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Studies on the interaction of matrix-bound inhibitor with Band 3, the anion transport protein of human erythrocyte membranes

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The effect of temperature and chemical modification on the interaction of the human erythrocyte Band 3 protein (the anion transport protein) with 4-acetamido-4′-isothiocyanostilbene 2,2′-disulfonate (SITS; $K_i = 10$ μM)-Affi-Gel 102 resin was studied. Band 3 binds to the affinity resin in two states; weakly bound, which is eluted by 1 mM 4-benzamido-4′-aminostilbene 2,2′-disulfonate (BADS; $K_i = 2$ μM), and strongly bound, which is eluted only under denaturing conditions by 1% lithium dodecyl sulfate (LDS). At 4°C, most of band 3 was present initially in the weakly bound form and very little in the strongly bound form. With longer incubations at 4°C, the weakly bound form was slowly converted to the strongly bound form. At 37°C, most of Band 3 was rapidly converted to the strongly bound form, with some Band 3 still remaining in the weakly bound form. Band 3 dimers, labelled with 4,4′-diisothiocyanostilbene 2,2′-disulfonate (DIDS) in one monomer, did bind to immobilized SITS but did not become tightly bound upon incubation at 37°C. Since the covalent attachment of DIDS to one monomer prevented the adjacent monomer from becoming tightly bound to immobilized SITS ligand, this observation suggests that the inhibitor-binding sites of the two adjacent monomers must be interacting with each other. When the inhibitor site of Band 3 was selectively modified by citrate in the presence of 1-ethyl-3-(3-azonia-4,4-dimethylpentyl)carbodiimide (EAC), Band 3 bound to the resin was more easily eluted by BADS, suggesting reduced affinity for immobilized SITS. However, citrate-modified Band 3 did become tightly bound upon incubation at 37°C.

Introduction

Band 3 is the major integral membrane protein of the erythrocyte and catalyzes the rapid exchange of anions across the membrane [1–3]. The anion-translocation site is located in the membrane-bound carboxyl-terminal domain (M_r = 55 000) of Band 3. Anion exchange is thought to occur via a conformational change that translocates the anion-binding site between external and cytoplasmic membrane interfaces [1,4]. Anion exchange is specifically inhibited by stilbene disulfonates, which bind at the external transport site of Band 3 [1,5]. This site is buried within the

Abbreviations: 5P8 buffer, 5 mM sodium phosphate (pH 8.0); BADS, 4-benzamido-4′-aminostilbene 2,2′-disulfonate; Band 3 extract, supernatant from KI-extracted solubilized ghosts in citrate buffer with 1% $C_{12}E_8$; $C_{12}E_8$, octaethylene glycol mono-*n*-dodecyl ether; citrate buffer, 228 mM sodium citrate (pH 8.0); DBDS, 4,4′-dibenzamidostilbene 2,2′-disulfonate; DIDS, 4,4′-diisothiocyanostilbene 2,2′-disulfonate; EAC, 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide; H_2-DIDS, 4,4′-diisothiocyanodihydrostilbene 2,2′-disulfonate; LDS, lithium dodecyl sulfate; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate.

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membrane [6,7] and has been located 35–40 Å from sulfhydryl residues on the cytoplasmic domain [7]. The kinetics of DBDS binding studied both by stopped-flow and temperature-jump experiments have suggested that a rapid initial binding step is followed by a slower conformational change that locks the inhibitor in place [8]. Intrinsic fluorescence quenching studies also suggest that a conformational change in the Band 3 protein accompanies the binding of BIDS [7]. Also, there have been some suggestions that inhibitor-binding sites on two adjacent monomers of Band 3 dimer may interact with each other [9,10].

We have designed and synthesized an affinity resin for purification of Band 3 protein [11]. Band 3 binds specifically to SITS-Affi-Gel 102 resin via its inhibitor binding site and the bound protein can be eluted specifically by free inhibitor ligands. We have also shown that upon initial binding to immobilized SITS, Band 3 becomes more tightly bound, presumably through a conformational change in the protein [11], as has been suggested from the previous kinetic studies [7,8].

The SITS-Affi-Gel 102 can be used to probe the binding characteristics of stilbene disulfonate to Band 3 protein. Immobilization of ligand in a fixed orientation onto a solid matrix makes separation of free protein from protein-ligand complex very easy, and elution profile of the bound protein gives direct indication of protein affinity for the immobilized ligand. Thus, effects of chemical modification on the affinity of Band 3 towards stilbene disulfonate inhibitors can be easily studied. We affirm that citrate modification of the inhibitor binding site on Band 3 reduced protein affinity for the immobilized ligand. Moreover, we provide evidence for intersubunit interaction between inhibitor binding sites of adjacent monomers.

Experimental procedures

Materials

Affi-Gel 102 was purchased from Bio-Rad. SITS was obtained from Pierce (Rockford, IL) and from U.S. Biochemical, Cleveland, OH. BADS was synthesized according to Kotaki et al. [12] as previously described [6]. Lithium dodecyl sulfate was a product of Boehringer-Mannheim, while $C_{12}E_8$ was from Nikko, Tokyo. All other chemicals were reagent grade or better. EAC was prepared as previously described [13] from the free base of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (purchased from Pierce) in anhydrous diethyl ether solution by the method of Sheehan et al. [14]. The white crystalline product was extensively washed with anhydrous diethyl ether to remove unreacted carbodiimide and methyl iodide. [^{3}H]H_2DIDS was purchased from Research Development Corporation, Hospital for Sick Children, Toronto, Ontario.

Methods

Details of erythrocyte ghost membrane preparation, solubilization of Band 3 and SITS-Affi-Gel 102 affinity resin preparations are given elsewhere [11]. Erythrocyte ghosts were prepared from outdated blood (kindly provided by the Canadian Red Cross) by hypotonic lysis in 5 mM sodium phosphate (pH 8.0).

Treatment of intact erythrocytes with EAC [13]. Erythrocytes were washed four times with 10–15 vol. of 0.9% (w/v) NaCl at 4°C to remove storage medium and white cells. In order to obtain chloride-free external medium, cells were further washed three times with 10 vol. of 28.5 mM sodium citrate (pH 7.4)/205.3 mM sucrose. Cells were then incubated with 8.7 mM EAC in 28.5 mM sodium citrate (pH 6.7)/205.3 mM sucrose for 5 min at 37°C. Cells were washed three times with 28.5 mM sodium citrate (pH 7.4)/205.3 mM sucrose prior to ghost membrane preparation. Anion transport was measured by following the uptake of [^{32}P]phosphate into chloride-loaded cells as described previously [13]. EAC modification produced about 60% inhibition of anion transport [13].

DIDS treatment of erythrocytes. Erythrocytes were washed three times with 150 mM NaCl/5 mM sodium phosphate (pH 8.0) at 4°C. Cells were suspended at a 25% hematocrit in the same buffer. [^{3}H]H_2DIDS or DIDS was added to various concentrations and the suspension was incubated at 37°C for 1 h. The cells were washed with the same buffer containing 0.5% bovine serum albumin at 4°C, followed by two washes at 4°C with the same buffer.

Binding assay. Unless stated otherwise, all steps were carried out at 0–4°C. Protein-binding assays were performed in 1.5 ml microfuge tubes. 25 μl of packed resin was washed twice with 250 μl of 228 mM sodium citrate (pH 8.0)/0.1% $C_{12}E_8$/1 mM dithiothreitol (citrate buffer) and was incubated with 1 ml of Band 3 extract (1.5–2 mg/ml protein; see Ref. 11) for 15 min. After removing the supernatant, the gel was washed at least three times with 250 μl of citrate buffer and the bound protein was then eluted by shaking the resin for 10 min with 110 μl of 1 mM BADS in 5 mM sodium phosphate/1% $C_{12}E_8$. Finally, the resin was washed at least twice with 10 vol. of citrate buffer and then extracted with 110 μl of 1% LDS/5 mM sodium phosphate for 10 min.

Analytical techniques. Protein assay was according to Lowry et al. [15]. Sodium dodecyl sulfate gel electrophoresis was performed according to Laemmli [16]. Protein bands were stained with Coomassie blue. Commassie-blue-stained gels were scanned in Joyce-Loebl Chromoscan 3 densitometer at 530 nm.

Results

Interaction of Band 3 with SITS-Affi-Gel 102

The interaction of Band 3 with immobilized SITS was studied at 4°C and 37°C. Fig. 1 shows that at 4°C the amount of Band 3 weakly bound to the resin increased in a time-dependent manner and reached a maximal binding after 30 min (Fig. 1a). At 4°C after 60 min the amount of Band 3 in the tightly-bound form was about half (15 units vs. 32 units) of the amount of Band 3 in the weakly-bound form (Fig. 1b).

In a parallel set of experiments, after the initial 15 min at 4°C, the resin was either incubated at 37°C or washed to remove free protein and then incubated at 37°C. As we have shown previously [11] removal of unbound protein and incubation of matrix-bound protein at 37°C resulted in rapid and quantitative conversion of Band 3 to the tightly bound form. This is shown by the rapid decrease in the amount of BADS-elutable protein with a concomitant increase in Band 3 in the LDS eluate (Fig. 1). In the presence of free protein, incubation at 37°C resulted in a decrease in BADS-elutable Band 3 with a concomitant increase in LDS-elutable Band 3 (Fig. 1). In this case, however, the conversion from the weakly-bound state to the strongly bound state was not as rapid. Moreover, even after a 40 min incubation at 37°C, a part of Band 3 was still present in the BADS-elutable state.

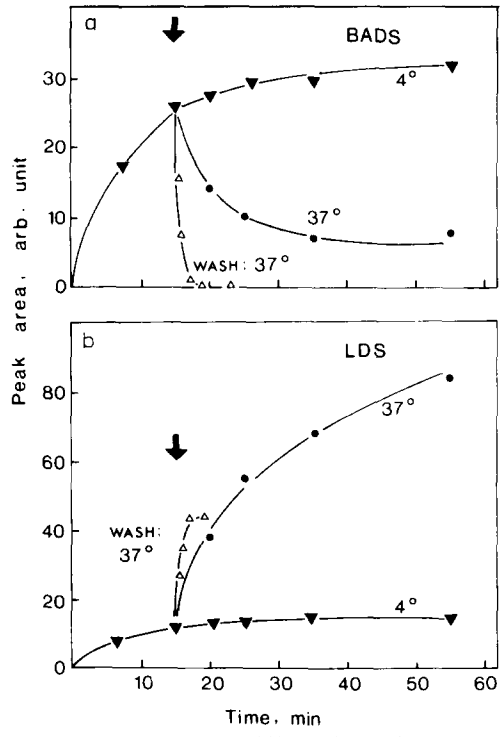

Fig. 1. Effect of temperature shift on the elution properties of Band 3 bound to the SITS-Affigel-102 affinity resin. Details of the experiment are given under Experimental procedures, except that following 15 min at 4°C, incubation was continued at 4°C (▼) or incubated at 37°C with (△) or without (●) washing off the unbound protein. 1 mM BADS-5P8 eluates (a) and 1% LDS-5P8 eluates (b) were electrophoresed on SDS-polyacrylamide gel, stained with the Coomassie blue stain and scanned in a Joyce Loebl Chromoscan 3 densitometer at 530 nm. The arrow shows the time at which incubation was shifted to 37°C.

At 4°C with prolonged incubation (up to 18 h), the amount of Band 3 in the weakly bound form remained constant. However, the amount of Band 3 in the strongly bound form continued to increase and was 3-fold higher than the amount of weakly bound Band 3 at 18 h (Fig. 2). This shows that at 4°C, there is a slow but constant conversion of the weakly-bound form to the strongly-bound form. Not surprisingly, attempts to purify

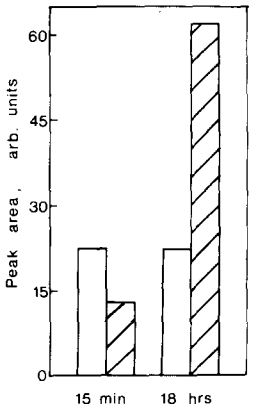

Fig. 2. Effect of prolonged incubation on Band 3 binding and elution. Binding assay was performed as described under Experimental procedures either for 15 min or for 18 h at 4° C. 1 mM BADS-5P8 elutes and 1% LDS-5P8 eluates were electrophoresed on SDS-polyacrylamide gel, stained with the Coomassie blue stain and scanned in a Joyce Loebl Chromoscan 3 densitometer at 530 nm. Open columns: 1 mM BADS-5P8 eluate; hatched columns: subsequent eluate with 1% LDS-5P8.

Band 3 by applying membrane extracts overnight at 4° C on a SITS-Affi-Gel 102 column gave poor yields of purified Band 3, although large amounts of protein were bound to the resin (data not shown). This was simply due to conversion of the bulk of bound Band 3 to the tightly-bound form not elutable by BADS.

Interaction of DIDS-labelled Band 3 with immobilized SITS

Band 3 exists mostly as a dimer in the membrane and in solutions of non-ionic detergents such as $C_{12}E_8$ [17]. Thus, Band 3 can bind to the immobilized SITS via one or both monomers. We studied this phenomenon by partially labelling Band 3 with [^{3}H]H_2DIDS or DIDS. Under these conditions we will have a mixture of Band 3 populations with both, one or no monomers covalently labelled by DIDS. Ghost membranes prepared from control or DIDS labelled erythrocytes were stripped with 0.2 mM EDTA (pH 7.5) followed by 1 M KI as described earlier [11]. The KI-extracted pellet was solubilized in citrate buffer with 1% $C_{12}E_8$ (Band 3 extract). The protein binding assay was performed at 4° C as described earlier [11] with a few modifications described under figure legends.

In order to determine the proportion of Band 3 dimers that have both inhibitor sites occupied by [^{3}H]H_2DIDS, protein-binding assays were performed with increasing amounts of resin. At the resin to sample ratio of 4, all of unlabelled Band 3 was bound to immobilized SITS (data not shown), suggesting that at this ratio of resin to protein the amount of ligand available is sufficient to bind all of the Band 3. Fig. 3 shows that when Band 3 was labelled with 3 μM [^{3}H]H_2DIDS no more than 70% counts were removed from the supernatant with increasing amount of resin. Thus, the remaining 30% represents labelled Band 3 with both sites occupied by [^{3}H]H_2DIDS and unable to bind to the resin. When Band 3 was labelled with 30 μM [^{3}H]H_2DIDS (conditions to label the protein fully), 80% of Band 3 did not bind to the resin. The 20% that bound represents protein with one site free and nonspecifically bound Band 3. The Band 3 that was bound to the resin and thus removed from supernatant was labelled only in one monomer with [^{3}H]H_2DIDS and bound to the resin via the unlabelled monomer. The Band 3 that did not bind to the resin and therefore remained in the supernatant must have had both monomers labelled by [^{3}H]H_2DIDS.

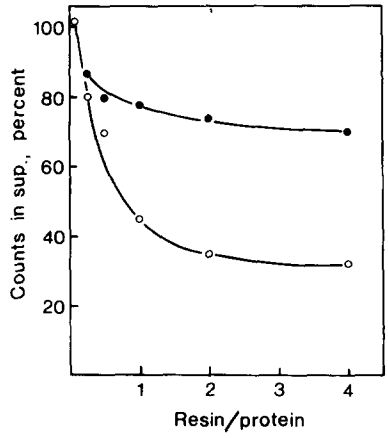

Fig. 3. Effect of increasing amount of the affinity resin on removal of Band 3 from supernatant. [^{3}H]H_2DIDS labelled Band 3 was incubated with SITS-Affi-Gel 102 resin for 15 min at 4° C. After letting the resin settle for 5 min, radioactivity was counted in the supernatant. A control with equal volumes of Sephadex 4B resin was also run. ○, Band 3 labelled with 3 μM [^{3}H]H_2DIDS; ●, Band 3 labelled with 30 μM [^{3}H]-H_2DIDS.

TABLE I

BINDING OF PARTIALLY [^{3}H]H_2DIDS-LABELLED BAND 3 TO SITS-AFFI-GEL 102 RESIN

Band 3 extract was prepared from erythrocytes labelled with 3 μM [^{3}H]H_2DIDS. 50 μl resin were shaken with 60 μl Band 3 extract (1 mg protein per ml) for 15 min at 4°C. Band 3 was eluted with 60 μl 1 mM BADS-5P8 followed by 60 μl 1% LDS-5P8.

Fraction	Sephadex 4B (dpm)	SITS-Affi-Gel 102 (dpm)
Total added	25171	25171
Supernatant	26725	19716
BADS eluate	–	2877
LDS eluate	–	1698

In order to see whether Band 3 labelled with [^{3}H]H_2DIDS in one monomer is bound specifically to the resin via the other monomer, bound Band 3 was eluted by BADS. Table I shows that Band 3 labelled with 3 μM [^{3}H]H_2DIDS bound to immobilized SITS and was eluted by 1 mM BADS. A portion of DIDS-labelled Band 3 was also eluted subsequently by 1% LDS. DIDS-labelled Band 3 did not bind to Sepharose 4B resin. This shows that Band 3 dimer labelled with [^{3}H]H_2DIDS in one monomer does bind to immobilized SITS and, moreover, this binding must be mediated via the stilbene-binding site of the unlabelled monomer, since the bound Band 3 can be eluted by BADS.

We have earlier shown that Band 3 bound to the SITS-Affi-Gel 102 resin becomes tightly bound upon incubation at 37°C and cannot be eluted with 1 mM BADS (Fig. 1, Ref. 11). When Band 3 dimers, labelled with 3 μM [^{3}H]H_2DIDS, were bound to immobilized SITS and temperature-shifted to 37°C after the removal of the free protein, up to 40% of counts were eluted by 1 mM BADS (data not shown). Under these conditions, unlabelled Band 3 is totally converted to a BADS-unelutable form. Thus, Band 3 labelled in one monomer with stilbene disulfonates is not quantitatively converted to the tightly bound form.

In order to see whether there was any correlation between the DIDS labelling and inability of Band 3 to acquire tight-binding (BADS-unelutable) form, a binding and temperature-shift experiment was performed with Band 3 covalently labelled with increasing amounts of DIDS. Fig. 4a shows that, as expected, with increasing DIDS label the amount of Band 3 bound to the resin decreased. Fig. 4b shows that, whereas unlabelled Band 3 was completely converted to the tightly bound form by incubation at 37°C, a part of the DIDS-labelled Band 3 was still present in the weakly bound form. Moreover, the proportion of Band 3 that remained in the weakly bound form increased with increasing DIDS label. When labelled with 2 μM DIDS, only 20% of Band 3 remained in the weakly bound form. When labelled with 50 μM DIDS, more than 90% of Band 3 that bound to immobilized SITS failed to acquire the tightly bound form. Thus, labelling of Band 3 with DIDS and the inability of such a molecule to acquire tight binding are strongly correlated. Since the tight binding is achieved through a conformational change in the protein molecule [8,11], binding of stilbene disulfonates in one monomer seems to affect the behaviour of the other monomer.

Interaction of citrate-modified Band 3 with immobilized SITS

Water-soluble carbodiimides which react with carboxyl groups under mild conditions [18] inhibit anion transport [19,20]. Band 3 extract was made from the erythrocytes that had been reacted with citrate and EAC under conditions that inhibited

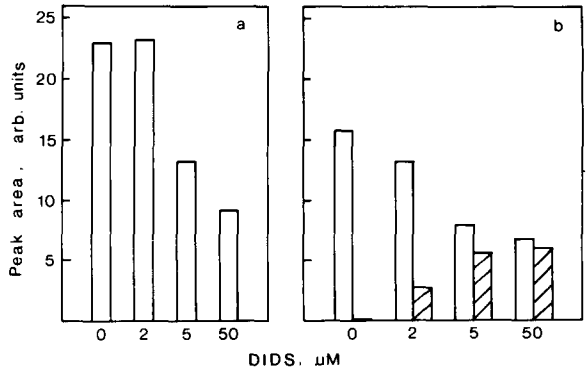

Fig. 4. Effect of DIDS labelling on Band 3 binding to immobilized SITS. Band 3 isolated from erythrocytes labelled with 0, 2, 5 or 50 μM DIDS was used in protein binding assay as described under Experimental procedures. (a) Total amount (BADS elute + LDS elute) of Band 3 bound to the resin; (b) open columns, amount of Band 3 eluted by BADS at 4°C; hatched columns, amount of Band 3 eluted by BADS upon incubation at 37°C for 10 min prior to elution.

the anion transport by 60%. This treatment resulted in incorporation of 1 mol of citrate per Band 3 dimer. EAC activates a carboxyl group of citrate which then covalently reacts with lysine 'a', which is involved in stilbene disulfonate binding (Werner, P.K., personal communication).

Band 3 extract was also made from control erythrocytes that were treated identically except that EAC was missing from the reaction mixture. 1 ml Band 3 extract was shaken with 25 μl SITS-Affi-Gel 102 resin for 15 min at 4°C and the bound protein was eluted with 250 μM BADS-5P8 followed by 1% LDS-5P8. Both control Band 3 and citrate-modified Band 3 were eluted by 250 μM BADS (Fig. 5, lanes 1 and 2) and a portion of Band 3 was also present in the subsequent 1% LDS eluate (lanes 5 and 6). For the temperature-shift experiment, Band 3 was initially bound to the affinity resin at 4°C for 15 min, free Band 3 was removed and the resin-bound protein was incubated at 37°C for 10 min. Following this treatment Band 3, from both control and citrate-modified Band 3 preparations was not eluted by BADS (lanes 3 and 4) and was present in 1% LDS eluate (lanes 7 and 8).

Fig. 5 also shows that the amount of Band 3 eluted by 250 μM BADS is not the same in control and citrate-modified Band 3 samples (with 1 mM BADS both control and citrate-modified Band 3 were eluted to the same extent). More of the citrate-modified Band 3 is eluted by 250 μM BADS as compared to that of control Band 3 (Fig. 5, lane 1 vs. lane 2; 184 units of citrate modified Band 3 vs. 100 units of control Band 3 by densitometry; data not shown), whereas subsequent elution by 1% LDS shows less citrate-modified Band 3 as compared to control Band 3 (120 units vs. 173 units respectively; data not shown). The affinity resin bound the same total amount of control and EAC-treated Band 3 (lanes 7 and 8); only their elution profile was different. In a separate experiment, a similar observation was made when the amount of protein was assayed in BADS and LDS eluted by the method of Lowry et al. [15] (data not shown). Since 250 μM BADS eluted more citrate-modified Band 3 than control Band 3, although the same amount of Band 3 was bound to the affinity resin, it indicates that modifying the inhibitor binding site with citrate results in weak binding and as a result citrate-modified Band 3 comes off more easily. It is interesting to note that citrate-modified Band 3, like control Band 3, becomes BADS-unelutable following temperature-shift to 37°C for 10 min. This means that although the citrate-modified Band 3 binds more weakly to the resin, it is still able to acquire the high-affinity form.

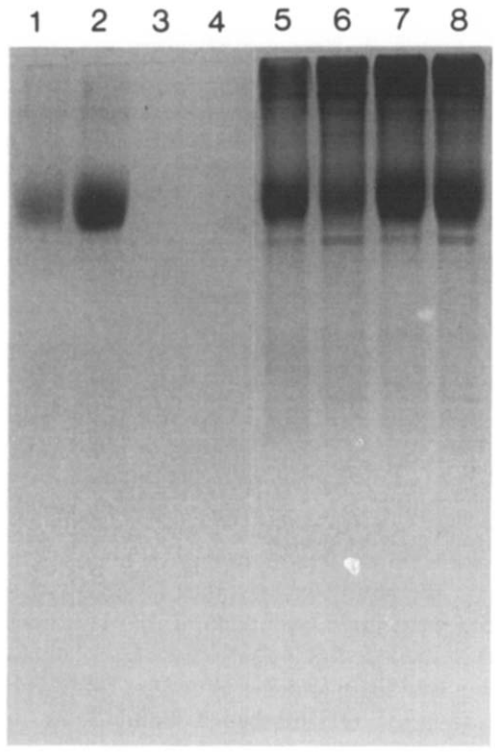

Fig. 5. SDS-polyacrylamide gel electrophoresis of stripped erythrocyte membrane proteins on a 10% gel prepared according to the procedure of Laemmli. Binding assay was performed as described under Experimental procedures. Lanes 1–4, 250 μM BADS-5P8 elutes; lanes 5–8, subsequent 1% LDS-5P8 eluates. Lanes 1, 5, control Band 3 and 4°C; 2, 6, citrate-modified Band 3 and 4°C; lanes 3, 7, control Band 3 temperature-shifted to 37°C following the initial binding; lanes 4, 8, citrate-modified Band 3 temperature-shifted to 37°C following the initial binding. Experimental details are given under Results.

Discussion

In order to use SITS-Affi-Gel 102 as a probe for studying stilbene disulfonate interaction with Band 3, it was important to establish that the

interaction of immobilized SITS does not significantly deviate from that of free ligand with Band 3. It has been suggested that free inhibitor ligand binding to Band 3 occurs in two steps [7,8]. The initial weak binding is followed by strong binding brought about presumably through a conformational change in the protein [8]. This may represent a partial translocation of the inhibitor mimicking substrate movement. The interaction of inhibitor (in this case SITS) with Band 3 can be schematically represented as follows:

$$\text{SITS} + \text{Band 3} \rightleftharpoons \text{SITS} \cdot \text{Band 3} \rightarrow \text{SITS} \cdot \text{Band 3}^*$$

Our results show that interaction of Band 3 protein with SITS immobilized onto a matrix is similar to free ligand–Band 3 interaction. Like free ligand, immobilized SITS molecules bind to Band 3 in two steps. Band 3 initially binds to immobilized SITS to give a SITS · Band 3 complex which at 4° C is slowly converted to SITS · Band 3*, Band 3* being the form of Band 3 that has high affinity for the inhibitor (BADS-unelutable form). When immobilized SITS is incubated with Band 3 for a prolonged period, most of the weakly bound Band 3 becomes converted to a strongly bound state. Our data (Fig. 1) also indicate that the initial binding step is fast at 4° C, suggesting a low activation energy barrier, whereas the conformational change step is very slow at 4° C but rapid at 37° C, suggesting a high activation energy barrier. This observation is in agreement with an earlier report by Verkman et al. [8].

It should be pointed out that we are studying the interaction of solubilized Band 3 with matrix-bound inhibitor. Direct comparisons with studies of Band 3 in membranes with free inhibitor cannot be made. For example, the half-time for the conversion of free inhibitor to the tight conformation is 4^{-1} s, while that half-time for conversion with matrix-bound inhibitor is much longer (about 1 min). Thus, taking these observations in consideration it can be said that our affinity resin can be used to study Band 3–stilbene disulfonate interaction and to draw valid conclusions regarding the general nature of such an interaction.

The interaction of DIDS-labelled Band 3 with immobilized SITS provides interesting clues as to the nature of the interaction between stilbene disulfonate binding site on adjacent monomers of Band 3 dimers. Our data clearly show that, while one monomer is covalently labelled with DIDS, the other monomer still retains its ability to bind SITS. However, upon binding to immobilized SITS only through one monomer, Band 3 dimer labelled with DIDS in the other monomer fails to acquire tight binding upon incubation at 37° C. This indicates that, although the inhibitor-binding sites on adjacent monomers act independently of each other in achieving initial binding with ligand, they must cooperate to achieve the tight binding that follows initial binding. Occupation of one site on a monomer by DIDS does not prevent the other monomer from binding an inhibitor ligand, but does prevent it from undergoing the conformational changes necessary to acquire tight binding.

There is a possibility that the tight binding is due to both subunits of the Band 3 dimer binding to two ligands on the resin and not due to a conformational change. The distance between two SITS molecules and their orientation has to be very precise for both monomers to bind to the resin. Assuming that immobilized ligands are uniformly distributed in space on the resin and located at each corner of units in a cubic lattice [21], the distance between neighboring ligands will be 65 Å at 12.5 mM ligand concentration (as is the case in SITS-Affi-Gel 102 resin; data not shown). The distance between stilbene-binding sites on two adjacent monomers was found to be only 28–52 Å [22]. Thus, the possibility that the tight binding is due to both monomers binding is unlikely. The quantitative conversion of the bound Band 3 to the tight-binding state also argues against such a possibility, since every Band 3 dimer must bind to two ligands. We attempted to obtain a clear answer by reducing the ligand density on the resin, thus making it extremely unlikely that a dimer could bind to two ligands simultaneously. Under these conditions, Band 3 binding was lost completely (most likely due to interference with free charged spacer-arms) and therefore the temperature-shift experiment could not be performed.

Verkman et al. [8] have suggested that after rapid binding of one DBDS molecule to one monomer of Band 3 dimer, a slower conformational change takes place which then permits the reaction of a second DBDS molecule to the other

monomer. Their studies, however, do not address the question of whether or not the second monomer upon binding DBDS molecule also undergoes the conformational change. Our data with DIDS, a less bulkier inhibitor than DBDS, suggest that the second monomer does not undergo conformational change upon binding to immobilized SITS. One explanation could be that during the sequential binding only the first monomer that binds inhibitor undergoes the conformational change. Thus, conformation change in one monomer automatically precludes conformation change in the adjacent monomer. Another possibility is that binding of DIDS in one monomer slows down the conformational change in the other monomer significantly, so that within the time frame of present studies (10 min) it fails to acquire tight binding. We could not test this possibility since with longer incubations (more than 60 min) in the absence of free Band 3, the bound Band 3 was detected to dissociate from the resin. Alternatively, following the binding of inhibitor ligand to one monomer, both monomers undergo the conformational change in concert. Under these circumstances, the unoccupied monomer can only bind an inhibitor ligand, and not lock in place. As a result, it can now bind to immobilized SITS only weakly and cannot become tightly bound upon incubation at 37° C. Finally, the DIDS-labelled subunit may not have undergone the conformational change that must precede a conformational change in its partner subunit. Whatever the mechanism may be, our data do show that inhibitor-binding sites on adjacent monomers interact with each other.

EAC, a water-soluble carbodiimide, reacts with carboxyl groups [18] and inhibits anion transport [19,20]. In the presence of citrate, however, it activates citrate carboxyls, which then form a covalent bond with a lysine residue involved in inhibitor binding. Data discussed above show that citrate modification decreases the affinity of Band 3 for immobilized SITS, since citrate-modified Band 3 is more easily eluted from the resin as compared to control Band 3. Recent fluorescent enhancement studies from this laboratory have shown that citrate modification of Band 3 in the presence of EAC reduces its affinity for BADS by 2-fold (Werner and Reithmeier, unpublished data). Under this condition, citrate is thought to modify a lysine residue involved in stilbene disulfonate binding and thus reduce the affinity of Band 3 for the inhibitor. Thus, SITS-Affi-Gel 102 resin can be used to study changes associated with chemical modification of the inhibitor binding site of Band 3. Our data also indicate that the lysine residue involved in inhibitor binding is not important in tight binding, since citrate-modified Band 3 can be rendered BADS-unelutable. Thus, our studies show that immobilizing a ligand onto a matrix not only provides a useful system for affinity purification of the desired protein, but also provides a simple and effective tool to study ligand–protein interaction.

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