Factors Determining the Conformation and Quaternary Structure of Isolated Human Erythrocyte Band 3 in Detergent Solution†

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ABSTRACT: Fluorescence spectroscopy was used to follow the kinetics of covalent binding of DIDS (4,4′-diisothiocyanato-2,2′-stilbenedisulfonate) to isolated band 3 in $C_{12}E_8$. We have discovered a dilution-induced loss in the ability of band 3 monomer to form a covalent adduct with DIDS. The loss in DIDS reactivity with dilution followed a 50:50 biphasic time course despite the use of a homogeneous preparation of band 3 oligomers. The loss in reactivity generally correlated with the association of band 3 dimers and tetramers to higher oligomeric structures. The final aggregated product was capable of binding BADS (4-benzamido-4′-amino-2,2′-stilbenedisulfonate) reversibly, but with an affinity nearly 30-fold lower than that of the starting material. Removal of the cytoplasmic domain of band 3 slowed the conformational interconversion of the integral domain by about 5-fold and inhibited the aggregation process. The conformational interconversion was slowed in the presence of 150 mM chloride but not in 90 mM sulfate. Covalent binding of DIDS inhibited the aggregation of band 3. Addition of 250 μM lipid inhibited both the loss of DIDS reactivity and the protein aggregation process. While several types of lipid offer protection, phosphatidic acid accelerated the decay process by eliminating the biphasicity. We conclude that the conformation of the integral domain of band 3 can be modulated allosterically by the addition of ligands, including various lipids. The results offer direct evidence for cooperative interactions between band 3 subunits during loss of activity, and they show that the cytoplasmic domain participates in the control of this transition.

Band 3 is an abundant, integral membrane protein found in the red blood cell membrane (Passow, 1986; Salhany, 1990). It is composed of an integral domain containing the transport function (Lepke et al., 1992) and a water-soluble, cytoplasmic domain which functions to bind cytosolic and certain cytoskeletal proteins (Salhany, 1990). Evidence has developed indicating that the two domains can interact (Salhany et al., 1980; Hsu & Morrison, 1983; Sami et al., 1992) and that such interactions may modulate the transport function under certain conditions (Ducis et al., 1988; Yamaguchi & Kimoto, 1992; Schofield et al., 1992). In addition, recent transport kinetic studies suggest that subunit interactions can play a significant role in modulating the monomeric transport activity (Salhany & Cordes, 1992). Yet, despite this evidence, the conformational basis for interdomain interactions is uncertain, and there is little conformational evidence to support a subunit interaction hypothesis.

In order to obtain functionally relevant conformational information, it is customary to isolate and purify a given protein and to study its ligand binding and spectroscopic properties. We have recently initiated studies with purified band 3 (Casey et al., 1989) in order to define structure/function relationships. In this report, we demonstrate a critical role for various band 3 ligands, including lipids, in stabilizing a conformation of the porter which is capable of forming a covalent adduct with DIDS.[1] Lipid-stabilized, soluble band 3 reacts with DIDS and binds BADS in a manner which is quantitatively similar to that found for band 3 in the red cell membrane. This work defines conditions for maintaining conformationally viable band 3 in detergent solution, thus establishing a rational foundation for future biophysical studies.

MATERIALS AND METHODS

L-α-Phosphatidylcholine, type III-B from bovine brain; L-α-phosphatidylethanolamine, type I-A from bovine brain; L-α-phosphatidyl-L-serine, from bovine brain; L-α-phosphatidyl-DL-glycerol, distearoyl, ammonium salt; L-α-phosphatidylinositol, ammonium salt from bovine liver; sphingomyelin, type I from bovine brain; L-α-phosphatidic acid, dioleoyl, sodium salt; L-α-lysophosphatidic acid, oleoyl, sodium salt; cholesterol, Sigma grade; cholesteryl myristate; tristearin, Sigma grade; TPCK–trypsin; phenylmethanesulfonyl fluoride (PMSF) and polyoxyethylene-8-lauryl ether ($C_{12}E_8$) were obtained from Sigma Chemical Co. 4,4′-Diisothiocyanato-2,2-stilbenedisulfonate, disodium salt (DIDS), was from Aldrich Chemicals. Globoside, type $GbOsE_4Cer$ from human erythrocyte, and ganglioside, type GM_3 from calf brain, were purchased from BioCarb Chemicals. Triton X-100, electrophoresis purity, was from Bio-Rad. 4-Benzamido-4′-amino-2,2′-stilbenedisulfonate (BADS) was synthesized as described by Kotaki et al. (1971). All other chemicals were of reagent quality.

Preparation of Band 3. Intact band 3 was purified as described by Casey et al. (1989) from in-date human red blood cells obtained from the Red Cross. In brief, ghost membranes were stripped of peripheral proteins with 2 mM

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[1] Abbreviations: DIDS, 4,4′-diisothiocyanato-2,2′-stilbenedisulfonate; BADS, 4-benzamido-4′-amino-2,2′-stilbenedisulfonate; DNDS, 4,4′-dinitro-2,2′-stilbenedisulfonate; DBDS, 4,4′-dibenzamido-2,2′-stilbenedisulfonate $C_{12}E_8$, polyoxyethylene-8-lauryl ether; PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; BS^3, bis(sulfosuccinimidyl) suberate.

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EDTA, pH 12, and solubilized in 1.0% $C_{12}E_8$. Band 3 was then separated from glycophorin on an (aminoethyl)-Sepharose 4B column (synthesized as described by Shalteil and Er-El (1973)) using a NaCl gradient. Fractions containing 4–15 μM band 3 were stored at 4 °C in the elution buffer (5 mM sodium phosphate, pH 8.0, containing 0.1% $C_{12}E_8$, 20 μg/mL PMSF, and approximately 60 mM NaCl). Examination of the fractions by gel filtration HPLC, as described by Casey and Reithmeier (1991), showed that the peak fractions contained primarily two species (see Figure 7, t = 0 trace). We will refer to the slower of these species as a dimer and the faster as a tetramer of band 3, after the assignments made by Casey and Reithmeier (1991). The trailing AE-Sepharose fractions contained only the larger of these two species. We used the trailing fractions directly as a convenient source of pure tetramer for some experiments.

The integral domain of band 3 was purified as described by Oikawa et al. (1985). In brief, ghost membranes were treated with TPCK–trypsin (5 μg/mL) at 0 °C for 1 h. The reaction was stopped with PMSF. The membranes were then stripped with 2 mM EDTA, pH 12, solubilized in 1.0% $C_{12}E_8$, and passed over a DEAE column to give pure integral domain.

Protein, Lipid, and Stilbenedisulfonate Concentrations. Absorbance was used to determine the concentration of intact band 3, 95 kDa, extinction at 280 nm equal to 77 000 M^{-1} cm^{-1} (Dorst & Schubert, 1979); band 3 integral domain, 55 kDa, extinction at 280 nm equal to 55 000 M^{-1} cm^{-1} (Maneri & Low, 1989); DIDS, extinction at 340 nm equal to 36 000 M^{-1} cm^{-1} (Eisinger et al., 1982); and BADS, extinction at 340 nm equal to 33 800 M^{-1} cm^{-1} (Kotaki et al., 1971). The concentrations of lipids from natural sources were estimated using an average molecular weight of 700, except for globoside and ganglioside which were estimated from an approximate molecular weight of 1260.

Fluorescence. All fluorescence measurements were made with a Perkin-Elmer, variable-wavelength fluorescence spectrophotometer, Model 650-40 using the ratio mode, 2-nm slits on both the excitation and emission monochrometers, and a fluorescence cuvette with a 4-mm excitation path length. Samples were equilibrated at 25 °C before measurement. Under these conditions, the signal-to-noise ratio was about 45 to 1.

Assay for Covalent Binding of DIDS to Band 3: Preliminary Considerations. We use DIDS reactivity to probe the structure of isolated band 3. DIDS is known to bind noncovalently to the band 3 monomer with a 1:1 stoichiometry and then react with a lysine to form a covalent adduct (Passow, 1986).

$$\text{DIDS} + \text{band 3} \rightleftarrows \text{DIDS·band 3} \xrightarrow{k} \text{DIDS·band 3*} \quad (1)$$

The asterisk (*) indicates the covalent adduct, and k is the first-order rate for adduct formation. The fluorescence of the DIDS·band 3 adduct is used to monitor its formation. Since it is known that light can cause isomerization of stilbenes about the central ethylene double bond, to give an equilibrium mixture of cis and trans forms (Jakobsen & Horobin, 1989; Lewis et al., 1940), it is necessary to consider photoisomerization in any assay using the intense light found in a spectrofluorometer. The trans form of DIDS is fluorescent while the cis form is not (Lewis et al., 1940). In agreement with the literature, we have found that the fluorescence of *trans*-DIDS is diminished when exposed to the xenon light of the spectrofluorometer. Under our conditions, a stable fluorescence is reached in 10 min. The final fluorescence suggests a 30:70 mixture of cis and trans isomers. Preliminary experiments indicate that only the trans isomer will form a covalent adduct with band 3. Since we use a 1.5-fold excess of DIDS over band 3, the final concentration of trans isomer is still greater than the concentration of band 3 monomers. Once the DIDS·band 3 adduct forms, its fluorescence is stable to xenon light. For the reaction in eq 1, the total fluorescent yield is defined as the difference between the final fluorescence after the reaction and the starting fluorescence of the free DIDS. This difference is directly proportional to the concentration of adduct formed.

Upon forming the adduct, the fluorescence maxima of DIDS are shifted from an excitation at 342 nm and emission at 415 nm to an excitation at 362 nm and emission at 448 nm. In addition, the maximum fluorescence of the covalent adduct is increased about 4-fold over that of the free DIDS. The fluorescence spectra for the DIDS·band 3 adduct in solution are very similar to those reported by Eisinger et al. (1982) for the DIDS·band 3 adduct in the red cell ghost membrane (excitation 350 nm, emission 450 nm). These properties of the DIDS adduct are unchanged when band 3 integral domain is used in place of intact band 3 or when the pure tetramer is substituted for the mixture of dimer and tetramer.

Experiments aimed at characterizing the change in band 3 reactivity toward DIDS used the following general protocol: Band 3 was diluted into buffers of various composition, incubated for various times, and then reacted with DIDS.

High-Pressure Liquid Chromatography. HPLC gel filtration was performed on a Varian Model 5000 liquid chromatograph using a 0.8 × 30 cm TSK-G4000SWXL column with guard (Supelco) equilibrated at room temperature in 5 mM sodium phosphate, pH 7.0, containing 0.1% $C_{12}E_8$ and 100 mM NaCl. Protein was eluted isocratically in the same buffer at a flow rate of 0.5 mL/min. Peaks were detected by absorbance at 215 nm using a Varian Model UV-50 variable-wavelength absorbance detector.

RESULTS

(A) Assay for Covalent Binding of DIDS to Band 3

(1) Rate of Covalent Adduct Formation. Mixing 0.70 μM intact band 3 with 1.17 μM DIDS (in 28.5 mM sodium citrate buffer, pH 7.35, containing 0.1% $C_{12}E_8$ at 25 °C) resulted in a monophasic rise in fluorescence, at an apparent first-order rate of 0.5 ± 0.05 min^{-1} (Figure 1A, t = 0 trace). Increasing the DIDS concentration 2-fold did not change this apparent rate, indicating that this is the limiting, first-order rate for adduct formation and that this reaction is preceded by a noncovalent complex between band 3 and DIDS that is fully saturated under these conditions (see eq 1). At pH 7.0, the apparent rate is about 0.2 min^{-1}. At pH 7.35, the pH most commonly used in the following studies, the rate has risen by 2.5-fold to 0.5 min^{-1}. By pH 8.5, the rate has risen to 4.8 min^{-1}. Janas et al. (1989) reported a rate of 0.14 min^{-1} for band 3 in resealed erythrocyte ghosts (2 mM potassium phosphate buffer, pH 7.3, containing 165 mM KCl, 25 °C).

The kinetic properties of the DIDS reaction are unchanged when the integral domain is used in place of intact band 3. Nor is there any difference when tetrameric band 3 is compared to dimer/tetramer mixtures. Formation of the noncovalent DIDS·band 3 complex is complete within the mixing time (about 15 s). No change in either the fluorescence or absorbance spectra of DIDS was detected consequent to formation of the noncovalent complex.

(2) Estimation of the DIDS Dissociation Constant. Complete saturation of band 3 occurs at a 1.5 molar excess of DIDS suggesting that the DIDS dissociation constant is about

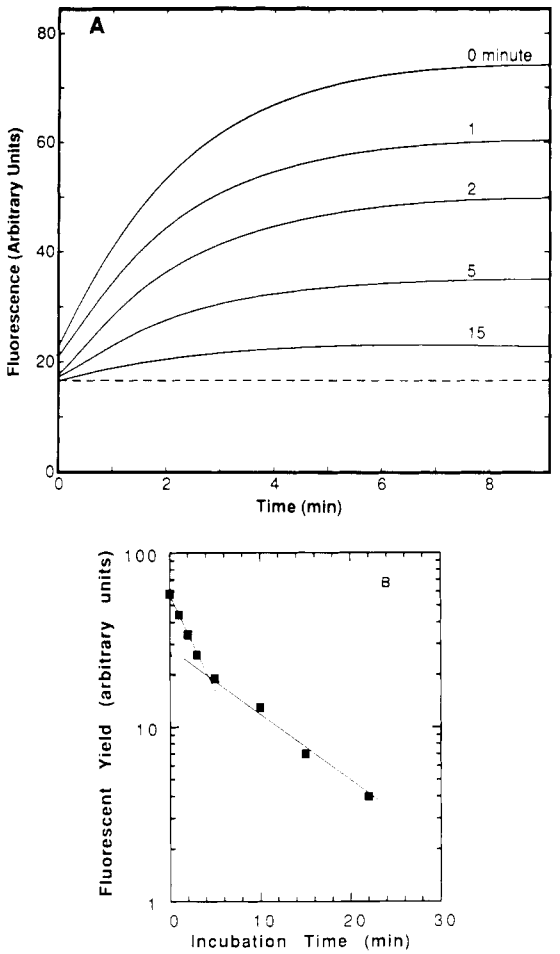

FIGURE 1: Reaction of DIDS with band 3 to form a covalent adduct. (A) Assay conditions: 50 μL of 14.4 μM intact band 3 (2-day-old) in 5 mM sodium phosphate buffer, pH 8.0, containing 0.1% $C_{12}E_8$ and 60 mM NaCl was mixed with 900 μL of 28.5 mM sodium citrate buffer, pH 7.4, containing 0.1% $C_{12}E_8$ and incubated at 25 °C, in the dark. At timed intervals after mixing, 10 μL of 117 μM DIDS was added and the progress of adduct formation was followed in the spectrofluorometer (excitation at 350 nm, emission at 460 nm). The incubation times, before DIDS was added, are indicated at the right of each curve in minutes. Some curves have been omitted for clarity. The dashed line is the fluorescence of starting DIDS, in the absence of band 3. The final pH of the reaction mixture was 7.35. (B) Squares: a semilog plot of the fluorescent yield (end-point fluorescence minus the starting DIDS fluorescence) versus incubation time. Data are taken from the traces in panel A. Dashed line: the fast phase of the biphasic time course corrected for the slow-phase contribution (Fersht, 1977).

100-fold smaller than the band 3 concentration or about 10^{-8} M. This is similar to the value (3×10^{-8} M at 0 °C) reported by Janas et al. (1989) for band 3 in red cell ghost membrane.

(B) Dilution Induced Loss in Band 3 Reactivity toward DIDS

(1) Reaction of Intact Band 3. The yield of DIDS adduct was lowered when dilute band 3 (0.70 μM) was incubated in the reaction buffer before addition of DIDS (Figure 1A). After 50 min, band 3 is no longer covalently reactive toward DIDS. Addition of 1.2 μM DIDS to unreactive band 3 caused quenching of the protein fluorescence indicative of complex formation.

The time course for loss of DIDS-reactive band 3 is given in Figure 1B. The loss of DIDS-reactive band 3 is biphasic with about 50% of the total accompanying each phase. The apparent rates (at pH 7.35) are 0.092 min^{-1} (slow phase) and 0.69 min^{-1} (fast phase). The same biphasic loss of DIDS-reactive band 3 occurs whether we use a mixture of dimer and tetramer or pure tetramer.

Aging the purified band 3 at 4 °C in the storage buffer slows the rate of loss of DIDS-reactive band 3. The apparent rates for both phases of the decay process slowed, while the amplitude of each phase remained at about 50%. This can be seen by comparing Figure 1B and Figure 4 (closed circles). We have not yet determined the mechanism involved in this phenomenon. However, we have found that addition of 0.3 mM EDTA to the storage buffer prevents it.

The fast phase rate of decay is comparable to the rate of formation of the DIDS band 3 adduct (k_{app} = 0.5 min^{-1}). In view of this, it is reasonable to ask if the decay process continues after the addition of DIDS to the mixture or if the noncovalent complex of DIDS with band 3 stops the decay. The effect of the noncovalent DIDS·band 3 complex on the decay process became apparent in a study of the pH dependence of the decay. The decay rates of both phases decreased as the pH was raised. This is just opposite to the effect of pH on the adduct formation rate. Thus, at pH 8.5, the fast phase decay proceeds at 0.045 min^{-1} and the slow phase at 0.006 min^{-1}. Since adduct formation proceeds at 4.8 min^{-1} at pH 8.5, there will be no loss of DIDS-reactive band 3 during adduct formation. At pH 7.35, where adduct formation and fast phase decay rates are comparable, there would be significantly less adduct formed if the decay process was ongoing in the presence of DIDS. However, the total amount of adduct formed is essentially identical at both of these pH values. Thus, decay of DIDS reactivity is immediately arrested by reversible binding of DIDS.

The rate of loss of DIDS-reactive band 3 is dependent on the concentration of the detergent. The rates of both phases of the reaction increase with increasing detergent concentration; e.g., the fast phase proceeds at 0.0039 min^{-1} in 0.01% $C_{12}E_8$ and at 0.990 min^{-1} in 0.4% $C_{12}E_8$. A similar detergent dependence of activity was observed by McIntosh and Ross (1985) for the Triton X-100 solubilized Ca^{2+}-ATPase. Despite this marked effect of detergent on the stability of band 3, the rate of adduct formation (k in eq 1) was nearly unaffected, rising at most 20% on going from 0.01% to 0.4% $C_{12}E_8$.

(2) Reaction of DIDS with Band 3 Integral Domain. The purified integral domain of band 3 reacts with DIDS at a rate of 0.60 min^{-1} at pH 7.44. The rate of adduct formation increased as the pH was raised. Incubation of dilute integral domain (0.70 μM at 25 °C) resulted in a slow, monophasic loss of DIDS reactivity (0.017 min^{-1} at pH 7.44) (Figure 2).

(3) Protection against Loss of DIDS Reactivity, Using Intact Band 3. Addition of 0.175 mg/mL or about 250 μM phosphatidylethanolamine to dilute band 3 (0.70 μM in 28.5 mM sodium citrate, pH 7.4, containing 0.1% $C_{12}E_8$) inhibited the loss of DIDS reactivity. About 95% of the initial DIDS reactivity remained after 1 h, compared to complete loss of reactivity after 30 min in the absence of phosphatidylethanolamine. Other lipids (see Table I) were also found to be stabilizing. Both phospholipids and phosphorus-free lipids were effective. Lipids have proven effective in stabilizing other integral membrane proteins in detergent solution, e.g., Ca^{2+}-ATPase (Vilsen & Andersen, 1987), nicotinic acetylcholine receptor (McCarthy & Moore, 1992), the ATP/ADP carrier from mitochondria (Horvath et al., 1990), and the glucose transporter (Lundahl et al., 1991). In addition to the lipids listed in Table I, we tried the triglyceride, tristearin, and cholesteryl myristate; neither stabilized the DIDS reactivity of band 3. Addition of either EDTA, EGTA (trace metal scavengers), mannitol (free radical scavenger), or PMSF

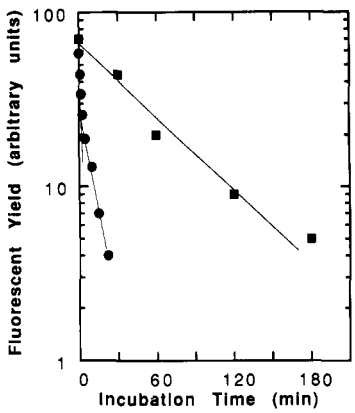

FIGURE 2: Time course for the loss of DIDS reactivity for band 3 integral domain. Conditions: circles, a repeat of intact band 3 data from Figure 1B, shown for reference; squares, 25 μL of 29 μM band 3 integral domain in 100 mM sodium phosphate buffer, pH 8.0, containing 0.1% $C_{12}E_8$ was mixed with 975 μL of 28.5 mM sodium citrate buffer, pH 7.4, containing 0.1% $C_{12}E_8$ and incubated at 25 °C and sealed. At defined time intervals, 10 μL of 117 μM DIDS, in water, was added and the progress of adduct formation was followed in the spectrofluorometer under continuous irradiation (excitation at 350 nm, emission at 460 nm). The final pH of the reaction mixture was 7.44.

(proteolysis inhibitor) was ineffective.

A measure of the effectiveness of each lipid at stabilizing DIDS reactivity was obtained by determining the amount of reactive band 3 remaining after a 1-h incubation in the presence of varying concentrations of lipid. Representative results for phosphatidylethanolamine and phosphatidylcholine are shown in Figure 3. The curve of fluorescence yield vs phospholipid concentration was saturating with a nonzero y-axis intercept. The nonzero intercept is most likely due to lipid which co-purified with the protein, now diluted about 20-fold. Saturation at high phospholipid concentration indicates that there is a specific interaction between phospholipid and band 3 and that stabilization is not due to some bulk effect of the phospholipid. We have reported the half-effect values for each lipid as a qualitative indication of their relative effectiveness at stabilization (see Table I). The half-effect value varies only 3-fold within this group of lipids, arguing that there is little selectivity among those lipids which are effective. Lipids also slow the limiting, first-order rate of adduct formation (Table I). Lipids with a net negative charge cause the largest rate reductions.

Chloride also stabilizes band 3 against loss of DIDS reactivity (Figure 4, triangles). The time course of reactivity loss remains biphasic with approximately 50% of the total appearing in each phase. That this stabilization is specific to chloride and not due to an increase in ionic strength was determined in preliminary experiments using various concentrations of chloride under iso-ionic conditions where apparent saturation behavior was observed. In addition, these experiments indicated that the half-effect value for chloride was approximately 50 mM. Sulfate will not substitute for chloride in stabilizing band 3 (Figure 4, squares).

Though the dilution-induced loss of DIDS reactivity could be inhibited by adding various ligands to band 3, the lost activity could not be recovered. If DIDS-unreactive band 3 were allowed to form, subsequent addition of lipids, chloride, or stilbenes did not restore DIDS reactivity.

(4) Stimulation of the Loss of DIDS Reactivity for Intact Band 3 by Addition of Phosphatidic Acid. Phosphatidic acid and lysophosphatidic acid accelerated the rate of loss of DIDS reactivity (Table I). In the presence of 240 μM phosphatidic acid, the loss of DIDS reactivity became linear (Figure 5, squares) with a rate equal to that of the fast phase loss when lipid was absent (Figure 5, dashed line). Absence of the second, slow phase allows the decay process to proceed to completion at the initial rate, effectively speeding up the overall decay process. Lysophosphatidic acid supports the same behavior.

(C) BADS Titration of DIDS-Reactive and DIDS-Unreactive Band 3

When 0.70 μM intact band 3 was titrated with BADS in the presence of 250 μM phosphatidylcholine, complex formation followed a simple, hyperbolic, saturation curve with a dissociation constant of 1.0–1.5 μM (Figure 6, circles). The dissociation constant was determined by fitting the data to the quadratic expression for ligand binding, using a nonlinear least-squares routine in the program Enzfitter (v 1.03 written by R. J. Leatherbarrow, published by Elsevier-Biosoft). This value is in good agreement with those reported for BADS binding to red cell membranes containing band 3: 1.3 μM (Macara & Cantley, 1981) and 1.0–1.3 μM (Lieberman & Reithmeier, 1983). It is also in good agreement with previously reported values for BADS binding to solubilized band 3: 1 μM in $C_{12}E_8$ (Lieberman & Reithmeier, 1983), 0.6 μM in $C_{12}E_8$ (Casey & Reithmeier, 1991), and 5 μM in Triton X-100 (Rao et al., 1979). Equivalent titrations were obtained with both the pure tetrameric band 3 and a mixture of dimeric and tetrameric band 3.

Unreactive band 3 formed a much weaker complex, with a dissociation constant of 15–30 μM (Figure 6, squares). This titration was not carried to saturation since the inner filter limit ($A_{280} > 0.1$) was reached at 14 μM BADS. We found no difference between pure tetrameric band 3 and dimer/tetramer mixtures.

Previous workers (Casey & Reithmeier, 1991) have reported a biphasic BADS titration for tetrameric band 3, with a dissociation constant for the high-affinity site of 0.6 μM. We obtained similar biphasic titrations when we diluted band 3 into buffer containing 0.1% $C_{12}E_8$ without lipid. The apparent dissociation constants for the two phases were about 0.5 μM and about 30 μM. The biphasicity in our case was artifactual. Such titrations take about 30 min to complete. Conversion of band 3 into the BADS low-affinity form, under these conditions, also takes about 30 min to complete. During the titration, those monomers not in complex with BADS would be expected to convert to the low-affinity form. Therefore, our biphasic titrations involve complex formation with high-affinity band 3 initially and then with low-affinity band 3 later on.

Titration of the integral domain with BADS was simplified by the slow rate of loss of DIDS reactivity (see Figure 2). The integral domain (in 28.5 mM sodium citrate buffer, pH 7.4, containing 0.1% $C_{12}E_8$ without added lipid) showed a simple, hyperbolic dependence on BADS with an apparent dissociation constant of 0.5 μM. If the integral domain were allowed to lose DIDS reactivity before being titrated with BADS, the binding was much weaker (10–30 μM).

(D) HPLC Studies on the Quaternary Structure of DIDS-Reactive and DIDS-Unreactive Band 3

The change in DIDS reactivity toward band 3 is accompanied by changes in the quaternary structure (Figure 7). At time zero, the HPLC elution profile is essentially the same as that described for pure band 3 by Casey and Reithmeier (1991). The tetramer elutes at 14 min and the dimer at 16 min. Following dilution, there is a time-dependent loss of the

Table I: Effect of Lipids on Band 3[a]

		experiment					
			I		II	III	
lipid	source	[lipid][b] (μM)	incubation time (min)	remaining reactive band 3 (%)	half-effect value (μM)	adduct formation rate (min^{-1})	pH[c]
none	N/A[d]	N/A[d]	30	0	N/A[d]	0.50	7.35
	N/A[d]	N/A[d]	10	22	N/A[d]	N/A[d]	7.35
phosphatidylethanolamine	bovine brain	250	60	95	20	0.31	7.24
phosphatidylcholine	bovine brain	250	170	86	75	0.39	7.28
phosphatidylserine	bovine brain	250	240	91	30	0.17	7.26
phosphatidylinositol	bovine liver	250	360	100	40	0.20	7.36
phosphatidylglycerol, distearoyl	synthetic	220	120	97	45	0.22	7.33
phosphatidic acid, dioleoyl	synthetic	240	10	0	N/A[d]	0.18	7.36
sphingomyelin	bovine brain	250	180	80	70	0.44	7.28
cholesterol	synthetic	250	60	20	N/A[d]	0.46	7.29
globoside	human erythrocyte	80	180	55	N/A[d]	0.72	7.62
ganglioside	calf brain	40	90	70	N/A[d]	0.48	7.35
lysophosphatidic acid, oleoyl	synthetic	250	15	0	N/A[d]	0.25	7.32

[a] Data from three types of experiment are shown in the table. Experiment I is summarized in columns 3–5. Band 3 (0.70 μM) was incubated at 25 °C in 28.5 mM sodium citrate buffer, pH 7.4, containing 0.1% $C_{12}E_8$ and the indicated lipid (at the concentration given in column 3) for a fixed time (given in column 4). Then the remaining DIDS-reactive band 3 (column 5) was determined by adding 1.17 μM DIDS to the incubation mixture. Experiment II is summarized in column 6. Band 3 (0.70 μM) was incubated for 1 h at 25 °C in 28.5 mM sodium citrate buffer, pH 7.4, containing 0.1% $C_{12}E_8$ and various concentrations of the indicated lipid. The half-effect value is the concentration of lipid in which 50% of the starting band 3 remains DIDS-reactive. Experiment III is summarized in column 7. Band 3 (0.70 μM) was reacted with 1.17 μM DIDS at 25 °C in 28.5 mM sodium citrate buffer, pH 7.4, containing 0.1% $C_{12}E_8$ and the indicated lipid (at a concentration given in column 3). The limiting, first-order rate of adduct formation (column 7) was monitored by the increase in fluorescence with time (excitation 350 nm, emission 460 nm). Saturation by DIDS was demonstrated with each lipid except for phosphatidylglycerol, ganglioside, cholesterol, and lysophosphatidic acid. [b] Concentrations of lipids isolated from natural sources were calculated using an approximate MW of 700, except for globoside and ganglioside which were calculated using an approximate MW of 1260. Those lipids which were supplied in chloroform or chloroform/methanol solutions were dried under nitrogen before dissolving in buffer. [c] Final measured pH for experiments involving the indicated lipid. [d] N/A indicates a value that is either not applicable or not available.

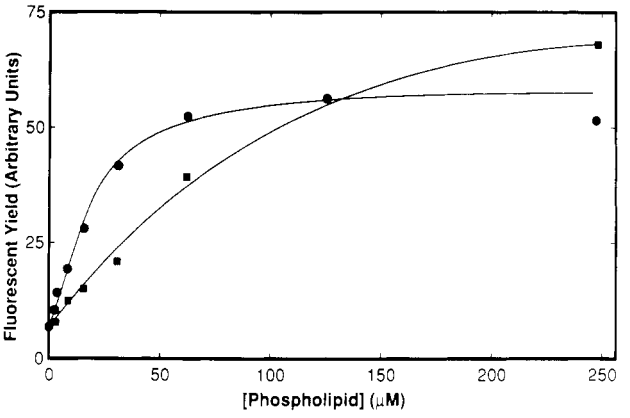

FIGURE 3: Dependence of DIDS reactivity on the concentration of phospholipid. Conditions: circles, 54 μL of 13.0 μM intact band 3 in 5 mM sodium phosphate buffer, pH 8.0, containing 0.1% $C_{12}E_8$ and 60 mM NaCl was mixed with 1 mL of 28.5 mM sodium citrate buffer, pH 7.4, containing 0.1% $C_{12}E_8$ and varying amounts of phosphatidylethanolamine (0–250 μM) and then incubated at 25 °C and sealed. After 1 h, 10 μL of 117 μM DIDS, in water, was added and the progress of adduct formation was followed in the spectrofluorometer under continuous irradiation (excitation at 350 nm, emission at 460 nm). Squares: the same protocol was used, with phosphatidylcholine substituted for phosphatidylethanolamine. The final pH values of the reaction mixtures were 7.24 and 7.28, respectively.

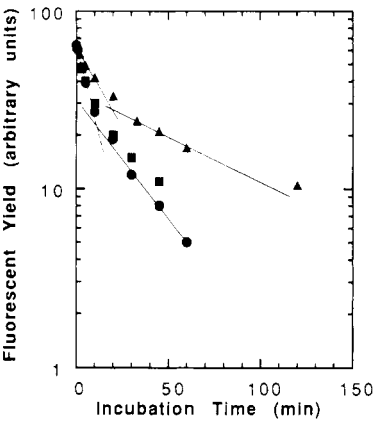

FIGURE 4: The effect of chloride and sulfate on the time course for loss of intact band 3 capable of forming a fluorescent adduct with DIDS. Conditions: circles, 51 μL of 13.6 μM intact band 3 (10-day-old) in 5 mM sodium phosphate buffer, pH 8.0, containing 0.1% $C_{12}E_8$ and 60 mM NaCl was mixed with 949 μL of 28.5 mM sodium citrate buffer, pH 7.4, containing 0.1% $C_{12}E_8$ and incubated at 25 °C and sealed. At timed intervals, 10 μL of 117 μM DIDS, in water, was added and the progress of adduct formation was followed in the spectrofluorometer under continuous irradiation (excitation at 350 nm, emission at 460 nm). The final pH was 7.30. Triangles: the same protocol as for the circles except that the sodium citrate buffer also contained 50 mM NaCl (subsaturating). Final pH was 7.26. Squares: the same protocol as for the circles except that the sodium citrate buffer also contained 90 mM sodium sulfate. Final pH was 7.19.

dimer peak and a broadening of the tetramer peak. Ultimately, a new peak becomes resolved which elutes slightly faster than the original tetramer peak (elution at 13 min). We have not been able to determine whether this new peak is a tetramer with an enlarged effective Stokes radius or a higher order oligomer. In the presence of phosphatidylethanolamine (70 μM), both the DIDS reactivity and the quaternary structure were preserved. Covalent binding of DIDS to band 3 also inhibits the change in quaternary structural following dilution.

Removal of the cytoplasmic domain from intact band 3 does not prevent the loss of DIDS reactivity following dilution (Figure 2), but it does prevent the change in quaternary structure which accompanies loss of DIDS reactivity for intact band 3. The DIDS-reactive integral domain eluted from the HPLC gel filtration column at the position of a dimer of 55-kDa monomers, in agreement with Casey and Reithmeier (1991). Its elution position did not change as DIDS reactivity was lost.

DISCUSSION

We have found that pure, $C_{12}E_8$-solubilized band 3 undergoes a rapid change in structure when diluted. This

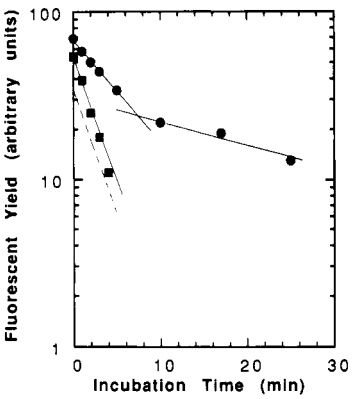

FIGURE 5: Effect of phosphatidic acid on the loss of DIDS reactivity for intact band 3. Conditions: circles, 47 μL of 15.0 μM intact band 3 (7-day-old) in 5 mM sodium phosphate buffer, pH 8.0, containing 0.1% $C_{12}E_8$ and 60 mM NaCl was mixed with 953 μL of 28.5 mM sodium citrate buffer, pH 7.4, containing 0.1% $C_{12}E_8$ and incubated at 25 °C and sealed. At timed intervals, 10 μL of 117 μM DIDS, in water, was added and the progress of adduct formation was followed in the spectrofluorometer under continuous irradiation (excitation at 350 nm, emission at 460 nm). Squares: the same protocol as for the circles except that the sodium citrate buffer contained 240 μM phosphatidic acid in addition to the $C_{12}E_8$. Dashed line: the fast phase of the biphasic time course with the slow phase contribution subtracted out (Fersht, 1977). Final pH was 7.36 for both studies.

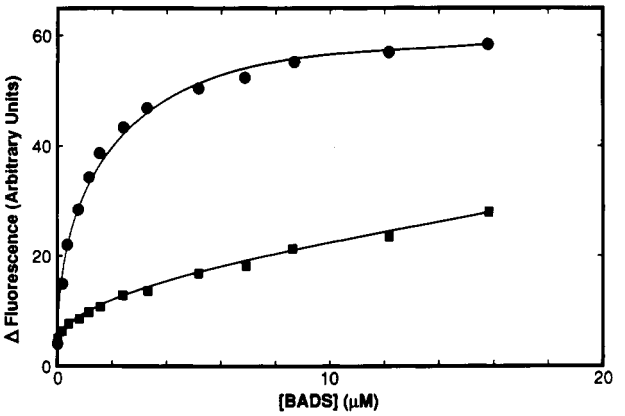

FIGURE 6: Titration by BADS of DIDS-reactive band 3, in the presence of phosphatidylcholine, and DIDS-unreactive band 3. Conditions: circles, 51 μL of 13.6 μM intact band 3 in 5 mM sodium phosphate buffer, pH 8.0, containing 0.1% $C_{12}E_8$ and 60 mM NaCl was mixed with 949 μL of 28.5 mM sodium citrate buffer, pH 7.4, containing 0.1% $C_{12}E_8$ and 250 μM phosphatidylcholine at 25 °C. Microliter amounts of BADS (from 0.188 mM or 1.88 mM stock solutions in water) were added and the fluorescence (excitation at 280 nm, emission at 460 nm) was measured. The measured fluorescence was corrected by subtracting the background fluorescence due to free BADS and is reported here as Δ fluorescence. The fluorescence of the free BADS was obtained from a parallel titration of buffer not containing band 3. All measurements were also corrected for dilution. Final pH was 7.28. Squares: the same protocol was used except that phosphatidylcholine was absent and the diluted band 3 was allowed to incubate at 25 °C until all covalent reactivity toward DIDS was lost before initiation of the BADS titration. Final pH was 7.30.

structural change leads to the elimination of covalent reactivity of the band 3 monomer toward DIDS (DIDS-reactive versus -unreactive conformations) and the lowering of the reversible affinity of BADS for the intramonomeric stilbenedisulfonate site (high affinity versus low affinity). The change in monomeric structure is associated with the interconversion of intact band 3 dimers and tetramers to an end state which is larger in size than the tetramer. Our data cannot distinguish whether this end state is an oligomer with more than four subunits (i.e., pentamer or hexamer) or whether it is a tetramer with altered monomeric conformation and larger Stokes radius.

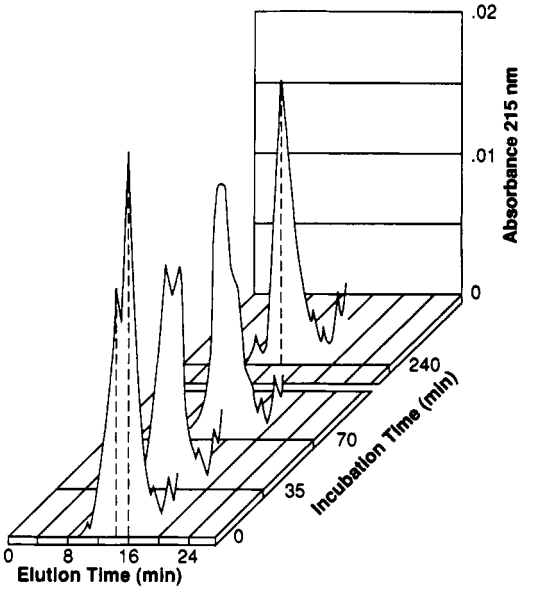

FIGURE 7: Time course for the change in quaternary structure of band 3 which accompanies the loss of DIDS reactivity, as determined by HPLC. Conditions: 100 μL of 13.8 μM intact band 3 (1 day after preparation) in 5 mM sodium phosphate buffer, pH 8.0, containing 0.1% $C_{12}E_8$ and 60 mM NaCl was mixed with 900 μl of 28.5 mM sodium citrate buffer, pH 7.4, containing 0.1% $C_{12}E_8$ and incubated at 25 °C and sealed. At defined time intervals, 35 μL of the mixture was removed and injected onto a TSK-G400SWXL HPLC gel filtration column equilibrated in 5 mM sodium phosphate buffer, pH 7.0, containing 0.1% $C_{12}E_8$ and 100 mM NaCl. The sample was eluted in the same buffer at 0.5 mL/min. Peaks were detected by absorbance at 215 nm.

The rate at which these dilution-induced changes take place depends critically on the type of band 3 ligand present in the buffer. The rate is slowed by addition to the dilution medium of certain ligands such as chloride (but not sulfate), lipids (but not phosphatidic acid), or DIDS. Ligands which stabilized the DIDS-reactive conformation of the monomer also greatly slow the rate of formation of the oligomeric end state. On the basis of the irreversible nature of the process which leads to this end state, and on the basis of the capacity of certain band 3 ligands to slow the rate of formation of that state, we can conclude that an intermediate must form between the ligand-bound, DIDS-reactive conformation and the unreactive end state:

$$\text{[DIDS-reactive band 3]} \overset{\text{ligands}}{\rightleftarrows} \text{[band 3 intermediate]} \rightarrow \text{[unreactive band 3]} \quad (2)$$

Two specific mechanisms can be proposed to explain these kinetics, which differ with respect to the assignment of the hypothetical intermediate state of the protein. In one mechanism, dimers and tetramers simply change conformation upon dissociation of stabilizing ligands consequent to dilution. Thus, altered oligomeric conformations are intermediates on the pathway to end-state formation. On the basis of the available data in the literature (Falke et al., 1984; Marsh, 1990), dissociation of anions and lipids can be assumed to be very much more rapid than the rate of conformational change. In the second mechanism, dilution of the protein would cause a similar rapid dissociation of stabilizing ligands. But here, a slower dissociation of band 3 oligomers to monomers is proposed to occur. The monomers so formed would then rapidly change conformation and irreversibly associate to the final oligomeric end state. In this mechanism, monomers constitute the transient intermediate.

Our data cannot discriminate between these two possible mechanisms. The HPLC data of Figure 7 show no evidence for monomer formation with dilution of the protein, in agreement with the findings and conclusions of Casey and Reithmeier (1991). Yet, Schubert's laboratory has presented ultracentrifugation data using dilute band 3 in detergent solutions which suggest the existence of a monomer/dimer/tetramer equilibrium (Pappert & Schubert, 1983). Our HPLC results could accommodate the formation of monomers with dilution of the protein if the hypothetical conformational change in the dissociated monomer and the formation of the irreversible end state were both very much faster than the rate of monomer formation per se. In that way, the monomeric species would not be significantly populated during the interconversion of dimers and tetramers to the final oligomeric end state.

The foregoing series of observations defines two forms of band 3 which can occur in detergent solution. Two similar forms of membrane-bound band 3 have been described previously in the literature. We have shown (Salhany et al., 1991) that treatment of red cells with DNDS and BS[3] (bis-(sulfosuccinimidyl) suberate) results in a population of transport active band 3 which can no longer form a covalent adduct with DIDS and a population which still can. Kampmann et al. (1982) found it necessary to propose both DIDS-reactive and DIDS-unreactive populations of band 3 in order to rationalize their observations on the kinetics of intramonomeric cross-linking of band 3 by DIDS. And, Passow (1986) found that the reactivity of the DIDS-reactive lysine toward fluorodinitrobenzene was enhanced by chloride but not by sulfate. These results indicate that the structure and reactivity of band 3 are dependent on the ligands to which it is bound and that to a first approximation only two conformational states of the porter monomer exist: DIDS-reactive and DIDS-unreactive.

The isolated integral domain undergoes reactivity and affinity changes on dilution which are similar to those found with intact band 3. However, the integral domain does not change quaternary structure. This argues that the quaternary structure changes are mediated by the cytoplasmic domain and that those changes are not obligatorily coupled to the reactivity/affinity changes at the stilbene binding site. However, removal of the cytoplasmic domain greatly slows the rate of change in conformation, suggesting that the two domains are conformationally interactive in intact band 3.

To this point in the discussion, we have freely compared results for membrane-bound band 3 to results for pure band 3 in detergent solution. This comparison assumes that isolation of band 3 in detergent has not significantly altered its properties. In order to justify that assumption, it is necessary to show that the properties of band 3 in solution are the same as those of band 3 in the membrane. Rao et al. (1979) compared the binding of DBDS, DNDS, and BADS to both solubilized and membrane-bound band 3. They found that these stilbenes bound to band 3 with equal affinity in both environments. We have confirmed the BADS binding observation. BADS affinity for phospholipid stabilized, soluble band 3 is 1.0–1.5 μM, which is in good agreement with values of 0.6–1.3 μM reported for ghost membranes (Lieberman & Reithmeier, 1983; Macara & Cantley, 1981; Casey & Reithmeier, 1991). In addition, we have found that formation of the covalent adduct between DIDS and soluble band 3 occurs at a rate of 0.2–0.5 min^{-1}, at 25 °C (depending on conditions, see Table I), which is in reasonable agreement with the value of 0.14 min^{-1} at 25 °C reported by Janas et al. (1989) for adduct formation in ghost membranes. We therefore feel that observations made on band 3 in solution are relevant to band 3 in the membrane.

One striking feature of our results is the importance of lipid in maintaining solubility of band 3 in a form which retains DIDS reactivity and high BADS affinity, characteristic of the membrane-bound protein. A wide selection of lipids is effective in this stabilization (Table I), and their half-effect values (20–50 μM) indicate a substantial degree of specificity in their association with band 3. We take this to indicate that lipids also play an important role in determining the structure and reactivity of band 3 in the membrane. Consistent with this conclusion, the literature provides several examples of the importance of lipids to the structural integrity and activity of band 3. First, lipids co-purify with band 3 (Maneri & Low, 1989; Ross & McConnell, 1978). Second, the transport activity of band 3 reconstituted into lipid vesicles has been shown to be sensitive to the lipid used in creating the vesicles (Köhne et al., 1983). Third, the thermal stability of band 3 reconstituted into lipid vesicles is sensitive to the lipids used in creating the vesicles (Maneri & Low, 1978). Thus, band 3 can be added to a growing list of integral membrane proteins which require lipids for the maintenance of structure and function (Marsh, 1990).

Despite this apparent requirement for lipid, there are several reports in the literature on the titration of solubilized band 3 with stilbenes (without added lipid) which give the high-affinity binding found for membrane-bound band 3: BADS (Boodhoo & Reithmeier, 1984; Lieberman & Reithmeier, 1983; Rao et al., 1979), DBDS (Rao et al., 1979), and DNDS (Rao et al., 1979). Three experimental factors should be kept in mind when these studies are compared to our work. The first is the time interval between diluting band 3 and obtaining the measurement. Loss of high-affinity stilbenedisulfonate binding is not immediate upon dilution. For example in 0.1% $C_{12}E_8$, loss of high affinity takes 30 min to come to completion. Measurements made within the first few minutes would be unaffected. Second is the concentration of detergent present during the measurement; the rate of affinity decrease is inversely related to the detergent concentration. At 0.01% $C_{12}E_8$, high affinity is retained for hours. Third is the concentration of chloride present during the measurement. Chloride, like lipid, stabilizes band 3. It is our contention that when these factors are considered, the early titration reports can be rationalized with our findings.

When purified band 3, in the absence of stabilizing factors such as lipid, is diluted into buffer, DIDS reactivity is lost. The time course for loss of intact, DIDS-reactive band 3 is biphasic (Figure 1B). The biphasicity remains effectively unchanged despite changes in the pH, the ionic strength, and the concentrations of $C_{12}E_8$, lipid, and chloride. The biphasicity is not due to a difference in decay rate for dimeric and tetrameric band 3 (which were frequently present in approximately equal amounts in our experiments) since the pure tetramer also decays biphasically. The biphasicity thus appears to be an intrinsic property of the protein. Phosphatidic acid can convert the biphasic time course into a simple, first-order time course (Figure 5). This demonstrates that those subunits, which would otherwise decay slowly, can be induced to decay rapidly, i.e., they are under negative control. This, in turn, is direct evidence for subunit interaction in the decay process. In the absence of phosphatidic acid, loss of DIDS reactivity in one subunit decreases the rate at which reactivity is lost in its partner. That we observe two phases, each amounting to about 50% of the total, strongly suggests that

the interactions are between monomers within a dimeric unit. This holds true even when the quaternary structure of band 3 is tetrameric, suggesting further that the tetramer might be more accurately viewed as a dimer of dimers rather than as a tetramer of monomers all of which interact with their neighbors in a symmetrically identical manner. Subunit interaction for band 3 in solution complements the abundance of evidence in support of subunit interactions for membrane-bound band 3 [see Salhany (1990) for a review and Salhany and Cordes (1992)]. It is important to note that the integral domain lost DIDS reactivity monophasically. This suggests that the biphasicity and hence the subunit interactions may be mediated by the cytoplasmic domain.

In summary, our results offer evidence that isolated band 3 is a conformationally flexible protein whose conformation is influenced strongly by ligands and by the attachment of the cytoplasmic domain. On the basis of these findings, we established a set of conditions under which isolated band 3 may be studied with a high degree of confidence as to its conformational integrity. Owing to the irreversible nature of the conformational change in the absence of lipids, it may be necessary to reinvestigate the various biophysical studies where dilute, isolated band 3 was used without added lipids. Furthermore, the irreversible change in conformation which we observe may prevent band 3 from forming suitable crystals for structural work. Problems in obtaining suitable crystals may be expected when the mother liquor lacks phospholipids and contains sulfate. The solution conditions outlined in the results section may be an important step in the successful crystallization of this transporter.

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