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Research article

INVOLVEMENT OF CARBOXYL GROUPS IN CHLORIDE TRANSPORT AND REVERSIBLE DIDS BINDING TO BAND 3 PROTEIN IN HUMAN ERYTHROCYTES

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Abstract: Noncovalent DIDS binding to Band 3 (AE1) protein in human erythrocyte membranes, modified by non-penetrating, water soluble 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)-carbodiimide iodide (EAC), was studied at 0°C in the presence of 165 mM KCl. Under experimental conditions applied up to (48 ± 5) % of irreversible chloride self-exchange inhibition was observed. The apparent dissociation constant, K_D, for "DIDS-Band 3" complex, determined from the chloride transport experiments, was (34 ± 3) nM and (80 ± 12) nM for control and EAC-treated resealed ghosts, respectively. The inhibition constant, K_i, for DIDS was (35 ± 6) nM and (60 ± 8) nM in control and EAC-treated ghosts, respectively. The reduced affinity for DIDS reversible binding was not a result of negative cooperativity of DIDS binding sites of Band 3 oligomer since Hill's coefficients were indistinguishable from 1 (within the limit error) both for control and EAC-treated ghosts. By using tritium-labeled DIDS, 4,4'-diisothiocyanato-2,2'-stilbenedisulfonate ([^{3}H]DIDS), the association rate constant, k_{+1} ($M^{-1}s^{-1}$), was measured. The mean values of (4.3 ± 0.7) x 10^5 $M^{-1}s^{-1}$ for control and (2.7 ± 0.7) x 10^5 $M^{-1}s^{-1}$ for EAC-treated ghosts were obtained. The mean values for K_D, evaluated from [^{3}H]DIDS binding measurements, were (37 ± 9) nM and (90 ± 21) nM for control and EAC-modified ghosts, respectively.

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Abbreviations used: DIDS – 4,4'-diisothiocyanato-2,2'-stilbenedisulfonate; DNDS – 4,4'-dinitrostilbene-2,2'-disulfonate; EAC – 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)-carbodiimide iodide; H_2DIDS – 4,4'-diisothiocyanodihydro-2,2'-stilbenedisulfonate; TEE – tyrosine ethyl ester; WRK, Woodward's reagent K – N-ethyl-5-phenylisoxazolium 3'-sulfonate

The results demonstrate that EAC modification of AE1 reduces about 2-fold the affinity of AE1 for DIDS. It is suggested that half of the subunits are modified near the transport site by EAC.

Key words: Band 3, Carbodiimide, Dissociation constant, Erythrocyte membrane, Stilbenedisulfonate

INTRODUCTION

Band 3 (AE1) protein (reviewed in [1-2]) belongs to a family of anion exchange proteins that mediate transport of chloride and bicarbonate ions across the plasma membrane. Human Band 3 consists of two structurally and functionally distinct domains. The 43-kDa NH_2-terminal domain faces the cytoplasm, where it interacts with cytosolic and cytoskeletal proteins. The 52-kDa COOH-terminal domain hydrophobically associated with the plasma membrane is essential for catalyzing the electroneutral exchange diffusion of inorganic anions. Human Band 3 protein is present in about 1.1×10^6 copies per cell and consists of 911 amino acid residues. The *in situ* studies of AE1 protein have indicated its oligomeric structural organization with the dimer species predominant (reviewed in [3]). The inorganic anion transport catalyzed by Band 3 follows the so-called ping-pong kinetics in which a single site reciprocates between inside- and outside-facing orientations.

The identification of amino-acid residues that are involved in the transport process is necessary for elucidation of the molecular mechanism of inorganic anion permeation across the erythrocyte membrane. Functionally essential, positively charged arginyl residues with pK~12 were found in studies of the effects of extracellular pH on the exchange function [3] and by using chemical modification methods [4-5]. The results of Janas *et al.* [6] indicate that the positively-charged lysine groups are not involved in the translocation step in anion exchange since the activation energy of sulfate transport is unchanged after reductive methylation of lysine residues of Band 3. The dependence of monovalent and divalent anion exchange on extracellular pH provided the first evidence for a functionally important carboxyl group(s). Protonation of a group of pK~5.2 inhibits chloride self-exchange [7-8] and accelerates Band 3-mediated sulfate ion influx [9].

Stilbenedisulfonates are potent inhibitors of the Band 3 anion exchange system [10]. The anion transport site and the stilbenedisulfonate binding site are structurally not coincident but they overlap. In the present paper, we examine the role of the carboxyl residues in reversible interaction of DIDS with Band 3 protein. We used water soluble, membrane-impermeable carbodiimide (EAC) for irreversible modification of carboxyl residues in Band 3 protein that are accessible from the extracellular solution only. The apparent dissociation constant and association rate constant for reversible DIDS binding to Band 3 were measured in control and EAC-treated ghosts. The Hill coefficients and the K_i value for inhibition of chloride transport by DIDS were determined.

MATERIALS AND METHODS

Radioactive isotopes

^{36}Cl as KCl, specific activity 500 μCi $mmol^{-1}$, was purchased from AEK (Risφ, Denmark). The tritiated form of DIDS, 4,4'-diisothiocyanato-2,2'-stilbenedisulfonate ([^{3}H]DIDS) with specific activity 30-60 mCi $mmol^{-1}$, was prepared as described previously [11]; this form is the tritiated DIDS itself, not dihydro-DIDS.

Chemical reagents

All media were prepared from reagent grade chemicals. EAC (1-ethyl-3-(4-azonia-4,4-dimethylpentyl)-carbodiimide iodide) was synthesized from EDC (1-ethyl-3-dimethylaminopropyl-carbodiimide), hydrochloride and methyl iodide as described previously [12]. Nonradioactive 4,4'-diisothiocyanato-2,2'-stilbenedisulfonate (DIDS) was prepared according to the method described previously [13]. The buffer salts, 2-N-morpholinoethanesulfonic acid (MES) and 2-amino-2-hydroxymethyl-1,3-propanediol (TRIS), were from Calbiochem-Behring Corp. (San Diego, CA).

Preparation of resealed RBC ghosts

Freshly drawn, heparinized human blood from healthy adults (blood was obtained in accordance with the ethics guidelines) was centrifuged at room temperature, the plasma and buffy coat were removed, and the cells were washed three times as described [14]. Resealed ghosts containing buffer A (165 mM KCl, 2 mM TRIS and 0.5 mM EDTA/EGTA) were prepared from human erythrocytes as described previously [14]. The pH ≈ 7.2 of the haemolysate was determined while the ghosts were still unsealed. After preparation, the ghosts were washed thrice in unbuffered 165 mM KCl medium and resuspended to a cytocrit of ~40%. Depending on the type of experiment, the resealed ghosts were either incubated with $^{36}Cl^{-}$ or further treated (see below) before incubation with the tracer. The ghost resealing was assessed using 14C-labeled inulin [14].

EAC treatment

The volume of 10 ml of resealed ghosts with an intracellular pH of ~7.2 (0°C) was pelleted (44,000g for 15 min. at 0°C). The ghosts were resuspended in unbuffered KCl to a cytocrit of ~80%. The volume of 10 ml of the resealed ghosts, suspension at 38°C, was mixed with 20 ml of EAC medium prewarmed to 38°C (165 mM KCl, 10 mM MES, 30 mM EAC, pH 5.8) for 20 min. The modification of the ghosts with EAC was interrupted by diluting the 30 ml of ghost suspension into 150 ml of ice-cold stopping solution (165 mM KCl, 2 mM TRIS, pH 10.3 at 0°C).

Cl^{-} exchange flux

After the final washing, control and EAC-treated ghosts were resuspended to a cytocrit of ~40%, labeled with ^{36}Cl (0.5 μCi/ml suspension), and packed by centrifugation for 10 min at 40,000g in slender nylon tubes. The efflux of

radioactive chloride from labeled ghosts was initiated by injecting a packed ghost sample (~200 mg of membranes) into 40 ml of the well-stirred flux medium thermostated at 0°C. Serial sampling of the cell-free extracellular solution was done with the filtering technique and the supernatant was assayed for $^{36}Cl^-$ by β-scintillation spectroscopy. The rate constant k (s^{-1}) for chloride efflux was calculated by linear regression analysis of $\ln[(a_\infty - a_t)/(a_\infty - a_0)]$ vs. time (t), where a_t and a_∞ are the extracellular radioactivity at time t and at isotopic equilibrium, respectively, and a_0 is the extracellular radioactivity at time zero, caused by trapping of isotope between the ghosts during the preparation procedure.

The dissociation constant (K_D) for reversible DIDS binding to Band 3 protein in erythrocyte ghosts

The technique and equations described previously [15] were used. The control or EAC-treated erythrocyte ghost suspension (cytocrit ~40%), cooled to 0°C, was injected into a 0°C thermostated reaction vessel containing flux medium (165 mM KCl, 2 mM K_2HPO_4, pH 7.3) with appropriate DIDS concentrations. The exposure lasted < 3 min and the ghosts were separated from the unbound DIDS in solution by a quick centrifugation at 0°C. The supernatants containing various free DIDS concentrations were kept on ice (in the dark) until use as chloride efflux media. The isolated ghosts were loaded at 0°C with ^{36}Cl and the tracer efflux was measured as described above.

By definition:

$$(K_D) = [S] \times [D] / [SD] \quad (1)$$

where [S], [D] and [SD] are the concentrations of the free form of binding sites in the membranes, the probe (DIDS molecules) and the complex form, respectively.

$$[D_T] = [D] + [SD] \quad (2)$$

$$[S_T] = [S] + [SD] \quad (3)$$

$$I = [SD] / [S_T] \quad (4)$$

where $[D_T]$ and $[S_T]$ are the total concentrations of the probe and the binding sites, respectively and I is the fractional inhibition of chloride transport. Rearranging equations 2-4, and substituting in equation 1, we obtain:

$$[D_T] / I = K_D (1-I)^{-1} + [S_T] \quad (5)$$

The fractional inhibition of chloride transport was determined from the equation:

$$I = 1 - (k_i / k_o) \quad (6)$$

where k_i is the rate coefficient for chloride self-exchange in the presence of DIDS, and k_o is the rate coefficient with no DIDS in the extracellular medium.

As can be seen from equation 5, the plot of $[D_T]/I$ against $(1-I)^{-1}$ shows the slope equal to K_D if $[S_T]$ is kept constant in each experimental series. This was fulfilled by keeping the hematocrit constant during flux measurements.

The association rate constant (k_{+1}) for reversible DIDS binding to Band 3 protein in erythrocyte ghosts

The experimental method and equations described previously [16] were applied for k_{+1} determination in control and EAC-treated erythrocyte ghosts. For this purpose tritium-labeled DIDS ([^{3}H]DIDS) was used. In the experimental protocol the volume of 1 ml of ghost suspension (cytocrit ~70%) chilled to 0^{o}C was mixed with 39 ml of well-stirred medium thermostated at 0^{o}C (165 mM KCl, 2 mM K_2HPO_4, pH 7.3). At zero time the volume of 0.6 ml of [^{3}H]DIDS solution (7.6 μM [^{3}H]DIDS, 165 mM KCl, 2 mM K_2HPO_4) at 0^{o}C was injected into the ghost suspension and the reaction of DIDS with Band 3 protein was initiated.

The reversible binding of [^{3}H]DIDS to Band 3 protein proceeds according to the scheme:

$$S + D \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} SD \qquad (7)$$

where [S], [D] and [SD] are the equilibrium concentrations of the free form of the binding sites in Band 3 protein, the probe ([^{3}H]DIDS molecules) and the complex form, respectively.

The rate of the reaction (7) can be written as:

$$d[SD] / dt = k_{+1} \times [S] \times [D] - k_{-1} \times [SD] \qquad (8)$$

By definition:

$$K_D = k_{-1} / k_{+1} \qquad (9)$$

where k_{+1} ($M^{-1}s^{-1}$) and k_{-1} (s^{-1}) are the association and dissociation rate constants for DIDS-Band 3 complex, respectively, and K_D (M) is the dissociation constant for the reaction (7).

Introducing equations (2), (3) and (9) into (8), and integrating, we obtain:

$$Y \equiv k_{+1} \times t = (1/\sqrt{A})\ln\left(\frac{2[SD]-[S_T]+[D_T]+K_D-\sqrt{A}}{2[SD]-([S_T]+[D_T]+K_D)+\sqrt{A}} \cdot \frac{[S_T]+[D_T]+K_D-\sqrt{A}}{[S_T]+[D_T]+K_D+\sqrt{A}}\right) \qquad (10)$$

where: $A = ([S_T] + [D_T] + K_D)^2 - 4[S_T][D_T]$.

Equation (10) describes the kinetics of reversible [^{3}H]DIDS binding to Band 3 protein under conditions where $[S_T]$, $[D_T]$ and K_D remain constant during the experiment. The association rate constant k_{+1} ($M^{-1}s^{-1}$) equals the numerical value of the slope of Y vs. time t.

Statistical analysis

The p-value calculations, based on t-statistics and testing the hypothesis of no linear relationship of the data in Fig. 3, were performed according to [17].

RESULTS

Effect of DIDS on chloride self-exchange in EAC-treated erythrocyte ghosts

As was established previously, DIDS reversibly inhibits chloride ion transport in intact erythrocytes at 0°C, if the time of cell exposure to the inhibitor is short enough to avoid covalent adduct formation (k_{cov} = 3.9 x 10^{-3} min^{-1} at 0°C) [15]. The side chain residues of Band 3 that are responsible for the reversible DIDS attachment and chloride flux inhibition are not known exactly. In this study we explored the role of extracellularly exposed side chain carboxyl residues of Band 3 protein in DIDS reversible interaction. For chemical modification of Band 3 protein we used EAC, which is known as an irreversible modifier of the protein carboxyl groups and membrane-impermeable agent [12]. Under the applied experimental conditions (30 mM EAC in 165 mM KCl, 10 mM MES, pH 5.8; 38°C) the conversion of the externally exposed carboxyl group(s) in Band 3 to N-acylurea probably occurred as a result of resealed erythrocyte membrane treatment by EAC [12]. Bjerrum *et al.* [12] have shown, by using [^{14}C]EAC, that ~15 x 10^6 EAC molecules were incorporated per membrane when the chloride exchange was reduced to ~50% of the control (under similar conditions of EAC treatment as in this study). Much higher selectivity of the labeling (~1 x 10^6 EAC molecules per membrane), resulting in ~50% inactivation of Cl^- exchange, was obtained when trypsin-treated membranes were reacted with EAC in the presence of DNDS.

Fig. 1 shows the representative data obtained from measuring the rates of chloride self-exchange in control (Fig. 1A) and EAC-treated (Fig. 1B) erythrocyte ghosts at 0°C in the presence of various DIDS concentrations in the extracellular medium. Chloride efflux measurements were performed under conditions of equilibrium between DIDS free in solution and reversibly bound to membrane; therefore all curves in Fig. 1 display linearity. As can be seen from Fig. 1A, the rate of the chloride ion efflux in control ghosts (measured as the slopes of the efflux curves) gradually decreased as the total DIDS concentration increased from 0 to 0.88 μM. The calculated fractional inhibition of chloride flux in control erythrocyte ghosts was up to 98%, indicating the high potency of DIDS for inhibition of chloride flux at 0°C.

Under the modification conditions applied, EAC inactivated irreversibly up to (48 ± 5)% of the control chloride exchange capacity in resealed ghosts. Fig. 1B shows the effect of DIDS on the residual chloride flux in EAC-treated erythrocyte ghosts. Similarly, as was observed for control ghosts (Fig. 1A), the rate of the chloride self-exchange in EAC-modified ghosts decreased as the DIDS concentration increased in the extracellular medium, meaning that the residual flux (~50% of control) is sensitive to DIDS. The fractional inhibition of the residual chloride efflux was within the range of (0-93)% at total DIDS concentration from 0 to 0.8 μM in the outside solution. The DIDS inhibition was reversible since albumin (0.5% solution) completely abolished the inhibitory

effect of DIDS on chloride self-exchange in both control and EAC-treated ghosts (data not shown).

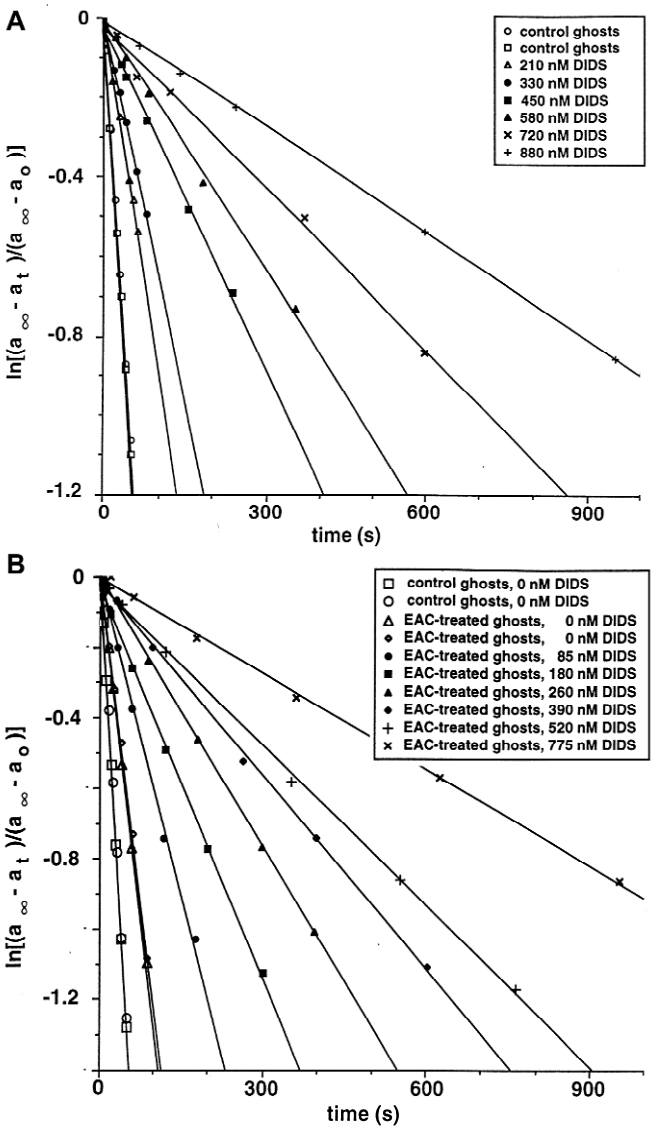

Fig. 1. Effect of DIDS on chloride self-exchange (0°C, pH 7.3, 165 mM Cl^-) in resealed human erythrocyte ghosts as measured under conditions of equilibrium between DIDS free in extracellular medium and reversibly bound to the membrane (representative plots). A – control ghosts, B – EAC-treated ghosts up to 52% of the control Cl^- exchange capacity. $^{36}Cl^-$ efflux was carried out in media used for pretreatment of ghosts with DIDS (total concentrations as indicated) at 0°C. The graphs show the fraction of $^{36}Cl^-$ remaining in the ghosts as a function of time. The slope of each straight line corresponds to the rate of $^{36}Cl^-$ self-exchange and was determined by using linear regression analysis.

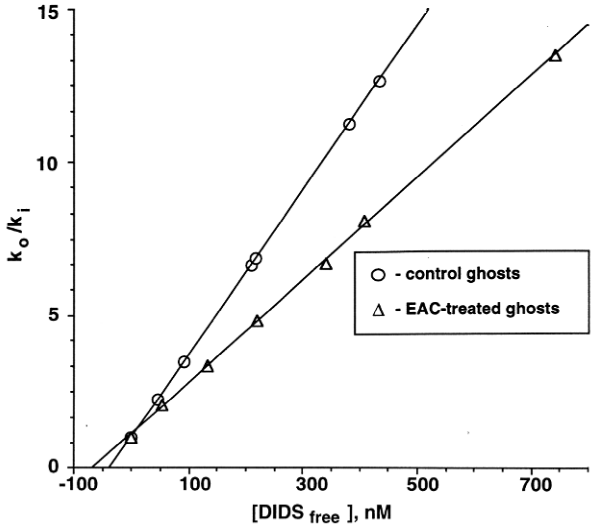

Fig. 2. Modified Dixon plots of chloride self-exchange measured at 0°C (pH 7.3, 165 mM Cl^-) in the presence of various DIDS concentrations in the extracellular medium for control (○) and EAC-modified (Δ) resealed erythrocyte ghosts. The ratio of k_o / k_i was plotted against the concentration of DIDS in the extracellular solution, $[DIDS]_{free}$. k_i (s^{-1}) and k_o(s^{-1}) are the rate constants of chloride self-exchange in the presence and absence of DIDS. The concentration of DIDS that produced 50% inhibition (K_i) is the intercept on the abscissa of the linear regression line. The data points were taken from Fig. 1A and 1B.

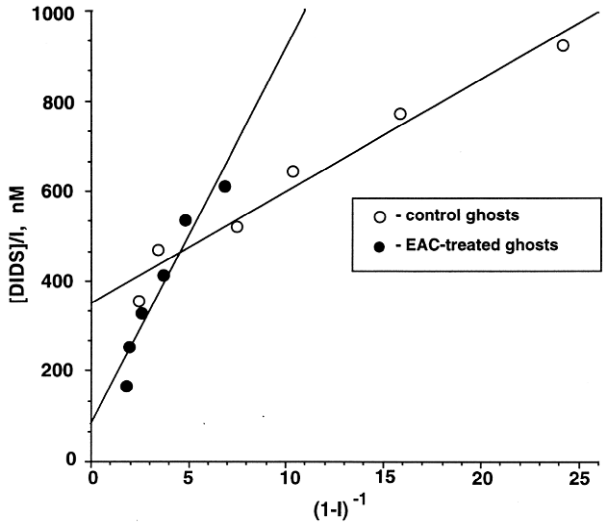

Fig. 3. Representative plots for determination of the dissociation constant, K_D (M), of reversible "DIDS-binding site" complex in control (○) and EAC-treated (●) resealed erythrocyte ghosts. The abscissa is the reciprocal of the residual fractional flux, and the ordinate is the ratio of DIDS total concentration ([DIDS], nM) and the fractional inhibition (I). The slope of each plot equals K_D, which is 33.5 nM for control ghosts and 80.3 nM for EAC-treated ghosts. The plots were made by means of the method of least-squares fitting.

On the basis of transport experiments, as in Fig. 1, modified Dixon plots were constructed (Fig. 2). As can be seen from Fig. 2 the linear plots of k_o / k_i versus $DIDS_{free}$ concentration in the extracellular solution were obtained (k_o and k_i denote the respective rate constant of chloride exchange in the absence and presence of a particular concentration of DIDS). The linearity of Dixon plots seen in Fig. 2 indicates that, similarly as in control ghosts, DIDS interacts with the single site after modification of Band 3 protein by EAC. The intercept of the curve with the abscissa gives the K_i value (K_i is the concentration of DIDS that produces 50% inhibition). We obtained average values (± standard error, three experiments) of (35 ± 6) nM and (60 ± 8) nM for K_i in control and EAC-treated resealed ghosts, respectively. The difference between these two means is statistically significant with the p value equal to 0.025. The evaluated value of K_i for control ghosts is in good agreement with the value of 40 nM estimated previously for intact erythrocytes [13]. The K_i value for DIDS binding increases by a factor of two in EAC-treated ghosts, indicating the reduction of DIDS affinity for binding to the site in modified Band 3 protein.

On the basis of chloride flux measurements, the values of the apparent dissociation constant, K_D, for reversible reaction of DIDS with Band 3 in control and EAC-modified resealed erythrocyte membranes was evaluated. The values of fractional inhibition of chloride transport, calculated for every total DIDS concentration applied, were used for plotting the linear relationship. Fig. 3 shows the representative plots for control and EAC-treated ghosts, respectively. The two linear relationships between DIDS concentration and $(1\text{-}I)^{-1}$ are statistically significant at the p level of 0.001, thus providing strong evidence that DIDS concentration and $(1\text{-}I)^{-1}$ are linearly correlated for both control and EAC-treated ghosts. The correlation coefficients of the data in Fig. 3 are equal to 0.96 (control) and 0.94 (EAC-treated). The apparent dissociation constant, K_D, was determined from these plots as the slopes of the obtained curves. The mean value (± standard error, three experiments) for the apparent dissociation constant, K_D, evaluated in our study was (34 ± 3) nM and (80 ± 12) nM for reversible DIDS interaction with control and EAC-treated erythrocyte ghosts, respectively. The difference between these two means is statistically significant with the p value of 0.01. Thus the reversible binding of DIDS molecules to Band 3 protein in membranes chemically changed by EAC proceeds with over two-fold lower dissociation constant in comparison to control ghosts.

To address the question whether the reduced affinity for reversible DIDS binding to Band 3 modified by EAC arises from modification of only one monomer, we constructed Hill plots on the basis of chloride equilibrium exchange measurements. Fig. 4A presents representative data obtained from measuring the extent of inhibition of chloride equilibrium self-exchange in the presence of increasing concentrations of DIDS. The hyperbolic saturation curve was obtained for both control and EAC-treated ghosts. From such measurements, Hill plots were constructed to determine the effect of the EAC treatment on the degree of cooperativity of the binding sites (Fig. 4B). Hill

coefficients (± standard error, three experiments) of 1.02 ± 0.07 and 0.98 ± 0.06 for control and EAC-treated ghosts were obtained, showing no cooperativity of DIDS binding sites in Band 3 oligomer both before and after EAC-treatment.

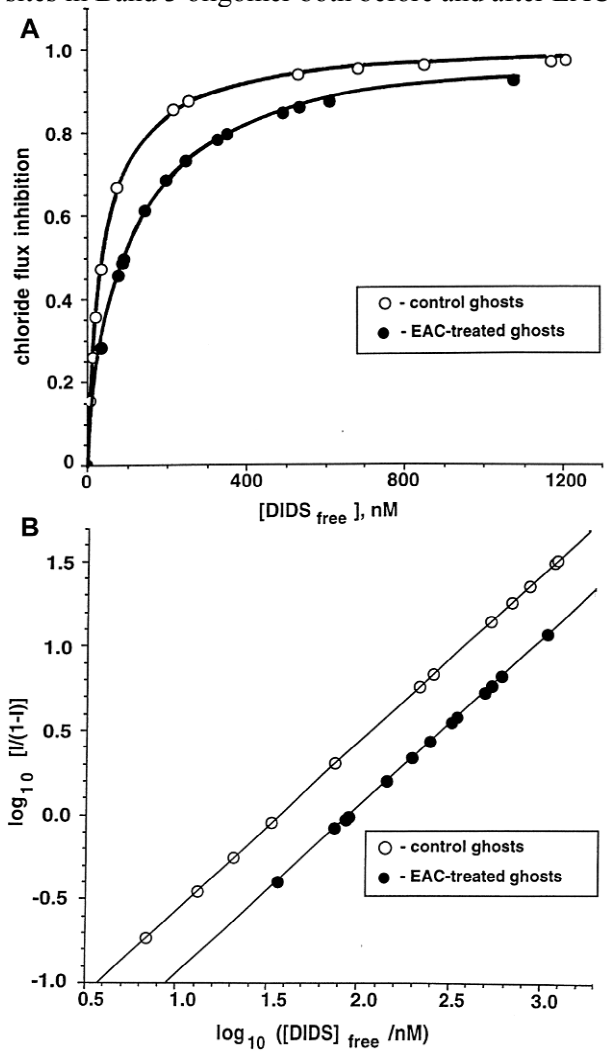

Fig. 4. Construction of Hill plots. A – DIDS inhibition of chloride self-exchange in control (○) and EAC-treated (●) resealed erythrocyte ghosts at 0°C, pH 7.3, in the presence of 165 mM Cl^- (representative plots). Resealed membranes were EAC-inactivated to 50% of the control chloride flux. The abscissa denotes the concentration of free DIDS molecules in the extracellular medium. The ordinate denotes the fractional inhibition of chloride flux caused by reversibly bound DIDS. B – Hill plots derived from chloride self-exchange measurements as in (A) for reversible DIDS binding to resealed ghosts. The abscissa represents the logarithm of DIDS free concentration (nM) in the extracellular medium during $^{36}Cl^-$ efflux measurements, the ordinate represents the logarithm of the ratio of fractional inhibition (I) and the residual flux (1-I). The slope of the curves corresponds to Hill coefficient: 0.99 for control and 1.00 for EAC-treated erythrocyte ghosts.

[^{3}H]DIDS binding to erythrocyte membranes

The aim of this study was to characterize the kinetics of reversible DIDS interaction with Band 3 protein chemically modified by EAC in resealed human erythrocyte ghosts. The experiments performed with tritium-labeled DIDS ([^{3}H]DIDS) provide a way of measuring the association rate constant k_{+1} ($M^{-1}s^{-1}$) for reversible DIDS binding to the sites on Band 3 protein. The binding process was initiated by injection of the radioactive DIDS solution to the suspension of control or EAC-treated ghosts. The binding experiments were carried out at 0^{o}C and 120 s duration. Samples of the extracellular medium, taken by filtration technique at appropriate time intervals, were assayed for tritium radioactivity.

As the binding of [^{3}H]DIDS molecules to Band 3 protein proceeded, the radioactivity of the extracellular solution decreased with time. The nonlinear (monophasic) decline of the tritium radioactivity from medium was observed either for control or EAC-treated ghosts (data not shown). The radioactivity measured for each taken sample was directly proportional to the concentration of unbound (free) [^{3}H]DIDS present in the extracellular solution. The number of [^{3}H]DIDS molecules taken up by each individual cell was calculated from the total [^{3}H]DIDS concentration added to the ghost suspension, from the measured concentration of [^{3}H]DIDS in the extracellular medium and counted number of red blood cell ghosts per unit volume. Fig. 5A presents the representative time course for [^{3}H]DIDS uptake in control and EAC-treated ghosts. As can be seen from Fig. 5A, the time-dependence of the association of [^{3}H]DIDS with the sites on Band 3 protein follows the profile of a single, saturable binding process in control and EAC-modified membranes, respectively. However, a lower level of saturation was observed for EAC-treated ghosts. [^{3}H]DIDS uptake curves reach an equilibrium (within ~60s) at the maximal level of about 5.8 x 10^5 and 3.2 x 10^5 [^{3}H]DIDS molecules bound per one control or EAC-treated ghost, respectively (Fig. 5A). According to Bjerrum *et al.* [12] the relation between residual exchange and covalent DIDS binding in EAC-treated membranes was nonlinear (in contrast to control membranes) and ~90% of the residual Cl^- exchange was inhibited by covalent binding of ~8 x 10^5 DIDS/membrane. The remaining 10% was inhibited by binding an additional ~4 x 10^5 DIDS/membrane. Therefore the total inhibition was achieved by binding of ~1.2 x 10^6 DIDS/membrane, which corresponds to the number of Band 3 molecules per cell. A similar number, ~1.2 x 10^6 DIDS/membrane, was obtained by Wieth *et al.* [7] for covalent binding of DIDS to control cells. In addition, Janas *et al.* [15] obtained the same value in the case of reversible DIDS binding to Band 3 protein in control cells.

The concentrations of [^{3}H]DIDS bound to the membrane (SD_∞), [^{3}H]DIDS free (D_∞) in the extracellular medium and the concentration of free binding sites (S_∞) at the equilibrium state (usually after 60 s duration of the binding experiment) were taken for calculation of the equilibrium dissociation constant (K_D) for reversible DIDS binding to Band 3 ($K_D = S_\infty \times D_\infty / SD_\infty$). The mean value

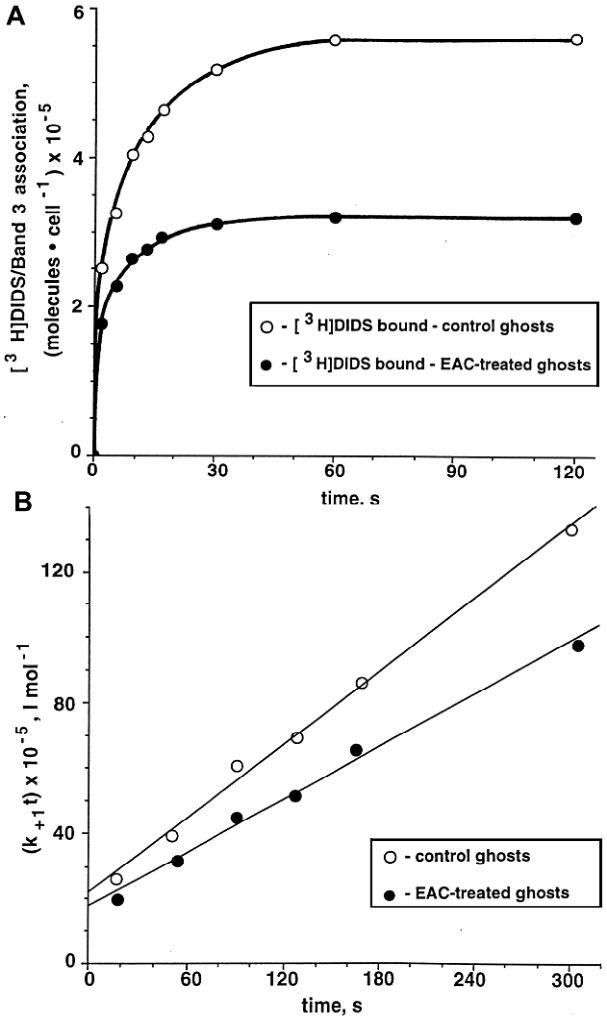

Fig. 5. [^{3}H]DIDS binding to erythrocyte membranes. A – Representative time courses of reversible [^{3}H]DIDS binding to Band 3 protein in control (○) and EAC-treated (●) resealed human erythrocyte ghosts. B – Plot of $Y = k_{+1}t$ as a function of time t. The values of Y were calculated according to equation 10 (for details see Materials and Methods section). k_{+1} is the association rate constant for reversible binding of [^{3}H]DIDS to Band 3 protein. The slope of each straight line corresponds to the value of k_{+1} (M^{-1} s^{-1}) and equals 3.93 x 10^5 $M^{-1}s^{-1}$ and 2.69 x 10^5 $M^{-1}s^{-1}$ for control and EAC-treated resealed ghosts, respectively.

(± standard error, four experiments) for K_D obtained from equilibrium [^{3}H]DIDS uptake measurements was (37 ± 9) nM and (90 ± 21) nM for control and EAC-modified ghosts, respectively. The difference between these two means is statistically significant with the p value of 0.01. These values are in good

agreement (within the error limit) with the values of K_D obtained independently in this study from chloride flux measurements (see previous section).
As described in Materials and Methods, the value of the association rate constant k_{+1} ($M^{-1}s^{-1}$) for "DIDS-Band 3" reversible complex formation can be evaluated on the basis of the time course of [^{3}H]DIDS uptake as it is presented in Fig 5A. Fig. 5B shows the [^{3}H]DIDS binding data replotted in the form of equation 10. The slopes of the resulting lines in Fig. 5B correspond to the values of the association rate constants, k_{+1}. The average values (± standard error, four experiments) of (4.3 ± 0.7) x 10^5 $M^{-1}s^{-1}$ and (2.7 ± 0.7) x 10^5 $M^{-1}s^{-1}$ were obtained for control and EAC-treated ghosts, respectively, showing that DIDS binding to EAC-treated membranes proceeds with a lower association rate constant in comparison to control ghosts. The difference between these two means is statistically significant with the p value of 0.025. The lower association rate constant for DIDS binding to Band 3 after modification by EAC implies that the binding reaches the equilibrium state at a lower level of DIDS bound per cell (according to equations 1 and 9, see Materials and Methods).

DISCUSSION

Treatment of red blood cells with carbodiimide compounds inhibits monovalent and divalent anion transport [7, 12, 18-20]. The covalent binding of DIDS [12] and H_2DIDS [20] to the extracellular binding site is essentially intact after EAC modification. Wieth *et al.* [7] observed an apparent loss of covalent DIDS binding sites after treatment with EAC. They found that in the EAC-treated membranes, chloride self-exchange decreased linearly with DIDS covalent binding, but complete inhibition of transport was obtained with about 6 x 10^5 DIDS molecules bound per cell, i.e. half of the number needed to label all Band 3 molecules in the control cells. In contrast, Bjerrum *et al.* [12] reported that in EAC-modified resealed ghosts, a nonlinear relation between the remaining exchange activity and the number of covalently bound DIDS molecules occurred. By extrapolation of the data they found that complete inhibition of the residual chloride transport in EAC-treated cells could be obtained with binding of ~1.2 x 10^6 DIDS molecules per cell, which corresponds to the number of DIDS binding sites per control membrane. This indicated that the stoichiometry of DIDS covalent binding to Band 3 protein remains unchanged after EAC modification and binding of DIDS proceeds more likely with the changed affinity. Moreover, Werner and Reithmeier [49] observed no reduction in H_2DIDS labeling after treatment with EAC. In addition, the linearity of Dixon plots in our experiments (Fig. 2) confirms the existence of a homogeneous population of DIDS reversible binding sites in EAC-treated cells and suggests a 1:1 stoichiometric relationship between the inhibitor and inhibition. Because ca. 1.5 EAC molecules, under applied experimental conditions, bind to one Band 3 protein [12], ca. one third of EAC molecules which bind Band 3 can modify the carboxyl groups near the transport site. DIDS affinity to Band 3 after EAC

treatment is 2-fold lower and less than two EAC molecules are bound per AE1 dimer, suggesting that half of the subunits are modified by EAC near the transport site and that half are much more weakly capable of binding DIDS.
As shown in this report, the modification of the carboxyl groups on band 3 can alter the structure of the membrane domain, contributing towards a reduced transport rate and reduced DIDS affinity. There are three possible hypotheses on how the structure of the membrane domain can be altered: the first – through modification of the stimulatory (modifier) site; the second – through modification of glutamates and aspartates near the KLIK binding motif for DIDS; the third – through indirect structural rearrangements.

Hypothesis on modification of the stimulatory site

The properties of the extracellular anion binding site of the transport system remain unchanged after EAC treatment as can be judged from the measurements of the extracellular chloride ion concentration at half-maximal flux [12]. This result strongly suggests that the carboxyl groups modified by EAC are not directly involved in chloride binding to the extracellular anion binding site. On the other hand, the existence of a stimulatory site was suggested, based on thermodynamic data, near the transport site on Band 3 protein [42]. According to this model, the binding of an anion to this stimulatory site can facilitate (through electrostatic repulsion interaction between two anions) the initial stage of the transmembrane movement of another anion from the transport site. A similar mechanism was proposed for the movement of ions within the pore of the KcsA K^+ channel from *Streptomyces lividans* [21]. As was previously proposed [16], one of the two sulfonate groups of the DIDS molecule binds to the transport site (located in the transmembrane α-helical segment of Band 3) whereas the second group (in *trans* configuration) interacts with the stimulatory site (located in the extracellular loops of Band 3 protein). The existence of a second chloride-binding site was also suggested, based on kinetic data, by Passow and coworkers, as a binding/transport site for chloride/proton cotransport" [1]; by Salhany and coworkers, as a "modifier site" [22]; and by Jennings, as a site at which "Cl^- may exert its stimulatory effects as a cofactor" [23]. The two-fold increase of the value of K_D for reversible DIDS binding to Band 3 (Fig. 3) after EAC treatment indicates that the free energy change (ΔG) of reversible DIDS binding at 0^oC decreases. This effect can result from the diminished interaction of one of the two sulfonate groups of the DIDS molecule with the modified (by EAC) carboxyl residues at the stimulatory site since the properties of the transport site remain unchanged after EAC treatment [12]. The results of Falke and Chan [24] suggesting that DIDS partially blocks access to the transport site are also consistent with the "stimulatory site" model, and indicate the importance of the stimulatory site in Band 3 function since irreversibly-bound DIDS does not completely inhibit binding of Cl^- to the transport site while it abolishes the translocation of anions across the membrane [7]. Therefore, the results presented in this paper suggest that EAC molecules modify the carboxyl

groups of the stimulatory site but do not change the carboxyl groups of the transport site on Band 3.

Hypothesis on modification of glutamates and aspartates near the KLIK binding motif for DIDS

The DIDS molecule has two negatively charged sulfonate groups and two isothiocyano groups that are capable of reacting covalently with free amines. In human Band 3 protein, the target of one covalent reaction of DIDS is the lysine at position 539 (K539) near the extracellular end of transmembrane segment 5 (TM 5). However, the second reaction can take place with a lysine which is close spatially to K539 but far away from K539 in linear distance along the polypeptide chain (reviewed in [25-26]). A potential DIDS-binding motif at the putative extracellular end of TM 5 was proposed to be KLIK (where the first K is K539 in human AE1) on the basis of homologies among AE1-AE3 [27]. DIDS probably reversibly binds before irreversibly reacting with the binding motif, as studied in the case of another member of the bicarbonate-transporter family (SLC4), NBCe1-A [28]. There are both glutamate and aspartate residues close to the KLIK motif (at a distance of just 3-4 amino acids from this motif) [26]. Therefore, the chemical modification of these acidic amino acids by EAC treatment can partially block the access of DIDS to the KLIK domain. There is most likely an overlap between the stimulatory site and the KLIK domain extended to the surrounding amino acids residues.

Hypothesis on indirect structural rearrangements

The changes in the transport rates and DIDS affinity may result from conformational changes in the Band 3 protein, consequent to the extracellular carboxyl group modification, which alters the pK of functionally important groups at the intracellular side of the protein. Therefore the EAC-modified carboxyl groups outside the extracellular anion binding site of the Band 3 protein may only be allosterically coupled to this site. A similar hypothesis was suggested by Bjerrum [12] based on the changes in the intracellular titration function.

In conclusion, the results demonstrate that EAC modification of AE1 reduces about 2-fold the affinity of AE1 for DIDS, and we suggest that half of the subunits are modified near the transport site by EAC.

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