Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 11844–11848, December 1995
Physiology

Differential sensitivity of stilbenedisulfonates in their reaction with band 3 HT (Pro-868 → Leu)

JAMES M. SALHANY†, LAWRENCE M. SCHOPFER†, MARGUERITE M. B. KAY‡§¶, DEBRA N. GAMBLE§¶, AND CHRISTINE LAWRENCE‖

†Veterans Administration Medical Center and Departments of Internal Medicine and of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE 68198; Departments of ‡Microbiology and Immunology and of §Medicine, and ¶Veterans Administration, University of Arizona College of Medicine, Tucson, AZ 85724; and ‖Division of Hematology, Department of Medicine, Bronx Municipal Hospital Center and The Albert Einstein College of Medicine, Bronx, NY 10461

Communicated by Joseph F. Hoffman, Yale University, New Haven, CT, August 10, 1995

ABSTRACT **Band 3 HT (Pro-868 → Leu) is a mutant anion exchange protein which has several phenotypic characteristics, including a 2- to 3-fold larger V_{max}, and reduced covalent binding of the anion transport inhibitor 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate (H_2DIDS). We have used fluorescence kinetic methods to study inhibitor binding to band 3 to determine if the point mutation in band 3 HT produces localized or wide-spread conformational changes within the membrane-bound domain of this transporter. Our results show that covalent binding of H_2DIDS by band 3 HT is slower by a factor of 10 to 20 compared with the wild-type protein. In contrast, no such difference in the kinetics was observed for covalent binding of 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS). In addition, the kinetics of H_2DIDS release from band 3 HT was abnormal, while the kinetics of 4,4′-dibenzamidostilbene-2,2′-disulfonate (DBDS) release showed no difference when compared with the wild-type protein. We conclude that substitution of leucine for proline at position 868 does not perturb the structure of "lysine A" in the membrane-bound domain of band 3 but rather produces an apparently localized conformational change in the C-terminal subdomain of the protein which alters H_2DIDS affinity. When combined with the observation of an increased V_{max}, these results suggest that protein structural changes at position 868 influence a turnover step in the transport cycle.**

The introduction of specific mutations in proteins provides an opportunity to test hypotheses concerning the relationship of structure to function. A rare, naturally occurring mutant has been described recently for band 3, the chloride/bicarbonate exchanger of the red blood cell membrane (1, 2). This mutant is known as band 3 HT (Pro-868 → Leu) (3–5). The initials HT stand for High Transport to indicate that the value of V_{max} for this mutant is 2- to 3-fold larger than normal (3–5). In addition to this phenotype, band 3 HT also shows reduced covalent binding of a potent inhibitor of anion exchange, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate (H_2DIDS) (Fig. 1). H_2DIDS is known to react rapidly with "lysine A" (lysine 539) (6), which is located in the 5th of 14 transmembrane helices which make up the membrane-bound domain of band 3 (7). Yet, proline 868 is located in helix 14, far from the primary H_2DIDS-binding site in the linear sequence of the transporter (7).

Two hypotheses can be proposed to explain the decreased reactivity of H_2DIDS: (*i*) The Pro-868 → Leu mutation in helix 14 may produce a widespread conformational change in the membrane-bound domain which propagates to the N-terminal portion of that domain, affecting the conformation of lysine A in helix 5; or (*ii*) the conformation of lysine A is normal, but binding of H_2DIDS to band 3 HT is abnormal due to unique interactions of this inhibitor with the C-terminal subdomain of the membrane-bound domain, in particular helices 13 and 14. It has been established that H_2DIDS also reacts with lysine 851 in or near helix 13 (6), which is close to Pro-868.

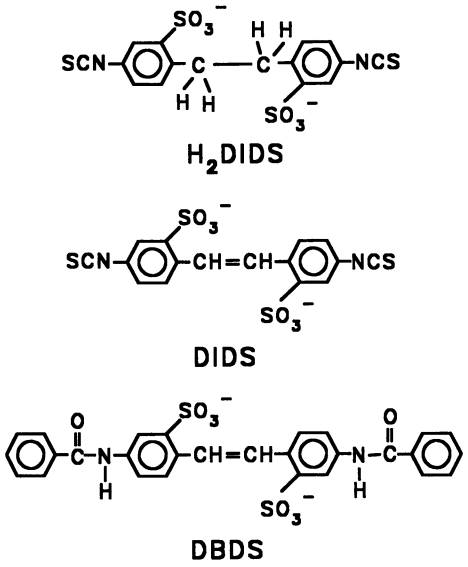

FIG. 1. Structures of the stilbenedisulfonate molecules used in this study. DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DBDS, 4,4′-dibenzamidostilbene-2,2′-disulfonate.

To discriminate between these hypotheses, we have compared the reaction of H_2DIDS with that of a homologous inhibitor DIDS (Fig. 1), both for wild-type band 3 and for band 3 HT. DIDS and H_2DIDS react with lysine A (1, 2, 8) in helix 5 (6). However, they differ significantly in their interactions with helix 13 (9). Thus, if conformational changes near helix 14 alter H_2DIDS binding to band 3 HT and thereby slow its rate of covalent reaction with lysine A, the binding of DIDS may not show such differences since it interacts differently with the C-terminal subdomain. On the other hand, if the conformation of helix 5 is altered, then both inhibitors would be expected to show an altered rate of covalent binding to band 3 HT.

MATERIALS AND METHODS

Reagents. DIDS, disodium salt (*trans* form), was purchased from Sigma and a stock solution was prepared in water as described (10). H_2DIDS was purchased from Molecular

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DBDS, 4,4′-dibenzamidostilbene-2,2′-disulfonate; bistris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

Probes, and a 40-μM stock solution was prepared in 150 mM NaCl/5 mM sodium phosphate, pH 7.4 (Buffer A) just prior to use, as described (11). DBDS was synthesized as described (12) and prepared in Buffer A or Buffer B (25 mM sodium citrate/5 mM sodium phosphate, pH 7.4).

Membrane Preparations. Red cells containing band 3 HT came from the proposita's father, who is heterozygous for the band 3 mutation (5). Venous blood was drawn in a standard citrate/phosphate/dextrose/adenine solution (CPDA1) and shipped to Tucson by overnight express. The blood was then separated into its component parts on Ficoll/Hypaque, as described (5). The red cells were suspended in Alsever's buffer (20.5 g of dextrose per liter/0.55 g of citric acid per liter/2 g of inosine per liter/4.2 g of NaCl per liter/8 g of trisodium citrate per liter/0.5 g of neomycin per liter/0.33 g of chloramphenicol per liter) solution, as described (3), and shipped to Omaha on ice by overnight delivery. Control blood was obtained from the Omaha Chapter of the American Red Cross as fresh but underweight units. Both control and mutant red cells were washed three times in phosphate-buffered saline (PBS) after removing the serum and buffy coat from control cells. Unsealed ghosts from both control and mutant red cells were prepared by lysing samples diluted 1:30 in ice-cold 5 mM sodium phosphate, pH 8, and then washing the lysed samples diluted in the same buffer (13). Ghosts were salt-stripped as described (10) to remove any membrane-associated hemoglobin or glycolytic enzymes. The concentration of band 3 in ghosts was determined directly by substoichiometric titration with DIDS by using a fluorescence covalent-binding assay (10, 14). The ghost preparations were normalized by measurement of total membrane protein with the bicinchoninic acid method (Pierce).

H_2DIDS/DIDS Replacement Reaction. We use the difference in protein fluorescence quenching due to H_2DIDS and DIDS reversible binding to band 3 to monitor the kinetics of replacement of H_2DIDS by DIDS, as described (11). This replacement reaction offers a direct measure of the rate of H_2DIDS release from its reversible complex with band 3 (11, 15).

The H_2DIDS/DIDS replacement reaction can also be used to monitor the rate of H_2DIDS covalent adduct formation. This is accomplished by measuring with the stopped-flow apparatus the total observed fluorescence change for the above replacement reaction at various times after mixing band 3 with H_2DIDS. As the H_2DIDS covalent adduct forms, the amplitude of the fluorescence change decreases. When all sites are covalently bound by H_2DIDS, mixing with DIDS produces no detectable change in protein fluorescence (11). A plot of the total fluorescence change versus elapsed time describes the time course of H_2DIDS covalent adduct formation. The rate of covalent adduct formation is sufficiently slow in unsealed ghosts to allow for accurate measurement of the rate of H_2DIDS covalent binding (see below).

DIDS Covalent Adduct Formation Reaction. The kinetics of DIDS covalent adduct formation were studied essentially as described (10). DIDS was added to ghosts in an amount sufficient to saturate all of the band 3 sites, and the kinetics were followed in a Perkin–Elmer spectrofluorometer model 650-40 at 25°C in 150 mM NaCl/50 mM *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (bistris) adjusted to pH 7.2 with acetic acid. The excitation wavelength was 360 nm, and the kinetics were observed by following the fluorescence emission at 450 nm.

DBDS/DIDS Replacement Reaction. The DBDS "off" rate constant was measured as described (15). The off rate constant was determined in either Buffer B, or after mixing DBDS-saturated band 3 in Buffer B with Buffer A to give a final chloride concentration of 75 mM, all at constant ionic strength.

Kinetics of Reversible Binding of H_2DIDS and DIDS to Band 3. The kinetics of H_2DIDS and DIDS binding to band 3 in unsealed ghosts were measured according to procedures given in previous reports (11, 16), where protein fluorescence quenching associated with inhibitor binding was followed. The reactions were performed at concentrations of DIDS and H_2DIDS between 2 to 5 μM and a band 3 concentration of 1 μM. The reactions were performed in Buffer A at 25°C.

Data Analysis. The time courses for inhibitor release were fitted to either single or double exponential equations to obtain the rate constants, while the time courses for inhibitor binding were fitted to a double exponential equation as described (11, 16).

Secondary plots of the observed rate constants for inhibitor binding were fitted according to the following model system. Inhibitor binding kinetic studies have shown that DBDS (17, 18) and H_2DIDS and DIDS (11, 16) follow a two-step binding mechanism:

$$S + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (SB) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (SB)^*, \quad [1]$$

where S is stilbenedisulfonate, B is a band 3 subunit, and (SB) and (SB)* are two conformational states of the complex. Two well-resolved kinetic phases are observed (11, 16, 18, 19). The apparent rates of these phases were sufficiently well separated to justify treating the two steps in Eq. **1** as uncoupled processes (20).

The fast phase apparent rate constants followed a classical second-order dependence and were fitted to the following equation:

$$k_{obs} = k_1(S) + k_{-1}. \quad [2]$$

The slow phase apparent rate constants followed a saturation dependence and were fitted to the following equation:

$$k_{obs} = \frac{k_2(S)}{K_1 + (S)} + k_{-2} \quad [3]$$

where $K_1 = k_{-1}/k_1$. At saturation, the slow-phase rate is equal to $k_2 + k_{-2}$ (20), which can be used to calculate k_2 by subtracting k_{-2} from the observed rate. The k_{-2} value was determined experimentally from the replacement reaction (11, 15). The rate constant from the replacement reaction was found to be virtually identical to the value of k_{-2} determined from a fit of Eq. **3** to the DBDS binding data in the presence of chloride (18). Under these conditions, k_{-2} is large enough to measure accurately using the forward-flow inhibitor binding reaction.

The values of K_2 and K_d were calculated from the above kinetic constants using the equations:

$$K_2 = k_{-2}/k_2 \quad [4]$$

and

$$K_d = \frac{K_1 K_2}{1 + K_2}. \quad [5]$$

All kinetic results were analyzed by using Sigma Plot (Jandel Scientific, San Rafael, CA).

RESULTS

H_2DIDS/DIDS Replacement Reaction. Fig. 2 shows the kinetics for the replacement of H_2DIDS by DIDS for wild-type band 3 (Fig. 2*A*) and for band 3 HT (Fig. 2*B*). The reaction for wild-type band 3 in unsealed ghosts was monophasic and exponential, with an apparent rate constant for H_2DIDS release of 0.24 ± 0.01 s^{-1}. This agrees with our previously reported number for band 3 in unsealed ghosts (0.23 s^{-1} at

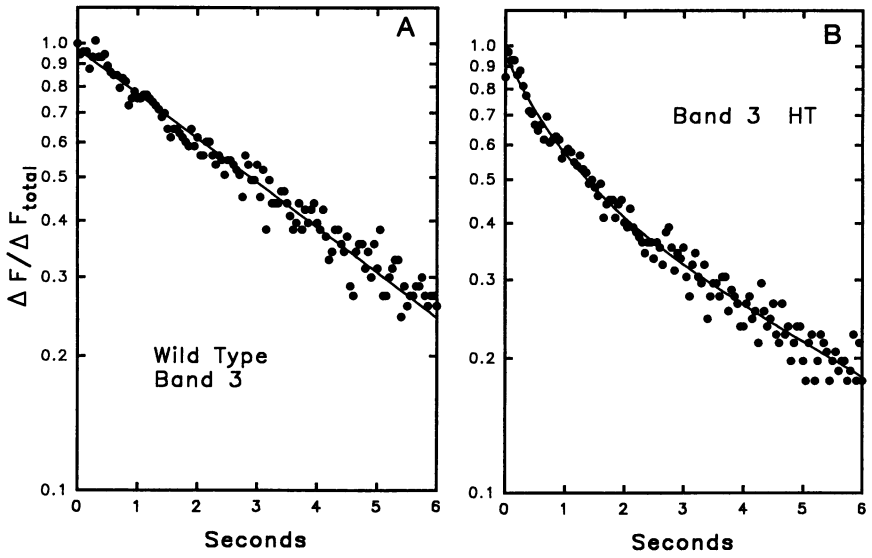

FIG. 2. H_2DIDS/DIDS replacement reaction for wild-type band 3 (*A*) and for band 3 HT (*B*). Reactions were performed at 25°C by premixing unsealed ghosts with H_2DIDS to yield concentrations of 1.4 μM band 3 and 6 μM H_2DIDS in Buffer A. This sample was then immediately (<1 min) mixed in the stopped-flow apparatus with 12.3 μM DIDS prepared in Buffer A. This is sufficient to totally displace reversibly bound H_2DIDS from band 3. The kinetics were measured by exciting the sample at 280 nm and following the fluorescence through a 315-nm cutoff filter. The term ΔF is defined as the difference between the final fluorescence and the fluorescence at any time. The term ΔF_{total} is defined as the difference between the final fluorescence and the fluorescence at time $t = 0$. A total of 1000 data points were collected per reaction, and the reaction time courses were fitted to either single or double exponential equations. Wild-type band 3 followed single exponential kinetics with an apparent off rate constant of 0.24 ± 0.01 s^{-1}. Band 3 HT membranes showed 50:50 biphasic time courses and had the following off rate constants: k_{fast} = 1.2 ± 0.17 s^{-1} and k_{slow} = 0.18 ± 0.02 s^{-1}.

23°C; ref. 15).†† In contrast, H_2DIDS release from band 3 HT ghosts showed 50:50 biphasic kinetics (Fig. 2*B*). The slow-phase rate constant was 0.18 ± 0.02 s^{-1}, which is quite close to the rate for wild type. The fast-phase rate constant was about 7-fold larger (1.2 ± 0.17 s^{-1}). Biphasic kinetics are consistent with this individual being heterozygous for the band 3 mutation (5). The similarity of the wild-type rate constant and the slow-phase rate constant in the mutant allows us to assign the slow phase for this heterozygous individual to wild-type band 3. This comparison indicates that band 3 HT has the faster rate of H_2DIDS release.

H_2DIDS Covalent Adduct Formation Rate. Once the H_2DIDS–band 3 reversible complex forms, the isothiocyano groups of H_2DIDS react with lysine A (8). To determine the rate constant for this H_2DIDS covalent adduct formation reaction, we measured the total fluorescence change (ΔF_{total}) for the H_2DIDS/DIDS replacement reaction as a function of the time elapsed after mixing unsealed ghosts with H_2DIDS (conditions described above). Fig. 3*A* (closed circles) shows that normalized ΔF_{total} for wild-type membranes decreases exponentially with time. The apparent rate constant for adduct formation was 0.017 ± 0.003 min^{-1}.

When the same reaction was performed with unsealed ghosts containing band 3 HT, a 50:50 biphasic time course was observed (Fig. 3*A*, open circles). The fast-phase rate constant (k_{fast} = 0.041 ± 0.006 min^{-1}) is comparable with wild type, while the slow-phase rate constant was slower by a factor of about 10 (k_{slow} = 0.002 ± 0.001 min^{-1}). This comparison suggests that the slow phase corresponds to H_2DIDS adduct formation with band 3 HT monomers. Such an assignment is consistent with the observation by Bruce *et al.* (4), who found that band 3 HT in membranes from a homozygous individual formed less H_2DIDS covalent adduct than wild type after a fixed incubation time.

DIDS Covalent Adduct Formation. The kinetics of DIDS covalent adduct formation were followed by observing the fluorescence increase after formation of the fully saturated DIDS–band 3 reversible complex (10). The results are shown as normalized fluorescence changes in Fig. 3*B*. Unlike the covalent reaction of H_2DIDS, we observed no difference in the kinetics between wild-type band 3- and band 3 HT-containing membranes. Titration of both types of membranes with DIDS showed the same number of DIDS binding sites when total membrane protein concentrations were matched (data not shown), in agreement with Bruce *et al.* (4).

We have found that the DIDS adduct formation rate is dependent on chloride binding to the transport site (10). When the chloride dependence of the DIDS adduct formation reactions for wild-type band 3 and band 3 HT membranes were compared, no heterogeneity was observed in the latter, and the two saturation curves were superimposable. These findings suggest that chloride binding to the transport site in the DIDS–band 3 reversible complex is normal in band 3 HT.

DBDS/DIDS Replacement Reaction. Since it is technically difficult to measure the DIDS off rate because of the rapidity of the covalent adduct formation reaction (10), we used our DBDS/DIDS replacement reaction as another example of stilbenedisulfonate release from band 3 (15). The reactions were all monophasic and exponential (see figure 4 of ref. 15), and there was no significant difference in the DBDS-release rate between wild-type band 3 and band 3 HT (Table 1). Furthermore, chloride accelerated the reaction to the same degree in both cases (Table 1). These results suggest that there is no difference in chloride binding between wild-type band 3

††It should be noted that wild-type band 3 in unsealed ghosts showed monophasic, exponential replacement reaction kinetics under all conditions, including after partial covalent labeling of the sample with H_2DIDS. In contrast, the same reaction for isolated band 3 in 0.01% [poly(oxyethylene-8-lauryl)ether ($C_{12}E_8$)/25 μM phosphatidylcholine/150 mM NaCl/5 mM sodium phosphate, pH 7.4, yielded biphasic kinetics after H_2DIDS reacted covalently with part of the band 3 monomer population (11). We have recently found that solubilization of band 3 in $C_{12}E_8$ increases the value of k_1 in Eq. 1 of the text ≈10-fold for both H_2DIDS and DBDS and increases k_{-2} ≈4- to 6-fold (11, 16, 18). These findings indicate that solubilization in $C_{12}E_8$ produces conformational changes in the vicinity of the stilbenedisulfonate-binding site on band 3. Thus, caution is necessary when comparing the inhibitor binding kinetics of band 3 in unsealed ghosts with the inhibitor binding kinetics of the solubilized transporter.

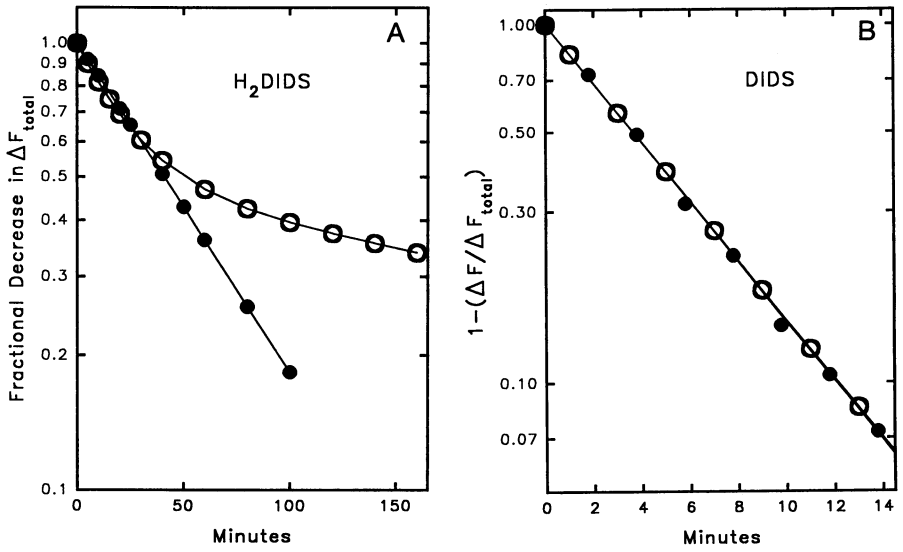

FIG. 3. Kinetics of H_2DIDS and DIDS covalent adduct formation with wild-type band 3-containing membranes (●) and with band 3 HT-containing membranes (○). (*A*) Adduct formation by H_2DIDS was followed in the stopped-flow apparatus by measuring the total observed change in fluorescence (ΔF_{total}) which occurs upon replacement of H_2DIDS by DIDS as a function of elapsed time after mixing with H_2DIDS. Replacement reactions were performed under exactly the same conditions as stated in the legend to Fig. 2. These ΔF_{total} values were normalized by dividing ΔF_{total} at time t in the covalent adduct formation reaction, by ΔF_{total} at time $t = 0$. The result is plotted as fractional decrease in ΔF_{total}. This value decreased exponentially for wild-type membranes, with an apparent rate constant for H_2DIDS covalent adduct formation of 0.017 ± 0.003 min^{-1}. The same reaction for band 3 HT membranes was 50:50 biphasic with the following apparent rate constants for each phase: k_{fast} = 0.041 ± 0.006 min^{-1} and k_{slow} = 0.002 ± 0.001 min^{-1}. (*B*) Reaction of DIDS with band 3 in unsealed ghosts was performed as described in the text. The concentration of wild-type band 3 was 1.1 μM, and the concentration of total band 3 in the HT-containing membranes was 0.7 μM. The concentration of DIDS was equal to 1.3 times the concentration of band 3 in each case. For the DIDS reaction, ΔF is the observed fluorescence at time t minus the background fluorescence at time $t = 0$. ΔF_{total} is the fluorescence at the end of the reaction minus the fluorescence at time $t = 0$. The DIDS covalent adduct formation reactions were purely exponential in each case, showing no evidence for heterogeneity. The apparent rate constant for covalent binding of DIDS was 0.19 ± 0.003 min^{-1}.

and band 3 HT, in agreement with our findings with DIDS mentioned above.

H_2DIDS and DIDS Binding Kinetics. The kinetics of reversible binding of H_2DIDS and DIDS to band 3 were followed by monitoring band 3 protein fluorescence quenching (excitation 280 nm) (11, 16). With wild-type band 3, biphasic time courses were observed for both stilbenedisulfonates, as reported previously (16). With band 3 HT, the time courses for both DIDS and H_2DIDS were essentially identical to their respective time courses with wild-type band 3. The fast-phase apparent rate constants followed a classical second-order dependence with respect to stilbenedisulfonate concentration, while the slow-phase rate constants showed saturation behavior (data not shown, but see figure 3 of ref. 16 for an illustration).

Table 1. Kinetic and equilibrium constants from the text for the reversible binding of various stilbenedisulfonates to band 3

Stilbene-disulfonate	Wild-type membranes	Band 3 HT membranes
DIDS		
k_1	(1.3 ± 0.3) × 10^6 $M^{-1}\cdot s^{-1}$	(1.4 ± 0.3) × 10^6 $M^{-1}\cdot s^{-1}$
k_{-1}	2.2 ± 0.3 s^{-1}	1.2 ± 0.4 s^{-1}
K_1	1.7 × 10^{-6} M	0.9 × 10^{-6} M
k_2	1.1 ± 0.02 s^{-1}	1.1 ± 0.03 s^{-1}
k_{-2}	—	—
K_2	—	—
K_D	—	—
H_2DIDS		
k_1	(2.2 ± 0.1) × 10^6 $M^{-1}\cdot s^{-1}$	(3.0 ± 0.5) × 10^6 $M^{-1}\cdot s^{-1}$
k_{-1}	0.5 ± 0.3 s^{-1}	2.0 ± 1.0 s^{-1}
K_1	0.23 × 10^{-6} M	0.67 × 10^{-6} M
k_2	1.8 ± 0.3 s^{-1}	1.3 ± 0.2 s^{-1}
k_{-2}	0.24 ± 0.01 s^{-1}	1.2 ± 0.2 s^{-1}
K_2	0.13	0.92
K_D	27 × 10^{-9} M	321 × 10^{-9} M
DBDS		
k_{-2} (citrate)	0.08 ± 0.005 s^{-1}	0.08 ± 0.005 s^{-1}
k_{-2} (chloride)	0.62 ± 0.04 s^{-1}	0.72 ± 0.05 s^{-1}

Experiments were performed in Buffer A except where citrate is indicated. In that case, Buffer B was used. The temperature was 25°C.

Table 1 lists the measured values for k_1, k_{-1}, and k_{-2}, and the calculated values for k_2, K_1, K_2, and K_D for H_2DIDS binding to wild-type band 3 and band 3 HT determined as described in *Materials and Methods*. For DIDS, the value of k_2 was taken directly from the slow-phase rate constant without correction. The value of k_{-2} is so small in this case that it can be ignored. The values of k_1, k_{-1}, and k_2 are not significantly different when wild-type band 3 is compared with band 3 HT for either stilbenedisulfonate. Only the H_2DIDS off rate constant (k_{-2}) shows a significant difference between wild-type and mutant. The effect of k_{-2} appears in the calculation of K_D, which shows that the H_2DIDS affinity for the mutant and the wild-type transporters differs by about 12-fold (Table 1).

DISCUSSION

Kay and coworkers discovered (3) and sequenced (4) a band 3 mutant known as band 3 HT (Pro-868 → Leu) which showed higher than normal anion transport and altered H_2DIDS binding, phenotypes that are specifically related to the membrane-bound domain of band 3. In the present study, we have demonstrated the unique ability of H_2DIDS reversible binding and covalent adduct formation to sense conformational changes in band 3 HT.

To understand the differential sensitivity of stilbenedisulfonates in their reactions with band 3 HT, it is important to consider briefly the known differences in the reactions of

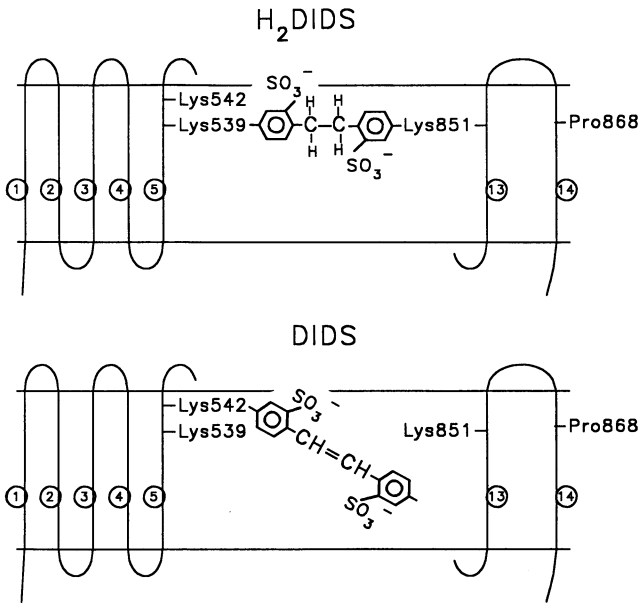

FIG. 4. Models for H_2DIDS– and DIDS–band 3 complexes. Transmembrane helices are numbered according to Tanner *et al.* (7), with helices 6–12 deleted for clarity.

DIDS and H_2DIDS with wild-type band 3. The membrane-bound domain of band 3 can be divided into two subdomains defined by digestion of unsealed ghosts with chymotrypsin (7). The N-terminal chymotryptic subdomain contains the first five transmembrane helices of band 3, while the C-terminal chymotryptic subdomain contains the remaining nine (7). Each subdomain contains lysines which can react with the isothiocyano groups of DIDS or H_2DIDS under certain conditions. The N-terminal subdomain reacts rapidly with DIDS, while the C-terminal subdomain does not react with DIDS at all at physiological pH (9). The pH must be raised to 12 or higher to initiate the reaction of DIDS with the C-terminal chymotryptic subdomain. In contrast, H_2DIDS reacts with both subdomains at very similar rates at physiological pH, the rate constant for the N-terminal subdomain being about 3-fold faster than the rate constant for the C-terminal subdomain (21). Furthermore, the covalent reaction of H_2DIDS with wild-type band 3 is about one order of magnitude slower than the covalent reaction of DIDS (Fig. 3 and ref. 8).

The lysines involved in the reaction of human band 3 with H_2DIDS have been identified as lysine 539 of helix 5 in the N-terminal subdomain (6, 22), and lysine 851 in or near helix 13 in the C-terminal subdomain (6). The lysines which react with DIDS have not been identified for human band 3, but lysine A has been identified as lys-539 for bovine band 3, which lacks lysine 542 (23). In contrast, Garcia and Lodish (24) have shown that lysine 539 of helix 5 is not essential for covalent binding of DIDS. This suggests that when lysine 539 is mutagenized to other residues, DIDS may react with lysine 542 in the same helix. It is possible that in wild-type band 3 DIDS can react with either lysine 539 or 542, while H_2DIDS reacts only with lysine 539 (6). Our results with band 3 HT, taken together with these observations from the literature, indicate that DIDS and H_2DIDS bind to the membrane-bound domain of human band 3 in significantly different configurations, such that they (*i*) interact differently with the C-terminal subdomain and that they (*ii*) have different rates of covalent adduct formation.

Fig. 4 shows schematic models of H_2DIDS and DIDS complexes with the membrane-bound domain of band 3. The key feature of these models lies in the different configurations depicted for H_2DIDS and DIDS within the stilbenedisulfonate site. To react with lysines 539 and 851 at their proposed locations on the outer aspect of band 3 (7), we place H_2DIDS in a position roughly parallel to the membrane plane in agreement with Okubo *et al.* (6). In contrast, we place DIDS in a more perpendicular position with respect to the membrane plane, so that its interaction with the C-terminal subdomain is different. We suggest that substitution of leucine for proline at position 868 produces a conformational change that is localized to the region of helix 13 and helix 14 and that specifically lowers the affinity of H_2DIDS for band 3 HT. We then suggest that this altered binding of H_2DIDS lowers the rate of covalent adduct formation. It seems highly unlikely that conformational changes have occurred in helix 5 consequent to a mutation at residue 868 since the kinetics of DIDS adduct formation remain unaltered (Fig. 3) and since the DBDS off rate was unchanged (Table 1). Further support for the view that the mutation in band 3 HT is localized to the C-terminal subdomain comes from the work of Kay *et al.* (5).

In conclusion, the results of this study indicate that the mutation in band 3 HT (Pro-868 → Leu) alters the conformation of the C-terminal subdomain, which may influence a turnover step in the transport cycle.

We thank Renee Sloan for performing gel electrophoresis studies; Karen Cordes for preparing unsealed ghosts and for help with the preparation of figures; and Monica Gustafson, Jeff Poulin, and Cathleen Cover for assisting in cell separation. This work was supported by the Medical Research Service of the Veterans Administration (J.M.S. and M.M.B.K.) and National Institutes of Health Grants AG08444 and AG09258.

1. Salhany, J. M. (1990) *Erythrocyte Band 3 Protein* (CRC, Boca Raton, FL).
2. Tanner, M. J. A. (1993) *Semin. Hematol.* **30,** 34–57.
3. Kay, M. M. B., Bosman, G. C. G. M. & Lawrence, C. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 492–496.
4. Bruce, L. J., Kay, M. M. B., Lawrence, C. & Tanner, M. J. A. (1993) *Biochem. J.* **293,** 317–320.
5. Kay, M. M. B., Bosman, G., Johnson, R. C., Poulin, J., Lawrence, C. & Goodman, J. (1994) *Exp. Clin. Immunogenet.* **11,** 209–221.
6. Okubo, K., Kang, D., Hamasaki, N. & Jennings, M. L. (1994) *J. Biol. Chem.* **269,** 1918–1926.
7. Tanner, M. J. A., Martin, P. G. & High, S. (1988) *Biochem. J.* **256,** 703–712.
8. Lepke, S., Fasold, H., Pring, M. & Passow, H. (1976) *J. Membr. Biol.* **29,** 147–177.
9. Kang, D., Okubo, K., Hamasaki, N., Kuroda, N. & Shimaki, H. (1992) *J. Biol. Chem.* **267,** 19211–19217.
10. Schopfer, L. M. & Salhany, J. M. (1995) *Biochemistry* **34,** 8320–8329.
11. Salhany, J. M., Cordes, K. A. & Schopfer, L. M. (1993) *Biochemistry* **32,** 7413–7420.
12. Kotaki, A., Naoi, M. & Yagi, K. (1971) *Biochim. Biophys. Acta* **229,** 547–556.
13. Steck, T. L., Weinstein, R. S., Straus, J. H. & Wallach, D. F. H. (1970) *Science* **168,** 255–257.
14. Van Dort, H. M., Low, P. S., Cordes, K. A., Schopfer, L. M. & Salhany, J. M. (1994) *J. Biol. Chem.* **269,** 59–61.
15. Salhany, J. M., Sloan, R. L., Cordes, K. A. & Schopfer, L. M. (1994) *Biochemistry* **33,** 11909–11916.
16. Salhany, J. M. (1995) *Biochem. Mol. Biol. Int.* **36,** 1067–1077.
17. Dix, J. A., Verkman, A. S. & Solomon, A. K. (1986) *J. Membr. Biol.* **89,** 211–223.
18. Salhany, J. M., Sloan, R. L., Cordes, K. A. & Schopfer, L. M. (1995) *Int. J. Biochem. Cell Biol.* **27,** 953–964.
19. Posner, R. G. & Dix, J. A. (1985) *Biophys. Chem.* **23,** 139–145.
20. Jackman, M. P., Parry, M. A. A., Hofsteenge, J. & Stone, S. R. (1992) *J. Biol. Chem.* **267,** 15375–15383.
21. Kampmann, L., Lepke, S., Fasold, H., Fritzsch, G. & Passow, H. (1982) *J. Membr. Biol.* **70,** 199–216.
22. Bartel, D., Hans, H. & Passow, H. (1989) *Biochim. Biophys. Acta* **985,** 355–358.
23. Moriyama, R., Nagatomi, Y., Hoshino, F. & Makino, S. (1994) *Int. J. Biochem.* **26,** 133–137.
24. Garcia, A. M. & Lodish, H. F. (1989) *J. Biol. Chem.* **264,** 19607–19613.