cdot 10^{-5}$ M. ——, EMI-ghost system (pH 6.3); ·····, EMI alone; - - -, EMI-ghost system (pH 7.4); -·-, EMI-ghost system (pH 7.4, resealed at 4 °C).

In this case, a positive CD band at about 552 nm, and two negative bands at about 523 and 505 nm were observed (Fig. 2B (solid line)). Such characteristic CD spectra could be observed at acidic pH. It should be noted that the weak shoulder in the absorption spectrum corresponds to the the wavelength of a crossover point of the CD spectrum. The displacement of the extremum of the CD spectrum is common for electronically forbidden transitions. The CD spectrum results partly from an exciton-coupling form [25,26]. When EMI was reacted with ghosts at pH 7.4, the CD spectrum changed drastically, so the formation of different type of the complex was suggested (Fig. 2B (broken line)). However, this CD spectrum was different from that shown in the previous report. Previously, a CD spectrum similar to the one-dotted broken line in Fig. 2 was observed [27]. This discrepancy was due to the difference in preparation of the resealed ghosts. When resealed ghosts were prepared at pH 7.4 from leaky ghosts by suspending them in 310 mosM phosphate buffer, and allowing them to stand overnight at 4 °C, the CD spectrum shown in one-dotted broken line was observed. On the other hand, the CD spectrum of the EMI-prelabeled ghosts prepared even at pH 6.0 (second system described above experimental) showed a weak negative band at about 560 nm and a positive band at about 535 nm, which was similar to that observed in the EMI-ghost system at pH 7.4 (Fig. 2B (broken line)). Furthermore, when the EMI-prelabeled ghosts were prepared at pH 7.4, a CD spectrum similar to that of the EMI-ghost system at pH 7.4 was observed.

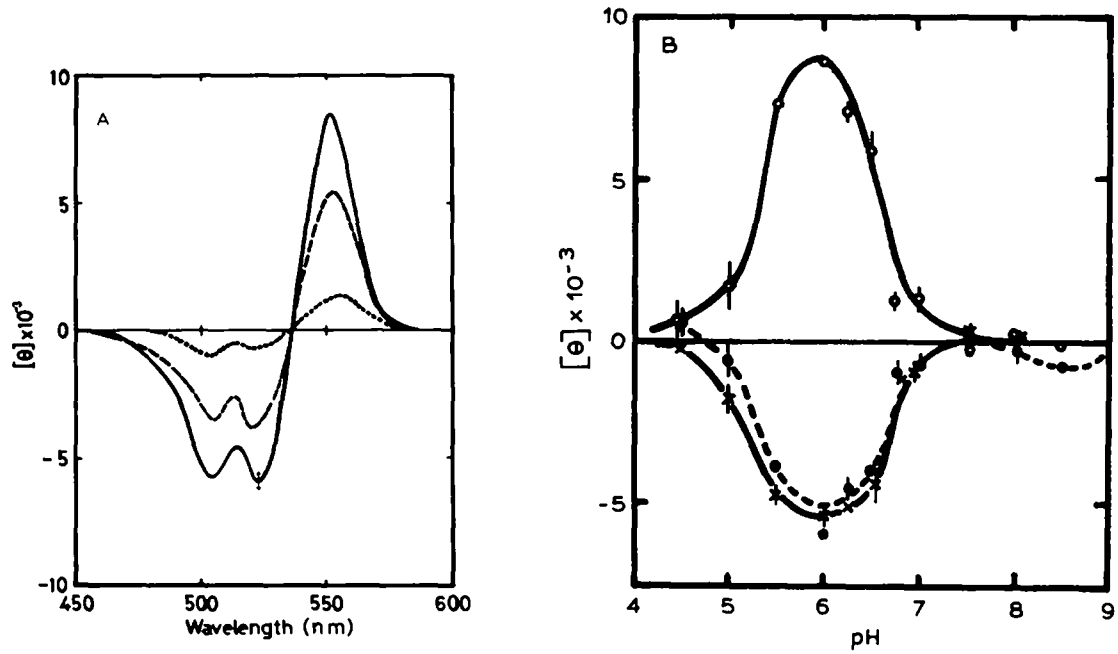

Fig. 3. (A) CD spectra of the EMI-ghost system at various pH. Samples were prepared by incubating the EMI-ghost system for 2 h at 37°C in buffers of the corresponding pH. An arrow indicates a signal/noise ratio. [ghost] = 0.13 mg proteins/ml (corresponding to $1 \cdot 10^{-3}$ M), [EMI] = $2 \cdot 10^{-5}$ M. ·····, pH 5.5; ———, pH 6.0; – – –, pH 6.5. (B) The CD intensities of three peaks at various pH. o———o, 552 nm; ×———×, 523 nm; ●– – –●, 505 nm. Each point represents the mean of at least three samples ± S.D.

The intensity of the CD bands was examined at various pH values (Fig. 3). In this case, the mixture of EMI and ghosts were incubated at various pH. As shown in the inset of Fig. 3, the intensities of the three CD bands were maximum at about pH 6. However, when the EMI-ghost complex was formed at pH 7.4 and then the pH was changed to 6.0, the spectrum showed little change in intensity and its pattern (data not shown). From these results, it can be postulated that at pH 6.0, the resealing of the ghosts may not be complete, so that EMI molecules can react with SH groups on cytoplasmic domains of ghost proteins. On the other hand, at pH 7.4, EMI molecules can react only with surface proteins because of complete resealing. In this case, the EMI CD is consistent with that of the EMI-prelabeled ghosts at pH 6.0 (Fig. 2 (broken line)).

It was reported that human band 3 contains six SH groups [17,23,28]. Three of the SH groups are present in the 43 K cytoplasmic N-terminal fragment of the protein, and the other two are located in the 35K C-terminal fragment. These five SH groups are cytoplasmic and can be competed by pCMBS against NEM and vice versa [29,30]. The sixth cysteine residue is cryptic and extracellular to the stilbene inhibition site [17]. The cryptic sixth residue is located in the membrane-associated 17K fragment.

Next, we examined characteristics of the EMI binding SH groups. It could be expected that inside-out ghosts might have shown intense EMI CD because of an exposure of the cytoplasmic five SH groups. Fig. 4 shows the CD spectra observed in the EMI-inside-out ghost system at pH 7.4 (dotted line) and 6.0 (solid line). In this system, the intensity of the band at pH 6.0 was considerably larger than that of the EMI-ghost system at pH 6.0. At pH 7.4, however, the intensity of the band was reduced. When the pH of this system was changed after incubation from 6.0 to 7.4, the intensity of the CD bands was weakened, but the spectral pattern was not

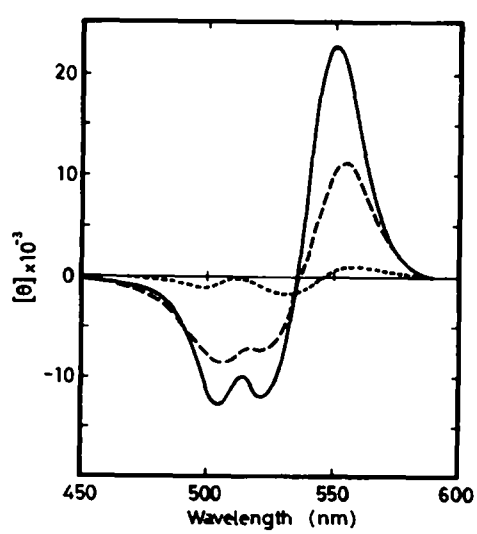

Fig. 4. CD spectra of EMI-inside-out ghost system at various pH. Samples were prepared by incubating the EMI-inside-out ghost system at the corresponding pH. [ghost] = 0.13 mg proteins/ml (corresponding to $1 \cdot 10^{-3}$ M), [EMI] = $2 \cdot 10^{-5}$ M. ———, pH 6.0; ------, pH 7.4; – – –, pH 7.4 (changed from pH 6.0 after incubation).

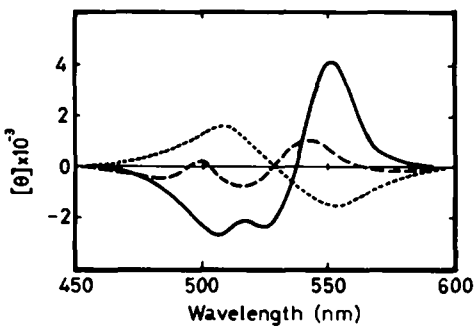

Fig. 5. Effect of pre-treatment of ghosts with pCMB on the CD spectra of the EMI-ghost system at pH 6.0. Samples were prepared by incubating the EMI-pCMB-treated ghost system at pH 6.0. [ghost] = 0.13 mg proteins/ml (corresponding to $1 \cdot 10^{-3}$ M), [EMI] = $2 \cdot 10^{-5}$ M. ———, [pCMB] = $2 \cdot 10^{-5}$ M; – – –, [pCMB] = $6 \cdot 10^{-5}$ M; - - - - -, [pCMB] = $1.2 \cdot 10^{-4}$ M.

changed (Fig. 4 (broken line)). From these results, the CD bands observed in the EMI-ghosts system around pH 6.0 were explained by the EMI molecules bound to the cytoplasmic SH groups. In other words, the exciton-coupling type CD should arise from the EMI molecules bound to the cytoplasmic SH groups. In addition, it can be said that the reaction of EMI with the cytoplasmic SH groups and the conformation around the SH groups are strongly dependent on pH.

Effects of pre-treatment of ghosts with SH-reagents on the EMI CD spectra

The interaction of several SH-modifying reagents with band 3 protein has been reported by many researchers [17,29–39]. NEM molecules bind to the five SH groups in the 35K and 43K fragments faced on the cytoplasmic side without affecting anion and water transport [29,30]. *p*-Chloromercuribenzoate (pCMB) and pCMBS modifies the all six SH groups of band 3 and inhibit red cell water transport without anion inhibitory effect [17,40]. Fig. 5 shows the pre-treatment effects of ghosts with pCMB on the EMI CD spectra at pH 6.0. At the lowest concentration of pCMB, the spectral pattern was not changed, but the intensity of the bands was reduced to one-half of their initial values (Fig. 5 (solid line)). This is attributed to the competitive binding to SH groups from EMI and pCMB. As the amount of pCMB increased further, the spectrum showed a somewhat complicated change (Fig. 5 (broken line)). This may be due to a conformational change of EMI binding sites, which arises from pCMB binding. At the higher concentration of pCMB, a typical exciton-coupling type CD was observed (Fig. 5 (dotted line)). These results indicate that the CD spectra originating from the bound EMI reflect a characteristic of an arrangement of the SH groups on the cytoplasmic domain of band 3. When ghosts were pre-treated with pCMBS, a change similar to the case of pCMB was observed. However, NEM and DTNB (5,5′-dithiobis(2-nitrobenzoic acid)), which is SH-modifying reagent, did not show such effects [41]. In the NEM- or DTNB-pretreated ghost system, the spectra showed complicated changes and the exciton-coupling type CD was not observed. The pre-treatment effect of DTNB seemed to be more complicated. However, further elucidation for the competitive effect of these SH reagents on EMI CD is difficult at present because of the lack of data.

We examined also pre-treatment effects of DIDS on the CD spectrum of the EMI-ghost system at pH 6.0. DIDS had no influence on the CD spectrum of EMI. This may indicate that the cryptic SH group is extracellular to the DIDS binding site or far from the DIDS binding site [35].

EMI binding proteins

In order to decide the EMI binding protein, the change of the CD intensity at 552 nm was examined at various P/D values (the mole ratio of erythrocyte membrane protein to EMI) (Fig. 6). The intensity of the CD band at 552 nm became maximal at P/D value of about 100. The concentration of ghost refers to the amino acid residue concentration, not of membrane or any particular protein. Further, ghost suspension of 0.13 mg protein/ml correspond to the concentration of about $1 \cdot 10^{-3}$ M. On the basis of these values and a conversion factor ($6.0 \cdot 10^{-10}$ mg protein/ghost) of Dodge et al. [21], the number of EMI molecules bound to one erythrocyte can be estimated to be about $2.8 \cdot 10^{7}$. This is larger than the number of SH groups on band 3 per ghost, i.e., $6 \cdot 10^{6}$ [17,23,28,42]. The residual EMI molecules should bind to proteins other than band 3.

For further characterization of EMI binding protein at pH 6.0, ghost proteins were solubilized and analyzed

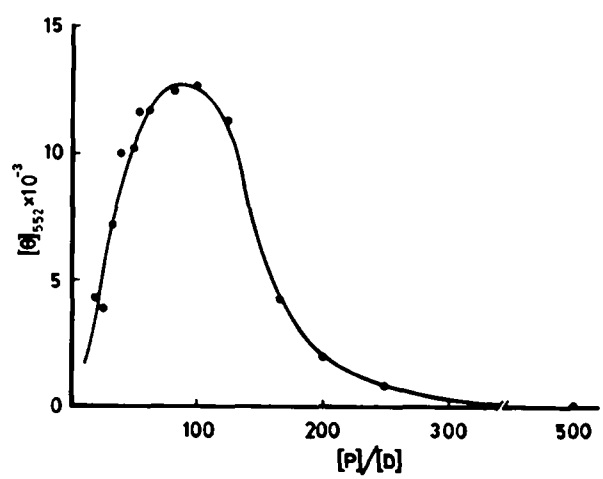

Fig. 6. Variation of CD intensity in the EMI-ghost system at various P/D values (the mole ratio of erythrocyte protein to EMI). Ellipticity was measured at 552 nm after incubation of the EMI-ghost system for 2 h at 37 °C in the Mes-KCl buffer (pH 6.0).

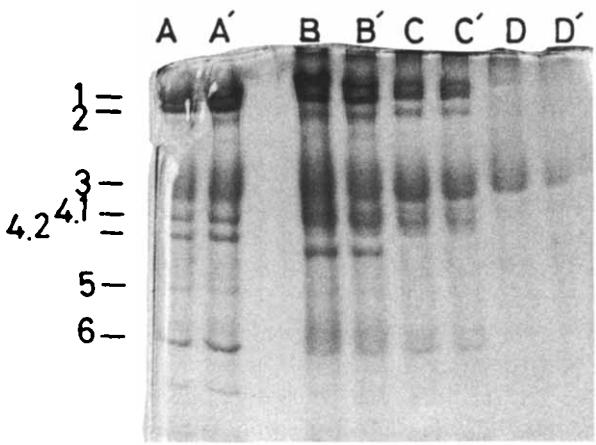

Fig. 7. SDS-PAGE of EMI-labelled ghosts. Lanes A and A', control; lanes B and B', treated with EMI; lanes C and C', EDTA-treated ghosts (EMI treated); lanes D and D', NaOH-extracted ghosts (EMI treated).

by SDS-PAGE. Fig. 7 shows the protein pattern at various stages of ghost treatments. Lanes B and B' are of EMI-bound ghosts. In these lanes EMI colour could be observed in the bands 1, 3, 4.2 and 4.5 regions, indicating reactions between EMI molecules and these proteins. In this connection, protein 4.5 contains SH groups [43]. Lanes C(C') and D(D') were obtained from ghosts after removing peripheral protein fragments by the extraction with EDTA and NaOH, respectively. It was observed that EMI-EDTA- or NaOH-treated ghost system gave the similar EMI CD to that of lane B(B') at pH 6.0. Thus, it can be said that the EMI molecules bound to the SH groups in band 3 exhibit the CD of the EMI-ghost system at pH 6.0. In order to decide the EMI binding region in band 3 protein which shows the CD spectrum at pH 6.0, we examined the effects of the cytoskeletal and peripheral proteins and tryptic digestion on the CD of the EMI-ghost system. It was observed that the depleted preparation of the cytoskeletal and peripheral proteins had little effect on the EMI CD. When the EMI-modified ghosts were digested by trypsin into two fragments, which were water-soluble 43K fragment and membrane-associated 52K domain, both fragments showed the EMI CD (Fig. 8).

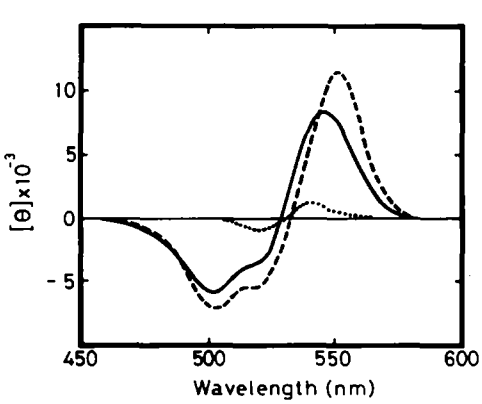

Fig. 8. CD spectra of the EMI-ghost systems and the EMI bound 43K fragment at pH 6.0. – – –, EMI-ghost system; ·····, EMI-ghost system prepared after trypsin digestion to the EMI-ghost system; ———, EMI-43K fragment prepared from soluble fraction after trypsin digestion to the EMI-ghost system.

From Fig. 8, it can be said that the characteristic CD spectrum observed for the EMI-ghost system at pH 6.0 originates exclusively from EMI molecules bound to the 43K fragment, though EMI molecules also bound to the proteins besides band 3. The wavelength of the positive band and the intensity of the CD bands of the 43K fragment were different from those of the EMI-ghost system. These differences may come from an alteration of EMI concentration and/or a disruption by lipid membranes.

Discussion

Binding of EMI to the cryptic SH group

Although EMI molecules inhibit the sulfate efflux as well as EITC, the EMI binding to the erythrocytes is more complex than that of EITC.

Nigg et al. [18] found 100% inhibition of sulfate exchange by binding of EMI at $1.1 \cdot 10^{-6}$ molecules per erythrocyte. This value agrees well with that of inhibitor such as DIDS, leading to the conclusion that EMI molecules bind to band 3 in the stoichiometry of 1 : 1. In this study, 90% inhibition was observed. From Fig. 1, the $2 \cdot 10^{-5}$ M EMI produces 10–25% inhibition of sulfate efflux, which is due to the EMI binding to the cryptic SH group. Solomon et al. [35] reported that the cryptic SH group is the key to their aqueous pore model for membrane transport of anions, cations, and small nonelectrolytes. Nevertheless, the binding of pCMB or pCMBS to the cryptic SH does not relate directly to the anion exchange. These results indicate that the surface-labeling properties of EMI are different from those of pCMB and pCMBS.

Previously, it was observed that in the EITC-ghost system the induced CD was pH dependent and that the inhibitory effect of EITC on the anion exchange was proportional to the intensity of the induced EITC CD bands [10]. In the EMI-erythrocyte system, however, the inhibitory effect of EMI on the sulfate efflux is maximum around pH 7. This is due to the fact that the pH dependence of the binding site of EMI and/or the conformation around the binding site, which is related to the condition of ionizable groups, are different from that of EITC. The inhibitory effect of EMI is independent of the intensity of the EMI CD. This result comes from the fact that EMI molecules can react with cryptic SH and/or cytoplasmic SH groups on band 3.

The spectral pattern of the EMI CD at pH 7.4 (Fig. 2 (broken line)) seems to be similar to that of the EITC CD [27]. This induced CD arises from an asymmetric conformation of the bound EMI (intrinsic optical activ-

ity) and/or an interaction of the bound EMI with an asymmetric environment (extrinsic optical activity). This indicates that the induced CD of EMI is caused primarily by the twisted conformation of the EMI bound to the cryptic SH. A rotameric state between xanthene and phenyl chromophores results in an inherent chirality in EMI. From the same consideration as the EITC CD, it can be said that the cryptic SH group on the 17K fragment, has a higher affinity for right-handed conformation of EMI molecules [27]. The chiral conformation of the bound EMI should be determined by the anchoring amino groups besides the SH group, which are positively charged residues. For the anion transport, these positive residues (functional amino groups) may play an important role. Since the EMI molecule bound to the cryptic SH is twisted in a right-handed sense, the functional amino groups and the SH group anchoring the EMI molecule may be a left-handed sense. (In the previous paper, we said that EMI bound to band 3 may be of left-handed conformation [27]. This presumption was obtained on the basis of the result observed under the different conditions from the present study as described in this paper.) These anchoring amino residues would satisfy a common requirement for a 'three point attachment model' [44]. The involvement of the cryptic SH groups in the anion, water or other solute transport has been reported by many authors [35,45–49]. The conformation around the cryptic SH group seems to be related to the condition of the burried ionizable groups. It is, however, difficult to give a more definite interpretation on a role of the cryptic SH group in the anion transport at this time.

Interaction of EMI with cytoplasmic SH groups

Although a high concentration of DTNB (5 mM) inhibits the uptake of phosphate and water flow into erythrocytes, the DTNB binding site does not react with either NEM or pCMBS [31,37,41]. The inhibition by DTNB is not due to reaction with an essential sulfhydryl group [41]. In this study, the different pre-treatment effects of pCMB and pCMBS on the EMI CD from those of NEM and DTNB were observed. This indicates that the competitive EMI binding is dependent upon the kind of SH reagents.

The CD spectrum of the EMI-prelabeled ghosts, which was not pH dependent, was different from that of the EMI-ghost system at pH 6.0. The binding site of EMI in the EMI-prelabeled ghost system or in the EMI-ghost system at pH 7.4 should be only cryptic SH group. On the other hand, in the EMI-ghost system at pH 6.0, EMI molecules can permeate into membrane and react also with SH groups in the cytoplasm-facing components of the membrane. Two explanations can be considered for the permeation: (i) at pH 6.0, EMI molecules are sufficiently protonated so that they can permeate into ghost or erythrocytes; (ii) at pH 6.0, the ghost resealing is not complete so that the ghosts are permeable to the probe. The latter is plausible, because the CD of the EMI-prelabeled ghost system is independent of pH, indicating that EMI cannot permeate into erythrocytes at pH 6.0. Lepke and Passow [50] found that the yield of resealed ghosts at pH 7.2 showed a maximum after hemolysis at pH 6. They said ionizable groups play an important role in the reconstitution of the membrane after hypotonic hemolysis. It seems that during resealing at pH 6 in this study, buried ionizable groups become exposed to the ambient medium, affecting molecular conformation. This results in the change of the hydrophobic interactions between membrane proteins and lipids [50]. Thus, the resealing at pH 6.0 may be incomplete.

A fraction of the leaky ghosts, f, was calculated on the assumption that the intensity of the EMI CD at pH 6.0 is proportional to the fraction. Further, if the inside-out ghosts used in this study contain 50% of rightside-out ghosts at pH 8.0 [22], it can be assumed that cytoplasm-facing components to which EMI molecules can bind increase by $0.5 + f$ at pH 6.0. The intensity of the EMI CD in the inside-out ghost system showed an increase of 2.7-times (Figs. 3 and 4). Thus, f is 0.29. This value is close to the percentage of leaky ghosts (Type III) presented Lepke and Passow [50] when the erythrocytes are hemolysed at pH 7.4.

Thus, it can be said that in the ghost system the specificity of labeling by EMI at different pH values is markedly different both quantitatively and qualitatively from those shown by Nigg et al. [18] in intact erythrocytes.

Configurational characterization of the cytoplasmic SH groups

As shown in Fig. 2A, at pH 6.0 the absorption spectrum of the EMI-ghost system reveals a weak shoulder at 536 nm. Furthermore, the CD spectrum seems to contain bands of equal intensity but of opposite sign, and a crossover point is close to the wavelength of the shoulder. These results suggest that the bound EMI molecules are not randomly distributed among the cytoplasmic SH groups, but occupy sites adjacent to one another and stack with each other, resulting in the exciton-coupling CD. That is to say, the CD of the EMI-ghost system at pH 6.0 may arise from dimeric or more aggregated EMI molecules. A Gaussian analysis, as shown in Fig. 9, for the CD curve of the EMI-ghost system at pH 6.0 suggests the presence of two couples of CD bands.

One couple consists of a positive band at 552 nm and a negative band at 522 nm (Fig. 9 (broken line)), the other is a couple of a positive band at 521 nm and a negative band at 509 nm (Fig. 9 (one-dotted broken line)). It should be noted that the former couple shows more intense bands. Besides the two coupled CD bands,

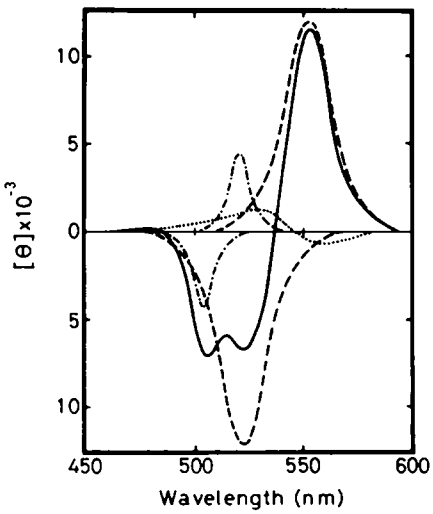

Fig. 9. CD spectrum of the EMI-ghost system at pH 6.0 (———), and its resolution into Gaussian bands (– – – and –·–). ·····, uncoupling CD band.

uncoupling type CD was revealed, which shows a minimum at 558 nm and a maximum at 531 nm (Fig. 9 (dotted line)). The uncoupling type CD is in fair agreement with the spectrum observed at pH 7.4 (Fig. 2 (broken line)). This indicates EMI molecules bound to the cryptic SH group contribute also to the CD at pH 6.0.

The electronic structure and spectrum of EMI seems to be similar to that of eosin Y [51]. The first intense band at 519 nm originates from the local excitation of the xanthene ring. The polarization direction of this transition is along the long-axis and of symmetry *B*. The shoulder at 536 nm in the absorption spectrum of the EMI-ghost system is closer to the crossover point of the CD spectrum. The CD spectrum seems to be coupling type. These results suggest a dipole–dipole interaction of the long-axis transition moments of EMI molecules bound to the cytoplasmic SH groups. According to the theory of the exciton-coupling [52], the coupling at the shorter wavelength region originates from a skew dimer, in which the xanthene rings stack with each other. On the other hand, the coupling at the longer wavelength region arises from a disymmetric alignment of the dimeric or trimeric EMI in a 'head to tail' arrangement of the transition dipole moment along the long-axis. In this case, EMI molecules may be trimeric, because the magnitude of the coupling bands is more intense than that at the shorter wavelength region and all of the three SH groups on the 43K fragment are occupied by EMI molecules. Since the longer wavelength bands of the coupling are positive in the two cases, the configuration of the EMI molecules is *R*-chirality. Thus, we can image that the EMI molecules bound to the cytoplasmic SH groups are shown aligned with the long axis of the molecule represented in both in-line and parallel profiles. The spatial arrangement of the SH groups on the 43K fragment may be a helical array, which forms a cluster of SH groups [53].

On the other hand, the EMI molecules bound to the 35K fragment are not in the dimeric form, because the EMI-trypsin-treated ghost system showed a small CD. It seems that each SH group on the 43K and 35K fragments plays a different role. The EMI CD can be usefully employed in investigating the role of SH groups on the 43K fragment. However, at present in the absence of information on the role of the cytoplasmic SH groups, the above consideration does not allow us to discuss more detail.

From the present study, it can be said that EMI CD can provide characteristics of the cryptic SH and the cytoplasmic SH groups in relation to the anion transport and the protein structure, respectively. Studies along this line are in progress.

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