Affinity chromatography of Band 3, the Anion Transport Protein of Erythrocyte Membranes*

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Affinity chromatography of Band 3 was performed using a series of affinity matrices synthesized with various inhibitor ligands and spacer arms. Hydrophilic spacer arms greater than four atoms in length were essential for Band 3 binding. An affinity resin prepared by reacting 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (Ki = 10 μM) with Affi-Gel 102 was found to be the most effective resin of the series tested. Solubilized proteins from human erythrocyte membranes were incubated with the affinity resin, and pure Band 3 was recovered by eluting with 4-benzamido-4'-aminostilbene-2,2'-disulfonate (BADS; Ki = 2 μM). Band 3 bound to the resin specifically in its stilbene disulfonate binding site, and optimal binding was achieved at pH 8 and at high ionic strength. At 4°C, up to 80% of the bound Band 3 could be eluted by 1 mM BADS, whereas the remainder could be eluted under denaturing conditions using 1% lithium dodecyl sulfate. At 22 or 37°C, the amount of BADS-elutable Band 3 was reduced with a concomitant increase of Band 3 in the lithium dodecyl sulfate elute. Thus, for successful affinity chromatography, the experiment must be carried out rapidly at 4°C. This procedure was also used to purify the Band 3 protein from mouse, horse, pig, and chicken erythrocytes.

Generally Band 3 is purified from hemoglobin-free erythrocyte membranes by "stripping" the membranes by addition of EDTA (9) or by exposure to extremes of pH (10) followed by selective extraction with nonionic detergents (11). Further purification may be achieved by anion exchange chromatography (11, 12) followed by column chromatography using an activated thiol gel (13) or a p-(chloromercuри)benzamido-ethyl agarose gel (11). This yields at least 95% pure Band 3.

Affinity chromatography has been used to purify various enzymes, and receptors (for a review see Ref. 14). The advantage of using immobilized ligand to purify a protein of interest over the other procedures is the specificity and rapidity of the procedure. Often, severalfold purification is achieved in a single step. However, no attempts to purify Band 3 using an inhibitor affinity resin have been reported. The difficulty arises due to the hydrophobic nature of Band 3 and its extensive association with the cytoskeletal network and other proteins. We here describe the design of an inhibitor affinity resin and experimental conditions of chromatography that allow biospecific purification of Band 3. The affinity resin has a high binding capacity and allows the rapid, convenient isolation of Band 3. We also provide a novel evidence for the two-stage binding of stilbene disulfonate inhibitors to Band 3 protein.

EXPERIMENTAL PROCEDURES

Materials

Affi-Gel 102 was purchased from Bio-Rad. Shaltiel hydrophobic chromatography kit with 4-, 6-, and 8-atom spacer arms was from Miles Scientific. SITS1 was obtained from Pierce Chemical Co., and 44'-diaminostilbene-2,2'-disulfonate (DADS) was obtained from Eastman. BADS was synthesized according to Kotaki et al. (15) as previously described (16). Aminoethyl-Sepharose 4E was synthesized according to Shaltiel and Er-El (17). Lithium dodecyl sulfate was a product of Boehringer Mannheim, and Cl2Es was from Nikko Chemical Co., Tokyo. All other chemicals were reagent grade or better.

Methods

Erythrocyte Membrane Preparation—Erythrocyte ghosts were prepared from outdated blood (kindly provided by the Canadian Red Cross) by hypotonic lysis in 5 mM sodium phosphate, pH 7.5, at 37°C for 30 min according to Bennett (19). All steps were carried out at 0–4°C, and membranes were recovered by centrifugation at 15,000 rpm in an SS-34 rotor in a Sorvall RC-5B centrifuge.

Solubilization of Band 3—Ghost membranes were stripped of cytoskeleton by 1 mM EDTA, 20 μg/ml phenylmethylsulfonyl fluoride, pH 7.5, at 37°C for 30 min according to Bennett (19). The stripped membranes were incubated with the affinity resin to be used for purification. The affinity resin was then bound to a column and washed to remove unbound material. Band 3 was eluted with 1 mM BADS, and the eluted material was applied to a column prepared with 4-, 6-, and 8-atom spacer arms. The eluate was collected in five fractions of 50 ml each. Fractions 2, 3, and 4 were pooled, dialyzed, concentrated, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the purity and yield of Band 3.

1 The abbreviations used are: SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate; BADS, 4-benzamido-4'-aminostilbene-2,2'-disulfonate; C12Es, octaethylene glycol mono-n-dodecyl ether; EDC, 1-ethyl-3-(dimethylaminopropyl)-carbodiimide hydrochloride; DADS, 4,4'-diaminostilbene-2,2'-disulfonate; DIDS, 4,4'-disothiocyanostilbene-2,2'-disulfonate; DNDS, 4,4'-dinitrostilbene-2,2'-disulfonate; LDS, lithium dodecyl sulfate; SPITC, 3-sulfophenyl isothiocyanate.

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membranes were then extracted with 1 M KI to remove other extrinsic membrane proteins. Stable heteromers of Band 3 with extrinsic proteins were observed if unstripped membranes were solubilized (19). The KI-extracted membranes were then solubilized (at protein concentrations of 1.5–2 mg/ml in 1% CI2E8 in 228 mM citrate buffer, pH 8, containing 1 mM dithiothreitol and 20 µg/ml phenylmethylsulfonyl fluoride (final  concentration). Following a 20-min incubation on ice, the solubilized membranes were centrifuged at 19,000 rpm for 30 min in an SS-34 rotor. The supernatant from KI-extracted solubilized ghosts in citrate buffer with 0.1% sodium azide.

with 5P8 buffer containing 0.1% CI2E8 overnight with at least two changes.

Labeling of Cells with DIDS—Erythrocytes at a 25% hematocrit in 5 mM sodium phosphate, pH 8, and 150 mM NaCl were reacted with 50 µM DIDS at 37 °C for 1 h. Cells were washed once with above buffer containing 0.5% bovine serum albumin and twice with buffer alone. Ghosts and Band 3-228C8 extract were prepared as above.

Affinity Resins—1 ml of Affi-Gel 102 resin (15 µmol of NH2/ml of settled gel) was washed with water followed by 100 mM sodium bicarbonate buffer, pH 8.5, and suspended in 1 ml of bicarbonate buffer. To this suspension was added 16.56 mg of SITS (30 µmol) dissolved in 2 ml of bicarbonate buffer. After adjusting the pH to 8.5, the suspension was shaken at 37 °C for 1 h. The resin was then washed with bicarbonate buffer followed by water and stored at 0–4 °C in a Joyce-Loebl Chromoscan 3 densitometer at 530 nm.

Affinity resins not directly purchased from commercial sources were prepared by conjugating various spacer arms to cyanogen bromide-activated Sepharose 6B according to Lukacovic et al. (11). Using radioactive glycine, the amount of spacer molecules on the resin was determined to be 3.1 µmol of resin. The inhibitor ligand was then coupled to 1 ml of these resins using 3 mmol of DADDS with 8 mmol of EDC (11).

Affinity Purification—Unless stated otherwise, all steps were carried out at 0–4 °C. Protein binding assays were performed in 1.0-ml microfuge tubes. 25 µl of packed resin was washed twice with 250 µl of 228 mM sodium citrate, 0.1% CI2E8, pH 8.0, (citrate buffer) and was incubated with 1 ml of solubilized membrane supernatant (1.5–2 mg/ml of protein) for 15 min. After removing the supernatant, the gel was washed at least three times with 250 µl of citrate buffer, and the bound material was then eluted by shaking the resin for 10 min with 110 µl of 1 mM BADDS in 5 mM sodium phosphate, 0.1% CI2E8. Finally, the resin was again washed at least twice with 10 volumes of citrate buffer and then extracted with 110 µl of 1% LDS in 5 mM phosphate for 10 min.

Analytical Techniques—Protein assays was according to Lowry et al. (21). Sodium dodecyl sulfate gel electrophoresis was performed according to Laemmli (22). Protein bands were stained with Coomassie Blue or Stains All (23). Coomassie Blue-stained gels were scanned in a Joyce-Loebl Chromoscan 3 densitometer at 530 nm.

RESULTS

Selection of Spacer and Ligand for Affinity Chromatography

For successful affinity chromatography, the choice of the ligand and the spacer molecule is of critical importance. The presence of a spacer arm sometimes becomes necessary to eliminate the steric hindrance of the matrix. Table I lists the names and the chemical structures of the different inhibitor ligands used in the present study. Table II summarizes the initial attempts to find a suitable spacer arm and ligand. For these studies Band 3-5P8 extract was used. 1 ml of affinity resin was packed in a 10 × 1.5-cm glass column equilibrated with 5P8 buffer containing 0.1% CI2E8. 0.5 ml of Band 3-5P8 extract was loaded on the column at 4 °C and washed with 5P8, 0.1% CI2E8. Band 3 bound to the column was then eluted with 0.3 M NaCl in 5P8, 0.1% CI2E8 and subsequently with 1% LDS in 5P8. Binding and elution of Band 3 was monitored on a chart recorder by ultraviolet absorption at 280 nm.

Hydrophobic Spacer Arms with DADDS—Band 3 did not bind either to Sepharose 6B matrix itself or to matrices with any of the hydrophobic spacer arms (listed in Table III) alone. Band 3 also did not bind to the column when DADDS was either attached directly to the matrix or via a short 2-atom spacer arm. With longer hydrophobic spacer arms (4 and 6 atoms in length), Band 3 did bind to the column. Band 3 bound to these columns could be eluted by increasing ionic strength of the buffer (up to 0.5 M NaCl) but not by 100 mM DADDS, 10 mM DND's, or 1 mM BADS in 5P8. Moreover, Band 3 covalently labeled with DIDS also bound to the column, suggesting that the binding was probably not entirely mediated via the inhibitor binding site. Attempts to minimize the nonspecific interaction by increasing CI2E8 concentration from 0.1 to 1% in the buffer did not meet with success. Adding 20% ethylene glycol or 10% glycerol to the buffer did not improve specific binding.

Hydrophilic Spacer Arms with DADDS—Since the use of hydrophobic spacer arms resulted in nonspecific interactions, hydrophilic spacer arms of equal or longer lengths were used in an attempt to minimize these interactions. Band 3 did not bind to the hydrophilic spacer arms alone. When DADDS was attached to either Gly-Gly or Gly-Gly-Gly spacer arms, Band 3 did not bind the columns. When DADDS was attached either to epoxy-Sepharose (oxirane spacer arm) or to aminoethyl succinyl spacer arm, then Band 3 did bind to the column. Interactions of Band 3 with these two affinity columns was more specific, since the binding could be prevented by covalently reacting Band 3 with DIDS. However, Band 3 binding to these columns was not always successful. One of the reasons for the irreproducibility could be a structural limitation of DADDS itself. DADDS has two amino groups (Table I) and it is possible that, during the carbodiimide coupling reaction (11), both ends of the DADDS molecule couple to spacer arms.

Spacer Arms with Different Ligands—To avoid the possibility of the ligand being coupled to the matrix through two sites, BADS instead of DADDS was coupled to a hydrophobic 6-atom spacer arm. BADS has a benzoyl group that blocks one amino group of DADDS. However, this makes BADS more hydrophobic and a stronger inhibitor than DADDS. Band 3 did bind to this column, but the binding was not prevented by covalently labeled DIDS. Another inhibitor, SPITC, has only one benzene ring and is a weak inhibitor as compared to DADDS. Affinity resin prepared by linking SPITC to the hydrophilic 6-atom spacer arm of Affi-Gel 102 was unable to bind Band 3 when Band 3-228C8 extract was shaken with the affinity resin at 4 °C (see “Experimental Procedures”). Finally, when affinity resin was prepared by linking SITS to Affi-Gel 102, Band 3 did bind to the column, and the binding was prevented by covalently labeling Band 3 with DIDS.

Interaction of Band 3 with SITS-Affi-Gel 102 Resin

To establish optimal conditions for Band 3 binding to the SITS Affi-Gel 102 affinity matrix and its subsequent elution in pure form, a small-scale assay was developed (see “Experimental Procedures”). This enabled us to examine several different conditions simultaneously. 1 ml of the Band 3-228C8 extract (Fig. 1, lane 3) was shaken with 25 µl of SITS Affi-Gel 102 for 15 min at 4 °C, and the affinity matrix was washed with citrate buffer containing 0.1% CI2E8. The bound protein was then eluted with 1 mM BADS in 5P8 buffer containing 1 mM dithiothreitol (lane 4). The affinity matrix was subsequently washed with 10 volumes of citrate buffer and eluted with 1% LDS in 5P8 buffer to elute the rest of the bound protein (lane 5).

Purity of BADS Eluate—The protein fraction eluted by 1 mM BADS when resolved on SDS-polyacrylamide gel electro-
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#### Table 1
Structure and inhibitory potency of various ligands

| Structure | Name                             | Abbrev | Ki (μM)\(^a\) |
|-----------|----------------------------------|--------|--------------|
| R\(_1\) = (-N=C=S), R\(_2\) = \((\text{CH}_3\text{CONH})\) | Stilbenedisulfonates | SITS   | -10          |
| R\(_1\) = R\(_2\) = (-NH\(_2\)) | 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate | DADS   | 1300         |
| R\(_1\) = R\(_2\) = (-NO\(_2\)) | 4,4'-dinitrostilbene-2,2'-disulfonate | DNDS   | 2            |
| R\(_1\) = R\(_2\) = (-N=C=S) | 4,4'-diisothiocyanostilbene-2,2'-disulfonate | DIDS   | 0.04         |
| R\(_1\) = (-NHCO\(_2\)) | 4-benzamido-4'aminostilbene-2,2'-disulfonate | BADS   | 2            |
| R\(_1\) = (-NH\(_2\)) | 3-sulfophenylisothiocyanate | SPI TC | -4000\(^b\)  |

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\(^a\)Concentration for 50% inhibition of chloride or sulfate flux (1).

\(^b\)Value is for isothiocyanate derivative of sulfanilic acid (25).

Phoresis showed a single Coomassie Blue-stained band of about \(M_\text{s} = 95,000\). Occasionally a minor band (\(M_\text{s} = 70,000\)) was also seen, especially if the Band 3-228C8 extract was more than a week old. When the SDS-polyacrylamide gel electrophoresis gels were stained with Stains All (see "Experimental Procedures") to detect glycoproteins, a small amount of glycophorin was observed together with Band 3 in the BADS elute (data not shown).

**Specificity of the Affinity Matrix**—A specific interaction between the protein and the immobilized ligand is desirable in affinity chromatography. The specificity of Band 3 interaction with immobilized SITS was examined in two ways. When the solubilized membrane proteins (Band 3-228C8 extract) were shaken with SITS-Affi-Gel102 in the presence of 200 μM DNDS, Band 3 did not bind to the matrix and remained in the supernatant (Fig. 2, lane 1 versus lane 2). Also, when the inhibitor binding site on the Band 3 molecules was blocked irreversibly by reacting cells with DIDS at alkaline pH, (DIDS-labeled Band 3-228C8 extract), Band 3 did not bind to the resin (lane 3).

**Characterization of Binding**

Fig. 3a shows the dependence of protein-affinity matrix interaction on the ionic strength of the buffer. Optimal binding was achieved only at fairly high ionic strength (228 mM sodium citrate, equivalent to about 0.7 M NaCl). In these studies, citrate buffer of increasing molarity was used rather than using NaCl, because chloride ion is a substrate for the anion transport and is known to compete with stilbene disulfonates (1). In order to achieve optimal binding, the SITS-Affi-Gel 102 matrix must be shaken with membrane proteins at an amount equal to or slightly below its maximal binding capacity (Fig. 3b). If low amounts of protein were used for binding, then Band 3 eluted with BADS was not detected on a Coomassie Blue-stained SDS-polyacrylamide gel. Reducing the amount of SITs ligated to Affi-Gel 102 to ½ and ⅛ of the normal amount (15 μmol of NH₂/ml of resin) resulted in the loss of Band 3 binding. Under the typical assay conditions, optimal binding of Band 3 was obtained when the affinity matrix was shaken with the protein sample for 30 min at 4 °C (Fig. 3c). Specific and optimal binding was obtained at pH 8. Excess free DNDS did not inhibit Band 3 binding to the matrix at pH 6, whereas the binding capacity of the matrix was reduced at pH 10 (data not shown). Band 3-228C8 extract, prepared from ghost membranes that were stripped at pH 12 with 2 mM EDTA rather than at pH 7.5, did not bind to the affinity resin.

**Characterization of Elution**

Band 3 bound to the affinity matrix could be eluted by various stilbene disulfonate derivatives dissolved in 5PBS buffer containing 0.1% C\(_{12}\)E\(_6\) and 1 mM dithiothreitol, pH 8 (Fig. 4). 1 mM BADS (lane 3) was a better eluent than 10 mM DNDS (lane 1) or 0.1 mM DIDS (lane 2). Band 3 was more effectively eluted by BADS at low ionic strength (5 mM phosphate) than at higher ionic strength (228 mM citrate; data not shown). Band 3 bound to the affinity resin could be partially eluted by lowering the ionic strength of the buffer, and addition of a stilbene disulfonate to the low ionic buffer improved the yield of Band 3. 0.1 mM BADS in 5PBS buffer eluted 40% of Band 3, whereas 1 mM BADS in 5PBS buffer eluted 80% of Band 3 that was bound to the affinity resin. Subsequent washing with 1% LDS eluted the remainder of
**Table II**

*Inhibitor affinity resins and their interaction with human erythrocyte Band 3*

Affinity chromatography was performed on 1-ml columns at 4 °C. 0.5 ml of Band 3-5P8 extract (1 mg/ml of protein in 5P8, 0.1% C12EB or 28.5 mM sodium citrate, 0.1% C12EB buffer) was applied to columns at a flow rate of 10 ml/h. Columns were washed with 5 ml of either buffer (phosphate or citrate) and eluted with 0.3 M NaCl, 0.1% C12EB followed by 1% LDS. Appearance of protein (monitored at 280 nm) in either of these two eluates was a criterion of binding.

| Spacer arm        | Length (atoms) | Inhibitor ligand | Protein binding | Specificity | Comments                      |
|-------------------|----------------|------------------|-----------------|-------------|-------------------------------|
| None              | -              | DADS             | -               | NA          |                               |
| Hydrophobic       |                |                  |                 |             |                               |
| Glycine           | 2              | DADS             | -               | NA          |                               |
| 4-Aminobutanoic acid | 4           | DADS             | +               |             |                               |
| 6-Aminobenzoic acid | 6            | DADS             | ++              |             |                               |
| Hydrophilic       |                |                  |                 |             |                               |
| Glycyl-glycine    | 6              | DADS             | -               | NA          |                               |
| Glycyl-glycyl-glycine | 8          | DADS             | +/−             | +/−         |                               |
| Oxirane epoxy-Sepharose | ~12        | DADS             |                 |             |                               |
| Aminoethyl succinyl | 8            | DADS             | +/-             | +/-         |                               |
| 6-Aminobenzoic acid | 6            | BADS             | ++              |             |                               |
| Affi-Gel 102      | 6              | SPITC            | −               | NA          |                               |
| Affi-Gel 102      | 6              | SITS             | +++             | +           | Band 3 eluted by BADS in 5P8 buffer |

*Plus sign denotes relative amount of protein that bound the columns and was eluted by chloride.

1 Biospecificity of the binding was determined by loading the protein sample on the column in presence of excess free ligand or by covalently labeling Band 3 with DIDS. Plus sign denotes specific binding; minus sign denotes nonspecific binding.

2 DADS was ligated directly to CNBr-activated Sepharose 6B.

3 NA, not applicable.

4 Spacer arms were coupled to CNBr-activated Sepharose 6B through their amino groups. DADS was ligated to the carboxyl group of the spacer arms using carbodiimide.

5 Epoxy-activated Sepharose 6B was obtained from Pharmacia.

6 Aminoethyl succinyl spacer arm was prepared according to Ikeda et al. (27).

Band 3 together with other proteins that may be bound to the resin nonspecifically (data not shown).

**Effect of Spacer Length on Band 3 Binding**

Affi-Gel 102 used in these studies is an agarose gel with a 6-atom hydrophilic spacer arm. Under the assay conditions, Band 3 did not bind to Affi-Gel 102 alone. Binding capacity of the affinity matrix is dependent on the length of the spacer arm used. The amount of Band 3 that is eluted by BADS decreased with reducing spacer length from 8 to 4 atoms (Fig. 5a). Also, the chemical nature of the spacer molecule is important since affinity matrix with a hydrophilic 6-atom spacer binds more Band 3 than the one with a hydrophobic spacer of equal length (Fig. 5b). Considering that both Affi-Gel 102 (hydrophilic 6-atom spacer) and ω-aminohexyl-agarose (hydrophobic 6-atom spacer) have equal amounts of spacer molecules (15 μmol/ml of beads) and that equal amounts of ligand (30 mM) were used during the coupling reaction, the data suggest that affinity matrix with hydrophilic spacer arms interacts more favorably with Band 3 and therefore result in increased binding capacity.

**Optimal Temperature for Protein Binding and Elution is 4 °C**

In order to establish optimal temperature conditions, protein binding and subsequent elution were carried out at different temperatures. When the experiment was carried out at 4 °C, most of the bound Band 3 could be eluted off the resin by 1 mM BADS and the rest of Band 3 with 1% LDS (Fig. 6). At room temperature, however, the amount of Band 3 in the 1% LDS eluate was increased. When the experiment was carried out at 37 °C, 1 mM BADS eluted very little Band 3, most of which could be eluted only with 1% LDS. These data suggest that at higher temperatures Band 3 binds more tightly to the resin and therefore cannot be eluted with 1 mM BADS effectively.

At 37 °C Immobilized SITS-Band 3 Complex Is Rapidly Converted to a Tightly Bound Complex

The process of affinity chromatography has two components: a binding component where Band 3 binds to the immobilized SITS and an elution component where free BADS competes with the immobilized SITS for the inhibitor binding site on the Band 3 molecule and elutes the bound protein from the resin. A change in temperature may affect equilibrium of these two processes in such a way that at higher temperatures Band 3 may exhibit a stronger affinity for the immobilized ligand, resulting in the inability of BADS to elute the protein from the affinity resin. To rule out this possibility, the following experiment was carried out. 1 ml of Band 3-
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**FIG. 1.** SDS-polyacrylamide gel electrophoresis of human erythrocyte membrane proteins on a 10% gel prepared according to the procedure of Laemmli. Lane 1, erythrocyte ghosts; lane 2, 1 mM EDTA-extracted ghosts, pH 7.5; lane 3, stripped ghost membrane (Band 3-228C8 extract); lane 4, protein fraction eluted from SITS-Affi-Gel 102 resin by 1 mM BADS; lane 5, protein fraction subsequently eluted by 1% LDS. The experiment was performed as described under "Experimental Procedures."

**FIG. 2.** SDS-polyacrylamide gel electrophoresis of human stripped ghost membrane proteins on a 10% gel prepared according to the procedure of Laemmli. In a microfuge tube, 100 μl of SITS-Affi-Gel 102 resin was incubated with 100 μl of stripped membrane preparation (protein concentration 1 mg/ml) in 114 mM sodium citrate buffer, pH 8, for 30 min at 4 °C. Supernatant was then collected and electrophoresed. Lane 1, incubation in absence of DNDS; lane 2, incubation in presence of 200 μM DNDS; lane 3, incubation with DIDS-labeled sample.

Band 3 was not released from the affinity resin (Fig. 7a). After 10 min at 4 °C, the bound protein was eluted with 1 mM BADS in 5%P8 buffer at 4 °C. The resin was finally eluted with 1% LDS in 5%P8 buffer after two washes of 250 μl of citrate buffer. Except for the incubation period of 37 °C, all other steps were carried out at 4 °C. As a result of incubation of 37 °C for 20 min, the BADS-elutable Band 3 was converted to BADS-unelutable form. Preincubation of the Band 3-228C8 extract alone at 37 °C did not alter its binding properties, suggesting that the conversion to BADS-unelutable form is not due to thermal denaturation and can occur only when Band 3 is already bound to SITS. Fig. 7b shows that the amount of Band 3 eluted by 1 mM BADS decreases, and that eluted by 1% LDS increases in a time-dependent manner as the resin-bound protein is incubated at 37 °C. Within 45 s, 50% of BADS-elutable Band 3 is converted to BADS-unelutable Band 3 (Fig. 7b). Since both the binding step and the elution step were performed at 4 °C, this observation suggests that, upon exposure to 37 °C, ligand-bound Band 3 becomes more tightly bound to the resin. It seems likely that the Band 3 eluted by 1 mM BADS is present in the initial weak binding conformation, whereas the Band 3 that is not eluted by 1 mM BADS but by 1% LDS is present in the tight binding conformation. These data also show that this change is slow at 4 °C but very fast at 37 °C.

 Binding Capacity and Stability of SITS-Affi-Gel 102 Resin

The binding capacity of SITS-Affi-Gel 102 resin was estimated from the amount of Band 3 protein eluted from the resin after it was saturated with Band 3-228C8 extract at 4 °C for 15 min. Table III shows that the resin has a binding capacity of 1.5 mg of Band 3/ml of resin. The binding capacity decreases slightly with the repeated use of resin (data not shown). When stored in citrate buffer with 0.1% sodium azide at 4 °C, SITS-Affi-Gel 102 affinity resin retained its full binding capacity over at least 3 months. When the same resin samples were repeatedly used, however, the binding capacity of the resin decreased slightly with each use. This is probably due to a small degree of irreversible adsorption of proteins to the resin, a phenomenon also observed with other affinity resins (24).

Affinity Purification of Band 3 from Other Sources

Ghost membranes were prepared from horse, chicken, pig, and mouse erythrocytes, and a crude extract was prepared by solubilizing the membranes in citrate buffer containing 1% C12E6. The solubilized membranes were then centrifuged in an SS-34 rotor at 19,000 rpm for 20 min. The supernatant was shaken with SITS-Affi-Gel 102 under the same experimental conditions used for purification of human Band 3. A major polypeptide of M, between 90,000 and 100,000 was eluted by 1 mM BADS from the affinity matrix shaken with crude extracts from these different sources (Fig. 8). Subsequent washing of the matrix with 1% LDS eluted the remainder of Band 3 and other nonspecifically bound proteins (data not shown).

**DISCUSSION**

Band 3 is an integral membrane protein and spans the erythrocyte membrane several times (2, 26). No successful attempts of purifying this protein employing the affinity chromatography technique have been reported. One of the difficulties is undoubtedly the hydrophobic nature of Band 3 that results in a high degree of nonspecific interactions with the affinity matrix. Stilbene disulfonates, being hydrophobic in nature, aggravate this problem. Also, stilbene disulfonates bear two negative charges which will promote ionic interac-
FIG. 3. **Protein binding characteristics of SITS-Affi-Gel 102 affinity resin.** In a, solubilized stripped membranes (1 mg of protein) were incubated with 50 µl of affinity resin in citrate buffer of increasing molarity, pH 8, at 4 °C for 30 min. Amount of protein disappeared from the supernatant is defined as the amount of protein bound to the resin. In b, the experimental details as described above except that increasing amount of stripped membrane protein in 228 mM citrate buffer, pH 8, with 0.1% C12E8 was added to the affinity resin. Amount of protein disappeared from the supernatant is defined as the amount of protein bound to the resin. For c, protein binding was performed as described under “Experimental Procedures.” Incubation was carried out for varied lengths of time. Fraction eluted with 1 mM BADS was electrophoresed on a 10% Laemmli gel and stained with Coomassie Blue, and the gel was scanned on a Joyce Loebl Chromoscan 3 densitometer at 540 nm. Area under Band 3 peak is defined as amount of Band 3 bound to the resin.

FIG. 4. **SDS-polyacrylamide gel electrophoresis of human erythrocyte membrane proteins on a 10% gel prepared according to the procedure of Laemmli.** Protein binding was performed as described under “Experimental Procedures.” Following incubation, resins were washed with 228 mM citrate buffer, pH 8, and eluted with 5 mM phosphate, 0.1% C12E8 buffer, pH 8, containing 10 mM DNDS (lane 1), 50 µM DIDS (lane 2), or 1 mM BADS (lane 3). Gels were stained with Coomassie Blue.

FIG. 5. **Effect of the spacer length on Band 3 binding to the affinity resin.** For panel a, 25 µl of SITS affinity resin with spacer arms of various lengths was shaken with 1 ml of Band 3-228C8 extract at 4 °C for 15 min, and the bound protein was eluted with 1 mM BADS. Other details were as given under Fig. 3C. Panel b shows amount of Band 3 eluted with 1 mM BADS from SITS affinity resin with a 6-atom hydrophobic spacer arm (A) or a 6-atom hydrophilic spacer arm (B).
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**Table III**

|                | Protein adsorbeda | Protein eluted by 1 mm BADSb | Protein eluted by 1% LDS |
|----------------|-------------------|------------------------------|-------------------------|
| **Resin**      | mg/ml             |                              |                         |
| Protein assay  | 5.1               | 1.5                          | 3.6                     |

a Amount of protein adsorbed was calculated from the amount of protein disappeared from the supernatant after 15 min incubation at 4°C. 

b BADS itself reacts with the Lowry reagent and gives color. Appropriate BADS blanks were included in the assay, and the amount of protein in BADS elute was recalculated.

**FIG. 6.** SDS-polyacrylamide gel electrophoresis of human stripped ghost membranes proteins on a 10% gel prepared according to the procedure of Laemmli. In a microfuge tube, 25 μl of SITS-Affi-Gel 102 resin was incubated with 1 ml of Band 3 extract (protein concentration ~1.5 mg/ml) in 228 mM citrate buffer, pH 8, for 20 min at 4, 22, or 37°C. After washing off the unbound protein, bound protein was eluted first with 1 mM BADS-5P8 (lanes 1-3) and subsequently with 1% LDS-5P8 (lanes 4-6) at respective temperatures. Lanes 1 and 2, 4°C; lanes 2 and 5, 22°C; lanes 3 and 6, 37°C.

**FIG. 7.** Panel a shows SDS-polyacrylamide gel electrophoresis of human stripped erythrocyte membrane proteins on a 10% gel prepared according to the procedure of Laemmli. Binding assay was performed as described under "Experimental Procedures" with the following modification. After removing the unbound protein with at least three washes, 100 μl of citrate buffer was added to the resin-bound protein and incubated at 37°C for up to 60 min. The supernatant (lanes 1 and 4) was removed, and the bound protein was then eluted with 1 mM BADS-5P8 (lanes 2 and 5) followed by 1% LDS-5P8 (lanes 3 and 6). Lanes 1-3, incubated at 4°C after the initial binding; lanes 4-6 incubated at 37°C for 5 min. Gels of samples incubated up to 60 min showed identical pattern as in lanes 4-6. Panel b shows the effect of temperature shift on the elution properties of Band 3 bound to the SITS-Affi-Gel 102 affinity resin. Details of experiment are given above except that incubation at 37°C was carried out only up to 8 min. 1 mM BADS-5P8 elutes and 1% LDS-5P8 elutes were electrophoresed on SDS-polyacrylamide gel, stained with the Coomassie Blue stain, and scanned in a Joyce Loebel Chromoscan 3 densitometer at 540 nm. ■■■■■■, Band 3 eluted with 1 mM BADS-5P8; ○—○○○, Band 3 subsequently eluted with 1% LDS-5P8.

**FIG. 8.** SDS-polyacrylamide gel electrophoresis of erythrocyte membrane proteins on a 10% gel prepared according to the procedure of Laemmli. 1 mM BADS eluate from SITS-Affi-Gel 102 resin shaken with 1% C2E8 extract from chicken ghosts (lane 1), mouse ghosts (lane 2), pig ghosts (lane 3), and horse ghosts (lane 4) is shown.

Summably due to an additional benzene ring in its structure that increases the hydrophobicity of the ligand, Band 3 did not bind to immobilized SPITC (Ki = 4 mM). Based on simple kinetic and equilibrium models of the affinity adsorption and desorption events, Graves and Wu (28) have predicted that in order to produce adequate affinity the immobilized ligand concentration should be more than 10 times the ligand-protein dissociation constant (Ki). Considering that concentration of immobilized SPITC was not more than 15 mM, it would not have adequate affinity to bind Band 3. We did not attempt to increase the concentration of immobilized SPITC to compensate for weak affinity because SPITC has a net negative charge, and it has been observed (6) that, with high ligand concentration, ionic interactions become dominant over biospecific interactions. We observed similar results upon increasing concentrations of immobilized DADS (data not shown).

SITS (Ki = 10 μM) immobilized at a concentration of 15 mM has proved to be satisfactory. Unlike DADS, one amino group of SITS is acetylated and therefore it cannot form "cross-bridges" on the solid support, and thus the ligand is probably present in a proper orientation for the binding. When the concentration of immobilized SITS was decreased...
from 15 to 3 or 1.5 mM, Band 3 did not bind to the affinity resin (data not shown). The lack of binding could be due to an increased K, value caused by immobilization of SITS, and therefore the lower ligand concentration results in inadequate affinity. It is also possible that, at lower ligand concentration, the excess free charged spacer arms interfere with the protein binding.

Steric hindrance is a commonly held rationale for the need of spacer arm extension (29). Thus a spacer arm would be needed if the ligand binding site is recessed deep within the interior of the protein. The present studies indicate that the stilbene binding site is not present on the surface of Band 3 but is recessed deep inside the protein molecule since Band 3 does not bind to the affinity matrix without a spacer arm or with short spacer arms. In order to achieve binding, a spacer arm of minimum 4-atom length is needed, and further elongation of the spacer arm results in increased binding capacity of the affinity matrix. Increase in binding capacity as a result of increasing spacer length has also been observed earlier (30).

It is conceivable that, upon binding to an immobilized ligand, the protein will also interact with the spacer arm, especially if the ligand binding site is recessed within the interior of the protein. This means that the chemical nature of the spacer arm is likely to influence protein-ligand interaction in such a case. It is generally observed that hydrophobic spacer arms increase nonspecific interactions, whereas hydrophilic spacer arms minimize this problem. The present studies with DADS attached to hydrophobic and hydrophilic spacer arms support the above view. It is interesting to note, however, that Band 3 does not bind to the spacer arms alone, and therefore binding of Band 3 to DADS-spacer-Sepharose column cannot be completely nonspecific. This is also supported by the fact that Band 3 bound to DADS affinity column can be eluted by increasing concentration of chloride (a substrate which presumably binds at or near the inhibitor binding site) but not by citrate ions which are not transported by Band 3 (data not shown).

Interaction of a protein with a series of ligand derivatives yields information about the chemical nature of the binding site. Similarly, studying protein interaction with a ligand immobilized via different spacer arms can give information about the microenvironment that surrounds the ligand binding site. For example, our results show that keeping the spacer length equal (to 6 atoms), introduction of hydrophilic groups on an otherwise hydrophobic spacer arm dramatically improves Band 3 binding to the affinity matrix and its subsequent recovery in the pure form. This indicates that the region that surrounds the stilbene binding site interacts more favorably with a hydrophilic rather than hydrophobic spacer arm. This suggests that the area surrounding the stilbene binding site has a fair degree of hydrophilicity, although the binding site itself is probably a hydrophobic cavity (1, 7, 8). Our results are particularly interesting since it has very recently been shown that, although heme pocket of myoglobin is lined with nonpolar amino acid residues, the binding site is strongly polar (31). On the basis of minor changes in acid dissociation constants of ionized groups inside certain proteins, it has been deduced that many protein cavities have a high effective polarity despite the lining of nonpolar amino acid residues (32).

The temperature-shift experiment demonstrates that, upon binding to the inhibitor ligand, Band 3 protein becomes tightly bound, presumably through a conformational change (33). Macara et al. (34) have suggested that the slow conformational change may represent a partial translocation of the inhibitor and that it occurs when the protein attempts to transport the inhibitor as it would a substrate and switches from an outward- to an inward-facing state. Thus, prior to the conformational change, the inhibitor binding site of the bound Band 3 is accessible to free ligand, and therefore the protein could be eluted with BADS. Upon conformational change the site becomes inaccessible (inward-facing state) to the free ligand and consequently cannot be eluted with BADS.

Our results have indicated two important points. First, when using SITS affinity column for purification of Band 3, it is imperative to carry out the experiment quickly at 4°C. Many workers have adopted to perform affinity chromatography at room temperature (30) or to load the sample very slowly on the column (35). Obviously, under these conditions, isolation and purification of Band 3 will not be very successful. This is important since it has recently been reported that the red cell glucose transporter can be bound to an inhibitor affinity column but cannot be eluted in native conformation (36) when the binding was performed at 4°C for 2 h. Thus, our finding may be of general importance in cases where a transport protein is being purified on an inhibitor affinity column. Second, from the reports published so far (33, 34) it has only been inferred that upon stilbene disulfonate binding to Band 3 the conformational change locks the inhibitor in place. We believe that our results provide a direct evidence that such a phenomenon does occur.

SITS-Affi-Gel 102 affinity matrix was also useful in purifying Band 3 protein from erythrocytes from different species. Further processing of the crude extracts of chicken, mouse, pig, and horse ghosts prior to affinity chromatography should yield Band 3 proteins of the same high purity as in the case of human Band 3 protein. We believe that this procedure will provide a quick and easy way to identify and isolate anion exchange proteins from various sources.

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