Interactions between Mutant and Wild-Type Band 3 Subunits in Hereditary Southeast Asian Ovalocytic Red Blood Cell Membranes†

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Received October 9, 1995; Revised Manuscript Received November 13, 1995⊗

ABSTRACT: Red cell membranes from individuals with Southeast Asian ovalocytosis (SAO) contain approximately equal proportions of wild-type band 3 and a mutant SAO band 3 which lacks residues 400−408. It is known that the V_{max} for anion exchange in SAO cells is reduced by about 50%, that SAO band 3 does not transport anions when expressed alone in a cellular expression system, that SAO band 3 does not bind stilbenedisulfonates, and that about 50% of the band 3 exists as wild-type/SAO heterodimers. In this report, we show that the kinetics of H_2DIDS (4,4′-diisothiocyanatodihydro-2,2′-stilbenedisulfonate) release from the wild-type band 3 in SAO membranes is biphasic. The two phases were present in about equal proportions, with rate constants differing by about 5-fold. In contrast, control cells showed monophasic, exponential kinetics with a rate constant comparable to that of the fast phase of SAO membranes. We assign the fast phase in SAO membranes to H_2DIDS release from wild-type subunits within homodimers and the slow phase to H_2DIDS release from the wild-type subunit within the heterodimer. No differences were observed in kinetic studies of H_2DIDS binding. These results suggest that the mutant band 3 subunit alters the conformation of its neighboring wild-type subunit within the heterodimer, resulting in about a 4-fold higher H_2DIDS affinity. Additional evidence suggesting that the interactions in the heterodimer may be confined to a region of the wild-type subunit containing the C-terminal subdomain is presented. The relationship of these subunit interactions to the observation of a reduced cellular anion transport function is discussed.

A hereditary ovalocytosis of Melanesian and Southeast Asian origin was first described by Lie-Injo (1965). The membranes of SAO[1] red blood cells show increased rigidity (Mohandas et al., 1984; Saul et al., 1984) and resistance to infection by malaria parasites *in vitro* (Kidson et al., 1981). This condition is associated with the deletion of nine amino acids (400−408) in the membrane-spanning domain of about one-half of the band 3 in SAO membranes (Jarolim et al., 1991; Mohandas et al., 1992; Schofield et al., 1992b). The deletion mutation is linked to a point mutation at amino acid 56 (Lys → Glu), known as band 3 Memphis (Mueller & Morrison, 1977; Ranney et al., 1990; Yannoukakous et al., 1991). SAO band 3 is incapable of transporting anions (Grovers et al., 1993) or binding stilbenedisulfonates either reversibly (Sarabia et al., 1993) or covalently (Schofield et al., 1992a). Since band 3 Memphis shows neither of these phenotypes, it has been concluded that the deletion mutation is responsible for the functional abnormalities (Moriyama et al., 1992).

SAO red cell membranes provide a unique opportunity to test questions concerning the significance of subunit interactions in band 3 function. Jennings and Gosselink (1995) have shown that band 3 heterodimers form in SAO membranes. The proportions of dimeric species are roughly consistent with a 1:2:1 ratio of wild-type homodimer, heterodimer, and SAO homodimer. Since the SAO homodimer is nonfunctional (Grovers et al., 1993), subunit interactions within the heterodimer would be established if it could be shown that only one-half of the wild-type band 3 in SAO membranes was functionally perturbed. In this report, we have used several of our recently described stilbenedisulfonate binding reactions (Salhany et al., 1993, 1994; Salhany, 1995; Schopfer & Salhany, 1995) to characterize the functional properties of the wild-type band 3 subunits in SAO membranes. Since stilbenedisulfonates do not bind either reversibly (Sarabia et al., 1993) or covalently (Schofield et al., 1992a) to SAO band 3, only the kinetic properties of the wild-type subunit will be detected. The stilbenedisulfonate release reactions (Salhany et al., 1994) and the covalent DIDS adduct formation reaction (Schopfer & Salhany, 1995) have been shown to be monophasic and exponential. Thus, if the properties of the wild-type subunits in the homodimer are different from those of the wild-type subunit in the heterodimer, the reaction time course will be 50:50 biphasic in studies with SAO membranes, in contrast to control.

Finally, we have also applied a newly described assay (Schopfer & Salhany, 1995) which monitors chloride binding to the transport site, since there is no information on K_m for SAO red blood cells.

† This work was supported by the Medical Research Service of the Veterans Administration.

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⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1995.

[1] Abbreviations: SAO, Southeast Asian ovalocytic; H_2DIDS, 4,4′-diisothiocyanatodihydro-2,2′-stilbenedisulfonate; DIDS, 4,4′-diisothiocyanato-2,2′-stilbenedisulfonate; BADS, 4-benzamido-4′-amino-2,2′-stilbenedisulfonate; DBDS, 4,4′-dibenzamido-2,2′-stilbenedisulfonate; PBS, 5 mM sodium phosphate (pH 8.0) plus 150 mM NaCl; 5P8, 5mM sodium phosphate (pH 8.0); bistris, [*N*,*N*-bis(2-hydroxyethyl)amino]-tris(hydroxymethyl)methane.

0006-2960/96/0435-251$12.00/0

EXPERIMENTAL METHODS

Materials. The sources of materials used in this study have been described before (Salhany et al., 1993, 1994; Schopfer & Salhany, 1995). Fresh underweight units of whole blood were donated by the Omaha Chapter of the American Red Cross. SAO blood was generously provided by Dr. David Liu at St. Elizabeth's Medical Center of Boston and shipped from Boston to Omaha on ice by overnight delivery.

Preparations. Unsealed ghosts from control and SAO red cells were prepared as described previously (Salhany et al., 1980). Membranes were stripped of associated peripheral proteins by washing twice in PBS and then three times in 5P8. Ghost concentrations were matched using light scattering. The amount of band 3 present was determined by fluorescence titration with DIDS (Van Dort et al., 1994; Schopfer & Salhany, 1995).

Inhibitor Release and Binding Kinetics. H_2DIDS release rates were measured by premixing H_2DIDS with ghosts and then immediately (<1 min) mixing that sample with excess DIDS. This reaction was monitored by observation of the change in protein fluorescence (excitation at 280 nm, with fluorescence emission measured through a 315 nm short wavelength cutoff filter) consequent to conversion from H_2-DIDS/band 3 to DIDS/band 3 reversible complexes (Salhany et al., 1993). DBDS release rates were also measured by rapidly mixing DBDS-saturated band 3 with excess DIDS. Here, the change in fluorescence between free and bound DBDS was monitored by excitation of the sample at 335 nm and observation of fluorescence through a 445 nm short wavelength cutoff filter. The experimental details and theory of these replacement reactions have been described in two previous reports (Salhany et al., 1993, 1994).

Kinetics of DIDS Covalent Binding to Band 3. DIDS covalent binding was monitored by observation of the increase in fluorescence after formation of a 1:1 DIDS/band 3 reversible complex (excitation at 360 nm, emission at 450 nm), as described previously (Schopfer & Salhany, 1995).

Kinetics of H_2DIDS Reversible Binding. The kinetics of H_2DIDS binding to unsealed ghosts were measured as described previously (Salhany, 1995), by observation of protein fluorescence quenching of band 3 consequent to reversible binding of H_2DIDS (Salhany et al., 1993). The time course of the reaction is biphasic, with the fast phase rate constant showing classical second-order kinetics and the slow phase rate constant showing saturation behavior (Salhany, 1995). These two phases were analyzed according to a two-step (Dix et al., 1986) uncoupled (Salhany, 1995) binding mechanism:

$$S + B \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} SB \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} SB \quad (1)$$

where S is stilbenedisulfonate, B is the band 3 subunit, and SB and SB* are two types of bound complexes. We have that

$$K_1 = k_{-1}/k_1 \quad (2)$$

and

$$K_2 = k_{-2}/k_2 \quad (3)$$

and that

$$K_d = \frac{K_1 K_2}{1 + K_2} \quad (4)$$

The H_2DIDS dependence of the fast phase rate constant was analyzed by fitting data using the equation

$$k_{fast} = k_1(S) + k_{-1} \quad (5)$$

For the slow phase

$$k_{slow} = \frac{k_2(S)}{K_1 + (S)} + k_{-2} \quad (6)$$

The values of k_{-2} were taken from the replacement reaction, since the values for H_2DIDS are too small to determine accurately in forward flow kinetic binding studies. Justification for this approach was presented elsewhere by comparison of the larger k_{-2} values for DBDS using both forward flow binding and replacement reactions (Salhany et al., 1995a).

RESULTS

H_2DIDS Release and Binding Kinetics. Figure 1A shows the time course for a typical H_2DIDS/DIDS replacement reaction for control ghosts in PBS. As reported previously (Salhany et al., 1994, 1995b), the time course is monophasic and exponential for >90% of the reaction. In contrast, SAO membranes showed grossly biphasic H_2DIDS release kinetics (Figure 1B). The two phases were present in about equal proportions, to within the errors associated with the fit, and the rate constants differed by about 5-fold. The fast phase rate constant was comparable to the rate constant for wild-type band 3 in control cells. Doubling the concentration of the replacing inhibitor (DIDS) had no effect on the kinetics, indicating that H_2DIDS release is rate-limiting (data not shown). Results like those in Figure 1B, were obtained in three out of three independent preparations from the same unit of SAO whole blood. Comparison of matched preparations of control and SAO membranes showed that the observed total fluorescence change for SAO membranes was about one-half of that of control (data not shown). This result is consistent with other evidence indicating that stilbenedisulfonates do not bind to SAO band 3 subunits (Sarabia et al., 1993; also see below). Thus, we can conclude that the observed heterogeneity seen in Figure 1B, arises from differences in the rates of H_2DIDS release from two types of functionally distinct wild-type band 3 subunits in the SAO membrane.

We have also measured the kinetics of H_2DIDS binding by observing the change in protein fluorescence upon mixing unsealed ghosts with various concentrations of H_2DIDS. The reaction is biphasic, with the fast phase following second-order kinetics, and with the slow phase showing saturation behavior (Salhany, 1995). We observed no significant difference between control and SAO membranes. Table 1 lists the observed rate constants determined in the forward flow kinetic binding experiments. Since there was no significant difference between SAO and control, and since SAO membranes showed well-resolved kinetic phases, we conclude that the values of k_1, k_{-1}, and k_2 are the same for all of the wild-type subunits in SAO membranes. Thus, wild-

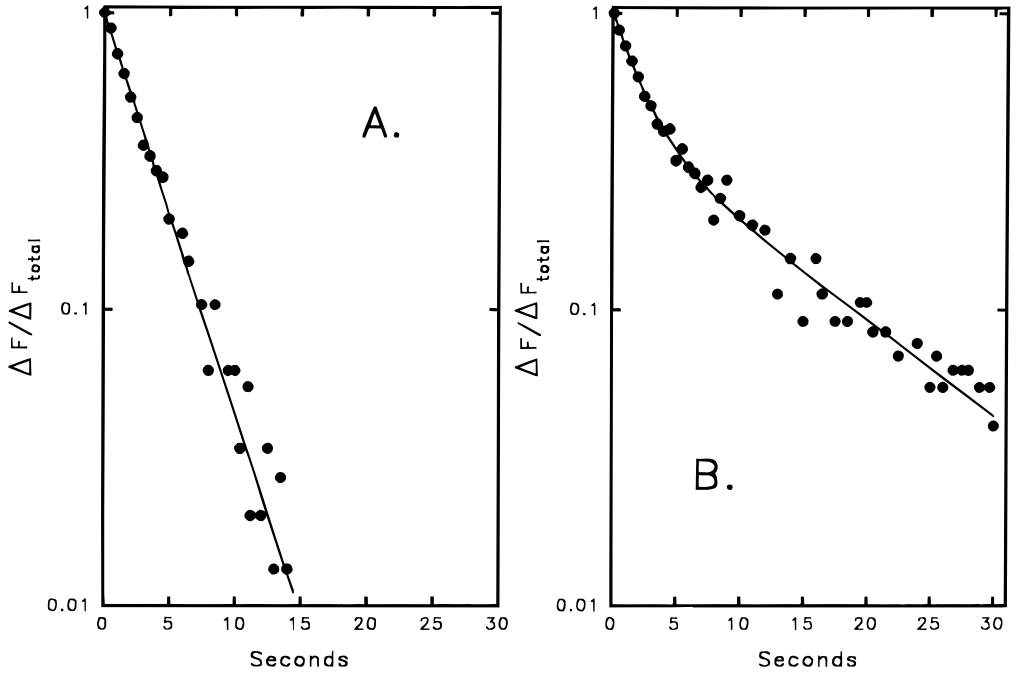

FIGURE 1: H_2DIDS/DIDS replacement reaction for wild-type band 3 subunits in control (A) and SAO (B) red cell membranes. Reactions were performed by premixing unsealed ghosts with H_2DIDS to yield concentrations of 2.2 μM band 3 and 6 uM H_2DIDS in 150 mM NaCl and 5 mM sodium phosphate (pH 7.4). Temperature = 25 °C. The sample was then mixed immediately (<1 min) in the stopped-flow with 13 μM DIDS prepared in the same buffer. Fluorescence was monitored by excitation at 280 nm and observation through a 315 nm short wavelength cutoff filter. 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 $t = 0$. Data points (1000) were collected, and the time courses were fitted to either single [$\Delta F/\Delta F_{total} = \exp(-kt)$ or double [$\Delta F/\Delta F_{total} = a_f \exp(-k_f t) + a_s \exp(-k_s t)$] exponential functions where a_f and a_s are the fractions of fast and slow phases, respectively. Control ghosts (A) gave a single exponential time course with $k = 0.31 \pm 0.05$ s^{-1}. SAO ghosts gave biphasic kinetics with $a_f = 0.6 \pm 0.05$ and $k_f = 0.4 \pm 0.1$ s^{-1} and $a_s = 0.4 \pm 0.1$ and $k_s = 0.08 \pm 0.01$ s^{-1}.

Table 1: H_2DIDS Binding to Unsealed Control and SAO Membranes

		SAO[a]	
	control	fast "off" rate	slow "off" rate
k_1 (M^{-1} s^{-1})	$(2.2 \pm 0.1) \times 10^6$	$(3.0 \pm 0.5) \times 10^6$	
k_{-1} (s^{-1})	0.5 ± 0.3	0.6 ± 0.2	
K_1 (μM)	0.23	0.2	
k_2 (s^{-1})	1.8 ± 0.3	1.7 ± 0.2	
k_{-2} (s^{-1})	0.31 ± 0.05	0.4 ± 0.1	0.08 ± 0.01
K_2	0.17	0.24	0.05
K_d (M)	33×10^{-9}	39×10^{-9}	10×10^{-9}

[a] Since there was no significant difference in H_2DIDS binding between control and SAO membranes, we assume that both populations of the wild-type monomers in SAO membranes [defined by the different values of k_{-2} (Figure 1 B)] have the same values of k_1, k_{-1} and k_2. These values and the respective values of k_{-2} were used to calculate K_d values for each wild-type band 3 population present in SAO membranes.

type subunits differ only in their H_2DIDS "off" rate constants. We have calculated overall K_d values for control membranes, and for each kinetic phase in the H_2DIDS "off" reaction for SAO membranes, using the values of k_1, k_{-1}, and k_2 given in Table 1 and using the various values of k_{-2} determined from the replacement reactions in Figure 1. These calculations show that the slow phase component in SAO cells has about a 4-fold higher H_2DIDS affinity, while the fast phase component has an H_2DIDS affinity which is essentially the same as that of control.

DBDS Release Kinetics. Sarabia et al. (1993) studied BADS binding to control and SAO membranes in matching concentrations. The total change in fluorescence was about one-half of normal for SAO membranes. However, there appeared to be no indication of heterogeneity in BADS binding. In a previous study using band 3 HT (Pro868 → Leu), we have shown that H_2DIDS binding is highly sensitive to mutations in the C-terminal subdomain of band 3 (Salhany et al., 1995b). DIDS and DBDS were not sensitive to conformational changes in this region of the protein. Thus, one possible explanation for the apparent difference between our results with H_2DIDS and the results of Sarabia et al. (1993) using BADS could be the lack of sensitivity of the latter stilbenedisulfonate to conformational changes in the C-terminal subdomain of the wild-type subunit of the SAO heterodimer. To test this hypothesis, we measured the kinetics of DBDS release from control and SAO membranes. DBDS is a close analogue of BADS (Cabantchik & Greger, 1992). Figure 2A shows the time course for the DBDS/DIDS replacement reaction in control membranes. The reaction is entirely monophasic and exponential as described previously (Salhany et al., 1994). The kinetics of the DBDS/DIDS reaction for SAO membranes is shown in Figure 2B, and was indistinguishable from control. These results and the results in Figure 1 illustrate once again the differential sensitivity between H_2DIDS and the other stilbenedisulfonates in their reversible binding to band 3 (Salhany et al., 1995b).

Effect of Chloride on the Kinetics of Covalent Binding of DIDS. We have shown that DIDS and chloride bind simultaneously to band 3 and that they interact allosterically within a ternary complex (Schopfer & Salhany, 1995). Chloride and various other anions accelerated the rate of covalent binding of DIDS to "lysine A" on band 3 (Schopfer & Salhany, 1995). The acceleration in the apparent rate

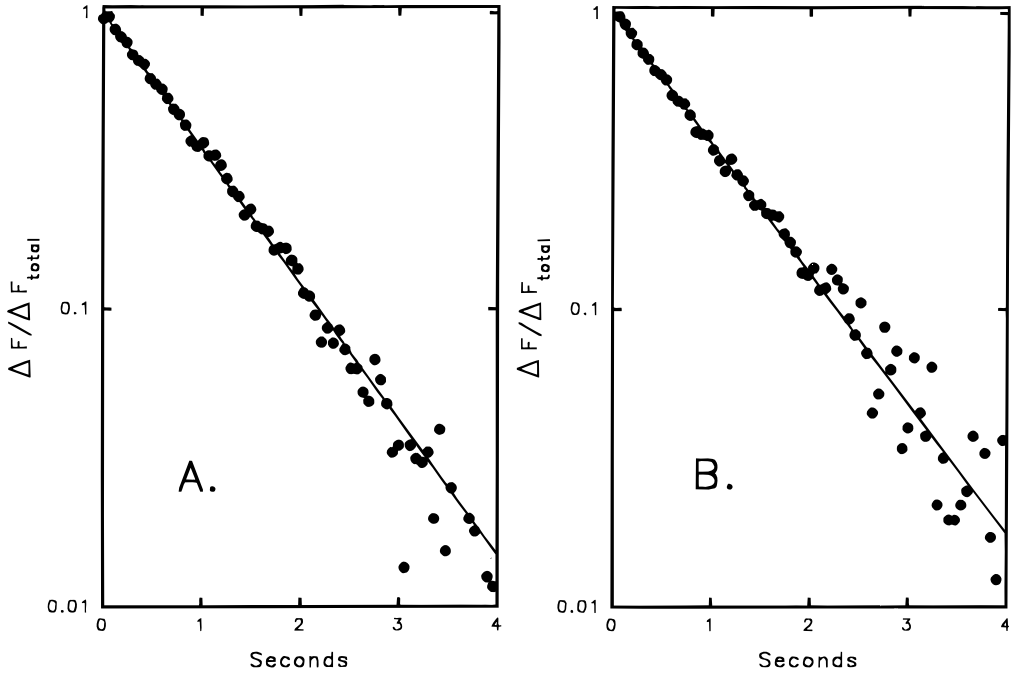

FIGURE 2: DBDS/DIDS replacement reaction for wild-type band 3 in control (A) and SAO (B) red cell membranes. Reactions were performed by premixing unsealed ghosts with DBDS to yield concentrations of 2 μM band 3 and 6 μM DBDS in 150 mM NaCl, and 5 mM sodium phosphate (pH 7.4). Temperature = 25 °C. The sample was then mixed in the stopped flow with 13 μM DIDS prepared in the same buffer. Fluorescence was monitored by excitation at 335 nm and observation of the emission through a 415 nm short wavelength cutoff filter. Data collection and analysis were as described in the legend to Figure 1. Control ghosts (A) gave a single exponential time course with a rate constant of 1.05 ± 0.01 s^{-1}. SAO ghosts showed indistinguishable kinetics with a rate constant of 1.01 ± 0.02 s^{-1}.

saturated as a function of anion concentration and gave an apparent K_d which was comparable to the K_m for chloride binding to the transport site on band 3. We have studied the effect of chloride on the kinetics of DIDS covalent adduct formation to "lysine A" on wild-type band 3 in SAO membranes. Titration of matching membrane preparations showed that the DIDS binding capacity was reduced by about one-half, consistent with the other evidence in the literature mentioned above, indicating that stilbenedisulfonates do not bind to SAO band 3. The time course of the DIDS covalent adduct reaction with wild-type band 3 was monophasic and exponential for >95% of the reaction (data not shown). Heterogeneity in chloride binding was assessed by studying the acceleration in DIDS adduct formation rate over a very wide range of chloride concentrations. This apparent rate constant is plotted versus chloride concentration in Figure 3. The data were fitted to a simple hyperbolic function. The fit showed a random distribution of residuals which is indicative of a good fit to the simple hyperbolic model. Furthermore, double reciprocal Benesi−Hildebrand plots (Benesi & Hildebrand, 1949) were linear. Thus, there appears to be no significant heterogeneity in chloride binding to the transport site on wild-type band 3 in SAO membranes.

DISCUSSION

The results of this paper show that wild-type band 3 exists in two functionally distinct states in SAO membranes, with each state having a different H_2DIDS affinity. That the proportion of the two states was about 50:50 is significant, since such a proportionality implies a specific perturbation in one-half of the wild-type subunit population. We suggest that the perturbed subunits are the wild-type subunits in the heterodimer population. Jennings and Gosselink (1995) established the existence of heterodimers and showed that the distribution of dimeric species was consistent with a 1:2:1 ratio of wild-type homodimer, heterodimer, and SAO homodimer. Thus, if the wild-type subunit in the heterodimer were functionally abnormal, we should expect to see 50:50 functional heterogeneity, and we do (Figure 1B). Furthermore, since the rate constant for the fast phase in SAO membranes (Figure 1B) is about equal to the rate constant for control (Figure 1A), we have assigned the fast phase in the reaction as H_2DIDS release from the wild-type homodimer. We have assigned the slow phase in the reaction as H_2DIDS release from the wild-type subunit in the heterodimer. Other measurements and calculations indicate that the wild-type subunit in the heterodimer has a 4-fold higher H_2DIDS affinity compared to the wild-type homodimer (Table 1).

While our results constitute the first evidence for functional heterogeneity in SAO membranes, there is other evidence in the literature which demonstrates subunit interactions between wild-type and SAO band 3. Moriyama et al. (1992) found that the calorimetric C-transition characteristic of the integral domain of wild-type band 3 (Davio & Low, 1982) was shifted to lower temperatures in SAO membranes (SAO band 3 did not have a C-transition). Covalent binding of DIDS both shifted the C-transition to higher temperature (Davio & Low, 1982) and eliminated the difference between SAO and control membranes (Moriyama et al., 1992). Moriyama et al. (1992) suggested that covalent binding of DIDS uncoupled interactions between dimers within a native state band 3 tetramer composed of wild-type and SAO band 3 homodimers. While such tetrameric effects of DIDS binding may exist in some instances (Salhany et al., 1990; Salhany 1992), we suggest that, in the case of SAO, a simpler hypothesis is that covalent binding of DIDS to the wild-type subunit alters the conformation of the heterodimer

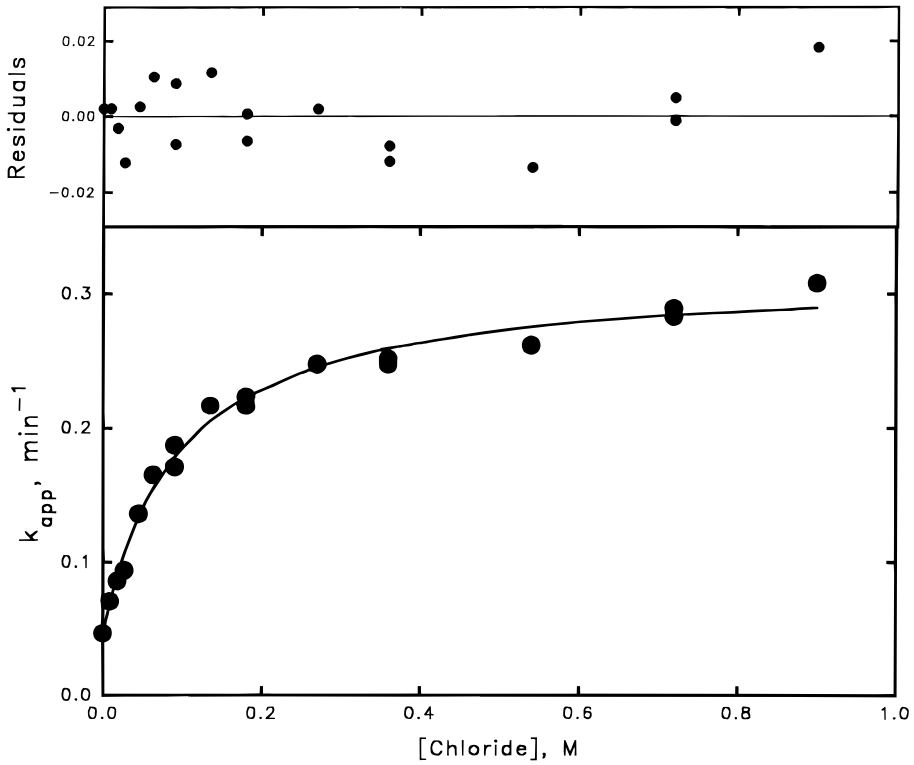

FIGURE 3: Dependence of the DIDS adduct formation rate constant (k_{app}) for SAO membranes on chloride concentration. One hundred microliters of unsealed ghosts was mixed with 900 μL of 50 mM bistris/acetate buffer (pH 7.2), containing various concentrations of sodium chloride and equilibrated at 25 °C. The reaction was initiated by addtion of 4.8 μL of 167 μM DIDS to give final concentrations of 0.8 μM DIDS and 1.1 μM wild-type band 3. The pH of all the reaction mixtures was 7.12 ± 0.03. All reactions were run to completion and apparent rate constants calculated from semi-log plots of ΔF (final fluorescence minus fluorescence at time t) versus time. Such plots were linear for all reactions to >95%. The data were fitted to a simple hyperbolic function, and an apparent K_d for chloride binding was determined to be 92 ± 11 mM. There was no evidence for heterogeneity in the data as illustrated by the random distribution of residuals.

(Jennings & Gosselink, 1995), thereby uncoupling subunit interactions within that structural unit (Salhany, 1990).

What is the significance of the altered H_2DIDS off rate constant from the heterodimer in light of the fact that neither DBDS nor DIDS showed such a difference? At first, it would seem that the H_2DIDS results should carry less weight in our considerations, owing to the larger number of negative results with related compounds. But we believe that a careful consideration of the literature and further consideration of newer results with two other band 3 mutants suggest strongly that the H_2DIDS results should be more heavily weighted in our considerations. This is because the other stilbenedisulfonates (DIDS and DBDS) have been found to be insensitive to conformational changes in the C-terminal subdomain of band 3. Lepke et al. (1976) first pointed out that H_2DIDS and DIDS have rather different characteristics, despite the fact that they behave as mutually competitive inhibitors and bind to the same site (Passow, 1986; Salhany et al., 1993). We have completed a study of band 3 HT (Pro868 → Leu) (Salhany et al., 1995b), where the differential sensitivity of H_2DIDS versus that of the other stilbenedisulfonates was dramatically illustrated. While H_2DIDS showed a 12-fold lower affinity for band 3 HT (due exclusively to an increase in k_{-2} of eq 1; i.e. the off rate constant) and showed a 10-fold slower rate of covalent adduct formation, neither DBDS nor DIDS could sense the conformational change induced by the mutation. In addition, we have recently completed a study of stilbenedisulfonate binding to band 3 Memphis variant II (Pro854 → Leu) (Hsu & Morrison, 1985; Bruce et al., 1994) where, once again, the H_2DIDS off rate constant was significantly altered (slowed) with no effect on the H_2DIDS on rate and no effect on DBDS or DIDS binding (J. M. Salhany, R. L. Sloan, and L. M. Schopfer, manuscript in preparation). Thus, H_2DIDS is clearly more sensitive to mutations within the C-terminal subdomain than is DIDS or DBDS. We believe that such differential sensitivity is related to differences in the structure of these inhibitors which cause them to assume different configurations within the stilbenedisulfonate site (Salhany et al., 1995b). DIDS differs from H_2DIDS in having an ethene linkage rather than an ethane linkage between the two phenyl rings. This difference may be expected to make DIDS more rigid than H_2DIDS.

The results discussed above suggest that the reason DIDS and DBDS do not sense conformational changes in the C-terminal subdomain of band 3 is that they interact differently with that subdomain. Thus, the failure of DIDS and DBDS to sense the conformational change in the SAO/wild-type heterodimer is not trivial. Rather, this failure directly suggests that the conformational change sensed by H_2DIDS is localized to the C-terminal subdomain of the wild-type subunit in the heterodimer. Furthermore, since the deletion mutation in SAO band 3 occurs in the N-terminal subdomain, it follows that deletion of the nine amino acids in the N-terminal part of the first α-helix perturbs a subunit contact between the N-terminal subdomain of the SAO subunit and the C-terminal subdomain of the wild-type subunit in the heterodimer. Finally, the exclusive effect of the conformational change in the heterodimer on k_{-2} of eq 1, with no effect on k_1, or k_{-1}, implies that the initial step in inhibitor binding is not changed. Rather, the stability of the H_2DIDS/band 3 complex has been increased as a result of a

perturbation to the secondary protein conformational step in eq 1. In our experience, the stilbenedisulfonate on rate constants are particularly insensitive to most changes in experimental conditions, with the exception of solubilization of membranes in $C_{12}E_8$ [poly(oxyethylene-8-lauryl ether)], but k_{-2} and the overall stability of the stilbenedisulfonate/band 3 complex are quite sensitive to such changes (Salhany et al., 1993, 1994, 1995a,b).

Are the subunit interactions sensed by H_2DIDS release likely to be involved in anion transport? More specifically, do subunit interactions involving conformational changes in the C-terminal subdomain influence the anion exchange function? The answer to this question will require determination of the three-dimensional structure of the band 3 dimer. At present, only a three-dimensional map at 20 Å resolution exists, which shows the presence of three subdomains per subunit (Wang et al., 1993) and what appears to be a central, dimeric access channel (Wang et al., 1994; Wang, 1994). Recent site-directed mutagensis studies have implicated conformational changes in the C-terminal subdomain as being apparently allosterically involved in the transport function (Mueller-Berger et al., 1995 a,b).

With regard to the SAO red blood cell, Jennings and Gosselink (1995) observed 50% reduction in cellular transport activity, measuring the kinetics at one presumably saturating sulfate concentration. On the basis of the results of Figure 3, we can suggest that the reduction in activity seen by Jennings and Gosselink (1995) is in fact due to a reduction in V_{max}, and not due to a large increase in K_m, since no heterogeneity was observed in chloride binding to the transport site for wild-type subunits in SAO membranes. A 50% reduction in the cellular V_{max} can have two explanations. (1) Monomers are independent functional units, and one-half of the monomers are functionally inactive, or (2) the dimer is the functional unit, exhibiting subunit interactions (Moriyama et al., 1992) in the heterodimer (Figure 1) such that the transport activity of the heterodimer is reduced by one-half. Then, 50% of the band 3 in such heterodimers would contribute 25% of the activity, while 25% of the wild-type homodimer would contribute the other 25%, yielding 50% cellular activity for SAO membranes. This situation is expected to lead to linear inhibition of transport in activity-labeling correlation plots for DIDS (Schofield et al., 1992a) or H_2DIDS. Even though H_2DIDS would tend to bind to the wild-type subunit of the heterodimer first, linear activity-labeling correlation plots would still be seen if the dimer were the functional unit, and if it had a V_{max} equal to one-half of that of the wild-type homodimer. Biphasic activity-labeling correlation plots would result only if the activity of the heterodimer were $<50\%$ (lag) or $>50\%$ (biphasic) of the homodimer activity. Thus, equilibrium exchange transport measurements cannot distinguish between monomeric and dimeric functional models. Other types of transport experiments, such as certain steady state (Salhany & Cordes, 1992) or transient state (Jennings, 1982; Salhany & Cordes, 1992) kinetic experiments, may be the only way to determine unequivocally whether the dimer or the monomer of band 3 is the basic functional unit for anion exchange.

In summary, our results with SAO membranes add to a growing body of evidence which demonstrates the existence of subunit interactions in the band 3 system [reviewed by Jennings and Gosselink, (1995)]. However, whether subunit interactions play a significant or essential role in anion exchange remains to be established definitively.

ACKNOWLEDGMENT

We thank Dr. David Liu for supplying a unit of SAO blood for this study and Ms. Karen Cordes for preparing the figures.

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BI952411B