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Amino acid residues complexed with eosin 5-isothiocyanate in band 3 protein of the human erythrocyte

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The amino-acid residues of band 3 protein taking part in the ionic interaction with an anion-transport inhibitor, eosin 5-isothiocyanate (EITC), were determined by pH titration. The plots of the absorbance of EITC-ghost system against pH reveal five equilibria, at pH 3.7, 6.4, 8.0, 11.0 and 13.1. Since the three equilibria, 3.7, 8.0 and 11.0, are representative of the EITC molecule, the others, 6.4 and 13.1, may be due to the interaction of EITC molecules with histidine and arginine residues, respectively. The same experiment using a reconstituted system of band 3-lipid vesicles gave results in good agreement with the EITC-ghost system. The intensity of the induced CD band at 530 nm of EITC molecules bound to ghosts was decreased by preincubation with arginine-specific reagents, phenylglyoxal and 1,2-cyclohexanedione, or histidine-specific reagents, diethylpyrocarbonate (DEPC) and *p*-diazobenzene sulfonate. The repression effects by these chemical modifiers were evaluated by measuring the concentrations which elicit 50% reduction. The histidine-specific reagents repressed the CD of EITC more effectively than the arginine-specific reagents. Furthermore, it was found that DEPC effectively inhibited the sulfate efflux from intact erythrocytes. These results suggest that the histidine residues participate in the anion-transport system of human red cells.

Introduction

Band 3 protein in human erythrocyte membrane is the major integral protein, which has an oligosaccharide chain at the extracellular portion of the peptide [1–4]. It is well known that band 3 is responsible for anion exchange across the erythrocyte membrane. Its molecular weight is about 95 000 and band 3 exists as a dimer of its subunits [5–8].

Much information about anion transport in erythrocytes has been obtained by using stilbenedisulfonate derivatives, which are strong inhibitors of anion transport from the external side of the membrane [9–11]. Other inhibitors, such as 2-[*N*-(4-azido-2-nitrophenylamino)]ethanesulfonate (NAP-taurine) [12,13], pyridoxal 5-phosphate [14] and eosin derivatives [15], have also been used for the investigation of the transport mechanism. Eosin derivatives are very interesting inhibitors, because their inhibitory action is strongly dependent on substituent groups, whereas stilbene derivatives do not show such a dependency [15]. However, the details of the interaction of eosin derivatives with band 3 have not yet been well established.

Abbreviations: $C_{12}E_9$, nonaethyleneglycol *n*-dodecylether; DEPC, diethylpyrocarbonate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EITC, eosine 5-isothiocyanate; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; NAP-taurine, 2-[*N*-(4-azido-2-nitrophenylamino)]ethanesulfonate; SDS, sodium dodecyl sulfate.

It has been proposed that in band 3 there are two kinds of binding site for the transport inhibitors [16]. One is a 'substrate site', which is the binding site of stilbenedisulfonate derivatives such as DIDS, and the other is called a 'modifier site', being the binding site of NAP-taurine. However, characterization of these binding sites has not been carried out. Cabantchik et al. [3] proposed that the stilbene disulfonate derivatives form three-point attachment features in the anion transport channel of band 3, and that they should be anchored by the cationic multipolar and nucleophilic residues in the anion recognition site. In the previous study, we compared the binding sites of eosin 5-isothiocyanate with that of DIDS using the induced circular dichroism method [17]. On the basis of the three-point attachment model, we have proposed that the binding sites for EITC and DIDS are different from each other. The present paper deals with the amino-acid residues participating in the binding with EITC molecules.

Materials and Methods

Materials

EITC was prepared by bromination of fluorescein isothiocyanate according to the procedure of Cherry et al. [18]. *p*-Diazobenzenesulfonic acid was synthesized by Frankel-Conrat [19]. Phenylglyoxal was obtained from Aldrich Chemical Co. 1,2-Cyclohexanedione was from Nakarai Chemicals. DEPC was from Tokyo Kasei Kogyo Co. $C_{12}E_9$ was presented from Nikko Chemical Co., Ltd. Egg-yolk phosphatidylcholine was from Sigma Chemical Co. Other chemicals were of analytical reagent grade.

Preparation of ghosts

Fresh human red blood cells (treated with sodium citrate anticoagulant) were obtained from Miyagi Prefectural Red Cross Blood Center. Human erythrocyte ghosts were prepared according to the procedure of Dodge et al. [20]. All operations were carried out at 0–4°C. Leaky ghosts were resealed in 310 mosM phosphate buffer (pH 7.4).

Ghosts labeled with EITC were prepared by following methods. The packed cells (10 ml) washed three times with isotonic phosphate buffer were incubated with EITC solution of $1 \cdot 10^{-5}$ M for 3 h at 37°C. The cells were then washed twice with the buffer to remove unreacted EITC molecules. The modified cells were hemolyzed and washed several times with 20 mosM phosphate buffer (pH 7.4) to obtain EITC-labeled ghosts.

Band 3 purification and reconstitution in liposomes

Band 3 was purified by the procedure of Lieberman and Reithmeier [21]. Band 3 was identified by SDS-polyacrylamide gel electrophoresis system of Laemmli [22]. The protein fraction eluted with 2-mercaptoethanol moved as one band corresponding to band 3 on SDS-polyacrylamide gel electrophoresis (data not shown). $C_{12}E_9$ was removed from the band 3 preparations by incubating with Bio-Beads SM2 overnight at 4°C. Band 3 fraction was incorporated into liposomes by a cholate dialysis method [23].

pH titration

Ghost (130 μg protein/ml) or liposome suspensions (32.5 μg protein/ml) were reacted with EITC ($2 \cdot 10^{-5}$ M) in the dark at 37°C for 2 h in 310 mosM phosphate buffer (pH 7.4). The pH of samples was adjusted by adding HCl or NaOH. The samples were then subjected to spectroscopic measurement.

Chemical modification of ghosts

Modificative reagents for amino-acid residues, except for 1,2-cyclohexanedione, were reacted with ghosts in 310 mosM phosphate buffer (pH 7.4) in the dark at 37°C for 2 h. 1,2-Cyclohexanedione was incubated with ghosts in sodium phosphate buffer containing sodium borate (150 mosM sodium borate/160 mosM sodium phosphate) (pH 8.0). After the preincubation, EITC ($2 \cdot 10^{-5}$ M) were added to the reaction mixture, this being followed by further incubation for 2 h.

Sulfate transport

Intact erythrocytes were washed twice with 310 mosM phosphate-buffered saline (pH 7.4) and once with phosphate buffer (pH 6.3). After preincubation of the cells in phosphate buffer (pH 6.3) for 2 h at 37°C, the cells (2.5% hematocrit) were incubated with sodium [^{35}S]sulfate (1 μCi/ml) for 2 h. The labelled cells were washed three times

with ice-cold phosphate buffer and the cells were resuspended in pre-warmed medium (2.5% hematocrit). After 1 h incubation at 37°C, sulfate efflux was measured. The isotope content in supernatant was measured after deproteinization with 2% trichloroacetic acid. To measure any effect of the chemical modifier, DEPC, on sulfate efflux, the labeled cells were mixed with DEPC solution and incubated for 10 min. The DEPC-modified cells were washed three times with ice-cold phosphate buffer and the efflux was measured as described above.

Spectral measurements

The absorption and circular dichroism (CD) spectra were taken with a Hitachi 220 spectrophotometer and a Jasco J-400X spectropolarimeter equipped with a data processor, respectively. The difference absorption spectra were measured using parallel cells of 5 mm pathlength. The measurements were carried out at room temperature.

The molar extinction coefficient, ε, was calculated on the basis of the initial concentration of EITC. The concentrations of protein in ghosts or liposomes were determined by the method of Lowry et al. [24] using bovine serum albumin as standard. When a mean residue weight of 130 was used to compute the ellipticity of the membrane proteins at 223 nm, the ghosts at $1 \cdot 10^{-3}$ M in 20 mosM phosphate buffer (pH 7.4) correspond to the concentration of about 0.13 mg protein per ml.

Results

pH titration of EITC-ghost system

The absorption spectrum of EITC shows a band at 522 nm with a shoulder at about 485 nm in 310 mosM sodium phosphate buffer (pH 7.4). Upon covalent binding of EITC to band 3, the absorption band shifts to 526 nm accompanied with the decrease in its intensity [25]. This reflects an interaction of EITC molecules with band 3 proteins. EITC molecules bound to band 3 are only responsible for the spectral change [17]. When the external pH of the EITC-ghost suspensions was changed, the band shifted to the short wavelength side with a fluctuation in intensity. Fig. 1 shows the changes in the intensity of the absorption peak plotted against pH in acidic region. Although the

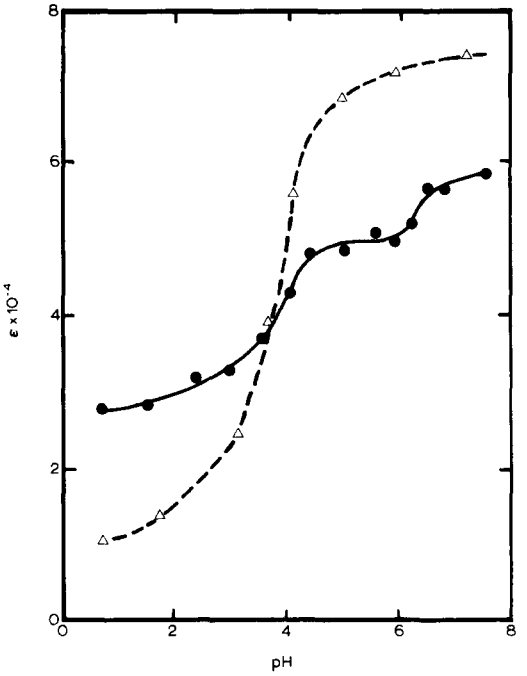

Fig. 1. pH titrations of EITC (△) and EITC-ghost (●) systems in the acidic region. A mixture of EITC and ghosts was incubated for 2 h at 37°C in the dark, and then the pH was adjusted using HCl. The absorption intensities were measured at maximum wavelengths. [EITC] = $2 \cdot 10^{-5}$ M; [ghost] = $1 \cdot 10^{-3}$ M (130 μg protein/ml).

pK value of EITC molecule in acidic region is about 3.7, the EITC molecules bound to ghost membranes gave a different pH-equilibrium profile showing pK values of about 3.7 and 6.4. Below pH 3.7, the hydroxy group of EITC is un-ionized. When the absorbance change of the ghost suspension was measured at 280 nm no gross changes in the turbidity of the ghosts were observed between pH 5 and 9. Thus, the equilibrium at pH 6.4 is not due to spurious absorbance changes. The equilibrium at pH 6.4 may reflect an electrostatic interaction between EITC molecules and amino-acid residues at the anion recognition sites in band 3. Since the pK value of the imidazole group of histidine is 6.0, a histidine residue seems to be responsible for the interaction of EITC in the anion recognition site.

Since the absorption spectra of the EITC-ghost system in alkaline pH region were slightly affected by variation of pH, difference spectra between

EITC and EITC-ghost system were measured. Fig. 2 illustrates the intensity change of the negative maximum in the difference spectra as a function of pH. The typical difference spectra are shown in the inset of Fig. 2. Three inflection points can be observed, at around pH 8.0, 11.0 and 13.1. From the titration curve of EITC (dotted line), the pK value of EITC molecule in alkaline region can be estimated to be 11.0. This corresponds to the dissociation of 6′-(or 3′-) hydroxy group in the xanthene skelton. Upon alkalinization of ghost suspensions, the absorbance at 280 nm increased progressively. This indicates an unfolding of membrane proteins. However, no inflection point could be observed at around pH 13. Furthermore, covalent damage between EITC molecules and band 3 was not revealed after exposure of the EITC-ghost system to high pH (pH 13.5). Therefore, the equilibrium at pH 13.1 may reflect interaction of EITC molecules with guanizino groups of arginine residues (pK = 12.5) [26]. The equilibrium at pH 8.0 comes from EITC molecules, but in the EITC-ghost system also a contribution of an weak interaction of EITC with lysine residues (pK = 10.4) or cysteine (pK = 8.0) of band 3 cannot be discounted.

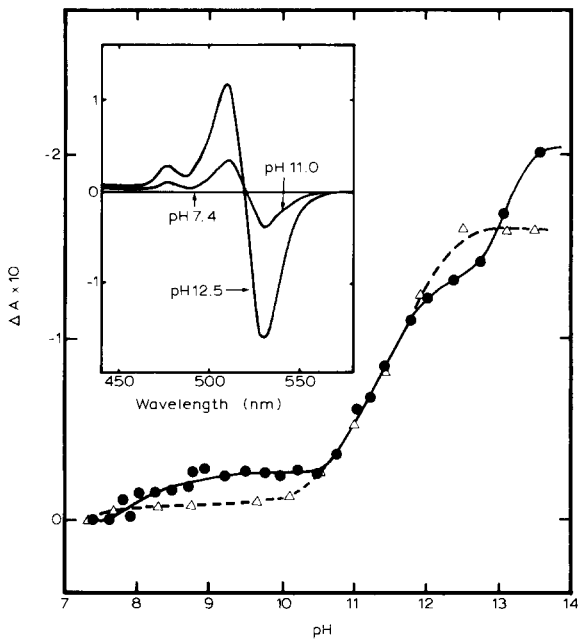

Fig. 2. pH titrations of EITC (△) and EITC-ghost (●) systems in the alkaline region. A mixture of EITC and ghosts was incubated for 2 h at 37°C in the dark, and then the pH was adjusted using NaOH. The intensities were measured at the negative peak in the difference spectra at various pH levels. [EITC] = $2 \cdot 10^{-5}$ M; [ghost] = $1 \cdot 10^{-3}$ M (130 μg protein/ml). Inset: Typical difference spectra between EITC and the EITC-ghost system at alkaline pH. (The difference at pH 7.4 is regarded as standard.)

In confirmation of the conclusion that EITC molecules bind exclusively to band 3 and that the new equilibrium points result from EITC-band 3 complexes, the same experiment was carried out using the isolated band 3 proteins incorporated into egg-yolk phosphatidylcholine liposomes. The labelling of EITC was done after the incorporation of band 3 into liposomes. By use of inside-out ghosts, it had been observed that only a few EITC molecules bind to cytoplasmic domains of band 3. Therefore, one can neglect influences of EITC molecules bound to cytoplasmic domains in the EITC-band 3 liposome system. The intensity changes of the peaks in the absorption and difference spectra at various pH levels are shown in Figs. 3a and b, respectively. New equilibria can be observed at pH 6.6 and 12.5, corresponding to 6.4 and 13.1 in the EITC-ghost system, respectively.

The solid lines in Fig. 3 as well as in Figs. 1 and 2 were determined so as to give the smallest mean-square error between the observed and theoretical values. The best fits were obtained when the two pK values were 3.6 and 6.6 in Fig. 3a, and 10.4 and 12.5 in Fig. 3b. At pH < 4, absorption intensities of the EITC-ghost systems were apparently higher than those of the EITC-band 3 liposome system. This is due to an increase in the turbidity of ghost suspensions in low acidic regions.

Effects of modification of amino-acid residues on induced CD of EITC

It is suggested that the arginine and histidine residues may participate in the interaction of EITC molecules with band 3 proteins. We examined the effects of chemical modifications of these residues on induced CD of the EITC-ghost system.

The EITC-ghost system shows an induced CD spectrum with a positive band at about 530 nm and negative bands below 450 nm at the wavelength in the region corresponding to the absorp-

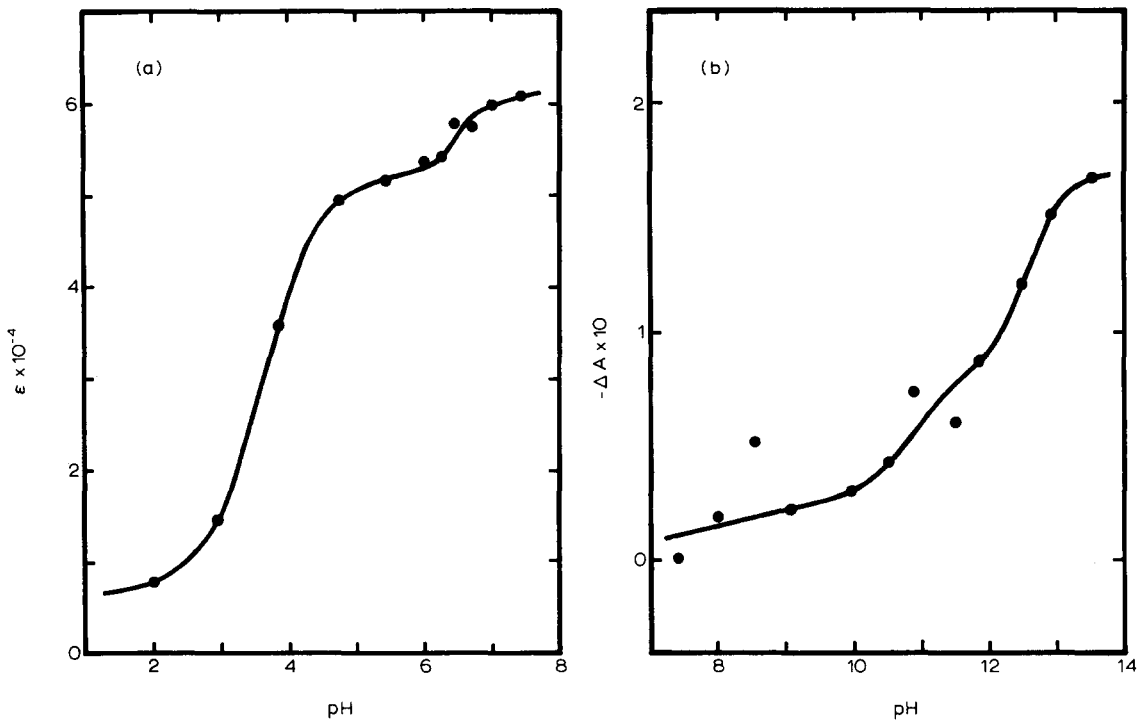

Fig. 3. pH titrations of EITC in reconstituted band 3-liposome system. (a) Plots of maximum peak intensity of absorption spectra in acidic region. The solid line is the sum of the two theoretical curves deriving from $\varepsilon = \varepsilon'/(1+10^{pK-pH})$. The values of ε' and pK were determined so as to give the smallest mean-square error between the observed (ε) and theoretical values. (b) Plots of negative maximum peak intensity of difference spectra in alkaline region. The solid line is drawn in a similar manner as in (A). Mixture of EITC and reconstituted band 3-liposome system was incubated for 2 h at 37°C in the dark, and then pH was adjusted by HCl or NaOH. [EITC] = $2 \cdot 10^{-5}$ M; [band 3] = $2.5 \cdot 10^{-4}$ M (32.5 μg protein/ml).

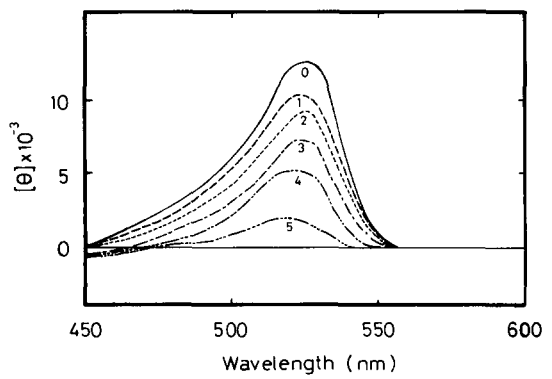

Fig. 4. Effect of the arginine-specific reagent, phenylglyoxal, on the CD spectra of the EITC-ghost system. Mixture of phenylglyoxal and ghosts was incubated for 2 h at 37°C in the dark, and then EITC was added to the mixture and incubated for 2 h. Curves 1–5 were recorded after adding $1 \cdot 10^{-5}$, $2 \cdot 10^{-5}$, $5 \cdot 10^{-5}$, $1 \cdot 10^{-4}$, $2 \cdot 10^{-4}$ M phenylglyoxal, respectively. [EITC] = $1 \cdot 10^{-5}$ M; [ghost] = $1 \cdot 10^{-3}$ M (130 μg protein/ml).

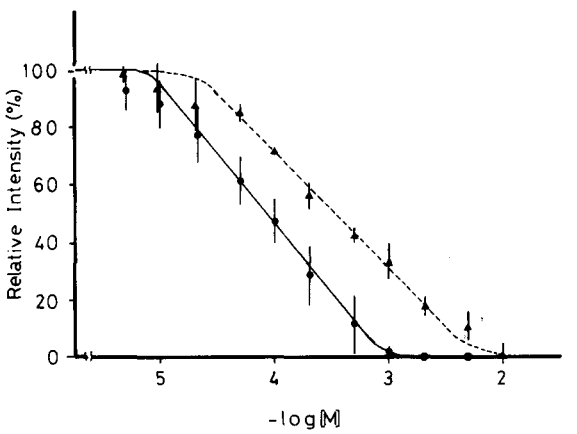

Fig. 5. Effect of arginine-specific reagents on the CD intensity of the EITC-ghost system. ●, phenylglyoxal; ▲, 1,2-cyclohexanedione. The plots are relative CD intensities at 530 nm in the presence of arginine-specific reagents. Bars show the standard deviations from at least three determinations.

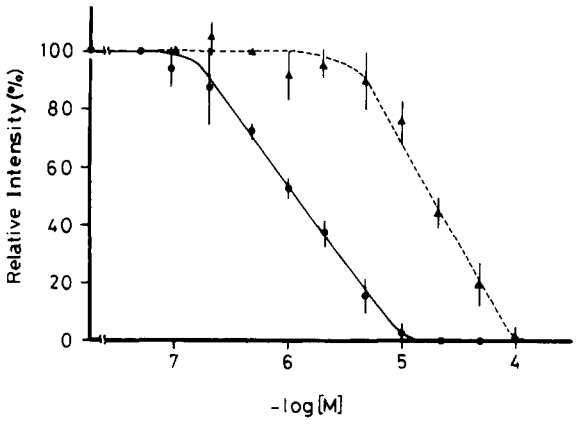

Fig. 6. Effect of histidine-specific reagents on the CD intensity of the EITC-ghost system. ●, DEPC; ▲, *p*-diazobenzene-sulfonate. The plots are relative CD intensities at 530 nm in the presence of histidine-specific reagents. Bars show the standard deviations from at least three determinations.

tion bands of EITC. Under the present conditions, although not all of the added EITC molecules bind to band 3, the induced CD in this system originates exclusively from EITC bound to band 3 [17]. The influences of unbound EITC molecules on CD spectra of the system can be neglected. Fig. 4 shows the effect of the arginine-specific reagent, phenylglyoxal [27], on the CD of the EITC-ghost system. The CD intensity at 530 nm decreases with increasing concentration of phenylglyoxal. The CD intensity at 530 nm was also decreased by the treatment with 1,2-cyclohexanedione, which is known as an arginine-specific reagent [28,29]. The dependence of the CD intensity of EITC at 530 nm on the arginine-specific reagents is shown in Fig. 5. The effects of histidine-specific reagents, DEPC [30] and *p*-diazobenzene sulfonate, on the CD spectra of the EITC-ghost system were also examined. The CD intensity at 530 nm decreased with increasing concentrations of DEPC and *p*-diazobenzene sulfonate (Fig. 6).

From these results it is obvious that both the arginine- and the histidine-specific reagents can effectively repress the induced CD of the EITC-ghost system. In contrast with these chemical modifiers, other compounds which bind specifically with amino-acid residues except for arginine and histidine, such as DTNB, which is a representative SH reagent, did not show such repres-

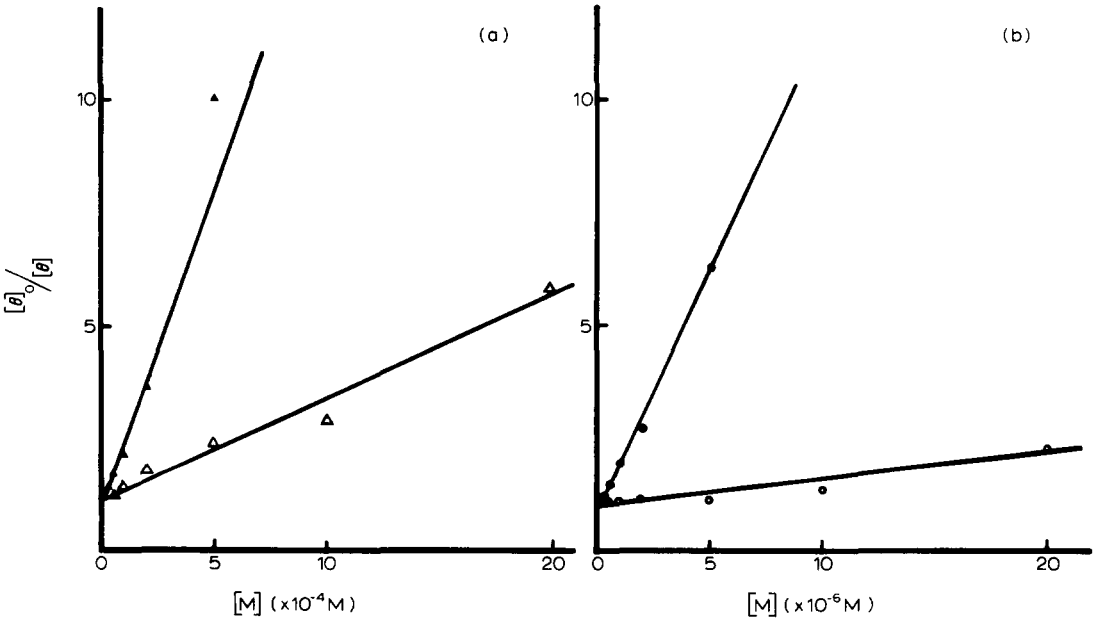

Fig. 7. Relative Dixon plots of induced CD represssion in the presence of chemical modifiers. (a) Arginine-specific reagents: ▲, phenylglyoxal; △, 1,2-cyclohexanedione. (b) Histidine-specific reagents: ●, DEPC; ○ *p*-diazobenzenesulfonate. The ratio $[\theta]_0/[\theta]$ was plotted against the concentration of the modifier, *M*. $[\theta]_0$ and $[\theta]$ are CD intensities of the EITC-ghost system in the absence and presence of modifiers, respectively. Data points were taken from Figs. 5 and 6.

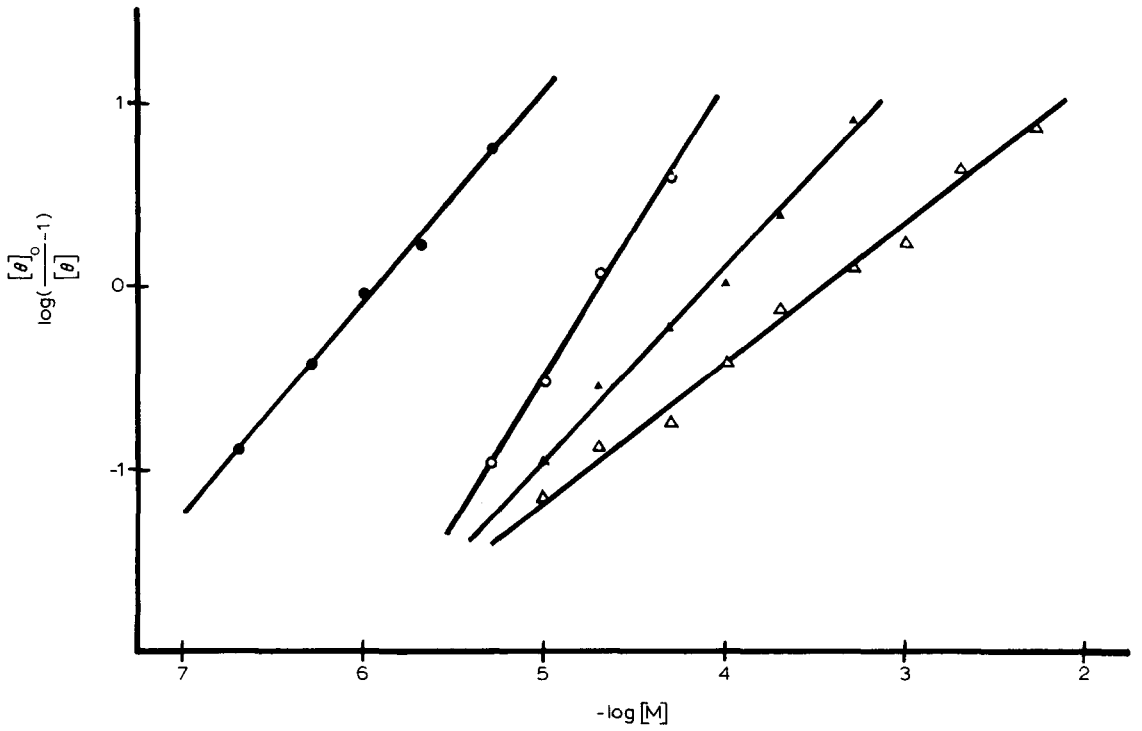

Fig. 8. Hill plots of induced CD represssion by chemical modifiers. ▲, phenylglyoxal; △, 1,2-cyclohexanedione; ●, DEPC; ○, *p*-diazobenzenesulfonate. Data points were taken from Figs. 5 and 6.

sion potencies or were required at very high concentration to repress. Thus, the arginine and histidine residues are unique in interacting with EITC molecules.

The curves in Figs. 5 and 6 are similar to those of the relation between the rate constant of anion exchange or inhibition and inhibitor concentration [10,31]. This may indicate that there is a direct relationship between modifier binding and the repression of the induced CD. From Figs. 7 and 8 we can estimate the values of I_{50}, which is the molar concentration reducing the initial CD intensity of the EITC-ghost system by 50%. A compilation of the I_{50} values is given in Table I. Although the repression potencies span a wide range of modifier compounds, the I_{50} values of the histidine-specific reagents, especially DEPC, are rather smaller than those of the arginine-specific reagents. Thus, it can be said that DEPC has a strong repression effects on the interaction of EITC with band 3. This suggests that the histidine residues play an important role in the interaction of the EITC molecule with band 3.

The rotatory strength at 223 nm is not affected by labelling with arginine- or histidine-specific reagents. This indicates that the α-helix content of the ghost proteins is not changed by these reagents and that the reactive region of the reagents is strictly localized.

TABLE I

REPRESSION POTENCIES OF CHEMICAL MODIFIERS

The values of I_{50}, concentrations that produced 50% repression of CD intensity, were taken from Fig. 7.

Modifier	Modified residue	I_{50} (M)
Phenylglyoxal	arginine	$7.6 \cdot 10^{-5}$
1,2-Cyclohexanedione	arginine	$5.2 \cdot 10^{-4}$
Diethylpyrocarbonate (DEPC)	histidine	$0.8 \cdot 10^{-6}$
p-Diazobenzenesulfonate	histidine	$1.5 \cdot 10^{-5}$

Effects of modification of amino-acid residues on the difference spectrum of the EITC-ghost system

Ehrenberg et al. [32] pointed out that DEPC also reacts with lysine, tyrosine, and cysteine residues in addition to histidine. In order to test the reactivity of DEPC with ghosts, the effects of DEPC on the difference spectrum of the EITC-ghost systems were examined. Fig. 9 shows the effect of addition of DEPC on the intensity of the difference spectra. It seems likely that the difference spectrum is slightly affected when the ghosts are first reacted with DEPC. This indicates that DEPC does not have a great effect on the reactivity of the isothiocyano group of EITC with ghosts. It is well known that there are lysine residues around the anion recognition site in band 3 [33,34]. The isothiocyano group of EITC covalently reacts with the ε-amino group of the lysine residue. Thus, it can be said that the lysine residues in band 3 do not react with DEPC molecules. On the other hand, the reactivity of DEPC molecules with tyrosine residues can be evaluated by measuring the decrease in the absorption at

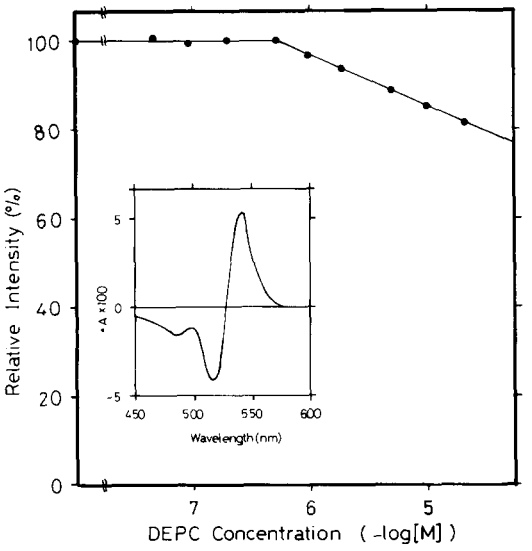

Fig. 9. Effects of DEPC on the difference spectra between EITC and the EITC-ghost system. The plots are relative difference absorption intensities of the positive peak at 540 nm in the presence of DEPC. A mixture of DEPC and ghosts was incubated for 2 h at 37°C in the dark, and then EITC was added to the mixture, this being followed by incubation for 2 h. Inset: typical difference spectrum between EITC and the EITC-ghost system at pH 7.4.

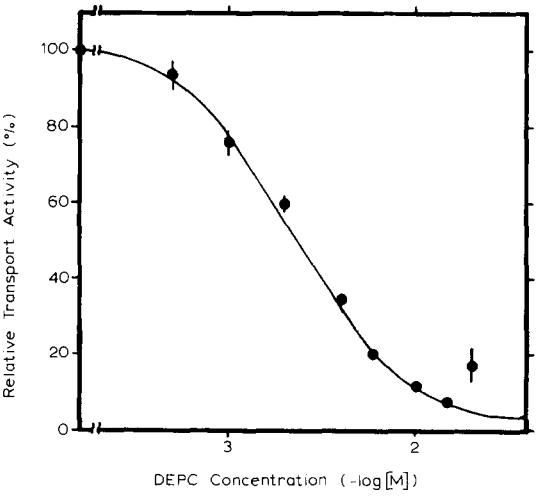

Fig. 10. Effect of DEPC on efflux of sulfate from intact erythrocytes. The plots show the percentage of the effluxed radioactivity in the presence of DEPC against the control. Bars show the standard deviations from at least three determinations. DEPC and cells were incubated for 10 min at 37°C in phosphate buffer, pH 6.3. [Erythrocyte] = 2.5% hematocrit.

278 nm [35,36]. The absorption intensity at 278 nm was not decreased by treatment of ghosts with DEPC. Comparing this with the results of the treatment of ghosts with DTNB, it was deduced that the cysteine residues were not responsible for the reaction with DEPC molecules (data not shown). Further, the induced CD of EITC could not be observed when the ghosts were first reacted with DEPC. These results imply also that the modification by DEPC is absolutely restricted to the special amino-acid residue.

Inhibition of anion transport by DEPC

It is well known that phenylglyoxal and 1,2-cyclohexanedione inhibit the anion transport in erythrocyte membranes [26–29,39]. We examined the effect of DEPC on anion transport.

Fig. 10 represents the residual transport of sulfates in erythrocytes after treatment with DEPC at pH 6.3. From Fig. 10, the concentration for 50% inhibition of sulfate efflux by DEPC can be estimated to be about 2.2 mM. At pH 7.0, 7.4 and 8.0, the same results were observed. The concentration of DEPC required for 50% inhibition is comparable to those of phenylglyoxal (2 mM) and 1,2-cyclohexanedione (5 mM) [28,29]. This result

suggests that the histidine residue should also play an important role in transport of sulfate across the red blood cell membrane.

Discussion

Interaction of EITC and band 3

In a previous report [17], we have demonstrated that the binding sites of EITC are different from the substrate sites for anion transport. We have considered the binding of EITC to band 3 based on the three-point attachment model by Aubert and Motais [37]: the isothiocyano groups at the phenyl ring in EITC molecules, which combine with amino groups in hydrophobic regions by covalent bonding: the other functional groups in EITC are the carboxyl anion at the phenyl ring and the oxyo anion at the xanthene skelton, which are expected to associate with amino groups in band 3 by ionic bonding. As shown in Figs. 1–3, there are at least two classes of titratable groups, showing equilibria at pH 6.4 and 13.1 in the EITC-ghost system. In view of the pK values around 6 and 13, the likely candidates seem to be the imidazole groups of histidine and the guanidino groups of arginine, whose pK values are roughly 6 and 12.5, respectively. The equilibria at pH 6.4 and 13.1 should be due to an electrostatic interaction between EITC molecule with imidazole groups of histidine and guanizino groups of arginine. According to the study of Barzilay et al. [10], the microenvironment of substrate recognition sites bears a positive multipolar character and possesses functionally essential groups with electron donor capacity embedded in a hydrophobic area. They pointed out that imidazole groups can be implicated as strong electron donors. Matsuyama et al. [38] propose a participation of histidine residue in the transport of inorganic phosphate and phospho*enol*pyruvate. Their results strongly support our present investigation.

The pK values of EITC molecule in alkaline pH region are about 8 and 11.0. They correspond to dissociation of the 6′- (or 3′-) hydroxy group and the 3′- (or 6′-) in the xanthene skeleton, respectively. Thus, it is likely that the 3′- (or 6′-) hydroxy groups interact with the guanizino groups. As a result, the carboxy groups of EITC bind to the imidazole groups. Considering that NH_2 rather than SH groups are involved in anion transport and that both DIDS and H_2DIDS covalently bind lysine residues on band 3, the covalent anchoring group for the isothiocyano group of EITC may be the NH_2 group of lysine [33]. Such considerations are consistent with the results of the CD experiment using the modifying reagents of amino acids.

Role of titratable groups

It is well known that there are titratable substrate and modifier sites in the anion transport system of human red cells [12,13,16]. From the titration data and the experiments of modification of arginine residue, Wieth et al. [26,27] and Zaki [28,29,39] proposed the participation of arginines in anion transport across the erythrocyte membranes. Bjerrum et al. [40] suggested that the arginine is the binding site of stilbene disulfonate derivatives. Our results in this study show the responsibility of arginine for the interaction of EITC with band 3 and for the sulfate transport. Such responsibility was also observed for the histidine residues in band 3. This implies the participation of imidazole group of histidine as titratable sites in the anion transport system.

Regarding to the identity of the titratable groups, the number of candidates is limited by the pK value around 6. Since the apparent pK of the transport regulatory groups changes very little between 0 and 38°C, the imidazole group of histidine was excluded because of its rather large enthalpy of ionization [40–43]. However, when the titratable positive group is located near histidine, the electrostatic work involved in binding the proton would lead to a decrease in the enthalpy of ionization [42]. It can be said that the titratable imidazole groups of histidine are located near the anion transport site. Such an image of the transport site is in conflict with the substrate site model [40].

Since the modifier site is also positively charged, the transport site which interacts with EITC may be the modifier site in view of its interaction with anionic substrates or inhibitors. The modifier sites may be located at the extracellular portion of the transport system [41].

According to the titratable carrier hypothesis, unloaded carrier or charged-form carrier cannot cross the membrane. To explain sulfate transport

by a monovalent anion carrier, a cotransport mechanism can be applied. The sulfate self-exchange flux is strongly pH-dependent and exhibits a pH maximum at pH 6.2 [44]. This is supposed to be due to a superposition of H^+/SO_4^{2-} co-transport and a protonation of the imidazole groups of histidine.

From the present study, it is suggested that both arginine and histidine residues in the binding sites of EITC molecules play an important role in the anion transport across the blood cell membranes.

Topological position of histidine responsible for transport

Although nothing is virtually known about the position of histidine in band 3 protein, Kopito and Lodish [45] have recently deduced the amino-acid sequence of murine band 3 from the nucleotide sequence analysis of a complementary DNA clone. The extensive homology between murine and human band 3 allows us to assign the topological position of the histidine under consideration [46]. According to Kopito and Lodish's predicted sequence of murine band 3, the protein possesses 12 membrane-spanning regions. A functional importance of this domain is a plasma membrane anion antiporter. There are five histidines in this domain. The positions of them are 669, 721, 752, 837 and 852. The former three histidines are located in membrane-spanning regions which contain both hydrophobic and polar residues, and cross the cell membrane as amphipathic helical structure. Such amphipathic helices might be combined to form an aqueous 'pore' and 'channel' for anion transport. The histidine residue at position 752 seems to be in the hydrophobic region. His-721 can be predicted to be endofacial. Thus, His-669 is located near the sites of oligosaccharide attachment, proteolysis (papain), and exofacial radioiodination.

A suppression of the induced CD of EITC was observed when the erythrocytes were pretreated with papain (data not shown). This indicates that the EITC-binding sites are located near the papain cleavage sites. Such suppression of the induced CD was also observed by the pretreatment of erythrocytes with chymotrypsin, implying that the binding sites are associated with α-helical segment. The topological position of His-669 is consistent with these observations. These considerations are also consistent with the possible location of the modifier sites.

References

1 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
2 Drickamer, L.K. (1978) J. Biol. Chem. 253, 7242–7248
3 Cabantchik, Z.I., Knauf, P.A. and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239–302
4 Jennings, M.L. (1984) J. Membrane Biol. 80, 105–117
5 Steck, T.L. (1974) J. Cell Biol. 62, 1–19
6 Liu, S.-C., Fairbanks, G. and Palek, J. (1977) Biochemistry 16, 4066–4074
7 Wang, K. and Richards, F.M. (1975) J. Biol. Chem. 250, 6622–6626
8 Steck, T.L. (1972) J. Mol. Biol. 66, 295–305
9 Cabantchik, Z.I. and Rothstein, A. (1974) J. Membrane Biol. 15, 207–226
10 Barzilay, M., Ship, S. and Cabantchik, Z.I. (1979) Membrane Biochem. 2, 227–254
11 Barzilay, M. and Cabantchik, Z.I. (1979) Membrane Biochem. 2, 255–281
12 Knauf, P.A., Ship, S., Breuer, W., McCulloch, L. and Rothstein, A. (1978) J. Gen. Physiol. 72, 607–630
13 Knauf, P.A., Breuer, W., McCulloch, L. and Rothstein, A. (1978) J. Gen. Physiol. 72, 631–649
14 Nanri, H., Hamasaki, N. and Minakami, S. (1983) J. Biol. Chem. 258, 5985–5989
15 Nigg, E., Kessler, M. and Cherry, R.J. (1979) Biochim. Biophys. Acta 550, 328–340
16 Dalmark, M. (1976) J. Gen. Physiol. 67, 223–234
17 Sato, Y., Chiba, T. and Suzuki, Y. (1986) Biochim. Biophys. Acta 856, 11–18
18 Cherry, R.J., Cogoli, A., Oppliger, M., Schneider, G. and Semenza, G. (1976) Biochemistry 15, 3653–3657
19 Frankel-Conrat, H. (1957) Methods Enzymol. 4, 247–269
20 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130
21 Lieberman, D.M. and Reithmeier, R.A.F. (1983) Biochemistry 22, 4028–4033
22 Laemmli, V.K. (1970) Nature 227, 680–685
23 Yu, J. and Branton, D. (1976) Proc. Natl. Acad. Sci. USA 73, 3891–3895
24 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
25 Sato, Y., Chiba, T. and Suzuki, Y. (1985) Chem. Pharm. Bull. 33, 3935–3944
26 Wieth, J.O. and Bjerrum, P.J. (1982) J. Gen. Physiol. 79, 253–282
27 Wieth, J.O., Bjerrum, P.J. and Borders, C.L. (1982) J. Gen. Physiol. 79, 283–312
28 Zaki, L. (1981) Biochem. Biophys. Res. Commun. 99, 243–251

29 Zaki, L. (1983) Biochem. Biophys. Res. Commun. 110, 616–624
30 Miles, E.W. (1977) Methods Enzymol. 47, 431–442
31 Cabantchik, Z.I. and Rothstein, A. (1972) J. Membrane Biol. 10, 311–330
32 Ehrenberg, S., Fedorosak, I. and Solymosy, F. (1976) Progr. Nucleic Acid Res. Mol. Biol. 16, 189–262
33 Ramjeesingh, M., Gaarn, A. and Rothstein, A. (1981) Biochim. Biophys. Acta 641, 173–182
34 Jennings, M.L. (1982) J. Biol. Chem. 257, 7554–7559
35 Burstein, Y., Walsh, K.A. and Neurath, H. (1974) Biochemistry 13, 205–210
36 Blumberg, S., Holmquist, B. and Vallee, B.L. (1973) Biochem. Biophys. Res. Commun. 51, 987–992
37 Aubert, L. and Motais, R. (1975) J. Physiol. 246, 159–179
38 Matsuyama, H., Kawano, Y. and Hamasaki, N. (1986) J. Biochem. 99, 495–501
39 Zaki, L. and Julien, T. (1985) Biochim. Biophys. Acta 818, 325–332
40 Bjerrum, P.J., Wieth, J.O. and Borders, C.L. (1983) J. Gen. Physiol. 81, 453–484
41 Knauf, P.A. (1979) Curr. Top. Membrane Transp. 12, 249–363
42 Brahm, J. (1977) J. Gen. Physiol. 70, 283–306
43 Wieth, J.O., Brahm, J. and Funder, J. (1980) Ann. NY Acad. Sci. 341, 394–418
44 Schnell, K.F., Gerhardt, S., and Schöppe-Fredenberg, A. (1977) J. Membrane Biol. 30, 319–350
45 Kopito, R.R. and Lodish, H.F. (1985) Nature 316, 234–238
46 Brock, C.J., Tanner, M.J.A. and Kempf, C. (1983) Biochem. J. 213, 577–586