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Kinetic evidence for modulation by glycophorin A of a conformational equilibrium between two states of band 3 (SLC4A1) bound reversibly by the competitive inhibitor DIDS

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ABSTRACT

Recent evidence has suggested that erythrocytes naturally deficient in glycophorin A (GPA) have a reduced V_{max} for monovalent anion exchange. Unanswered is whether miss-folding of band 3 during biosynthesis, or the absence of GPA modulation of properly folded band 3 is responsible. Here, I determine the effect of selective depletion of GPA on the kinetics of reversible binding of the competitive transport inhibitor DIDS (4,4′-diisothiocyanato-2,2′-stilbenedisulfonate) to properly folded band 3. Reversible binding of DIDS follows biphasic kinetics: a fast phase {DIDS + band 3 ⇔ (DIDS − band 3), k_1, k_{-1}} and a slower phase {(DIDS − band 3) ⇔ (DIDS − band 3)*, k_2, k_{-2}}. Selective depletion of GPA was accomplished by pretreating membranes with Triton X-100, over a range where erythrocyte hemolysis is inhibited by the detergent (0% to 0.03%, v/v). Pretreatment with sublytic Triton X-100: (a) virtually completely depleted GPA, (b) did not deplete membrane-bound band 3, and (c) slowed the overall rate of reversible binding of DIDS to band 3. Data analysis and model simulation studies indicated that the decrease in the rate of binding of DIDS was due exclusively to a decrease in k_{-2}, with no change in the initial rate of binding. Thus, depletion of GPA does not alter the native conformation of band 3 at the DIDS binding site, but rather modulates a conformational equilibrium between two states of the binary complex formed by the competitive inhibitor DIDS, reversibly bound to properly folded band 3.

Introduction

Glycophorin A (GPA) and band 3 (SLC4A1) are the most abundant integral membrane proteins of the human erythrocyte membrane, with the number of copies of each being approximately equal. While the multiple functions of band 3 are well defined (anion exchange, anchorage of the cytoskeleton and binding of glycolytic enzymes and hemoglobin, among other functions) [1–6], the exact function of GPA has been somewhat more obscure. Recent evidence has suggested that GPA interacts with band 3 within the lipid bilayer of the human erythrocyte membrane, and also provides a chaperone-like function for the membrane expression of band 3 [7].

One important unresolved question is whether the interaction of band 3 and GPA within the membrane serves to modulate the anion transport or protein binding functions of band 3. There is evidence that the presence of GPA influences the amount of oxyhemoglobin bound to the erythrocyte membrane [8] at the cytoplasmic domain of band 3 [9]. Newer evidence has indicated that cells deficient in GPA have a lower band 3-mediated anion exchange activity. This lower activity is manifest by a decrease in V_{max} for monovalent anion exchange, without changing either K_m or K_d, but by an increase in K_m for divalent anion transport [10,11]. Since the monovalent anion exchange function of band 3 was not totally inhibited in GPA deficient cells, it was suggested that band 3 can exist in at least two activity states [10]. However, it was not possible to determine whether these changes in the function of band 3 were mediated indirectly by the absence of GPA during band 3 biosynthesis, or whether the functional differences resulted from the absence of an effect of GPA on the conformation of mature, properly folded band 3 within the red cell membrane [10].

Here, I attempt to address this issue by studying the reversible binding of DIDS (4,4′-diisothiocyanato-2,2′-stilbenedisulfonate) to band 3 using isolated native erythrocyte membranes selectively depleted of GPA by treatment with so-called "sublytic" concentrations of Triton X-100 on ice, according to the method of Yu et al. [12]. With this approach, membranes selectively depleted of GPA would be expected to have copies of band 3 which were properly folded during biosynthesis. Furthermore, the effect of sublytic and lytic concentrations of Triton X-100 on the erythrocyte membrane have been studied extensively [13–20], thus facilitating interpretation of the DIDS reversible binding data.

Interpretation of the data in this study requires that the mechanism of DIDS reversible and covalent binding to band 3 be understood. DIDS is a member of the stilbenedisulfonate (SD) class of competitive

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inhibitors of band 3-mediated anion exchange [21]. All members of this class of inhibitors bind reversibly to an inhibitory site which is distinct from, but allosterically linked to the transport site on band 3 [22–26]. The kinetics of SD reversible binding to band 3 have been studied extensively, and detailed aspects of the binding process characterized [23,24,27–30]. The kinetic time courses for SD molecules DBDS (4,4′-dibenzamido-2,2′-stilbenedisulfonate) and H_2DIDS (4,4′-diisothiocyanatodihydro-2,2′-stilbenedisulfonate) binding to band 3 are biphasic, involving an initial second-order binding step followed by a "conformational transition" within the SD/band 3 binary complex [23,24,27–30].

Studies of DIDS binding to band 3 have been somewhat more controversial [29,31]. One paper [31] suggested that reversible binding of DIDS followed a monophasic time course at 0 °C, where formation of the covalent adduct is slowed [1]. The question concerning the presence of biphasic kinetics for reversible binding of DIDS was explored experimentally and theoretically in a subsequent study [29]. Biphasic kinetics were observed at 0 °C when [DIDS]>[band 3]. Model simulation studies showed that the use of a very low concentration of DIDS in the former study [31] caused the first step in the reaction to become rate limiting, thus explaining the observation of monophasic kinetic time courses. It was concluded [29] that reversible binding of DIDS to band 3 follows a biphasic kinetic rate law and obeys the mechanism shown in Eq. (1):

$$\mathrm{DIDS} + \mathrm{B3} \Leftrightarrow (\mathrm{DIDS}-B3) \Leftrightarrow (\mathrm{DIDS}-B3)^{*}\ k_1, k_{-1}, k_2, k_{-2} \qquad (1)$$

where $k_f = k_1\ [\mathrm{DIDS}] + k_{-1}$, $k_s = \{(k_2[\mathrm{DIDS}])/(K_1 + [\mathrm{DIDS}])\} + k_{-2}$, with B3 = band 3, $K_1 = k_{-1}/k_1$ and $K_2 = k_{-2}/k_2$, and with $K_d = (K_1 * K_2)/(1 + K_2)$.

When using DIDS, it is necessary to consider the kinetics of formation of the covalent adduct with "lysine A" [1]:

$$(\mathrm{DIDS}-B3)^{*} \Rightarrow (\mathrm{DIDS}-B3)^{+} \quad k_3 \qquad (2)$$

where $(\mathrm{DIDS}-\mathrm{B3})^{+}$ represents the DIDS − band 3 covalent adduct involving "lysine A". The kinetics of DIDS covalent adduct formation have been studied in detail under a wide range of conditions [32]. The contribution of covalent binding to the reversible binding reactions can be addressed in model simulation studies using the mechanisms given in Eq. (1) and (2) and the value of k_3 established in our earlier study [32].

Materials and methods

In-dated human erythrocytes were obtained from the Omaha Chapter of the Red Cross. Hemoglobin-free unsealed erythrocyte membranes (ghosts) were prepared as described previously [33] and washed in 5 mM sodium phosphate pH 8.0 (5P(8)). Resealed ghosts were prepared in 150 mM NaCl, 5 mM sodium phosphate pH 7.4 (PBS) as described [34]. Ghosts prepared by this method are sealed to anion leaks, and contain about 4 mM hemoglobin (heme basis) [34]. DIDS (disodium salt) (*trans*-form) was obtained from Sigma. A stock solution of 12.8 mM was prepared in water, protected from light, and stored at −20 °C until used. Triton X-100 was obtained from Bio-Rad. All other chemicals were of reagent grade or A.C.S. certified.

Selective depletion of GPA from human erythrocyte membranes using sublytic concentrations of Triton X-100 was performed exactly as described by Yu et al. [12]. Briefly, one volume of packed ghosts in 5P(8) was mixed with 5 volumes of 56 mM Na borate, pH 8.0 (ionic strength ≈ 0.008) containing 0%, 0.01% or 0.03% (v/v) Triton X-100, and incubated for 20 min on ice. After centrifugation, the ghost fraction was collected, and washed in 5P(8) and stored for electrophoresis. The other portion of the ghost fraction was washed in PBS and stored at 4 °C until used for the DIDS reversible binding kinetic studies.

The effect of increasing Triton X-100 concentration on membrane integrity was tested using a resealed ghost hemolysis assay. Resealed ghosts were washed in PBS and then suspended at ~7% hematocrit in the same buffer containing increasing amounts of Triton X-100 (0% to 0.2% (v/v)). The samples were then incubated for 20 min on ice, and then centrifuged at 4 °C. Each supernate was collected, quantitatively diluted if necessary, into a cuvette for absorbance measurements. A constant weight of dithionite crystals was added to the cuvette, which was then caped with a rubber stopper. The absorbance was measured at the 555 nm, which is an absorbance peak for deoxyhemoglobin. Percent hemolysis was calculated using a 100% hemolysis endpoint, which involved measuring the absorbance of the supernate from resealed ghosts hemolysed by 1:50 dilution in 5P(8).

SDS-PAGE was preformed as described [35], on samples with matching concentrations of ghosts, determined by light scattering [33]. After electrophoresis, duplicate gels were stained either with Coomassie Blue or periodic acid Schiff (PAS) staining procedures as described [35]. Gels were scanned using a Bio-Rad model 1650 transmittance/reflectance densitometer as described [35].

The kinetics of DIDS reversible binding to band 3 were performed as described [29], using a Gibson-Durrum stopped-flow apparatus interfaced to a computer containing the OLIS data acquisition system (Athens, GA). The reactions were performed at 25 °C using ghost suspensions matched on the basis of light scattering measurements [33]. The reaction was followed by observing band 3 protein fluorescence quenching through a 315 nm cutoff filter placed at right angle to the flow cell. The excitation wavelength was set at 280 nm. Studies have shown that band 3 protein fluorescence quenching by DIDS is linearly related to fractional saturation of reversibly bound DIDS under stoichiometric binding conditions [24].

Model simulation studies were performed using "Chemical Kinetics, Version 1.01", provided on-line by the IBM Almaden Research Center, as described previously [29]. Curve fitting of both experimental and theoretical data and other quantitative analyses and graphic presentations were performed using Sigma Plot (SPSS Science, Chicago, IL).

Results

Effect of Triton X-100 concentration on the hemolysis of resealed ghosts under isoosmotic conditions in PBS at pH 7.4

Fig. 1 shows the effect of increasing Triton X-100 concentration on the hemolysis of resealed ghosts under isoosmotic conditions. The profile is clearly biphasic. The insert shows that between 0 and 0.04% Triton X-100, the amount of background hemolysis actually decreases. This result is similar to that seen in the literature [13], where it was demonstrated that such sublytic concentrations of Triton X-100 actually inhibit osmotic hemolysis. Increasing the concentration of Triton X-100 beyond 0.04% induces hemolysis of resealed ghosts as expected from previous studies using intact red blood cells [13]. Thus, the membrane permeability barrier of our resealed ghost preparation is maintained and even stabilized over the 0% to 0.04% Triton X-100 concentration range.

Evidence for selective depletion of GPA from isolated red blood cell membranes pretreated with sublytic Triton X-100 (0% to 0.03% (v/v))

Fig. 2 shows the effect of pretreatment with increasing Triton X-100 concentration over the sublytic range on membrane protein composition, as determined by both PAS (Fig. 2A) and Coomassie Blue (Fig. 2B) staining of matching SDS-PAGE gels. Both gel patterns were the same as the classical patterns published by Yu et al. [12], and are presented in Fig. 2 over narrower migration ranges in order to focus on changes, or the lack thereof, in the peak intensities of GPA in the PAS staining pattern and spectrin through band 3 in the Coomassie Blue staining pattern.