Characterization of the Stilbenedisulfonate Binding Site of the Band 3 Polypeptide of Human Erythrocyte Membranes†

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ABSTRACT: The interaction of [^{14}C]-4-benzamido-4′-aminostilbene-2,2′-disulfonate (BADS), a fluorescent inhibitor of anion transport in human erythrocytes, with the band 3 polypeptide (M_r 95 000) of the plasma membrane was characterized. Equilibrium binding studies with intact cells and membranes revealed that there were 1.2×10^6 [^{14}C]BADS binding sites per cell and 2.4 nmol of [^{14}C]BADS bound/mg of membrane protein, corresponding to one BADS site per band 3 molecule. [^{14}C]BADS bound to band 3 in cells and membranes with an affinity of ~ 1 μM, equal to the K_i of inhibition of [^{32}P]phosphate uptake. The binding constant for BADS to membranes measured by enhancement of BADS fluorescence was dependent upon ionic strength (K_d = 1.3 μM at physiological ionic strength). Pretreatment of cells with 4-benzamido-4′-isothiocyanostilbene-2,2′-disulfonate (BIDS) or 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) prevented BADS binding to membranes. Perturbation of band 3 by (1) removal of associated band 6 proteins, (2) cross-linking of band 3 to a covalent dimer by oxidation with copper(II) *o*-phenanthroline, or (3) removal of the cytoplasmic domain (M_r 41 000) by mild trypsin digestion resulted in no change in the binding affinity of BADS. Extraction of membranes with 0.1 N NaOH or 0.1 N acetic acid to remove extrinsic membrane proteins resulted in irreversible loss of the BADS binding site, while extraction with 2 mM ethylenediaminetetraacetic acid, pH 12, had no effect. Chymotrypsin treatment of cells, which cleaved band 3 into amino-terminal 60 000-dalton and carboxyl-terminal 35 000-dalton fragments had no effect on BADS binding, while papain treatment of cells which produced two smaller fragments (M_r 59 000 and 30 000) greatly reduced the BADS binding affinity. Solubilization of the membrane with Ammonyx LO (lauryldimethylamine oxide), $C_{12}E_8$ (octaethylene glycol *n*-dodecyl monoether), or octyl glucoside had no effect on the binding of BADS, while the use of sodium deoxycholate or sodium dodecyl sulfate resulted in loss of the stilbenedisulfonate binding site. Band 3 purified in the presence of $C_{12}E_8$ bound BADS with high affinity (K_d = 1 μM). Band 3 purified from [^{14}C]BIDS-labeled cells contained 8–10 nmol of [^{14}C]-BIDS/mg of protein, equivalent to one stilbenedisulfonate binding site per band 3 molecule. The [^{14}C]BIDS attachment site was localized to a 19 000-dalton membrane-spanning domain of band 3.

The band 3 polypeptide of human erythrocyte membranes catalyzes the exchange of anions across the plasma membrane (Knauf, 1979). This 95 000-dalton glycoprotein spans the membrane with a 41 000-dalton amino-terminal domain in the cytoplasm and a 55 000-dalton carboxyl-terminal domain in the membrane (Steck et al., 1976). Anion transport in red blood cells is inhibited by a large number of compounds including stilbenedisulfonates (Knauf, 1979; Knauf & Rothstein, 1971; Cabantchik & Rothstein, 1972). 4,4′-Diisothiocyanostilbene-2,2′-disulfonate (DIDS) inhibits anion transport when applied from the outside of the cell and binds to one site per band 3 molecule (Knauf & Rothstein, 1971; Cabantchik & Rothstein, 1972, 1974; Cabantchik et al., 1978; Lepke et al., 1976; Ship et al., 1977; Ramjeesingh et al., 1980, 1981). This site has been localized to a lysine residue located in the carboxyl-terminal domain about 6000 daltons from the junction of the two domains (Ramjeesingh et al., 1980, 1981). The dihydro analogue of DIDS at 0 °C and other stilbenedisulfonates can inhibit anion transport by binding reversibly to band 3 (Shami et al., 1978; Barzilay et al., 1979; Rao et al., 1979; Dix et al., 1979; Macara & Cantley, 1981a,b; Froehlich, 1982; Forman et al., 1982).

We have synthesized a radioactive, reversible inhibitor of anion transport, [^{14}C]-4-benzamido-4′-aminostilbene-2,2′-disulfonate (BADS) and have characterized its interaction with cells, membranes, and purified band 3. A radioactive, irreversible inhibitor, [^{14}C]-4-benzamido-4′-isothiocyanostilbene-2,2′-disulfonate ([^{14}C]BIDS) (Rao et al., 1979; Macara & Cantley, 1981a,b) was also synthesized and was used to covalently label a portion of the stilbenedisulfonate binding site in band 3.

Experimental Procedures

Materials

4,4′-Diaminostilbene-2,2′-disulfonate was purchased from Eastman. [^{14}C]Benzoyl chloride (11.5 mCi/mmol) was purchased from Amersham. [^{14}C]BADS (1000 cpm/nmol in 10 mL of Aquasol) and [^{14}C]BIDS were synthesized according to Kotaki et al. (1971) and Maddy (1964), respectively, as previously described (Rao et al., 1979). Ammonyx LO (lauryldimethylamine oxide) was a gift of Oryx Chemical Co.; octaethylene glycol *n*-dodecyl monoether ($C_{12}E_8$) was purchased from Nikko Chemical Co., Tokyo; octyl glucoside and sodium deoxycholate were obtained from Calbiochem; sodium dodecyl sulfate, high purity, was a product of British Drug House. All other chemicals were reagent grade.

Methods

Membrane Preparations. Unless stated otherwise, 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-5 centrifuge. Erythrocyte ghosts were prepared from outdated blood (kindly provided by the Red Cross) by hypotonic lysis in 5 mM sodium phosphate, pH 8.0, as described by Dodge et al. (1963). Membranes were extracted with 10 volumes of 2 mM ethylenediaminetetraacetic acid (EDTA), pH 12, 0.1 N NaOH, or 0.1 N acetic acid at 0 °C (Steck et al., 1976). Membranes were recovered by centrifugation and

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were washed twice with 28.5 mM sodium citrate, pH 7.4, prior to the measurement of BADS binding.

Purification of Band 3. Band 3 was purified by a modification of established procedures (Lukacovics et al., 1981; Yu & Steck, 1975; Nakashima & Makino, 1980). Ghost membranes were extracted with 10 volumes of phosphate-buffered saline at 0 °C for 20 min. The membranes were recovered by centrifugation, washed once with 5 mM sodium phosphate, pH 8.0, and then extracted with 1 volume of 5 mM sodium phosphate, pH 8.0, containing 1% $C_{12}E_8$ at 0 °C for 20 min. After centrifugation at 19000 rpm for 20 min, the supernatant was applied to a 1 × 25 cm column of aminoethyl-Sepharose, prepared according to Shaltiel & Er-El (1973). After application of the sample, the column was washed with 1 column volume of 5 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, and 0.1 % $C_{12}E_8$. Band 3 was eluted with the same buffer containing 200 mM NaCl. Alternatively, band 3 was eluted with a gradient of NaCl from 0 to 200 mM in the same buffer. The peak containing band 3 was applied to a 1 × 5 cm column of [[*p*-(chloromercuri)-benzamido]ethylene]amino-Sepharose prepared according to Lukacovics et al. (1981). The column was washed with the same buffer and then 1 M KCl, followed by 28.5 mM sodium citrate, pH 7.4, and 0.1% $C_{12}E_8$. Band 3 was eluted by the same buffer containing 0.1% 2-mercaptoethanol.

Labeling of Cells with [^{14}C]BIDS. Cells at a 50% hematocrit in 5 mM sodium phosphate, pH 7.4, and 150 mM NaCl were reacted with 100 μM [^{14}C]BIDS at 37 °C for 2 h. Cells were washed once with phosphate-buffered saline containing 0.5% bovine serum albumin and twice with buffer alone. Ghosts and purified band 3 were prepared as above. Transport of control and BIDS-labeled cells was as described above.

Proteolytic Modification. Cells, at a 50% hematocrit in 5 mM sodium phosphate, pH 7.4 (Steck et al., 1976; Jennings & Passow, 1979), and 150 mM NaCl, were treated with chymotrypsin or papain (1 mg/mL) at 37 °C for 2 h. Digestion with chymotrypsin was terminated with 1 mM phenylmethanesulfonyl fluoride, and digestion with papain was terminated with 2 mM iodoacetamide. Cells were washed at 4 °C with phosphate-buffered saline containing 0.5% bovine serum albumin and then twice with buffer alone. Membranes, prepared from control, chymotrypsin-treated, or papain-treated cells, were suspended in 5 mM sodium phosphate, pH 8.0, at 1 mg/mL and digested at 4 °C for 1 h with 5 μg/mL trypsin. The reaction was stopped by addition of phenylmethanesulfonyl fluoride, and the membranes were washed with buffer or stripped with 2 mM EDTA, pH 12. Membranes were washed twice with 28.5 mM sodium citrate prior to the measurement of BADS binding.

Transport (Ho & Guidotti, 1975). Erythrocytes were suspended to a 50% hematocrit in 28.5 mM sodium citrate and 205.3 mM sucrose, pH 7.4 at 23 °C. At zero time, a small volume of 0.13 M sodium phosphate, pH 7.4, containing sodium [^{32}P]phosphate was added. The suspension was shaken slowly at 23 °C. At appropriate times up to 4 h, 200-μL aliquots of the suspension were removed and centrifuged immediately in a microfuge at 4 °C. Aliquots of 50 μL of the supernatant were removed and counted. For the inhibition studies, BADS was added at various concentrations before the addition of phosphate.

Binding of [^{14}C]BADS to Cells and Ghosts. Cells at a 50% hematocrit were incubated with various concentrations of [^{14}C]BADS for 1 h at 23 °C in 28.5 mM sodium citrate, pH 7.4, and 205.3 mM sucrose. Cells were sedimented in a microfuge, and aliquots of the supernatant were counted in a liquid scintillation counter to determine the concentration of free [^{14}C]BADS. The total concentration of [^{14}C]BADS was corrected for the volume occupied by cells by determining the volume of the suspension available to [^{3}H]inulin. Ghosts at a concentration of 1 mg/mL were incubated with various concentrations of [^{14}C]BADS for 1 h at 23 °C in 28.5 mM sodium citrate, pH 7.4. An aliquot of the incubation mixture was counted to determine the total concentration of reagent. Membranes were removed by centrifugation at 100000*g* for 30 min, and an aliquot of the supernatant was counted to determine the concentration of free [^{14}C]BADS. Binding data were plotted according to Scatchard (1949).

Fluorescence Assay. Fluorescence measurements (Rao et al., 1979) were made with a Perkin-Elmer Model MPF fluorometer thermostated to 20 °C. A 0.3 × 0.3 cm microcuvette was used to minimize the inner filter effect. Fluorescence data were corrected for dilution, self-quenching of the probe, and background fluorescence. Samples (5 μL) were diluted into 200 μL of 28.5 mM sodium citrate, pH 7.4. The effect of ionic strength on binding of BADS to ghosts was determined by adding membranes to solutions of sodium citrate, pH 7.4, at various concentrations. The effect of detergents on BADS binding was determined by diluting 10–20 μL of membranes into 200 μL of 28.5 mM sodium citrate, pH 7.4, containing various types and concentrations of detergents. Fluorescence was measured by exciting the probe at 340 nm and measuring fluorescence at 450 nm or by exciting the protein at 280 nm and measuring fluorescence at 450 nm by energy transfer (Rao et al., 1979). Excitation of BADS directly at 340 nm gave a high background fluorescence in the presence of detergents, presumably due to the interaction of BADS with the detergent.

Sodium dodecyl sulfate–polyacrylamide gels were run according to Laemmli (1970). Protein was determined according to Lowry et al. (1951), except that all samples were solubilized with 1% sodium dodecyl sulfate.

Results and Discussion

Binding of [^{14}C]BADS to Cells and Membranes. [^{14}C]-BADS inhibited phosphate uptake into red blood cells previously loaded with chloride with a K_i of 1.5 μM in agreement with previous results (Barzilay et al., 1979; Rao et al., 1979). The inhibition by BADS could be completely reversed by washing BADS-treated cells with isotonic buffer containing 0.5% bovine serum albumin, indicating that the BADS binding site is on the outside surface of the cell.

The binding of [^{14}C]BADS to intact cells is shown in Figure 1. The curved Scatchard plot could be resolved into two components. Subtraction of the low-affinity component from the data revealed a single class of binding sites with an affinity of 1.4 μM in agreement with the K_i of inhibition of phosphate transport. A stoichiometry of 1.2×10^6 [^{14}C]BADS sites per cell was calculated. The second class of sites of much lower affinity represents BADS sites unrelated to transport, possibly due to BADS binding to lipid.

The high-affinity BADS site (K_d = 1.5 μM) could also be detected in ghost membranes (Figure 2). The stoichiometry is 2.4 nmol of BADS bound/mg of membrane protein. Under the assumption that band 3 makes up 25% of the membrane protein, there is 0.96 mol of BADS/mol of band 3. This is equivalent to 1.03×10^6 sites per cell when a conversion factor of 1.41×10^9 cells/mg of membrane protein is used (Cabantchik et al., 1978). The number of band 3 monomers per cell has been estimated to be 1.2×10^6 (Knauf, 1979); therefore, there is one BADS binding site per band 3 molecule. The affinity of band 3 for BADS is dependent on the ionic

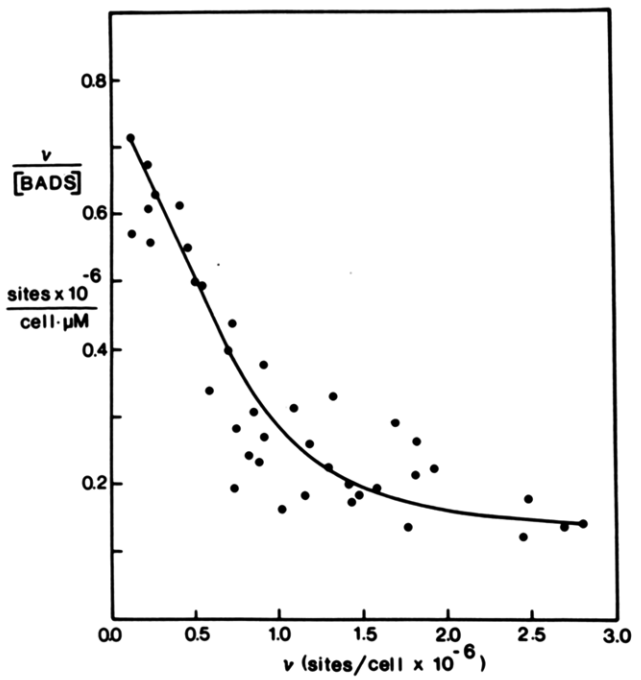

FIGURE 1: Scatchard plot of [^{14}C]BADS binding to red blood cells. ν, the number of sites per cell, was calculated from the difference between the total concentration of [^{14}C]BADS (corrected for the volume occupied by the cells) and the free concentration of [^{14}C]-BADS. The number of cells per milliliter was calculated from the hematocrit.

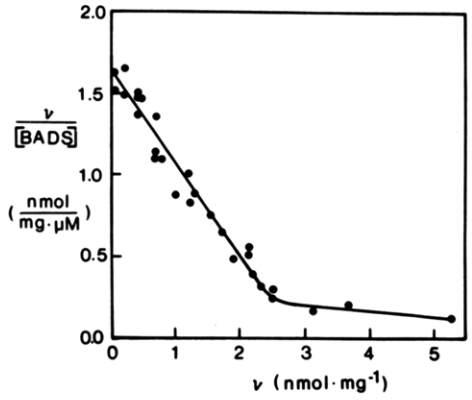

FIGURE 2: Scatchard plot of [^{14}C]BADS binding to membranes. ν, the nanomoles of [^{14}C]BADS bound per milligram of membrane protein, was calculated from the difference between the total concentration of [^{14}C]BADS and the free concentration of [^{14}C]BADS and the amount of protein in the suspension of membranes.

strength of the solution (Table I). At physiological ionic strength, the affinity (K_d = 1.3 μM) measured by fluorescence enhancement (Rao et al., 1979) agreed with the affinities measured directly on cells and ghost membranes and with the K_i of inhibition. Pretreatment of cells with BIDS or DIDS eliminated the high-affinity binding site for BADS. This shows that BADS is binding to the BIDS/DIDS binding site on band 3. All of the above results clearly show that there is a single stilbenedisulfonate site on band 3 and that this site binds BADS with an affinity of ~1 μM at physiological ionic strength.

Effect of Modification of the Amino-Terminal Cytoplasmic Domain of Band 3 on BADS Binding. A large number of proteins bind to the amino-terminal cytoplasmic domain of band 3 (Knauf, 1979). The effect of the removal of these proteins on BADS binding was determined. Removal of band 6 by extraction with 150 mM NaCl (Steck et al., 1976) had no effect on BADS binding (K_d = 1.05 μM) nor did extraction

Table I: Effect of Ionic Strength on BADS Binding to Membranes

sodium citrate concn (mM)	K^a (μM)	sodium citrate concn (mM)	K^a (μM)
0[b]	8.4	14.25	2.9
3.5	6.0	28.5	1.3
7.1	3.5	77.0	0.67

[a] Binding constant measured by the fluorescence assay. [b] Membranes suspended in distilled water.

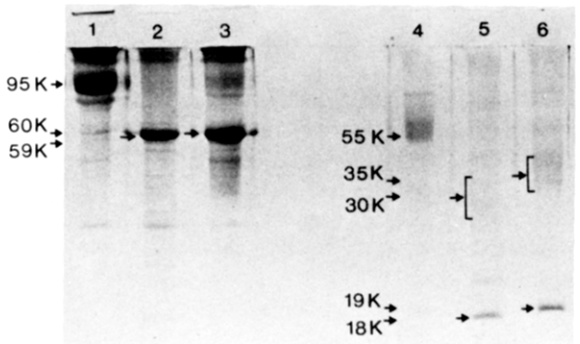

FIGURE 3: Sodium dodecyl sulfate gel electrophoresis of alkali-stripped membranes prepared from (1) control cells, (2) papain-treated cells, (3) chymotrypsin-treated cells, (4) trypsin-treated membranes, (5) trypsin-treated membranes prepared from papain-treated cells, and (6) trypsin-treated membranes prepared from chymotrypsin-treated cells. 95K, band 3; 60K, 60 000-dalton chymotryptic fragment; 59K, 59 000-dalton papain fragment; 55K, 55 000-dalton tryptic fragment; 35K, 35 000-dalton fragment; 30K, 30 000-dalton fragment; 19K, 19 000-dalton fragment; 18K, 18 000-dalton fragment.

of spectrin and actin with 0.1 mM EDTA (K_d = 1.06 μM) (Steck et al., 1976). Stripping of the membranes of peripheral proteins (Steck et al., 1976) with 5 mM EDTA at pH 12 did not destroy the BADS binding site (0.97 μM). However, no binding could be detected after stripping with 0.1 N NaOH or 0.1 N acetic acid, suggesting that these procedures irreversibly denature the protein and are not suitable for further studies of the native protein. Cross-linking of band 3 to a dimer by oxidation of a pair of sulfhydryl groups located in the cytoplasmic domain (Steck et al., 1976) did not alter the affinity of band 3 for BADS (K_d = 1.05 μM). Trypsin treatment of ghosts results in cleavage of the band 3 molecule into two domains (Steck et al., 1976). The N-terminal half of the molecule (M_r 41 000) can be removed from the protein without loss of the BADS binding (K_d = 1.1 μM). These results suggest that perturbation of the cytoplasmic domain of band 3 or indeed its complete removal had no effect on the binding of BADS. DIDS binding to band 3 has, however, been reported (Salhany et al., 1980) to affect the binding affinity of hemoglobin to the membrane. Removal of the cytoplasmic domain by chymotrypsin treatment of inside-out vesicles did not inhibit anion transport (Grinstein et al., 1978).

Effect of Protease Treatment of Cells on BADS Binding. In order to further characterize the BADS binding site, proteolytic dissection of the membrane domain band 3 was carried out. Chymotrypsin treatment of intact cells (Steck et al., 1976) cleaved band 3 into an N-terminal 60 000-dalton and C-terminal 35 000-dalton fragment (Figures 3 and 4). The BADS binding constant was unaffected by this chymotrypsin treatment (K_d = 1.6 μM). Cleavage of band 3 at the external chymotrypsin site (Figure 4) has little effect on the structure of the protein since the two chymotryptic fragments remain closely associated in the membrane (Jennings & Passow, 1979; Reithmeier, 1979) and the BADS site remains intact. Papain treatment on the other hand, which cleaved band 3 into a

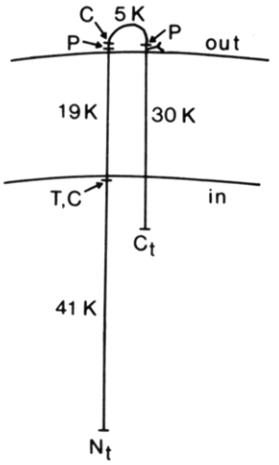

FIGURE 4: Model showing the effect of proteases on band 3 structure. C, chymotrypsin-sensitive sites; P, papain-sensitive sites; T, trypsin-sensitive site; N_t, amino terminal; C_t, carboxyl terminal; 41K, amino-terminal 41 000-dalton fragment; 19K, membrane-associated 19 000-dalton fragment; 5K, portion removed by papain treatment of cells; 30K, carboxyl-terminal 30 000-dalton fragment; Y, carbohydrate attachment site. The exact location of the carboxyl terminal is not known, and it is possible that papain removes a portion from this end of band 3.

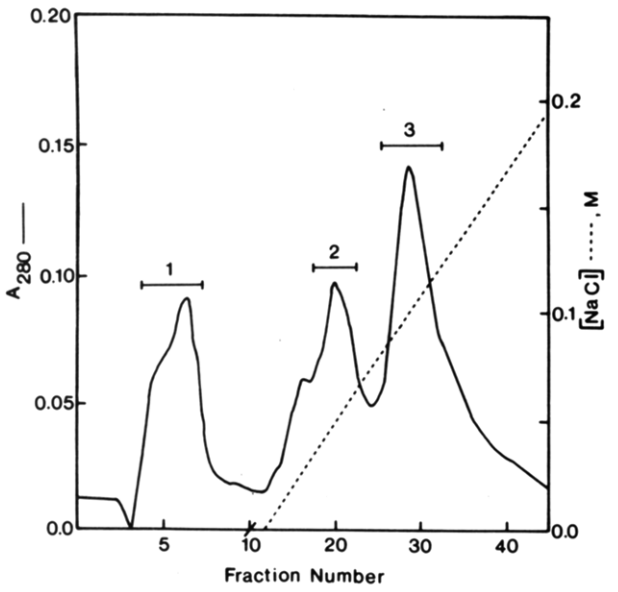

FIGURE 5: Chromatography of the $C_{12}E_8$ extract on a column (1 × 20 cm) of aminoethyl-Sepharose 4B at 4 °C. The extract (15 mL) containing 0.6 mg/mL protein was applied to the column. The column was washed with 2 column volumes of 5 mM Tris-HCl, pH 7.4. A linear gradient of 0–200 mM NaCl (100 mL of each buffer) in the same buffer was applied. Peak 3, which contained band 3, was pooled and applied directly to a column of [[*p*-(chloromercuri)benzamido]ethylene]amino-Sepharose 4B. The effluent was measured at 280 nm on a Pharmacia UV monitor. All buffers contained 0.1% $C_{12}E_8$.

59 000- and 30 000-dalton fragment (Jennings & Passow, 1979; Figures 3 and 4), completely prevented BADS binding. This suggests that the portion of band 3 removed by papain is required for BADS binding or that removal of this portion of band 3 resulted in a conformational change which caused loss of the BADS binding site.

BADS Binding to Purified Band 3. The ability of band 3, purified in the presence of detergents to bind BADS, was determined. Ammonyx LO, $C_{12}E_8$, and octyl glucoside selectively solubilized band 3 from the membranes as reported for Triton X-100 (Yu et al., 1973). BADS bound to solubilized preparations of band 3 with normal affinity (K_d = 0.9,

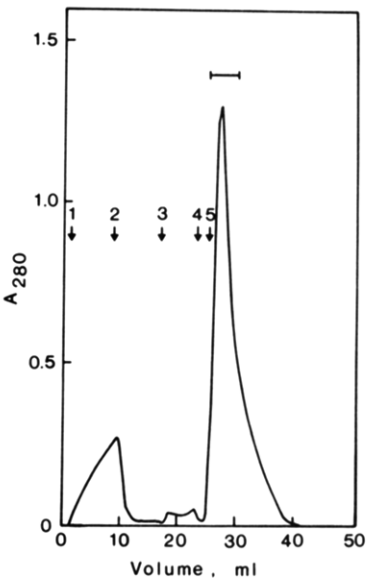

FIGURE 6: Chromatography of band 3 on [[*p*-(chloromercuri)benzamido]ethylene]amino-Sepharose 4B at 4 °C. (1) Sample applied; (2) washed with sample buffer; (3) washed with 1 M KCl; (4) washed with 28.5 mM sodium citrate, pH 7.4; (5) band 3 eluted with 28.5 mM sodium citrate, pH 7.4, and 0.1% 2-mercaptoethanol. The effluent was measured at 280 nm on a Pharmacia UV monitor. All buffers contained 0.1% $C_{12}E_8$.

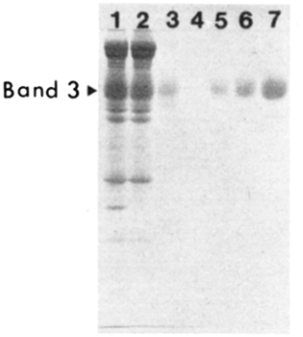

FIGURE 7: Sodium dodecyl sulfate gel showing various stages of purification of band 3. (1) Ghost membranes; (2) band 6 depleted membranes; (3) $C_{12}E_8$ extract; (4) peak 1 of aminoethyl column (Figure 5); (5) peak 3 of aminoethyl column; (6) purified band 3, 10 μg; (7) purified band 3, 25 μg.

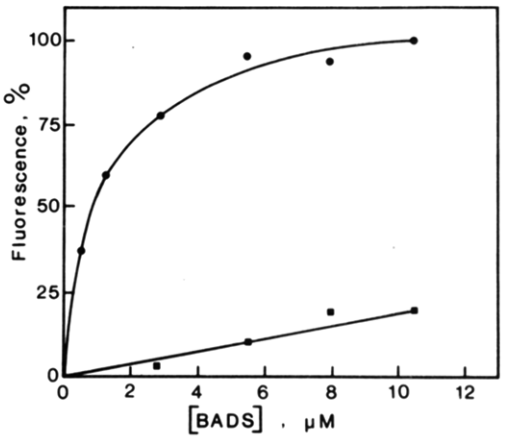

FIGURE 8: Fluorescence enhancement of BADS binding to purified band 3 in the presence of 0.1% $C_{12}E_8$ and 28.5 mM sodium citrate, pH 7.4. (●) Purified band 3; (■) band 3 purified from DIDS-pretreated cells.

1.3, and 1.0 in 0.1% $C_{12}E_8$, Ammonyx LO, and octyl glucoside, respectively), showing that the binding site is maintained in these detergents. A lower affinity binding (K_d = 2.0 μM) could

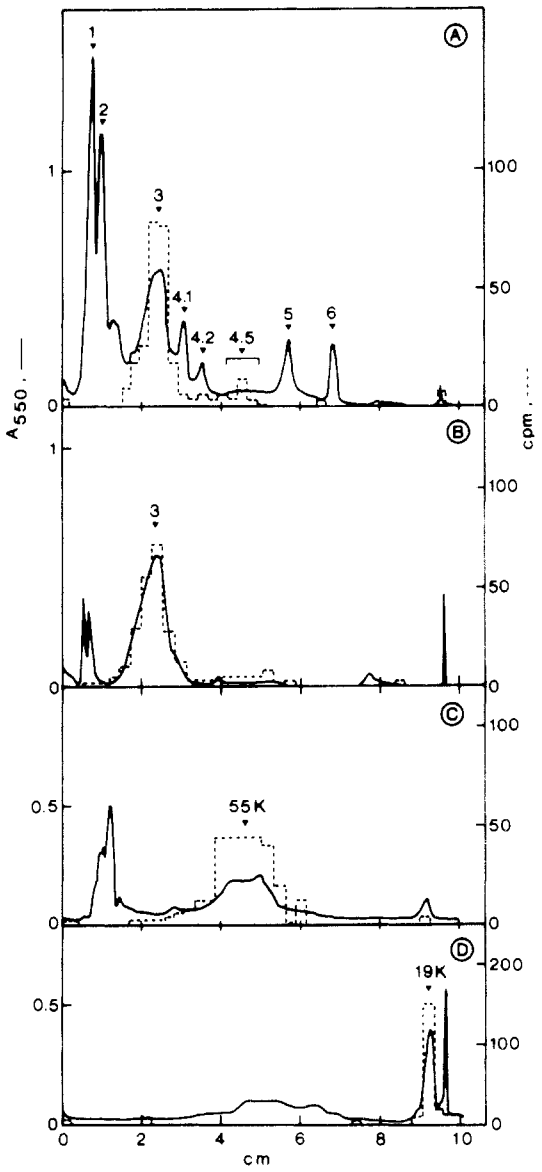

FIGURE 9: Scans of sodium dodecyl sulfate gels of [^{14}C]BIDS-labeled band 3 and proteolytic subfragments. (A) Ghosts; (B) alkali-stripped ghosts; (C) alkali-stripped, trypsin-treated ghosts; (D) alkali-stripped, chymotrypsin-treated ghosts. Gels were scanned at 550 nm in a Gilford gel scanner. Gels were sliced, solubilized with 0.4 mL of 30% hydrogen peroxide at 55 °C for 18 h, and counted in 10 mL of Aquasol. Protein bands are identified according to Steck et al. (1976). 55K, 55 000-dalton fragment produced by trypsin treatment of membranes; 19K, 19 000-dalton fragment produced by chymotrypsin treatment of membranes.

be measured in 0.1% deoxycholate. Sodium dodecyl sulfate at 0.1 or 1% and sodium deoxycholate at 1% in contrast completely solubilized the membrane, and the resulting band 3 was unable to bind BADS.

Band 3 was purified in the presence of $C_{12}E_8$ (Figures 5–7). This detergent has a low absorption at 280 nm which allowed us to monitor directly the purification of this protein (Figures 5 and 6). A cruder form of this detergent, Lubrol PX, has also been used in the purification of band 3. $C_{12}E_8$ offers considerable advantages over the more commonly used Triton X-100, which absorbs strongly at 280 nm, interferes with Lowry assays, and is chemically heterogeneous. Band 3 purified in the presence of $C_{12}E_8$ was capable of binding BADS with an affinity equal to that found for band 3 in the membrane (Figure 8). Since these preparations are devoid of phospholipid (Yu & Steck, 1975), phospholipids are not essential for BADS binding or for the maintenance of the native structure of the protein. The ability to assay the binding of BADS to band 3 in the purified state enables us to characterize the binding site in the absence of lipids and other proteins. For example, chemical modification studies that are under way have shown that tryptophan, tyrosine, lysine, and carboxyl groups, but not sulfhydryl groups, may be involved in BADS binding, and also in anion transport. The enhancement of fluorescence of BADS upon binding to purified band 3 is a very useful tool. Other than this fluorescence assay, the activity of band 3 in solutions can only be assayed by more tedious methods such as reconstitution.

Labeling of Band 3 with [^{14}C]*BIDS*. The number of stilbenedisulfonate sites on band 3 was also determined by labeling cells with [^{14}C]BIDS such that complete inhibition of anion transport was obtained. The band 3 protein was then purified, and the amount of BIDS bound could be accurately determined from the known specific activity of the probe. Ghosts irreversibly bound 2.52 nmol of BIDS/mg of protein, equivalent to 1.08×10^6 sites per cell. Two preparations of purified band 3 contained 8 and 10 nmol of [^{14}C]BIDS/mg of protein, respectively. This is equivalent to approximately one BIDS site per band 3 molecule. The [^{14}C]BIDS covalent attachment site was localized in a proteolytic fragment of band 3 (Figure 9). The BIDS site is almost exclusive to band 3, the 60 000-dalton chymotryptic fragment, and the 19 000-dalton chymotryptic fragment (Figure 6). No label was found in the amino-terminal 41 000-dalton fragment or the 35 000-dalton carboxyl-terminal fragment. The covalent labeling reagent DIDS is bifunctional. At neutral pH, it labels the same proteolytic fragment as BIDS (Knauf, 1979). However, at alkaline pH the second isothiocyano group reacts with a second site in band 3 in the 35 000-dalton carboxyl-terminal fragment (Jennings & Passow, 1979). BIDS therefore has the advantage of labeling at a single site without the concern of reaction of a second isothiocyano group with the protein or subsequently during further characterization of the protein. The sequence of amino acids around the [^{14}C]BIDS attachment site will provide valuable information concerning the stilbenedisulfonate binding site of band 3, and possibly the mechanism of anion transport.

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Registry No. BADS, 25431-18-9; BIDS, 78543-24-5.

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Kinetic Comparison of Bovine Blood Coagulation Factors IXaα and IXaβ toward Bovine Factor X†

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ABSTRACT: The V_{max}/K_m (μM^{-1} min^{-1}) for bovine factor X activation by bovine factor IXaα, in the presence of sufficient [Ca^{2+}] to saturate the initial reaction rate, was 0.007. When factor IXaβ was substituted for factor IXaα in this reaction, the V_{max}/K_m decreased to 0.001, suggesting that factor IXaα was a more potent catalyst under these conditions. When phospholipid (PL) vesicles (egg phosphatidylcholine/bovine brain phosphatidylserine, 4:1 w/w) were added to these same systems, at levels sufficient to saturate their effects, little change in the V_{max}/K_m occurred when factor IXaα was the enzyme. However, when factor IXaβ was employed, the V_{max}/K_m dramatically increased to 0.023, demonstrating that factor IXaβ responded to PL addition to a much greater extent than did factor IXaα. Upon addition of thrombin-activated factor VIII (factor VIIIa,t), at a suboptimal level, to the above systems, the V_{max}/K_m for factor X activation by factor IXaα/Ca^{2+}/PL/factor VIIIa,t was increased to 1.0, whereas this parameter for factor X activation by factor IXaβ/Ca^{2+}/PL/factor VIIIa,t under the same conditions was found to be 27.3. During these studies, it was discovered that the factor X which became activated to factor Xa during the course of reaction participated in several feedback reactions: activation of factor X, activation of factor VIII, and conversion of factor IXaα to factor IXaβ. All feedback reactions, which are capable of complicating the kinetic interpretation, were inhibited by performing the studies in a system which contained a rapid factor Xa inhibitor, Glu-Gly-Arg-CH_2Cl, thus allowing kinetic constants to be accurately determined. The results show that while factor IXaα is a more efficient enzyme than factor IXaβ toward factor X activation in the absence of cofactors, the response of factor IXaβ to the reaction cofactors, PL and factor VIIIa,t, is much greater than that of factor IXaα.

Factor IXa is an enzyme which possesses proteolytic, amidolytic, esterolytic, and thioesterolytic activity. This enzyme exists in the plasma in an inactive form, factor IX, which is activated through a series of proteolytic events. One activator of factor IX is the protease factor XIa in the presence of Ca^{2+} (Schiffman et al., 1963; Kingdon et al., 1964). Here, two steps are involved (Fujikawa et al., 1974). First factor XIa catalyzes cleavage of an Arg_{146}–Ala_{147} peptide bond in single chain (M_r 55 400) factor IX, yielding factor IXα. Next, the Arg_{181}–Val_{182} bond is cleaved, liberating an activation glycopeptide of molecular weight of approximately 10 000 and yielding a two-chain enzyme, factor IXaβ, of M_r ~44 400. Factor IX is also activated by a protease (RVV-X),[1] present in the venom

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[1] Abbreviations: RVV-X, the coagulant protein from the venom of Russell's viper; $NaDodSO_4$, sodium dodecyl sulfate; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; Tris, tris(hydroxymethyl)aminomethane; PEG, poly(ethylene glycol); EDTA, ethylenediaminetetraacetic acid.

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