Characterization of Matrix-bound Band 3, the Anion Transport Protein from Human Erythrocyte Membranes*

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The Band 3 polypeptide (Mr, 95,000) of the human erythrocyte membrane catalyzes the one-for-one exchange of anions, such as bicarbonate, across the plasma membrane (1). Band 3 is arranged as a dimer or perhaps a tetramer in the membrane and in solutions of nonionic detergents (2-9). The binding of Band 3 monomers was formed by dissociation of the dimers with 2-mercaptoethanol or (6) dissociating agents such as the sodium or lithium salts of dodecyl sulfate or guanidine hydrochloride. Complete removal of the denaturants allowed formation of refolded Band 3 monomers since the matrix-bound subunits could not reassociate. These refolded Band 3 monomers were unable to bind BADS. Release of the monomers from the matrix with 2-mercaptoethanol allowed reformation of dimers with recovery of the BADS binding sites. These results suggest that the dimeric structure of Band 3 is required for BADS binding and that the BADS binding sites may be at the interface between the two halves of the Band 3 dimer.

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EXPERIMENTAL PROCEDURES

Materials

[14C]BADS was synthesized using [14C]benzyl chloride (11.5 mCi/mmol, Amersham Corp.) as described previously (16). C12E8 was purchased from Nikko Chemical Co., Tokyo. Aminoethyl Sepharose 4B was synthesized according to Shaltiel and Er-El (17). A series of pCMB-Sepharose beads were synthesized as described by Lukacovics et al. (18). The different amounts of the ligand pCMB were attached to the beads by limiting the amount of cyanogen bromide used in the initial activation of the Sepharose. The beads were used within 3 months of synthesis since the capacity of the beads to bind protein decreased with age.

Methods

Attachment of Band 3 to pCMB-Sepharose—Unless stated otherwise, all steps were carried out at 0-4°C. Band 3 was purified as described previously (16) by selective extraction of ghost membranes with 0.5% C12E8 and chromatography on aminoethyl Sepharose in the presence of 0.1% C12E8. Band 3 in 0.1% C12E8 was applied to 1- to 5-ml columns (1-cm diameter) of pCMB-Sepharose at a flow rate of 1-2 ml/h. The effluent was monitored continually at 280 nm with a Pharmacia uv monitor. Band 3 was applied to the column until the matrix was saturated as judged by a plateau in the absorbance reading. The column was washed with 5-10 column volumes of sample buffer (usually 0.1% C12E8, 50 mM sodium phosphate, pH 8.0), then with the same buffer containing 150 mM NaCl, and finally with 0.1% C12E8 in citrate buffer (28.5 mM sodium citrate, pH 7.4). The matrix was removed from the column and 0.5- to 1-ml aliquots were placed in fresh columns. Band 3 was eluted at a flow rate of 3 ml/h from the matrix with either (a) 0.1% 2-mercaptoethanol to quantitate the total amount of protein attached to the matrix and the purity of Band 3 or (b) dissociating agents such as the sodium or lithium salts of dodecyl sulfate (0.1-1.0%), or guanidine HCl (6 M). After the protein had been eluted with the dissociating agent, the column was re-equilibrated with 20 column volumes of 0.1% C12E8 in citrate buffer. Refolded Band 3 monomers could be released from the matrix with the same buffer containing 0.1% 2-mercaptoethanol.

[14C]BADS Binding to Matrix-bound Band 3—Matrix-bound Band 3 was diluted 10 volumes with citrate buffer (C12E8 final concentration, 0.02%). Various amounts of [14C]BADS were added, and the suspension was mixed and incubated at room temperature for 1 h. Duplicate aliquots of the suspension (200 μl) were counted to determine the total concentration of [14C]BADS. The remainder of the suspension was centrifuged for 2 min in a microfuge. Duplicate aliquots (200 μl) of the supernatant were counted to determine the concentration of free [14C]BADS. The data are plotted according to Scatchard (19).

[14C]BIDS Labeling of Matrix-bound Band 3—Band 3 dimers (2.15 mg of protein) attached to pCMB-Sepharose (4.3 mg of protein/ml of bead) were labeled with 50 μM [14C]BIDS (1300 cpm/nmol) at 37°C for 1 h in 0.1% C12E8, 20 mM sodium phosphate, pH 7.6. The suspension was washed 3 times with 30 volumes of the same buffer at 0°C until no counts above background were detected in the washes.
supernatant. The matrix-bound Band 3 was suspended to 8 ml in 0.25% dodecyl sulfate in 0.1% C12E8, 20 mM sodium phosphate, pH 7.6, for 1 h at 0 °C. The supernatant was removed after centrifugation of the suspension and assayed for protein and radioactivity. The beads were resuspended to 8 ml in 0.1% 2-mercaptoethanol to release the protein covalently attached to the matrix. After centrifugation, the protein and radioactivity content of the supernatant was measured.

Analytical Techniques—BADS binding to Band 3 in solution was determined by the enhancement of BADS fluorescence (16, 20). Sodium dodecyl sulfate gel electrophoresis was performed according to Laemmli (21). Nondenaturing polyacrylamide gels were run in the presence of C12E8 as described by Nakashima and Makino (7). Protein was determined according to Lowry et al. (22) except that all samples were solubilized with 1% sodium dodecyl sulfate. Circular dichroism spectra were made on a Cary model 6001 circular dichroism attachment to a Cary model 80 recording spectropolarimeter.

RESULTS

Characterization of the Oligomeric Structure of Band 3 Using Matrix-bound Protein—We have previously reported (34) that Band 3 can be reversibly dissociated to monomers by dodecyl sulfate at 0 °C. Complete dissociation was not complete until relatively high (0.5%) concentrations of detergent were employed. The monomeric form of Band 3, in the presence of dodecyl sulfate, was not able to bind the anion transport inhibitor BADS. Immediate and rapid removal of the detergent by dialysis or dilution resulted in reformation of the dimer with the reappearance of the BADS-binding site. In order to characterize the BADS-binding site in Band 3 monomers, these preparations must be free of the dissociating agent to allow the subunits to refold. This is not possible in solution since the protein reassociated upon removal of the dissociating agent.

A useful approach for studying subunit interactions in enzymes is to immobilize subunits on a matrix to prevent reassociation (23–25). In this method, oligomers of the protein are covalently attached to Sepharose beads via a single subunit only (Fig. 1). This is accomplished by limiting the amount of reactive groups on the matrix. The oligomeric structure can be disrupted by a dissociating agent, and the noncovalently attached subunits can be removed from a column of the matrix. The protein cannot reform oligomers upon the removal of the dissociating agent since the subunits are covalently attached to a matrix. The result is matrix-bound refolded monomers.

While the original studies (23–25) employed cyanogen bromide-activated Sepharose as a matrix, we have used pCMB-Sepharose. This matrix has a significant number of advantages. First, since the cysteine content of most proteins is low, the probability of attaching oligomers via one subunit only is increased. Second, in order to quantitate the amount of protein attached to cyanogen bromide-activated Sepharose, the entire matrix must be hydrolyzed and the protein quantitated by amino acid analysis. With pCMB-Sepharose, an aliquot of the matrix-bound protein is treated with 2-mercaptoethanol to elute the protein which can then be readily quantitated by absorbance at 280 nm or by another protein assay. Third, the protein can be released from the matrix as a native dimer, denatured monomer, or refolded monomer and examined in solution.

Preparation of Matrix-bound Band 3 Dimers and Monomers—Band 3 contains six sulphydryl groups per subunit (26, 27) and can be attached to pCMB-Sepharose (Refs. 16, 18, and 28 and Fig. 2). Band 3 purified in the presence of C12E8 was loaded onto a column of pCMB-Sepharose until a plateau in the absorbance at 280 nm was reached (Fig. 2). At this point, the column was saturated with protein. The column was then washed with a series of buffers until the absorbance of the effluent reached zero. No additional protein could be eluted with low ionic strength or high salt (1 M) buffers.

In order to attach Band 3 oligomers to the matrix via one subunit only, the amount of ligand on the matrix must be limited. A series of pCMB-Sepharose matrices was prepared using various amounts of cyanogen bromide to activate the beads (Table I). The maximum capacity of the beads for Band 3 was approximately 7 mg/ml of bead. Both subunits of Band 3 were covalently attached to Sepharose beads activated with 250 mg of cyanogen bromide/ml of bead since no protein was eluted with 0.25% dodecyl sulfate. By reducing the amount of cyanogen bromide used to activate the beads, the amount of Band 3 eluted by dodecyl sulfate could be increased. At 1.25 mg of cyanogen bromide/ml of bead, the capacity of the column was less than maximal (5 mg/ml); however, 45% of the Band 3 could now be eluted with dodecyl sulfate. Further reduction of the amount of cyanogen bromide used to activate the beads did not increase the amount of Band 3 eluted with dodecyl sulfate. This finding is consistent with Band 3 being primarily in the dimeric state in solutions of C12E8 at protein...
plied to a 1.5-ml column of pCMB-Sepharose until a plateau was reached in the absorbance at 280 nm. The column was washed with: 2, sample buffer (0.1% C~ZER, 50 mM sodium phosphate, pH 8.0); 3, the same buffer with 150 mM NaCl; and 4, 0.1% C~ZER, 28.5 mM sodium citrate, pH 7.4.

Concentrations greater than 1 mg/ml. For example, if Band 3 were primarily a tetramer in solution, three-fourths of the protein would have been eluted by the dissociating agent at low ligand concentrations. The fact that the amount of Band 3 released by the dissociating agent was always less than 50% may be due to the presence of some Band 3 monomers in the C~E~ solution (7, 9) which also attached to the matrix.

It was essential that the column be fully saturated with protein; otherwise, the dissociated protein could rebind to the matrix. Reducing the amount of protein eluted from the column (Table I). To confirm that the protein eluted by dodecyl sulfate was due to dissociation of Band 3 dimers, Band 3 covalently cross-linked to a dimer with Cu"-o-phenanthroline (2, 4, 29) was attached to the matrix. Elution of a column of matrix-bound cross-linked dimer with dodecyl sulfate released only 5% of Band 3. We are, therefore, confident that dodecyl sulfate is indeed dissociating Band 3 dimers and that the protein left attached to the matrix is a dissociated monomer.

pCMB-Sepharose, prepared with 1.25 mg of cyanogen bromide/ml of bead was saturated with Band 3 as described in Fig. 2. The matrix which contained Band 3 dimers covalently attached by one subunit was removed from the column and divided into three equal aliquots. One aliquot was eluted with 2-mercaptoethanol directly to determine the amount of protein on the matrix (3.3 ml/ml of bead) and to check the purity of Band 3 (Fig. 3A). The Band 3 used in these experiments was greater than 95% pure as determined by sodium dodecyl sulfate-gel electrophoresis (Fig. 3A, inset). Elution of an aliquot of the matrix with 0.25% dodecyl sulfate released 2.2 mg of Band 3 per ml of bead. After re-equilibration of the column with a nondenaturing buffer, subsequent elution with 2-mercaptoethanol released a further 2.8 mg/ml. The sum of the amount of protein in the peak eluted by the dissociating agent and the peak subsequently eluted with 2-mercaptoethanol (5.0 mg/ml) agrees with the amount of protein eluted with 2-mercaptoethanol directly (5.3 mg/ml). No significant losses of protein had, therefore, occurred during any of the elution or renaturation procedures. 6 M guanidine HCl eluted the same amount of protein (2.1 mg/ml) as 0.25% dodecyl sulfate (Fig. 3C). After renaturation, a further 2.8 mg/ml of protein could be eluted with 2-mercaptoethanol (total protein, 4.9 mg/ml).

Higher concentrations of dodecyl sulfate did not increase the amount of Band 3 eluted. In fact, 0.1% dodecyl sulfate eluted as much Band 3 as higher concentrations of detergents, the peak eluted with 0.1% being slightly broader than with 0.25% dodecyl sulfate. The concentration of detergent required to dissociate Band 3 dimers from the matrix (0.1%) is considerably less than the amount required to completely dissociate the protein in solution (0.5%). This is because the continual elution of the column does not allow Band 3 to reassociate under these nonequilibrium conditions.

Inhibitor-binding Properties of Matrix-bound Band 3—Band 3 dimers were attached to pCMB-Sepharose via one subunit as described in the previous section. An aliquot of matrix-bound dimers was used to test the BADS-binding properties of this preparation. A curved Scatchard plot of [14C]BADS binding to matrix-bound Band 3 was obtained (Fig. 4). The plot could be resolved into a saturable and a nonsaturable component. The nonsaturable component was observed in all experiments and may be due to the interaction.

![Graph](http://www.jbc.org/)
of BADS with protein-bound detergent or perhaps low affinity BADS sites on Band 3. Band 3 covalently modified with the irreversible anion transport inhibitor DIDS was purified and attached to the pCMB-Sepharose. This preparation did not contain a high affinity BADS site, and only a nonsaturable component could be detected (Fig. 4). These results show that the matrix-bound Band 3 contains the BADS site which is irreversibly blocked with DIDS. Subtraction of the nonspecific component revealed that matrix-bound Band 3 dimers bound BADS with an affinity of 3.5 nmol/mg of protein at a stoichiometry of 7.0 nmol of BADS/mg of Band 3. Assuming a molecular weight of 95,000 for Band 3, there are 0.7 BADS molecule bound/Band 3 monomer. These results are in reasonable agreement with the affinities and stoichiometries measured for Band 3 in cells, membranes, and after purification in the presence of C$_{12}$E$_{6}$ (16).

The expected stoichiometry for Band 3 dimers is 16 nmol of BADS/mg of protein. The ability of both subunits of Band 3 to bind stilbene disulfonates was tested by labeling matrix-bound Band 3 with [14C]BIDS. It was found that the overall stoichiometry of labeling was 7.42 nmol/mg of protein at saturating concentrations of BIDS. 0.25% dodecyl sulfate released 35% of the protein in this experiment. This fraction of Band 3 contained 9.51 nmol of [14C]BIDS/mg of protein. The remainder of Band 3 released by 2-mercaptoethanol contained 4.82 nmol of [14C]BIDS/mg of protein. The lower amount of labeling of the subunit attached to the matrix may be due to reaction of pCMB with a sulphydryl in the stilbene disulfonate site (31). The amount of Band 3 released by dodecyl sulfate was less than 50% which suggests that some Band 3 monomers present in the C$_{12}$E$_{6}$ solution (7, 9) attached to the matrix. Matrix-bound Band 3 monomers, as shown below, are incapable of binding stilbene disulfonate. This would also account for the lower stoichiometry. These experiments show that both subunits of matrix-bound Band 3 are capable of binding stilbene disulfonates.

Refolded Band 3 monomers were formed in three ways. Native Band 3 dimers were attached to pCMB-Sepharose by one subunit. The protein was dissociated with either 0.25% dodecyl sulfate or 6 M guanidine HCl (as in Fig. 3). The denatured matrix-bound monomers were refolded by washing the columns with 20 column volumes of nondenaturing buffer. The matrix-bound Band 3 monomers formed using either dodecyl sulfate or guanidine HCl were totally incapable of binding BADS (Fig. 4). Band 3 monomers formed in solution with 0.5% dodecyl sulfate could also be covalently attached to pCMB-Sepharose. The maximum capacity of the matrix for this preparation was only 3 mg of Band 3/ml of bead. This is about half the normal capacity confirming that native Band 3 dimers are attached via one subunit only. After removal of the dodecyl sulfate, this preparation of matrix-bound monomers was also unable to bind [14C]BADS.

In order to check that the refolded Band 3 monomers attached to the matrix did not contain any residual dodecyl sulfate, the protein was dissociated with [35S]dodecyl sulfate. The detergent was removed by washing the column with buffer. Refolded Band 3 was released from the column with mercaptoethanol. No counts above background were detected in the peak of Band 3 protein, indicating that no dodecyl sulfate was left associated with the refolded monomers.

As shown in Fig. 3, refolded Band 3 could be released from the matrix with 2-mercaptoethanol. When the eluted Band 3 was examined by nondenaturing gel electrophoresis, the preparation behaved as a dimer (Fig. 5). The refolded monomer when released from the matrix was, therefore, capable of reassociating to a dimer. The BADS-binding properties of this preparation were examined and compared to native Band 3.

![Figure 4](http://www.jbc.org/)  
**FIG. 4.** Scatchard plot of [14C]BADS binding to matrix-bound Band 3. 1, dimers; 0, dimers prepared from DIDS-labeled cells; X, monomers refolded after dissociation with 0.25% dodecyl sulfate; and □, monomers refolded after dissociation with 6 M guanidine HCl. The points for Band 3 dimers (1) are from five different preparations of matrix-bound Band 3.

![Figure 5](http://www.jbc.org/)  
**FIG. 5.** Molecular weight determination of Band 3-detergent complexes by gel electrophoresis in the presence of C$_{12}$E$_{6}$. Standard proteins are: 1, thyroglobulin (M$_{r}$ = 669,000); 2, ferritin (M$_{r}$ = 440,000); 3, catalase (M$_{r}$ = 252,000); 4, bovine serum albumin dimer (M$_{r}$ = 136,000); and 5, bovine serum albumin monomer (M$_{r}$ = 68,000). Dimer is native Band 3 in C$_{12}$E$_{6}$ monomer is Band 3 dissociated with dimethyl maleic anhydride; renatured Band 3 is formed by release of the refolded monomer from pCMB-Sepharose by 0.1% 2-mercaptoethanol. The gel was a linear 4-20% polyacrylamide gradient in 0.1% C$_{12}$E$_{6}$, 10 mM Tris, 80 mM glycine, pH 8.3, and was run at 4 °C for 20 h.
3 dimers and Band 3 eluted with 0.25% dodecyl sulfate (Fig. 6). Native Band 3 dimers bound BADS with an affinity of 1.2 μM as shown previously (16). Band 3 eluted with 0.25% dodecyl sulfate bound BADS with much lower affinity (Fig. 6). The refolded Band 3 thus was released from the matrix-bound BADS with only a slightly less affinity (3 μM) than native Band 3 dimers. This preparation has, therefore, recovered most of this native structure.

Dissociation of Band 3 dimers by 0.25% dodecyl sulfate was not accompanied by large changes in the conformation of Band 3 as determined by circular dichroism (Fig. 7). The ability to reform dimers in solution may be due to the resistance of Band 3 to denaturation. The CD spectrum of refolded Band 3 released from the matrix was very similar to the spectrum of native Band 3 dimers (Fig. 7). As judged by CD, Band 3 had regained its native secondary structure after removal of the detergent and release from the matrix.

**DISCUSSION**

The results in this paper clearly show that refolded matrix-bound Band 3 monomers were unable to bind BADS. These monomers could reassociate when released from the matrix with recovery of the BADS-binding sites. Although there is one BADS site/Band 3 monomer, the dimeric structure is required for BADS binding. It may be that the dimeric structure is required to regain the native BADS binding site in each monomer. For example, a conformational change may occur upon dimerization that opens inhibitor binding sites in each subunit. The binding sites (two per dimer) may also be at the interface between the two halves of the dimer. Soluble enzymes such as glucose-6-phosphate isomerase (32) and phosphofructokinase (33) contain binding sites for substrates and effectors that are at the interface between adjacent subunits. In the case of phosphofructokinase, there is a solvent-filled hole approximately 7 Å in diameter that passes through the center of the tetramer (33). Stilbene disulfonates bind to a hydrophobic site in Band 3 (8, 20) and are able to cross-link two regions within the Band 3 monomer (15). This indicates that the stilbene disulfonate-binding site may be a deep cleft in the Band 3 protein. This cleft can accommodate two stilbene disulfonates per dimer. The two sites are in close proximity as determined by fluorescence quenching and energy transfer experiments (8, 30). These binding sites may define a channel facing the outside of the cell (1) that allows access to an anion-binding site.

The role(s) of the oligomeric structure of membrane proteins such as the anion transport protein of human erythrocyte membranes in transport is unknown. We propose that a channel exists between the two halves of the band 3 dimer and that this channel is blocked by transport inhibitors such as stilbene disulfonates. Dissociation of the dimer to a monomer would, therefore, result in destruction of this channel, predicting that monomers of Band 3 would be incapable of catalyzing the translocation of anions across lipid bilayers. This problem is being explored by reconstitution experiments.

**REFERENCES**


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**Fig. 6.** Enhancement of BADS fluorescence by binding to: ○, native Band 3; □, Band 3 eluted from pCMB-Sepharose with 0.25% dodecyl sulfate; and ○, refolded Band 3 released from pCMB-Sepharose with 0.1% C12E8, 28.5 mM sodium citrate, pH 7.4.

**Fig. 7.** Circular dichroism spectra of: C, refolded Band 3 released from pCMB-Sepharose with 2-mercaptoethanol; ◆, Band 3 released with 0.25% dodecyl sulfate; and —, native Band 3. All buffers contained 0.1% C12E8, 5 mM sodium phosphate, pH 8.0. The pathlength was 0.0105 cm and the spectra were taken at 27 °C. The protein concentrations were between 0.2 and 1.2 mg/ml.
Matrix-bound Band 3

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