Kinetics of Conformational Changes Associated with Inhibitor Binding to the Purified Band 3 Transporter. Direct Observation of Allosteric Subunit Interactions†

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ABSTRACT: Subunit interaction effects were identified for isolated human erythrocyte band 3, the anion exchanger, by observing both static and stopped-flow kinetic protein fluorescence changes associated with inhibitor binding to the intramonomeric stilbenedisulfonate site. We measured the the rate of conformational changes associated with reversible binding of H_2DIDS (4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate). The rate of H_2DIDS release was also measured. As a test for subunit interactions, we studied the effect of partial labeling of the band 3 monomer population with H_2DIDS on the equilibrium and kinetics of H_2DIDS reversible binding to the remaining monomers. The results showed biphasic kinetics for control band 3, with a pseudo-first-order ligand dependence for the fast phase followed by a slow ligand-independent relaxation. A second-order "on" rate constant for the fast phase was determined to be $(1.2 \pm 0.1) \times 10^7$ M^{-1} s^{-1}, while the associated "off" rate constant was found to be 1.1 ± 0.5 s^{-1}. From these kinetic constants, we calculated a K_d value of 95 ± 50 nM, which is in excellent agreement with the K_d value determined at thermodynamic equilibrium (110 ± 9 nM). Covalent labeling of 75% of the band 3 monomer population with H_2DIDS changed the kinetics of the fast phase, slowing the apparent rate by changing the order of the reaction from pseudo-first-order to zero-order. Partial labeling did not affect the ligand-independent relaxation. Separate measurements of the H_2DIDS "off" rate also showed a biphasic time course, with a 20-fold difference in apparent rate constants. Covalent labeling of 75% of the band 3 monomer population increased the ratio of fast to slow phases for H_2DIDS release from the remaining monomers. This change in the "off" reaction occurred after about 50% of the band 3 monomer population was labeled. A similar nonlinear effect was observed for the apparent K_d value for H_2DIDS binding to band 3. In this case, partial labeling caused a 5-fold increase in K_d. On the basis of these findings, we conclude that covalent labeling of one band 3 monomer with H_2DIDS significantly alters H_2DIDS reversible binding to the second monomer in the dimer. These inhibitor binding results, when taken together with our transport kinetic findings [Salhany, J. M., & Cordes, K. A. (1992) *Biochemistry 31*, 7301–7310], offer strong support for an allosteric subunit interaction hypothesis for band 3 structure–function.

Electroneutral anion exchange is one of several transport modes used for the facilitated diffusion of chloride and bicarbonate across cell membranes (Knauf, 1986). This type of transport activity is mediated by a family of integral membrane glycoproteins known collectively as the anion exchange (AE) transporters (Kopito, 1990). Band 3 (AE1) is the best characterized member of this family, being found in abundance in human red cell membranes ($\sim 1 \times 10^6$ copies per cell) (Passow, 1986; Salhany, 1990). Besides facilitating anion exchange, band 3 also functions to bind certain cytoskeletal and cytosolic proteins, such as ankyrin, glyceraldehyde-3-phosphate dehydrogenase, and hemoglobin (Bennett, 1985; Low, 1986; Salhany, 1990).

A major issue in the study of membrane transporters such as band 3 (Salhany & Swanson, 1978; Jennings, 1984, 1989; Salhany, 1990, 1992) and the glucose transporter (Hebert & Carruthers, 1991, 1992; Lundahl et al., 1991; Burant & Bell, 1992) has been the role of quaternary structure and subunit interactions in the transport mechanism. Band 3 is known to exist as dimers and tetramers in situ (Steck, 1972; Weinstein et al., 1980; Staros, 1982; Salhany et al., 1990), and these structures can be maintained as stable, functionally viable forms when the protein is isolated in $C_{12}E_8$[1] detergent solution (Casey & Reithmeier, 1991; Schopfer & Salhany, 1992). We have suggested that allosteric subunit interactions may serve to couple anion binding sites so as to accomplish anion exchange via a two-site mechanism involving the formation of a ternary complex (Salhany & Swanson, 1978; Salhany & Rauenbuehler, 1983; Salhany, 1990). There now seems to be general agreement that the AE family of transporters follows a two-site exchange mechanism (Salhany & Rauenbuehler, 1983; Benaroch & Gunn, 1992; Restrepo et al., 1989, 1991). Furthermore, there is both structural (Salhany et al., 1990, 1991; Salhany, 1992) and transport kinetic (Salhany & Swanson, 1978; Salhany & Cordes, 1992) evidence favoring an allosteric subunit interaction hypothesis. However, the exact relationship between the transport mechanism and the subunit interaction effects has not been established.

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[1] Abbreviations: $C_{12}E_8$, poly(oxyethylene-8-lauryl ether); BADS, 4-benzamido-4′-amino-2,2′-stilbenedisulfonate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; BIDS, 4-benzamido-4′-isothiocyanostilbene-2,2′-disulfonate; DBDS, 4,4′-dibenzamidostilbene-2,2′-disulfonate; $H_2(NBD)_2DS$, 4,4′-bis(4-nitro-2,1,3-denzoxadiazolyl)dihydrostilbene-2,2′-disulfonate; H_2DBDS, 4,4′-dibenzamidodihydrostilbene-2,2′-disulfonate.

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This article investigates the effect of partial covalent labeling of the band 3 monomer population with H_2DIDS on the kinetics and equilibrium of reversible H_2DIDS binding to the remaining monomers. H_2DIDS reacts with band 3 at "lysine A", with a 1:1 monomer stoichiometry. "Lysine A" is believed to be either lysine 539 (Bartel et al., 1989) or lysine 542 (Garcia & Lodish, 1989) of the human band 3 sequence (Tanner et al., 1988). H_2DIDS binding measurements were made using isolated band 3 in mixed phospholipid–detergent micelles under conditions which have been shown to stabilize the transporter (Schopfer & Salhany, 1992). In addition, we have devised a novel method to measure H_2DIDS off rate constants, involving the replacement of bound H_2DIDS by DIDS. Our results support a subunit interaction hypothesis for band 3 structure–function.

EXPERIMENTAL METHODS

Band 3 was isolated from potassium iodide stripped membranes as described previously (Casey & Reithmeier, 1991; Schopfer & Salhany, 1992). Protein concentration was determined on the basis of the absorbance at 280 nm using an extinction coefficient of 77 000 M^{-1} cm^{-1} (Dorst & Schubert, 1979). The isolated protein was studied in buffer A [150 mM NaCl, 5 mM sodium phosphate (pH 7.4), plus 25 μM phosphatidylcholine and 0.01% $C_{12}E_8$] at 22 °C. This buffer system stabilizes band 3 in a state exhibiting high-affinity stilbenedisulfonate binding comparable to that seen for binding to band 3 in situ (Schopfer & Salhany, 1992). The band 3 generated by the potassium iodide stripping method also has a high affinity for hemoglobin at the cytoplasmic domain (Shaklai et al., 1977; Salhany et al., 1980; Cassoly, 1983) [based on our preliminary observations using a fluorescence quenching assay for hemoglobin binding (Shaklai et al., 1977)].

H_2DIDS and DIDS were obtained from Molecular Probes (Eugene, OR) and Aldrich (Milwaukee, WI), respectively, and were prepared in buffer A. Stilbenedisulfonate concentrations were determined spectrophotometrically using extinctions: DIDS, 36 000 M^{-1} cm^{-1} at 340 nm (Eisinger et al., 1982); H_2DIDS, 39 000 $M^{-1}cm^{-1}$ at 286 nm (by weight).

H_2DIDS covalent adduct formation proceeds at a considerably slower rate than formation of the DIDS adduct (Lepke et al., 1976; Schopfer & Salhany, 1992). To attain partial H_2DIDS covalent labeling of the band 3 monomer population, isolated band 3 in buffer A was mixed with substoichiometric concentrations of H_2DIDS. Both control and experimental samples were allowed to react for 150 min at 22 °C. The amount of covalently bound H_2DIDS was determined indirectly by measuring the remaining reactivity of a given sample toward DIDS, by following the formation of the DIDS covalent adduct in the fluorometer (Perkin-Elmer Model 650–40; excitation, 350 nm; emission, 460 nm) [see Schopfer and Salhany (1992) for complete details]. In buffer A, band 3 was stable for the duration of this incubation. That is, no loss in DIDS reactivity occurred for samples incubated in the absence of H_2DIDS.

Stopped-flow kinetics was performed in a Gibson-Durrum stopped-flow apparatus interfaced to a Northstar computer with software provided by OLIS (Bogart, GA). We followed changes in the protein fluorescence of band 3 by excitation at 280 nm and observed the fluorescence through a 315-nm cutoff filter with the photomultiplier tube mounted at a right angle to the flow cell. All stopped-flow reactions were performed in buffer A.

Static fluorescence titrations were performed using 90 nM band 3 in 1 mL of buffer A at 22 °C. Microliter volumes of concentrated stilbenedisulfonate were added to the protein, and complex formation was followed by the loss of protein fluorescence (excitation, 280 nm; emission, 335 nm), corrected for dilution. The maximum dilution correction was 6%. Titrations were stopped when the absorbance at 280 nm reached 0.1, in order to minimize inner filter effects. Titrations were corrected for inner filter effects by generating a correction factor as described by Rao et al. (1979), using 2 μM D-tryptophan and various concentrations of H_2DIDS up to 4 μM. These control experiments were performed in the same buffer and with the same machine settings as the titrations. Equilibrium binding curves were analyzed using the corrected fluorescence data, as described by Heyn and Weischet (1975).

Data analysis was performed using the Enzfitter program for the IBM PC (Leatherbarrow, 1987).

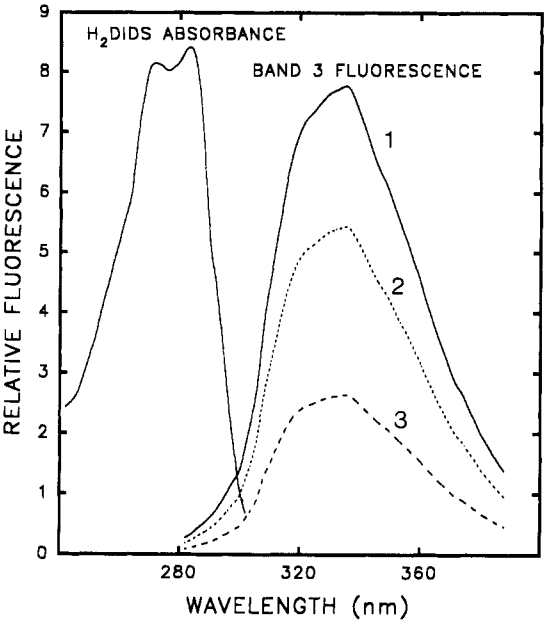

FIGURE 1: Absorbance spectrum of H_2DIDS and protein fluorescence spectrum of (1) isolated control band 3, (2) H_2DIDS covalently labeled band 3, and (3) DIDS covalently labeled band 3. The percent reduction in peak protein fluorescence relative to control band 3 is 30% in 2 and 66% in 3. The absorbance spectrum has been arbitrarily normalized to the fluorescence spectrum. Covalent labeling with inhibitors was performed as described in the Experimental Methods section.

RESULTS

Preliminary Considerations

Oligomeric Structure and Measured Function. The method we used to prepare band 3 (Casey & Reithmeier, 1991) yields a mixture of stable dimers and tetramers in a $C_{12}E_8$ solution when phospholipid is present (Schopfer & Salhany, 1992). No monomers are observed in these preparations, nor is there evidence for any equilibration between the two oligomeric forms (Casey & Reithmeier, 1991; Schopfer & Salhany, 1992). We have found that there is no difference in the BADS binding properties of isolated band 3 dimers and tetramers (Schopfer & Salhany, 1992). The kinetics presented below also showed no significant difference between band 3 dimers and tetramers.

Absorbance Spectrum of H_2DIDS and Changes in the Protein Fluorescence of Band 3 upon Binding H_2DIDS or DIDS. The absorbance spectrum of H_2DIDS and the protein fluorescence spectra of band 3 in various states are shown in Figure 1. It can be seen that labeling >95% of band 3 monomers with H_2DIDS causes a 30% reduction in the protein

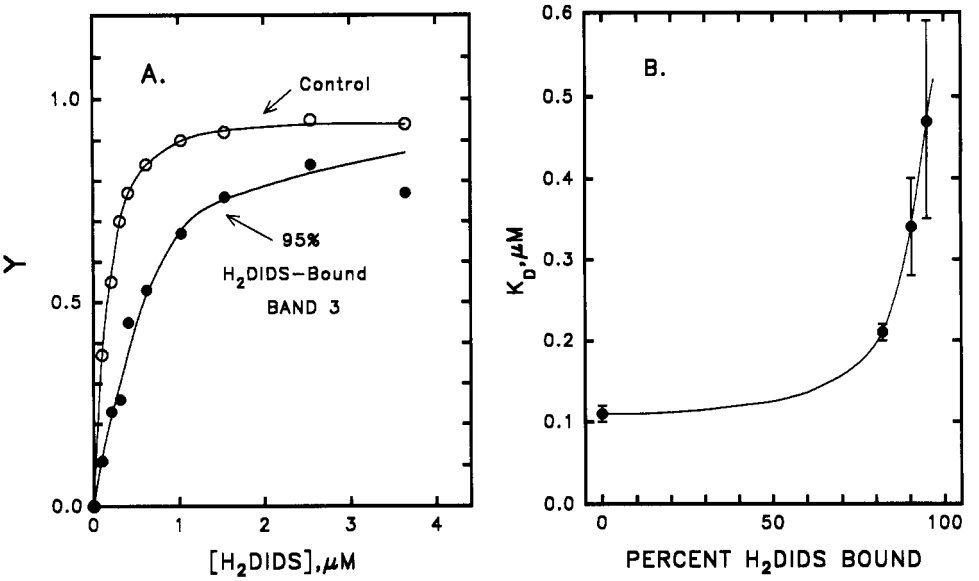

FIGURE 2: Effect of covalent labeling of the band 3 monomer population with H_2DIDS on H_2DIDS reversible binding to the remaining monomers measured at thermodynamic equilibrium. The concentration of band 3 was 90 nM, and the titrations were performed in buffer A at 22 °C. (A) Plot of fractional change in fluorescence ($Y = \Delta F/\Delta F_{total}$) versus total H_2DIDS concentration. Y was determined from fluorescence measurements corrected for inner filter effects and equilibrium binding constants were calculated, as described in the Experimental Methods section. The K_d value for control band 3 was 110 ± 9 nM, while that for band 3 with 95% of the monomers labeled with H_2DIDS was 467 ± 117 nM. (B) Plot of the fitted K_d values and the associated standard deviations for H_2DIDS reversible binding versus the fraction of H_2DIDS covalently bound to the band 3 monomer population.

fluorescence (spectrum 2 of Figure 1). In the case of DIDS, resonance energy transfer (Rao et al., 1979) causes greater quenching of the protein fluorescence, about 66%, for the covalently labeled protein (spectrum 3 of Figure 1). Furthermore, comparison of the amount of fluorescence quenching caused by reversible binding of the ligands to the quenching caused by covalent adduct formation showed that about 80–90% of the spectral change shown in Figure 1 (curves 2 or 3) arises from the reversible inhibitor binding step. Covalent adduct formation is a much slower process (0.02 min^{-1} for H_2DIDS at pH 7.4) compared to reversible binding (see below), and it could be followed directly.

The mechanism causing the H_2DIDS-induced fluorescence quenching involves a conformational change in band 3 (Macara et al., 1983). The evidence supporting this view is as follows. Firstly, there is virtually no spectral overlap between the absorbance of H_2DIDS and the fluorescence of band 3 (Figure 1), thus ruling out resonance energy transfer as a likely mechanism for the spectral change. Secondly, there is evidence that H_2DBDS produces a conformational change in band 3 which extends to the cytoplasmic domain (Macara et al., 1983). Finally, Kleinfeld et al. (1980, 1982) used resonance energy transfer between band 3 tryptophans and fatty acid probes in the lipid bilayer to map the relative distribution of band 3 tryptophans (Kleinfeld, 1988). They found that H_2DIDS binding induces a conformational change resulting in a change in the distribution of tryptophans toward the cytoplasmic side of the membrane. In the studies described below, we use the decrease in protein fluorescence (excitation at 280 nm, emission at 340 nm) to measure H_2DIDS reversible binding to isolated band 3 for the first time.

DIDS and H_2DIDS compete with each other for a single monomeric stilbenedisulfonate binding site (Jennings & Passow, 1979). Since DIDS quenches band 3 fluorescence to a greater extent than H_2DIDS, it is possible to follow the replacement of H_2DIDS by DIDS (decrease in fluorescence; Figure 1), provided the experiment is performed sufficiently rapidly so as to minimize H_2DIDS covalent adduct formation. The adduct formation rate is very slow for H_2DIDS at room temperature (see above) and thus poses no significant problem in these studies. The kinetic theory for such replacement reactions is well-established (Gibson & Roughton, 1955; Antonini & Brunori, 1971) and is not restated here.

Equilibrium Binding of H_2DIDS to Isolated Band 3 in Buffer A at 22 °C. Loss of protein fluorescence was used to measure the equilibrium binding of H_2DIDS to isolated band 3 (Figure 2A). Binding of H_2DIDS was apparently hyperbolic, and the value of the binding constant for control band 3 was 110 ± 9 nM. Labeling 95% of the band 3 monomers with H_2DIDS lowered the affinity of the remaining monomers by about 5-fold (Figure 2A). There was a nonlinear increase in K_d with increased fractional labeling (Figure 2B). The majority of the change occurred after >50% of the band 3 monomers were labeled.

We have also measured DIDS reversible binding to isolated band 3 in buffer A using the same methods. Monophasic binding was observed, with K_d values of 57 ± 8 nM in physiological chloride and 20 ± 3 nM in the absence of chloride.

Forward-Flow Kinetics of H_2DIDS Reversible Binding to Band 3

The On Kinetics for Control Band 3. Mixing H_2DIDS with isolated band 3 in the stopped-flow apparatus resulted in a biphasic decrease in protein fluorescence (Figure 3). Prelabeling band 3 fully with H_2DIDS eliminated all post-dead-time changes, while mixing either unlabeled or H_2DIDS-labeled band 3 with buffer A alone did not produce detectable changes in band 3 fluorescence (data not shown). The kinetic trace in Figure 3 is well-described by an equation representing the sum of two exponentials with about a 30-fold difference in rate between the fast and slow phases.[2]

In order to determine the mechanism involved in H_2DIDS binding, we studied the H_2DIDS concentration dependence of the apparent rate constants for each phase (Figure 4 A,B). The fast phase showed a classical plot indicative of apparent pseudo-first-order kinetics with respect to H_2DIDS concen-

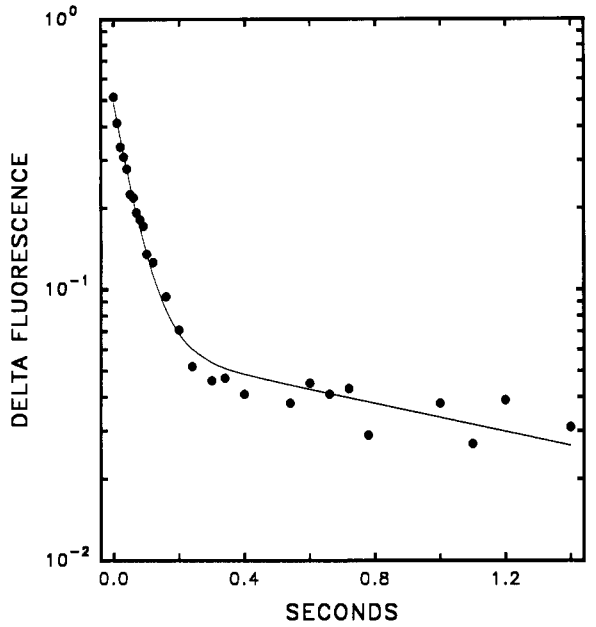

FIGURE 3: Stopped-flow kinetics of H_2DIDS reversible binding to isolated band 3. Isolated band 3 (2.5 μM) was prepared in buffer A and mixed 50:50 with 3 μM H_2DIDS in the stopped-flow apparatus at 22 °C. The solid line is a nonlinear least-squares fit to the equation, $\Delta F = \Delta F_1 \exp(-k_1 t) + \Delta F_2 \exp(-k_2 t)$, with $\Delta F_1 = 0.43 \pm 0.01$; $k_1 = 16.9 \pm 0.8$ s^{-1}, $\Delta F_2 = 0.06 \pm 0.01$, and $k_2 = 0.6 \pm 0.2$ s^{-1}. ΔFluorescence is defined as the difference in fluorescence between the final stable fluorescence and the fluorescence at the indicated time points and is shown as the absolute value of the change, in this figure and in the other figures in the article. The fluorescence scale has arbitrary units.

tration (Figure 4A). The slope of the line yields a second-order on rate constant of $(1.2 \pm 0.1) \times 10^7$ M^{-1} s^{-1}, while the intercept yields a value for the associated off rate of 1.1 ± 0.5 s^{-1}. These numbers allow the calculation of an apparent initial K_d for H_2DIDS binding of 95 ± 50 nM. This value is in excellent agreement with the value determined in the equilibrium binding measurements described above for control band 3 (Figure 2, $K_d = 110 \pm 9$ nM). The slow phase showed no significant dependence on H_2DIDS concentration (Figure 4B).

On Kinetics for Band 3 Solutions Whose Monomer Population Was Partially Labeled Covalently with H_2DIDS. Figure 5 shows the effect of partial labeling on the kinetics of H_2DIDS binding to the remaining monomers. While both traces in Figure 5 are biphasic, it is apparent that the fast phase rate has decreased considerably after labeling 75% of the band 3 monomer population with H_2DIDS. The apparent rate for this phase shows no significant dependence on H_2DIDS concentration (Figure 6A) over the same range where a classical pseudo-first-order dependence was observed for unlabeled band 3 (compare Figures 4 and 6). The slow phase was unaffected by partial labeling (Figure 6B). This change in the order of the reaction for the fast phase constitutes direct evidence for allosteric subunit interactions in the band 3 dimer.

H_2DIDS/DIDS Replacement Reaction

H_2DIDS Off Rate from Control Band 3. The top trace in Figure 7 shows typical results for experiments involving premixing band 3 with enough H_2DIDS to saturate all sites and then immediately mixing the ligand-bound material in the stopped-flow apparatus with DIDS. The kinetics is represented by two phases with about 11% fast phase ($k_{obs} = 20.4 \pm 2$ s^{-1}) and about 89% slow phase ($k_{obs} = 1.1 \pm 0.02$ s^{-1}). We studied the dependence of this reaction on the concentration of the replacing inhibitor DIDS (data not shown) and found that neither the rate constants nor the extent of the reaction varied significantly with increasing DIDS. This finding supports the view that the reaction is proceeding to near completion and that the rate-limiting step is H_2DIDS release (Gibson & Roughton, 1955; Antonini & Brunori, 1971).

H_2DIDS Off Rate Measurements from Band 3 Solutions Whose Monomer Population Was Partially Labeled with H_2DIDS. When band 3 is partially labeled with H_2DIDS such that about 75% of the monomers are bound, the total change in fluorescence for the H_2DIDS/DIDS replacement reaction decreases (Figure 7, bottom trace). Now, Δfluorescence accounts for about 24% of the fluorescence change seen with unlabeled band 3, as expected for titration of 75% of the lysine A sites. But, the proportion of the two phases of the reaction has changed significantly. Instead of 11% fast phase and 89% slow phase as seen with unlabeled band 3, there now is about 60% fast phase and 40% slow phase for partially labeled band 3.

In order to quantitate the relationship between the off kinetics and fractional labeling of the band 3 monomer population, we studied the replacement reaction at several levels of fractional labeling (Figure 8). Fractional labeling was determined on the basis of the size of the Δfluorescence for the complete reaction (Figure 7). The plot in Figure 8A shows only a slight variation in the off rate constant for the fast phase of the reaction. There was no change in the slow phase rate constant (see legend to Figure 7). However, the fraction of the phases changed significantly, but only after more than 50% of the monomers were labeled (Figure 8B). This latter finding seems to correspond to the type of nonlinear dependence seen for K_d (Figure 2B). Thus, as fractional covalent H_2DIDS labeling of the band 3 monomer population increases, the affinity of the remaining monomers for H_2DIDS decreases; the fraction of fast phase in H_2DIDS release increases, and these changes occur with relatively little change in the absolute values of either the fast phase or the slow phase off rate constants.

DISCUSSION

The results of this article support an allosteric subunit interaction hypothesis for band 3 structure–function. Partial labeling of the band 3 monomer population with H_2DIDS (a) lowered the affinity of remaining monomers for H_2DIDS (Figure 2), (b) increased the fraction of the fast phase in H_2DIDS release experiments (Figures 7 and 8), and (c) changed the order of the reaction for the fast phase in forward-flow H_2DIDS binding experiments (compare Figure 4A with Figure 6A). The first two functional transitions occurred after >50% of the band 3 monomer population was labeled.

[2] Since we were restricted to H_2DIDS and band 3 concentrations between about 1:1 and 4:1 H_2DIDS/band 3 monomer molar ratios, the exponential analysis of the time course described in the legend to Figure 3 may be expected to yield some biphasicity due to lack of adherence to strict pseudo-first-order conditions. We have simulated this situation both for a reversible and for an irreversible second-order reaction (not shown) and find that biphasicity can develop for the irreversible case. But this amounts to only about a 2.5-fold difference in apparent rate between the two phases over the range of ligand to protein ratios we use. Furthermore, both apparent rate constants showed a linear dependence on ligand concentration in the simulation and gave negative k_{obs} y-axis intercepts. The biphasic plot seen in Figure 3 cannot be explained by such a second-order assumption, since the rates of the two phases differ by at least 30-fold (Figure 3), and since the slow phase rate shows no significant ligand concentration dependence, while both phases give positive k_{obs} y-axis intercept values (Figure 4).

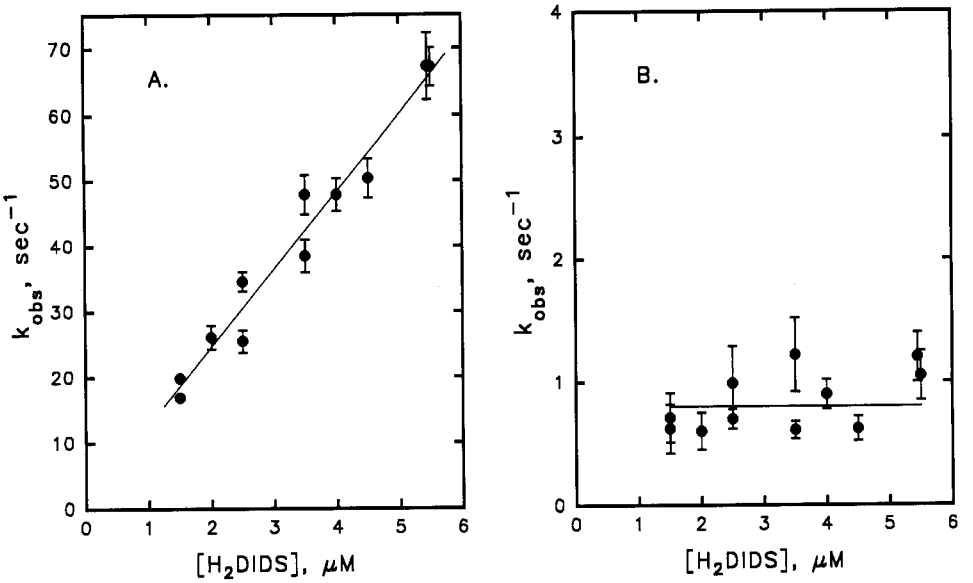

FIGURE 4: Plots of the observed rate constants for reversible H_2DIDS binding to unlabeled band 3, as a function of H_2DIDS concentration for the fast phase of the reaction (A) and for the slow phase of the reaction (B). The conditions are the same as those given for Figure 3. The line drawn in A was based on a value for the on kinetic constant of $(1.2 \pm 0.1) \times 10^7$ M^{-1} s^{-1} and a value for the off kinetic constant from the intercept of 1.1 ± 0.5 s^{-1}. A linear correlation coefficient of 0.9817 was obtained for the fit, with a p value of <0.001. On the basis of these rate constants for the fast phase, we calculate an initial equilibrium K_d for H_2DIDS binding of 95 ± 50 nM at 22 °C. The observed slow phase rate constant showed no significant dependence on H_2DIDS concentration (linear correlation coefficient = 0.4135, and $p > 0.2$). The line shown represents the mean value of the slow phase rate constants (0.8 ± 0.2 s^{-1}).

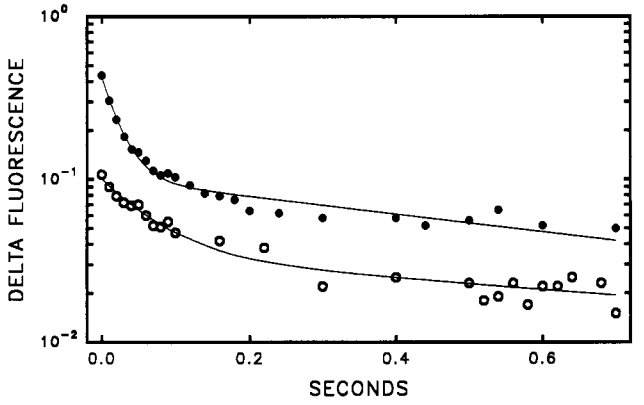

FIGURE 5: Stopped-flow kinetics of H_2DIDS reversible binding to unlabeled band 3 (●) and to band 3 whose monomer population was 75% labeled with H_2DIDS (○). Band 3 was incubated in buffer A, without and with a substoichiometric amount of H_2DIDS as described in the Experimental Methods section. At the end of the reaction period, there was no change in the DIDS binding capacity for the band 3 sample incubated in the absence of H_2DIDS, but the H_2DIDS-treated band 3 showed only about 25% of the original DIDS covalent binding capacity. These samples, containing a total of 5 μM band 3 monomer each, were mixed in the stopped-flow spectrophotometer with 10 μM H_2DIDS in buffer A at 22 °C. Note that ΔF_{total} for partially labeled band 3 is about one-fourth that of unlabeled band 3, in excellent agreement with the fraction of reactive monomers expected from the DIDS covalent binding assay. Both time courses are biphasic and were fit to the equation given in Figure 3, with the following values. Unlabeled band 3: $\Delta F_1 = 0.34 \pm 0.005$; $k_1 = 56 \pm 1.5$ s^{-1}; $\Delta F_2 = 0.08 \pm 0.004$; $k_2 = 0.6 \pm 0.06$ s^{-1}. Partially labeled band 3: $\Delta F_1 = 0.07 \pm 0.004$; $k_1 = 13.6 \pm 1.4$ s^{-1}; $\Delta F_2 = 0.03 \pm 0.004$; $k_2 = 0.6 \pm 0.14$ s^{-1}.

Within the context of the H_2DIDS release experiment, such a nonlinear dependence rules out sample heterogeneity as a possible explanation of the data. A simple mixture of two band 3 species with different affinities and kinetics would be expected to show a linear dependence for the loss of the high-affinity component as a function of increased labeling of the monomers in the sample. Furthermore, equilibrium binding curves would have to be biphasic for control band 3 if sample heterogeneity were a problem, yet no such biphasicity was observed.

Our equilibrium binding measurements support the work of Macara and Cantley (1981), who found that partial covalent labeling of the band 3 monomer population with BIDS lowered the affinity of $H_2(NBD)_2DS$ for the remaining monomers. Although steric hindrance arguments were used to explain that data (Macara & Cantley, 1981), such an explanation is inappropriate for H_2DIDS. A simple steric hindrance mechanism would predict that ligand binding to the second site should also show pseudo-first-order kinetics. The observed conversion of the kinetics to zero-order (Figure 6A) is not consistent with expectations from a steric hindrance model. In addition, evidence from both electron spin resonance (Anjaneyulu et al., 1988) and resonance energy transfer fluorescence measurements (Macara & Cantley, 1981) suggests that the distance between the two stilbenedisulfonate sites on a band 3 dimer is >16–20 Å. This distance is too large to support a steric hindrance argument for a molecule as small as H_2DIDS.

While kinetic and equilibrium constants are comparable for control band 3 and yield virtually identical K_d values (Figures 2 and 4A), such a comparison is not possible after labeling 75% of the band 3 monomer population with H_2DIDS. In this case, there was a loss of H_2DIDS concentration dependence in the forward-flow kinetic experiment (Figure 6A). It is interesting to consider how such a change in the order of the reaction could be explained. One model to consider suggests that partial labeling causes a conformational change which alters the mechanism of H_2DIDS binding. For example, the pseudo-first-order plot for the fast phase of control band 3 (Figure 4A) can be rationalized by a two-step mechanism where a slower ligand binding step precedes a much faster conformational change:

$$A \underset{k_{-1}}{\overset{(X)\,k_1}{\rightleftharpoons}} B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C \qquad (1)$$

The latter conformational change, B ↔ C, accounts for the change in fluorescence when H_2DIDS is the ligand. With

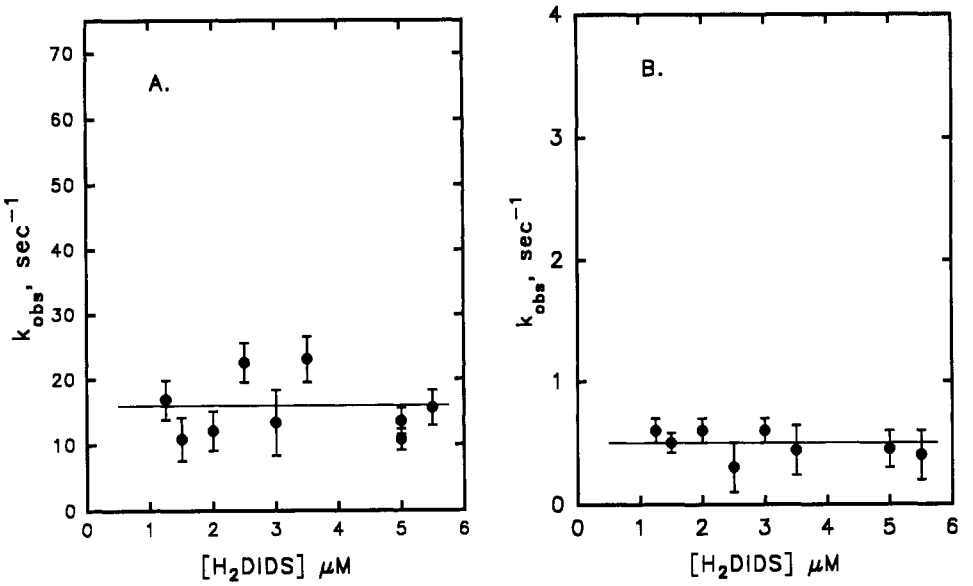

FIGURE 6: Plots of the observed rate constant for H_2DIDS binding to band 3 whose monomer population was 75% labeled with H_2DIDS, as a function of H_2DIDS concentration, for the fast phase (A) and for the slow phase (B). There was no significant dependence of the rate constant on H_2DIDS concentration for either the fast phase (linear correlation coefficient = 0.08, $p > 0.5$) or the slow phase (linear correlation coefficient = 0.2595, $p > 0.5$). The lines shown represent mean values of the rate constants for the fast phase (16 ● 5 s^{-1}) and for the slow phase (0.5 ± 0.1 s^{-1}).

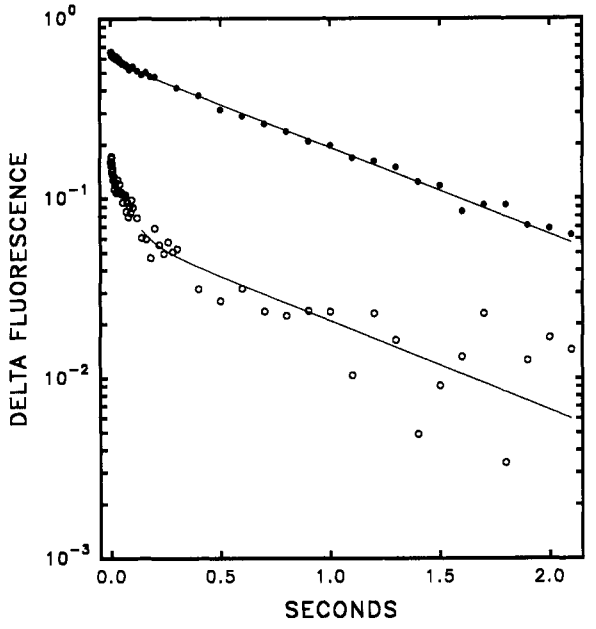

FIGURE 7: H_2DIDS/DIDS replacement reaction for unlabeled band 3 (●) and for band 3 with about 75% of the monomers labeled covalently with H_2DIDS (○). Covalent labeling was performed by premixing band 3 with H_2DIDS, allowing a specified time to lapse, and then mixing with DIDS in the stopped-flow apparatus. The after-mixing concentration of band 3 monomer was 2.5 μM, H_2DIDS was 5 μM, and DIDS was 5 μM. The equation given in Figure 3 was used to fit this data. Unlabeled band 3: $\Delta F_1 = 0.07$ ● 0.01; $k_1 =$ 20.4 ● 2 s^{-1}; $\Delta F_2 = 0.58 \pm 0.01$; $k_2 = 1.1 \pm 0.02$ s^{-1}. Partially labeled band 3: $\Delta F_1 = 0.09 \pm 0.007$; $k_1 = 14.4$ ● 2 s^{-1}; $\Delta F_2 = 0.065 \pm 0.01$; $k_2 = 1.1 \pm 0.2$ s^{-1}. The reactions were performed at 22 °C in buffer A. ΔFluorescence is defined in the legend to Figure 3.

partial labeling, a change in the conformation of the protein dimer may occur such that *conformational isomerization now precedes ligand binding* along a new kinetic pathway:

$$A' \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} B' \overset{(X)\,k_3}{\underset{k_{-3}}{\rightleftharpoons}} C' \qquad (2)$$

In reaction 2, the A′ ↔ B′ transition produces the change in fluorescence. Schopfer et al. (1988) have shown that a reaction like eq 2 can yield zero-order kinetics with respect to the concentration of X when $k_2 = k_{-3}$. In this regard, it is worth noting that the off rate for the fast phase in Figure 7 (20 s^{-1}) is approximately equal to the on rate for the fast phase of the partially labeled species (16 s^{-1}; Figure 6A) and that partial labeling with H_2DIDS increases the fraction of the 20 s^{-1} fast phase in the replacement reaction (Figure 7).

To rationalize the H_2DIDS release kinetics (Figures 7 and 8) and the equilibrium binding data (Figure 2), one may propose that band 3 can exist in two *dimeric* conformational states: one with an off rate of about 1 s^{-1} from both monomers and with a high affinity for H_2DIDS, and one with an off rate of about 20 s^{-1} from both monomers and with a low affinity for H_2DIDS. If the rate of interconversion between states is slow compared to the rate of H_2DIDS release, one will observe two kinetic phases in the H_2DIDS release reaction. When band 3 is fully saturated by *reversibly* bound H_2DIDS, the allosteric equilibrium would be shifted toward the high-affinity, 1 s^{-1} state. Covalent labeling of one monomer of the dimer would shift the allosteric equilibrium in the opposite direction, toward the low-affinity, 20 s^{-1} state.

This model is similar to the allosteric model of Monod, Wyman, and Changeux (MWC) (Monod et al., 1965). Although one might also consider a ligand-induced type of allosteric model as a possibility (Koshland et al., 1966), it is important to note that unlabeled band 3 showed *both* fast and slow H_2DIDS release phases. This would suggest that both dimeric conformational states exist prior to covalent labeling, as would be predicted by the MWC model but not by the Koshland model in its original form. Furthermore, a strict interpretation of the MWC allosteric model predicts no subunit interactions within a given dimeric conformational state. Subunit interactions arise during the concerted transition between states. The virtual lack of variation in the observed fast and slow phase off rate constants with partial labeling (Figure 8) seems consistent with this expectation of the model.

In the equilibrium H_2DIDS binding experiments, shifting the allosteric equilibrium toward the low-affinity state by labeling one monomer of the dimer can explain the observed lowering of the overall affinity of the system. Such a functional

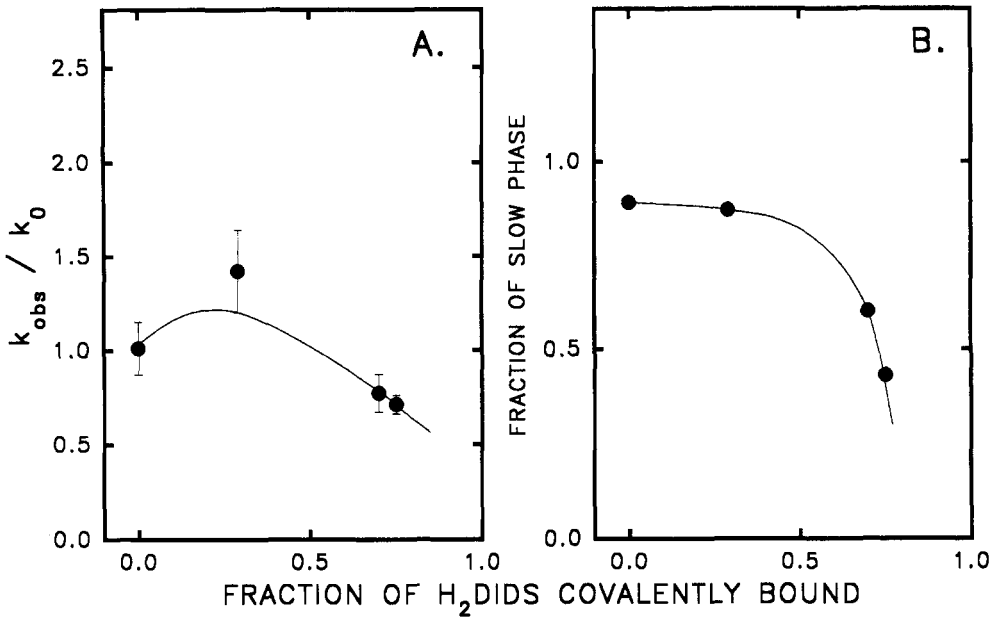

FIGURE 8: Plot of (A) the ratio of fast phase rate constant for H_2DIDS release and (B) the fraction of slow phase versus the degree of partial labeling of the band 3 monomer population. H_2DIDS covalent labeling was performed as described in the legend to Figure 7. The replacement reactions were performed under the conditions given in Figure 7, and the data points and error bars represent the mean and standard error of three determinations.

result predicts that one should be able to isolate two quaternary conformational states of the band 3 dimer. There is a growing body of structural evidence suggesting that band 3 can exist in two alternate dimeric conformational states and that covalent binding of DIDS favors one conformation of the dimer over the other (Salhany et al., 1990, 1991; Salhany, 1992; Tomida et al., 1988; Makino & Moriyama, 1989; Moriyama et al., 1992). It should be noted that such an allosteric model allows for the observation of positive cooperativity in inhibitor binding at equilibrium. However, it may not be technically possible to detect such cooperativity with a high-affinity ligand, if the cooperative inflection point in the binding curve occurs within the first 20% of binding, as might be expected for an allosteric two-site dimer model (Segel, 1975).

The demonstration of subunit interactions for the isolated band 3 dimer in nonionic detergent raises questions about the existence of such interactions for band 3 in situ. Our present results show that covalent modification of one band 3 monomer with H_2DIDS changes the inhibitor binding properties of the remaining monomer within a dimeric functional unit. A qualitatively similar effect was demonstrated recently by Salhany and Cordes (1992) in both steady-state and transient-state kinetic measurements of band 3 anion exchange. These authors showed the presence of a hysteretic transient during dithionite ($S_2O_4^{2-}$) transport. The type of transient observed depended on the pH or the type of coanion present. At pH 6, no transient was observed, showing that the observation of transients in the kinetics of dithionite transport is a property of the transporter protein and not some type of artifact. In addition, it was confirmed that dithionite influx exchange shows apparent negative cooperativity, in agreement with our previous results (Salhany & Swanson, 1978; Salhany & Gaines, 1981) and other results using more conventional substrates, such as sulfate and phosphate (Schnell & Besl, 1984) and more recently, chloride and bicarbonate (Fuhrmann et al., 1992). We suggested that the transient phase and the negative cooperativity were both due to an allosteric hysteresis (Neet & Ainslie, 1980) in band 3 involving a slow transition between two transport-active oligomeric conformational states (Salhany & Cordes, 1992). Support for such an allosteric hysteresis hypothesis was obtained by demonstrating that partial covalent labeling of the band 3 monomer population with DIDS greatly slowed the rate of the hysteretic transition, changed the substrate dependence of that transition, and eliminated any detectable indication of negative cooperativity for the substrate dependence of the steady-state velocity for dithionite transport (Salhany & Cordes, 1992).

When these transport results are taken together with our present kinetic and thermodynamic inhibitor binding studies, we can see that covalent labeling of lysine A on one monomer changes considerably the functional properties of the unlabeled monomer within a band 3 dimer. However, the exact role of subunit interactions in the band 3 transport mechanism (catalytic or regulatory) remains to be established.

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