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Characterization of the anion transport channel protein in human erythrocytes. Induced circular dichroism of inhibitors bound to the anion transport channel

Yukio Sato, Tadahiko Chiba and Yasuo Suzuki

Pharmaceutical Institute, Tohoku University, Aobayama, Sendai 980 (Japan)

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The induced circular dichroism (CD) of erythrocyte ghosts with anion-transport inhibitors has been studied. A ghost-EITC (eosin 5-isothiocyanate) system shows an induced CD spectrum at the wavelength region corresponding to the absorption bands of EITC. Also a ghost-EMI (eosin 5-maleimide) system shows induced CD, but has bands of opposite sign to the EITC system. From the change of the CD intensity, the number of EITC molecules bound to one erythrocyte was estimated to be about $1.4 \cdot 10^6$, being close to the number of band 3 copies per ghost. The CD spectra of EITC and EMI systems show that a configurational structure of the moiety anchoring the EMI molecule is the reverse to that of EITC. The preferred conformation of bound EITC may be twisted in a right-handed sense. From the signs of the induced CD bands in ghost-stilbene disulfonate systems, the chirality of twisted stilbene derivatives seems to be a left-handed sense, as is the case for the EMI derivative. The CD spectra of EITC in the presence of DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonate) shows that the binding site of EITC may not be identical with that of DIDS. The results observed in this study reflect the ternary arrangement of the functional amino groups in anion recognition sites.

Introduction

Band 3 protein is responsible for anion exchange across the membrane of the red blood cell [1,2]. A molecular basis of the transport mechanism has been assessed largely from kinetic studies of anion transport by the use of inhibitory chemical probes such as DIDS, NAP-taurine and EITC. Anion transport mediated by band 3 consists of an obligatory one-for-one exchange which is believed to be a 'ping-pong' mechanism. Many ideas about the molecular mechanism of anion transport have been proposed [3–10]. For further understanding of the mechanism, optical spectroscopic methods are useful, especially in the structural study of protein.

In a previous series of experiments we studied the interaction of EITC with erythrocyte ghosts, and it was proposed that the EITC-binding site on band 3 might be identical with a modifier site [11]. The complexation of EITC molecules with band 3 should be consistent with a 'three-point attachment model', which can be said to be a specific structural requirement governing the transport [12]. To gain further information about the arrangement of the functional amino groups, positively charged and nucleophilic residues in the anion-re-

Abbreviations: EITC, eosin 5-isothiocyanate; EMI, eosin 5-maleimide; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DADS, 4,4′-diaminostilbene-2,2′-disulfonate; NAP-taurine, *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate; FITC, fluorescein isothiocyanate; P/D, the ratio of the ghost concentration to the EITC concentration when the concentration of the ghost suspension is expressed in terms of mol/l; SITS, 4-acetoamide-4′-isothiocyanostilbene-2,2′-disulfonate; BIDS, 4-benzamido-4′-isothiocyanostilbene-2,2′-disulfonate.

cognition site, we examine the binding sites of EITC and DIDS by studying induced circular dichroism of the inhibitors complexed with band 3.

Materials and Methods

Materials. DADS was purchased from Wako Pure Chemical Industries, Ltd. EITC, DIDS and SITS were synthesized according to the procedures of Cherry et al. [13] and Cabantchik and Rothstein [14,15], respectively. All steps in these preparations were cnducted with minimum exposure to light. The purities of the resulting compounds were confirmed by thin-layer chromatography. NAP-taurine was obtained from Pierce Chemical Co. EMI was from Molecular Probes, and FITC from Dojin Co., Ltd. They were used without further purification.

Preparation of labeled ghosts. Resealed erythrocyte ghosts were prepared by the standard method of Dodge et al. [16]. Erythrocyte ghosts were incubated with various concentrations of inhibitors in the dark (37°C in 310 mosM phosphate buffer, pH 7.4). At appropriate incubation times, the solutions were subjected to spectral measurements.

Spectral measurements. The absorption and CD spectra were measured with a Hitachi 220 spectrophotometer and a Jasco J-400X spectropolarimeter equipped with data processors, respectively. The mesurements were carried out at room temperature, unless otherwise specified. The molar extinction coefficient, ϵ, was calculated on the basis of the initial concentration of inhibitors. The observed CD was expressed in terms of molar ellipticity, $[\theta]$, in deg · cm^2/dmol.

The protein concentration of the ghost membrane was determined by the method of Lowry et al. [17] using bovine serum albumin as the standard. When a mean residue weight of 130 was used to compute the ellipticity of the membrane proteins, ghost suspension of 0.13 mg proteins/ml correspond to about $1 \cdot 10^{-3}$ M [18]. In this study, the experiments of ghosts were carried out with a final protein concentration of 0.13 mg/ml in phosphate buffer (pH 7.4), unless otherwise specified. It is worthwhile to stress that the ghost concentration refers to the amino acid residue concentration. Each spectrum is plotted as the average of at least three protein samples, and the spectrum of each was recorded at least twice.

Results

Induced circular dichroism in ghost-eosin derivative systems

EITC is known as an inhibitor of sulfate exchange in the erythrocyte [9]. Although EITC has a chirality due to steric hindrance of the caboxylic group on the phenyl ring, it is racemic and optically inactive when it is free in solution. However, we can expect a perturbation of the enantiomer distribution upon binding to band 3 protein. The induced CD and absorption spectra of the ghost-EITC system is shown in Fig. 1. A negative CD band at about 560 nm and positive band at about 530 nm with shoulder at 490 nm, and four negative bands below 450 nm are observed. It has been reported that the CD photomultiplier tube is strongly implicated as the source of the artifact [20]. For a symmetrically scattering sample, the artifact varies with changes in the sample cell position, even though the photomultiplier position is held constant. In order to test for scattering distortions of the induced CD, we measured the spectra by changing the distance between the sample cell and the detector (end-window photomultiplier tube). The main positive CD band was not affected by changing the sample cell position, meanwhile a little fluctuation was observed in the negative CD bands at short wavelength region

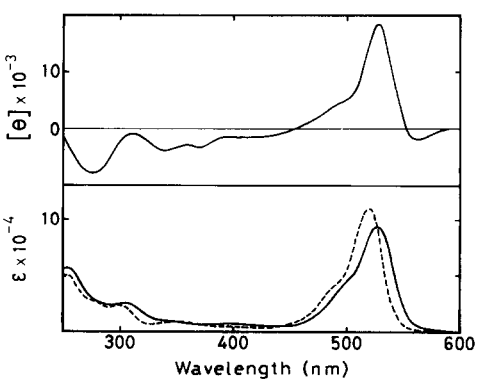

Fig. 1. CD and absorption spectra of ghost-EITC system after incubation for 3 h at 37°C in 310 mosM phoshate buffer (pH 7.4). [ghost] = $1.5 \cdot 10^{-3}$ M; [EITC] = $1 \cdot 10^{-5}$ M; - - - - - -, EITC alone.

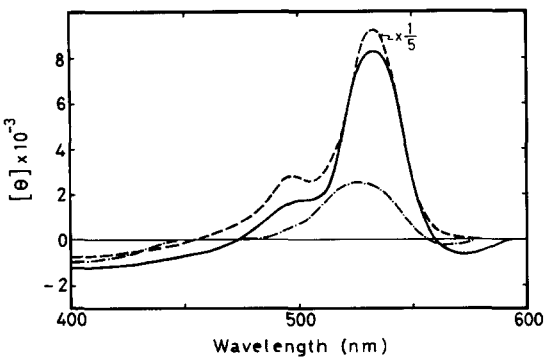

Fig. 2. CD spectra of ghost-EITC system at various concentrations of ghost in 310 mosM phoshate buffer. The spectra were obtained after incubation for 3 h at 37°C. [EITC] = $1 \cdot 10^{-5}$ M; (-·-·-): [ghost] = $5 \cdot 10^{-4}$ M; (————): [ghost] = $1 \cdot 10^{-3}$ M; (- - - - -): [ghost] = $1 \cdot 10^{-2}$ M.

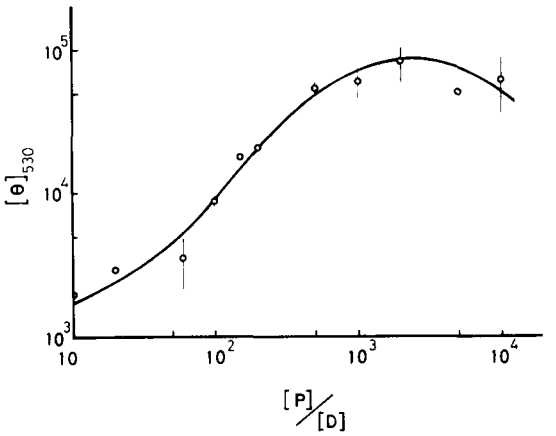

Fig. 3. Variation of CD intensity of ghost-EITC system in 310 mosM phosphate buffer (pH 7.4). Ellipticity was measured at 530 nm peak after incubation for 3 h at 37°C. Bars represent the standard deviation from 2–4 determinations.

(data not shown). Thus, it seems that the scattering distortions can be neglected.

As the amount of ghosts increases, these bands gradually increase in intensity, but the spectral pattern is unchanged, except at a high concentration of ghosts (fig. 2). At high concentrations of ghosts, the band at 560 nm disappears. The incrase of mole ratio of ghost protein to EITC appears to result in the disappearance of the 560 nm band. In regard to the CD band at 560 nm, there is no corresponding absorption band. Although an assignment of the 560 nm band is still unclear, two explanations are possible. First, an interaction among EITC molecules may cause the band at the long wavelength region. Second, the 560 nm band may be due to the differential scattering of circularly polarized light [21]. It can be ruled out that the 560 nm band results from charge-transfer transitions [22], because this band could not be observed at high concentrations of ghosts.

Two types of binding environments for EITC molecules are considered [11]. One is binding to band 3 and the second to the other constituents of ghost membrane. Although EITC binds almost exclusively to band 3 [23,24], other proteins or glycolipids in the ghosts should be also considered.

Fig. 3 shows the CD intensity of the 530 nm band as a function of the value of P/D (the mole ratio of erythrocyte protein to EITC). Again, it should be noted that the concentration of ghost refers to the amino acid residue concentration, not that of membranes, or any particular protein. The intensity becomes maximal at P/D value of about $2 \cdot 10^3$. On the basis of this value and a conversion factor ($6.0 \cdot 10^{-10}$ mg protein/ghost) of Dodge et al. [16], we can estimate the number of EITC molecules bound to one erythrocyte to be about $1.4 \cdot 10^6$. This is close to the number of band 3 copies per ghost, i.e., $1 \cdot 10^6$ [25]. This result supports that the induced CD can be related to the interaction between band 3 and EITC. Furthermore, it was observed that the induced CD spectrum of EITC in reconstituted band 3-lipid vesicle system was similar to that of the ghost-EITC system (data not shown). On the other hand, the EITC-lipid vesicle system showed no CD spectrum. Thus, the induced CD in the ghost-EITC system originates exclusively from EITC bound to band 3.

For comparison with the EITC system, the CD spectrum of the ghost-FITC system was examined. FITC does not inhibit anion transport. Fig. 4 shows the induced CD of the ghost-FITC system. It is noteworthy that the intensities of the CD bands are small in comparison with the EITC system, in spite of the similarity in their structures. The CD spectrum is of positive sign and similar to the absorption spectrum. In this system, the 560 nm band could not be observed. When the ghosts were first reacted with FITC and successively with EITC, the induced CD resulted from EITC decreased. The concentration of FITC required for

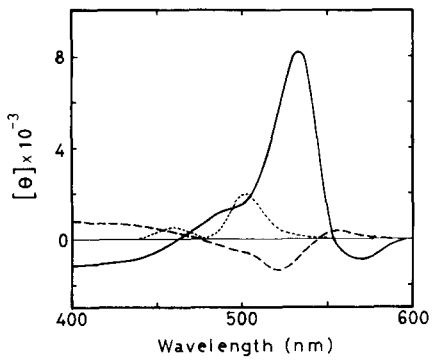

Fig. 4. CD spectra of fluorescein derivatives complexed with ghosts in 310 mosM phosphate buffer (pH 7.4). The spectra were obtained after incubation for 3 h at 37°C. ————, EITC; - - - - - -, FITC; — — —, EMI.

the reduction of the CD intensity induced by $1 \cdot 10^{-5}$ M of EITC to one-half was about $7 \cdot 10^{-5}$ M. The binding site of FITC molecules may be the same as that of EITC. However, when ghosts were incubated with the mixture of EITC and FITC, the suppressive effect of FITC on the induced CD of EITC was scarcely observed. This may be due to the fact that hydrophobic interaction plays an important role in the band 3-EITC complex formation [11]. The differences in hydrophobicity and/or size between EITC and FITC molecules might influence also their orientation or configurational structure at the binding site. This is due to the fact that the absence of four bromine atoms in the FITC molecule results in a different hydrophobicity and the lack of anionic characteristics at physiological pH [19]. Thus, the FITC system results in appearance of the small positive CD.

As seen in Fig. 4, an induced CD could be detected also in the ghost-EMI system. EMI is known as an extremely powerful inhibitor of anion exchange in erythrocytes [19,23,26]. Nigg and Cherry [27] reported that EMI binds exclusively to band 3. Upon covalent binding to ghost proteins, the absorption maximum shifted from 516 to 517 nm accompanied by a decrease in its intensity. The induced CD in this system exhibits a positive band at about 555 nm, negative bands at 520 nm with a shoulder at about 490 nm, and positive bands around 400 nm. It should be noted that the signs of each CD band are opposite to those in the EITC system. Also in the EMI systems, the induced CD may arise from an asymmetric twisted conformation of the chromophores. The opposite sign of the EMI CD bands suggests that the chirality of the asymmetric conformation of bound EMI is the reverse to that of EITC.

Induced circular dichroism in ghost-stilbene disulfonate systems

Many studies about the mechanism of anion transport have been investigated by the use of stilbene disulfonate derivatives which potentially inhibit the anion-exchange from the external side of the membrane [28–36]. The absorption spectrum and an induced CD spepctrum for a ghost-DIDS derivatives were also examined after incubation with ghosts for 1 h at 37°C (Fig. 5). Although the free DIDS molecule is optically inactive in solution, an induced CD could be detected in the ghost-DIDS system. The A band of DIDS at 342 nm, corresponding to that of the trans isomer, is composed of vibrational sub-bands termed the α, β, γ, and δ bands [37]. As the incubation time increased, the absorption intensity decreased. Two isosbestic points were observed at 305 and 384 nm. These observations suggest a reaction of DIDS molecules with band 3 [30]. The induced CD may arise from an asymmetric conformation of the bound DIDS, intrinsic optical activity, and/or an interaction of the bound DIDS with an asymmetric environment, extrinsic optical activity [38,39]. It was found that DADS and SITS showed a type

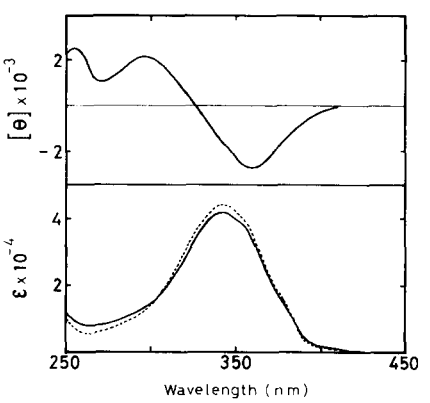

Fig. 5. CD and absorption spectra of ghost-DIDS system in 310 mosM phosphate buffer (pH 7.4). [ghost] = $1 \cdot 10^{-3}$ M; [DIDS] = $5 \cdot 10^{-5}$ M; - - - - - -, DIDS alone.

of induced CD spectra similar to that of DIDS. Stilbene disulfonate derivatives seem to exhibit induced CD in the wavelength region corresponding to the main absorption band, in which negative and positive CD bands are observed from the longer wavelength side.

Binding sites of EITC and DIDS molecules

In the previous report, we proposed that the binding sites for EITC and DIDS may be different from each other [11]. In order to obtain information about the binding sites for EITC and DIDS molecules, ghosts were incubated in the presence of both inhibitors. CD spectra similar to a combination of each individual spectrum could be observed in the corresponding regions (Fig. 6). Such CD spectral patterns could be observed also when the concentration of either inhibitor was increased 2- or 3-fold. Thus, it can be said that the binding sites for EITC and DIDS molecules are different from each other. The binding site for DIDS is known as the anion-binding site (substrate site) [1]. A preincubation of ghosts with EITC had little influence on the appearance of induced CD of DIDS. On the contrary, a preincubation of ghosts with DIDS resulted in decreasing of induced CD of EITC (Fig. 7). The amplitude of the CD bands progressively decerased with increasing of DIDS concentration (Fig. 7 (inset)). The DIDS concentration required to reduce the CD intensity of EITC at a concentration of $2 \cdot 10^{-5}$ M to one-half was about $2 \cdot 10^{-7}$ M, being an extremely lower concentration than that of FITC. Three explanations are possible for this result. One is that the EITC-binding site exists on the interior side of the cell rather than the DIDS-binding site. Second, a conformation change accompanied by DIDS binding reduces affinity of EITC binding to band 3. Third, DIDS can bind also to the EITC-binding-site at a low speed of reaction. Macara and Cantley [26] presented evidence that the binding site of EMI and BIDS is an external transport site of band 3. On the other hand, the existence of different binding sites for EITC and EMI was also reported [27]. Furthermore, we observed an exclusive binding of NAP-taurine to the EITC molecule. This evidence leads us to the presumption that the binding site of EITC is a modifier site. This consideration is consistent with the third explanation. It is considered that the modifier site is located at the extracellular end of the protein of the transport system [1c]. Thus, the EITC-binding site in band 3 is a second competing site, to which FITC and DIDS molecules also can bind. The binding activity of these molecules may be correlated with the hydrophobic character of the molecules.

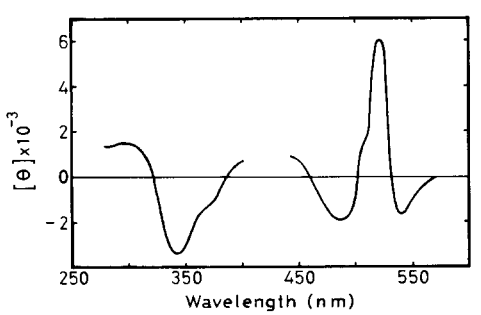

Fig. 6. CD spectra of ghost-EITC-DIDS system in 310 mosM phosphate buffer (pH 7.4). The spectra were obtained after incubation for 2 h at 37°C. [ghost] = $1 \cdot 10^{-3}$ M; [EITC] = $2 \cdot 10^{-5}$ M; [DIDS] = $5 \cdot 10^{-5}$ M.

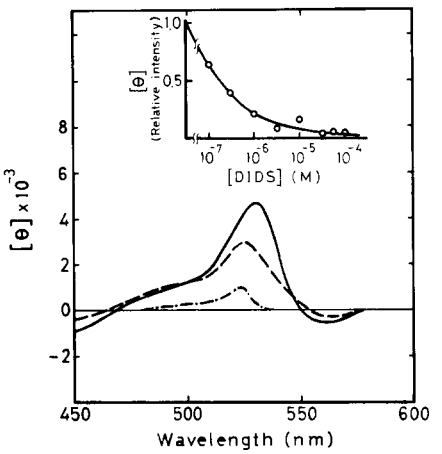

Fig. 7. Effects of preincubation of DIDS with ghosts on EITC CD in 310 mosM phosphate buffer (pH 7.4). The samples were prepared by incubation of EITC with ghosts for 2 h at 37°C. The ghosts were previously treated with DIDS for 1 h at 37°C. [ghost] = $1 \cdot 10^{-3}$ M; [EITC] = $2 \cdot 10^{-5}$ M; ————, in the absence of DIDS; — — —, [DIDS] = $1 \cdot 10^{-7}$ M; - · - · -, [DIDS] = $1 \cdot 10^{-6}$ M. Inset: Dependence of CD intensity of EITC on DIDS concentration. The CD intensities were measured at the peak of positive band.

Discussion

Induced CD in ghost-inhibitor systems

When optical measurements are made for turbid suspensions derived from biological membranes, it is necessary to take into account the distortions arising from the particulate nature of the samples [22,40–42]. Glaser and Singer [43] analyzed in detail the CD spectra of suspensions of intact erythrocyte membranes, and showed that the value of $[\theta]_{222}$ is not significantly altered by optical artifacts. The main distortions are concentration obscuring and differential scattering, which lead to a decrease in the measured CD. The flattening decreases absorption but the scattering increases. The differential scattering of the left and right circularly polarized light complicates the CD of optically active particulate systems. In the case of induced CD in the presence of biological membranes, the artifacts have not yet been discussed. In this study, however, these distortions were neglected because of the following reasons, in addition to the effect of the changing the distance between the sample cell and the detector. (i) The addition of ghost suspension used in this study did not affect the optical activity of other molecules such as riboflavin. This indicates that under the present conditions the differential scattering has little influence on primary CD. (ii) Concerning the main CD bands in the ghost-EITC system, the magnitude of the bands was not affected by rotating the sample cell in the optical axis. This indicates that the induced CD in the present systems exhibits little liquid-crystal-type behavior, or that there is no macroscopic anisotropy. (iii) When the CD spectrum was compared with that of the same sample containing 2% Tween-80 for solubilization, little discrepancy was observed. (iv) A pathlength dependence was not observed within cell length used (0.1–1 cm). (v) In an experiment with sonicated ghosts, no detectable changes in the CD spectra were observed. These results imply that the encountered particles have little influence on the induced CD. Although minor involvement of linear dichroism cannot be denied, the observed induced CD spectra must be real. Thus, in the present study, the scattering effect at the wavelength region corresponding to the main absorption bands of EITC was negligible.

Bustamante et al. [21] showed that the differential scattering must be taken into account before a quantitative interpretation of the CD is attempted whenever an apparent CD is measured outside the absorption bands of the sample. At the appropriate conditions, however, an apparent CD was not observed at wavelengths above region of the inhibitors. Furthermore, it was confirmed that the magnitude of the positive CD bands was hardly influenced under the various conditions at constant P/D value. These results, again, suggest that the differntial scattering effects can be neglected under the ghost concentrations used in this study. The induced CD can provide information about the conformation of the inhibitors in erythrocytes. Finally, the utility of the induced CD in the study of the anion-transport mechanism is evident. Although more detailed information is required, the present data can be used for a qualitative discussion.

Configurational characterization of the functional groups

The induced CD in the ghost-EITC system indicates that EITC is rigidly bound to band 3. The functional amino groups seem to be anchors for EITC molecules, and determine a conformation of bound EITC. Thus, the induced CD spectra contain information describing topographic characters of the functional amino groups in the anion-recognition sites. As mentioned above, the induced CD in the present systems may arise from both intrinsic and extrinsic origins. The chiral characterization cannot be determined strictly until the preference of either of two origins is resolved. To infer the relative importance of intrinsic and extrinsic contributions to the optical activity, a theoretical approach is necessary for the elucidation of its origin. Although further investigation along this line is now progressing, a single and most probable interpretation of predominant conformation of the bound inhibitors will be put forward. The absorption maximum of the ghost-EITC system is located at the slightly shorter wavelength side than the induced CD maximum. However, the sign of the CD spectrum is a single one (+) (Fig. 1). Therefore, the induced CD of EITC is caused primarily by the twisted conformation of the bound chromophore, though the in-

duced CD by an interaction of EITC with the protein cannot be denied [39]. Inherent chirality in EITC is due to rotameric states between xanthene and phenyl chromophores, and the conformation of the molecule can be pictured as either a right-handed or left-handed twisted molecule. According to the generally accepted rule that correlates the chirality with the sign of the lowest energy CD band, the sign of the CD band is positive when the chromophore (of C_2 symmetry and with a long-wavelength transition of symmetry B) is right-handed [44]. The direction of the long-wavelength transition of xanthene chromophore is along the long-axis and of symmetry B. The sign of the main long-wavelength CD band of the ghost-EITC system should reveal the handedness of the bound EITC molecules. According to Hirano [22], the first intense band at 522 nm originates from the local excitation of the xanthene ring. The polarization direction of this transition is along the long-axis. This transition is accompanied by a substantial amount of charge-transfer from the carbonyl oxygen and from the bromine substituents into the xanthene ring. The shoulder at 485 nm may be ascribed to the singly ionized species. The second weaker band around 300 nm is regarded as the overlap of the two different types of transition originating from the excitation of benzene and xanthene rings. The polarization direction of them is along the short-axis. The sign of the 560 nm band is opposite to that of the main CD band. This may indicate also that the bound EITC is right-handed. Thus, the present data suggest that the binding site of EITC in the band 3 has a higher affinity for right-handed conformation of EITC molecules. The functional amino groups in band 3 determine that chiral conformation of EITC because of their three-point attachment configuration. We can illustrate the arrangement of the functional amino groups. Since it is likely that EITC molecules bound to band 3 are twisted in a right-handed sense, the functional groups anchoring the EITC molecule may be a left-handed sense. On the other hand, EMI and stilbene disulfonates bound to band 3 may be of left-handed conformation, because the sign of the main long-wavelength CD bands is negative. Therefore, the arrangements of the functional groups anchoring these inhibitors, which are considered to bind to the transport site or substrate site of band 3, are right-handed. Further, it can be assumed that SH- and NH_2-groups are located close to each other, because the SH- rather than the NH_2-groups are involved in coupling of EMI with band 3, whereas the NH_2-group is a covalent-anchoring group for stilbene derivatives [1c,3,45].

Cousin and Motais [46] have reported that flufenamate binds to a site which presents a positive charged group at the water/protein interface, whereas the hydrophobic part of the molecule is inserted into a hydrophobic and electron-donor region of the protein. In the flufenamate system, the CD spectrum was similar to the absorption spectrum of flufenamate, being of negative sign. This may suggest that little specific steric arrangement of each chromophore is needed in the flufenamate molecule. Interestingly, the sign of the CD spectrum of the FITC system is opposite to that of the flufenamate system. It is likely that there is a correlation between the CD sign and inhibitory action. This may reflect the specific environment in the neighborhood of the transport channel. The details are at present unknown.

Again, the CD spectra of inhibitors contain information about the spatial arrangement of the functional amino groups in the anion-recognition sites. This arrangement should characterize the anion-transport mechanism in erythrocytes. In view of our presents tudy, information from CD studies and the results of kinetic studies or other technical analyses combine to give a more detailed interpretation of anion transport. In conclusion, this study suggests that the induced CD of the ghost-inhibitor systems can be usefully employed in investigating the mechanism of anion transport in erythrocyte. An identification of the functional amino groups for EITC will be reported elsewhere.

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