Transient-State Kinetic Evidence for Intersubunit Allosteric Hysteresis during Band 3 Anion Exchange†

James M. Salhany* and Karen A. Cordes

The Veterans Administration Medical Center and The Departments of Internal Medicine and Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska 68105

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ABSTRACT: On-line, dual-wavelength stopped-flow spectroscopy was used to follow continuously, the uptake of dithionite ($S_2O_4^{2-}$) into human erythrocyte resealed ghosts. We describe the general characteristics of a pre-steady-state transient phase measured both with chloride and sulfate as co-anions. We then quantitatively characterize the dithionite concentration dependence of the amplitude factor and relaxation constant (k_R) for the transient phase measured in sulfate medium. We also study the dithionite dependence of the steady-state velocity. Our results suggest that dithionite induces a slow conformational change in band 3 leading to hysteresis in the transport velocity. As many as 25 turnovers of the transport cycle per monomer can occur prior to attainment of steady state. Both k_R and the amplitude factor for the transient phase were dithionite concentration dependent. In addition, the steady-state velocity showed apparent negative cooperativity. To discriminate between monomeric and intersubunit allosteric hysteresis, we performed a series of critical kinetic tests with cells labeled partially with 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS). Coverage of 85% of the band 3 monomer population with DIDS caused k_R to decrease about 10-fold, the dithionite concentration dependence of k_R to change significantly, and the apparent negative cooperativity for the steady-state velocity to be eliminated. These results suggest that intersubunit allosteric hysteresis makes a significant contribution to dithionite transport by band 3.

Human erythrocyte band 3 facilitates anion exchange and is involved in the binding of cytoskeletal and cytosolic proteins to the red cell membrane (Passow, 1986; Salhany, 1990). An important question about the function of this porter concerns the molecular basis for the observation of non-Michaelian kinetics in the transport of both divalent and monovalent anions (Salhany, 1990). We originally demonstrated the presence of apparent negative cooperativity in an influx exchange experiment using dithionite ($S_2O_4^{2-}$) (Salhany & Swanson, 1978; Salhany & Gaines, 1981). Subsequently, Schnell and Besl (1984) showed that sulfate and phosphate gave similar patterns in influx exchange experiments. We investigated the origin of cooperativity in dithionite transport by showing that partial labeling of the band 3 population with SITS[1] resulted in hyperbolic kinetic patterns for the remaining active monomer (Salhany & Swanson, 1978). Since titration of all copies of band 3 with DIDS inhibits 99.9% of dithionite transport (Salhany et al., 1987), we suggested that the apparent negative cooperativity and the conversion from negative cooperative to hyperbolic kinetic patterns are a consequence of some type of intersubunit allosteric interaction mechanism (Salhany & Swanson, 1978; Salhany, 1990, 1992). Within the context of this hypothesis, monomers of band 3 oligomers (Salhany et al., 1990; Casey & Reithmeier, 1991) function as transport units (Lindenthal & Schubert, 1991) with intersubunit homotropic allosterism serving to modulate the monomeric activity (Monod et al., 1965; Koshland et al., 1966; Salhany, 1990).

As a general hypothesis, porter allosterism can explain many aspects of band 3 transport kinetics (Salhany, 1990). However, it is not clear why apparent negative cooperativity should be observed in the influx exchange experiment (Salhany & Swanson, 1978; Salhany & Gaines, 1981; Schnell & Besl, 1984) while positive cooperativity followed by partial substrate inhibition is observed when anion exchange is measured at equilibrium (Schnell & Besl, 1984; Stadler & Schnell, 1990). It may be possible to reconcile these apparently contradictory findings by suggesting that a hysteretic transition (Frieden, 1979; Neet & Ainslie, 1980) occurs in band 3 during the influx exchange experiment due to the rapid imposition of an inwardly directed anion gradient. The possibility of band 3 hystersis is suggested by our earlier finding of a slow pre-steady-state transient in the time course of dithionite uptake (Salhany & Swanson, 1978; Salhany & Gaines, 1981). Such transients and negative cooperativity are often related indications of hysteretic transitions (Frieden, 1979; Neet & Ainslie, 1980). In the present study, we investigate the possibility that the transient phase, and the apparent negative cooperativity of dithionite transport, arise from band 3 hysteresis and that the particular type of band 3 hysteresis is intersubunit allosteric hysteresis (Mouttet et al., 1974; Kurganov et al., 1976).

EXPERIMENTAL PROCEDURES

A. Sample Preparation and Measurement of Transport. Materials, methods, and the theory for the dithionite transport assay have been described previously (Salhany & Swanson, 1978; Salhany & Gaines, 1981; Salhany, 1990). We use metHb resealed ghosts prepared as described (Salhany & Swanson, 1978). Red cells were lysed 1:5 and then resealed using the same hemolysate to yield a final internal hemoglobin concentration of about 4 mM.

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* To whom correspondence should be sent: Research Service, VA Medical Center, 4101 Woolworth Ave., Omaha, NE 68105.

[1] Abbreviations: SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; bistris, *N*,*N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; tris, tris(hydroxymethyl)aminomethane; CHES, 2-(*N*-cyclohexylamino)ethanesulfonate; CAPS, 3-(cyclohexylamino)-1-propanesulfonate; NBD-taurine, *N*-[(2-aminoethyl)sulfonyl]-7-nitrobenz-2-oxa-1,3-diazole.

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Two buffer systems were used. Buffer A contained 1 mM Mg and 25 mM each of bistris, tris, CHES, and CAPS, with either 90 mM sulfate or 150 mM chloride present at the time of resealing. Buffer B contained 200 mM sucrose, 1 mM $MgSO_4$, and 50 mM bistris, adjusted to pH 7 with sulfuric acid to yield about 6 mM total sulfate (Salhany & Gaines, 1981).

Buffer A was used to study the pH dependence of transport. metHb resealed ghosts were washed four times in buffer A adjusted to the appropriate pH (1:50 dilution with each wash). Included was one 15-min room-temperature equilibration step. Cells were suspended in the respective buffer for 0.5 h at room temperature prior to the transport assay. The absorbance of metHb was used to confirm the change in intracellular pH (Antonini & Brunori, 1971).

Buffer B was used to study the dithionite dependence of the transient and steady-state kinetic parameters. The osmotic pressure was kept nearly constant by substituting dithionite for sucrose (Salhany & Gaines, 1981). In all cases, the cells contained 200 mM sucrose and 50 mM bistris, thus offering intracellular buffering capacity for both pH and osmotic pressure. We have noted previously that variation in sucrose concentration has no effect on transporter function (Salhany & Gaines, 1981).

The dual-wavelength stopped-flow spectrophotometric transport assay was described previously (Salhany & Swanson, 1978; Salhany & Gaines, 1981). Typically, 250 data points were collected for each run at both a reaction wavelength and a reaction isosbestic for metHb and deoxyHb. Split time-base data collection was employed to improve the accuracy of each progress curve in the initial portion of the reaction.

B. Analysis of Kinetic Transients Related to Hysteretic Behavior. Any burst or lag in product formation involving a single-exponential decay which is followed by a linear steady state can be analyzed using one of two types of equations to describe the data through the linear portion of the progress curve (entire progress curves are not analyzed). Equation 1 describes the change in instantaneous velocity (V_t) as a function of time beginning at some initial velocity (V_0) and culminating in a steady-state rate V_s:

$$V_t = V_s - (V_s - V_0) \exp(-k_R t) \quad (1)$$

where k_R is the observed rate for the transition between the initial state and the final steady state. The integrated form of this equation is

$$Q = [(V_0 - V_s)/k_R]\{1 - \exp(-k_R t)\} + V_s t \quad (2)$$

where Q is the concentration of product formed during the reaction. While eq 1 can be used to describe the transient approach to a steady state, Neet and Ainslie (1980) stated explicitly that "The more straightforward and statistically more proper procedure is to use a parametric fit of the product vs time data directly rather than to fit the velocities that were derived from those data". Thus, for purposes of fitting, we parameterize eq 2 by setting $[(V_0 - V_s)/k_R] = P$ to yield

$$Q = P\{1 - \exp(-k_R t)\} + V_s t \quad (3)$$

From eq 3, it is obvious that when $V_0 > V_s$ (burst kinetics), P will be positive, and when $V_0 < V_s$ (lag periods), P will be negative. In all experiments, Q is directly proportional to $\Delta A/\Delta A_{tot}$ for the reduction of intracellular metHb by extracellular dithionite (Salhany & Swanson, 1978). We fit eq 3 to each time course when it applies (see below for exceptions) in order to obtain P, k_R, and V_s. The equation was fit to the data using the Enzfitter program (Leatherbarrow, 1987).

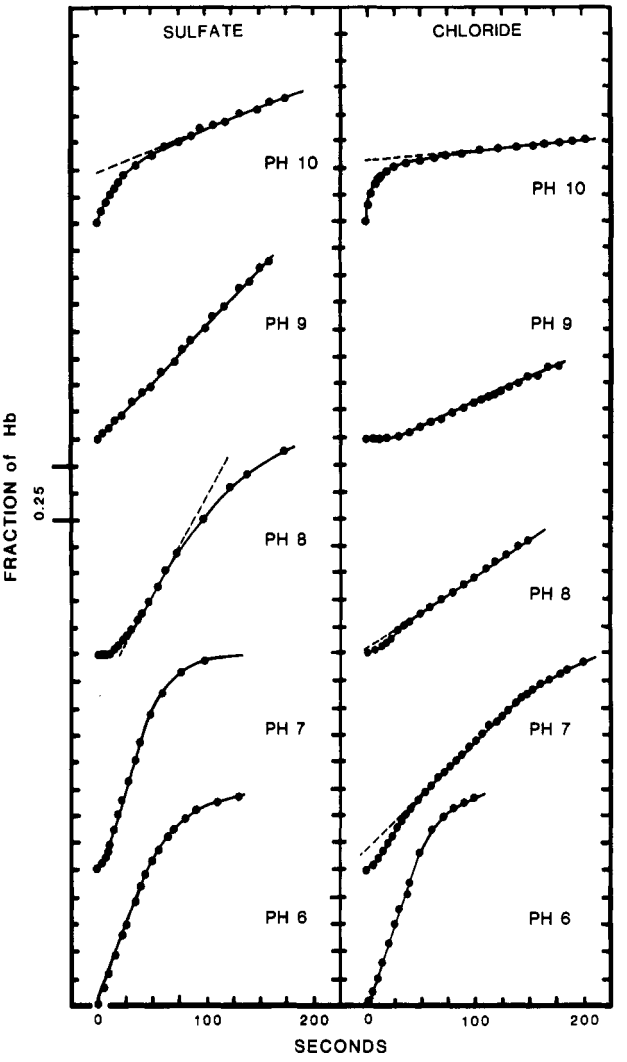

FIGURE 1: pH and co-anion dependence of the time course of dithionite uptake into resealed ghosts. Cells were in buffer A plus either 90 mM sulfate or 150 mM chloride at 1.6% hematocrit. Dithionite was 10 mM in all cases. The temperature was 31 °C. Ghosts were resealed as described in the text so as to contain the same buffer inside and outside. The y-axis is marked in increments of 0.25 unit of deoxyHb per two hatch marks. Each time course proceeds from 0 to 1 with respect to deoxyHb formation. The dashed lines indicate the steady-state portion of the progress curve.

Velocities were calculated as described previously (Salhany & Swanson, 1978).

RESULTS

A. General Characteristics of Dithionite Uptake Kinetics. 1. pH and Co-Anion Dependence of the Kinetics: Measurements in Sulfate Medium. There is no observable transient when transport is measured at pH 6 in sulfate (Figure 1). Increasing the pH causes a lag to develop between pH 7 and 8. At pH 9, no transient is observed, while at pH 10, a "burst" phase is seen which lasts for about 50 s, reaching a well-defined, linear steady state. The simplest qualitative interpretation of the sulfate results, within the context of eq 2, is to suggest that at pH 6 there is no transient phase because k_R is too fast to observe, while raising the pH slows k_R. The slowing of k_R is supported by quantitative fits of eq 3 to the data at pH 7, 8, and 10. The results, expressed as $1/k_R$ vs pH, are shown in the top portion of Figure 2. The interconversion from lag to burst between pH 7 and 10 in sulfate implies a change in sign for the amplitude factor of eq 3. At pH values between 7 and 8, $V_0 < V_s$, yielding lags, while at

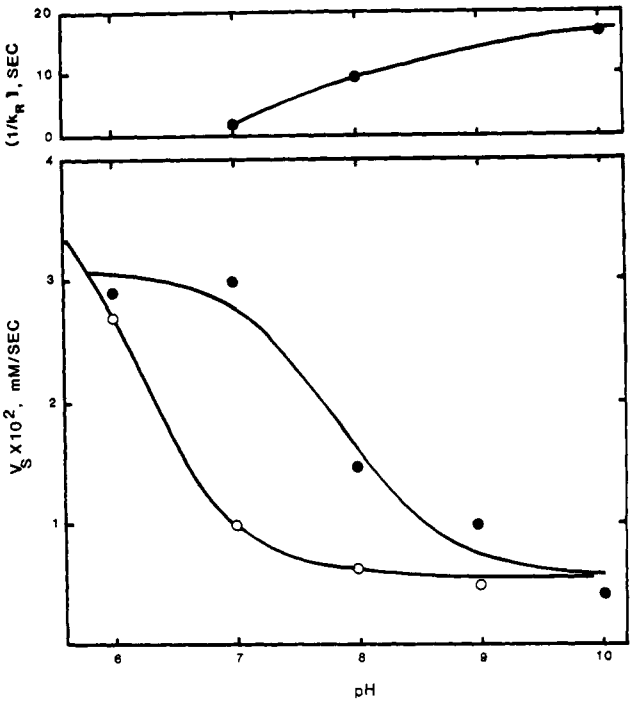

FIGURE 2: pH dependence of k_R and V_s for dithionite uptake into resealed ghosts. Experimental conditions were the same as in Figure 1. (Top) Plot of $1/k_R$ calculated for the sulfate data in Figure 1 using eq 3 of the text. (Bottom) The steady-state velocities (V_s) are shown for chloride and sulfate as co-anions. The steady-state velocities were calculated based on the slope of the linear portion of the progress curve after the transient (Salhany & Swanson, 1978). A single pK titration curve was fit to the velocity data using the following parameters: (chloride, open circles) pK = 6.14 ± 0.16, upper limit (4.24 ± 0.56) × 10^{-2} mM/s, lower limit (0.56 ± 0.04) × 10^{-2} mM/s; (sulfate, closed circles) pK = 7.8 ± 0.3, upper limit (3.2 ± 0.3) × 10^{-2} mM/s, lower limit (0.57 ± 0.26) × 10^{-2} mM/s.

pH values greater than 9, $V_0 > V_s$, accounting for bursts. While V_0 may or may not increase with increasing pH, V_s definitely decreases (Figure 1; also see below). Our data base is not sufficient to establish the pH dependence of V_0 quantitatively.

Measurements in Chloride Medium. While generally similar results are obtained for dithionite transport measured in chloride (Figure 1), the transient phase in this medium is much more complex (Figure 1). Although no transient is observed at pH 6, raising the pH produces a complex burst phase at pH 7 and 8 which cannot be characterized using eq 3. This type of transient is best described as a "succession of transients" going from an initial lag to a burst, followed by a slower linear steady state (Figure 1). Such a succession of transients is indicative of a complex transport mechanism (Wierzbicki et al., 1990). Raising the pH above 8 eliminates this complexity, leading to a simple lag or burst at pH 9 and 10, respectively.

This comparison between sulfate and chloride suggests that the transient phase is a true feature of the transporter, since the samples only differ experimentally in that one contains a physiological concentration of sodium chloride while the other contains a nearly osmotically equivalent amount of sodium sulfate. Furthermore, it is well established that raising the pH slows considerably the steady-state transport velocity for divalent anions (Passow, 1986; Salhany, 1990). This type of pH dependence is evident with both chloride and sulfate as co-anions (Figure 1). However, the final steady-state velocity (i.e., the slope of the linear portion of the progress curve) is considerably slower in chloride due to a lower apparent pK (Figure 2). A similar difference in apparent transport pK was observed by Eidelman and Cabantchik (1983) for NBD-taurine efflux from symmetric chloride cells vs symmetric sulfate cells.

2. Temperature Dependence of the Kinetics. We have studied the temperature dependence of k_R in buffer B (data not shown). At 42 °C, no transient was observed. Between 24 and 32 °C, we observed an increase in k_R yielding a Q_{10} of about 2.9, with a ΔH^* of about 20 kcal/mol. Such a high enthalpy of activation is not consistent with diffusion processes such as unstirred layer effects as an explanation for the lag (Verkman & Dix, 1984). Rather the data suggest that k_R reflects a property of the transporter protein itself, either substrate binding, protein conformational change, or some combination of the two processes.

3. Calculation of the Number of Turnovers of the Transport Cycle during the Transient Phase. To identify the physical basis for the transient phase, it is necessary to consider whether that phase represents a single turnover of the transport cycle (i.e., a catalytic transient) or whether multiple turnovers occur prior to attainment of the linear steady state. At pH 10 in sulfate, the burst comprises about 25% of the total reaction. At the hematocrit used (1.6%), a total of 64 μM metHb is reduced (i.e., 4000 μM heme for packed resealed ghosts times 1.6% for the dilution of cells). With a stoichiometry of two hemes reduced for every $S_2O_4^{2-}$ that enters the cell (Salhany & Swanson, 1978), we have a total of 32 μM dithionite entering the cell during the period of observation. The amount of dithionite entering during the burst phase is then 25% of 32 μM or 8 μM. There are about 20 μmol of band 3 monomer/L of packed red cells (Fairbanks et al., 1971). This would give about 0.32 μmol of band 3/L of cells (i.e., 0.32 μM) at 1.6% hematocrit. Thus, the burst phase represents about 25 turnovers of the transporter in this case. Such calculations suggest strongly that the transient phase is not catalytic.

B. Dithionite Dependence of the Transient-Phase Kinetic Parameters and of the Final Steady-State Velocity Measured in Sulfate Medium. If the transient phase is too large to be catalytic, it may be a ligand-induced hysteretic effect. Such transients are expected to have substrate-dependent kinetic parameters (Frieden, 1979; Neet & Ainslie, 1980). We have studied the dithionite concentration dependence in buffer B where the transient occurs in the form of a lag at dithionite concentration above 1 mM (see below). Figure 3 shows the dithionite concentration dependence of the transport time course (Figure 3A) and of the steady-state velocity (Figure 3B). No transient phase is detectable at 1 mM dithionite (Figure 3A, line 1). Raising the dithionite concentration reveals a lag period (Figure 3A, curves 2 and 3). This transition shows that the lag is dithionite concentration dependent. It also rules out the possibility that the transient involves a "second consumer" of intracellular dithionite. Such a second consumer hypothesis would require the observation of lag at all dithionite concentrations.

The steady-state velocity extracted using eq 3 shows apparent saturation behavior (Figure 3B), which agrees with our previous work (Salhany & Swanson, 1978). However, inspection of Lineweaver–Burk plots of the data at two pH values (Figure 4A) reveals that there is a distinct biphasic or apparent negative cooperative substrate dependence for the steady-state velocity. These findings confirm our previous work showing a skewed or shallow biphasicity in the Lineweaver–Burk plot at neutral pH but a strongly curving biphasicity at alkaline pH (Salhany & Swanson, 1978; Salhany & Gaines, 1981).

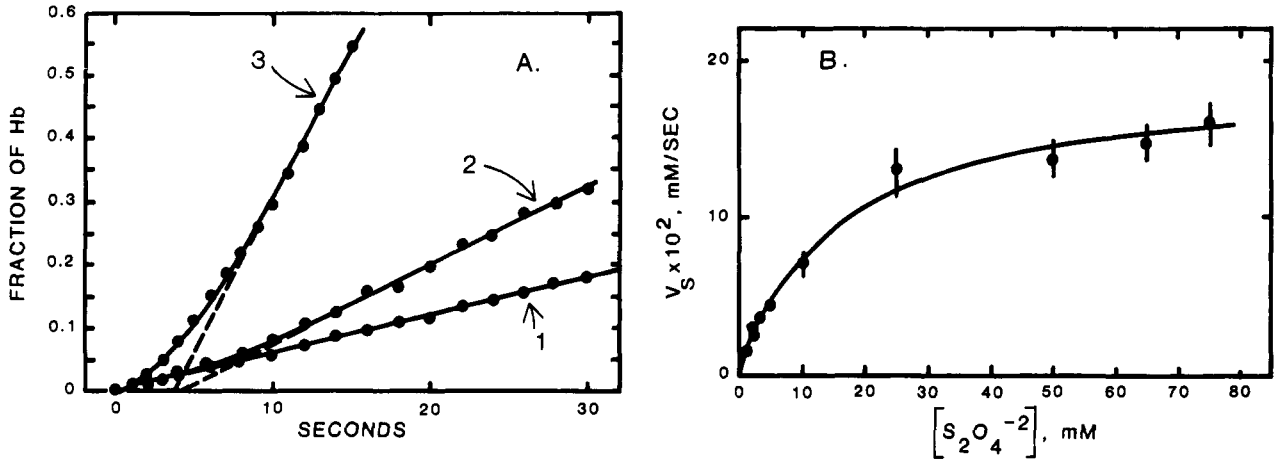

FIGURE 3: Concentration dependence of the time course (A) and steady-state velocity (V_s) (B) for dithionite uptake into resealed ghosts. Cells were in buffer B. Sucrose and dithionite were exchanged to maintain near isoosmotic conditions. Temperature, 32 °C. Equation 3 was used to fit the data (except line 1). The concentration of dithionite for each time course in (A) was as follows line 1, 1 mM; line 2, 2 mM, and line 3, 25 mM.

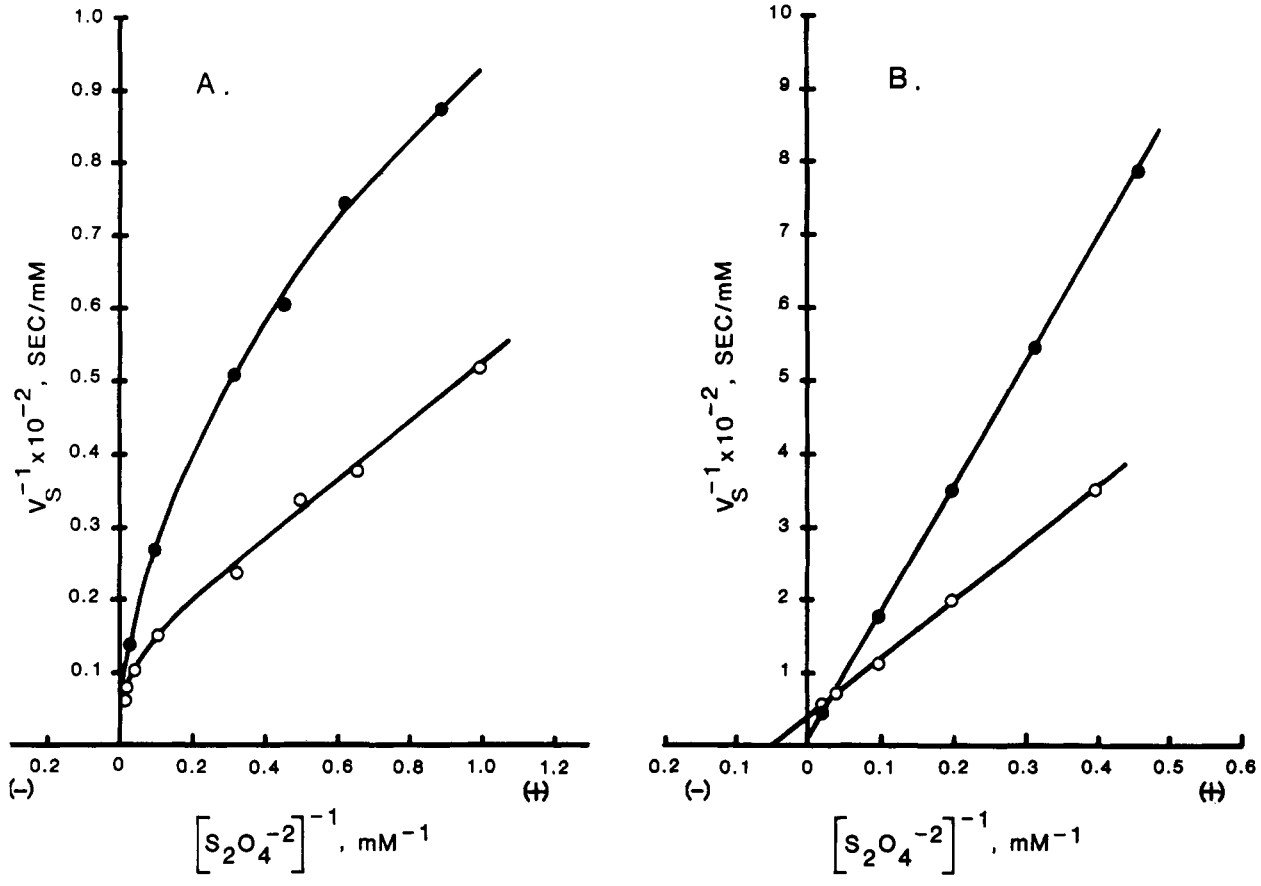

FIGURE 4: Lineweaver–Burk plots for control resealed ghosts (A) and for ghosts partially inhibited with DIDS (B). Ghosts were either in buffer B at pH 7 (open circles) or in 200 mM sucrose plus 50 mM tris at pH 8 (closed circles). Resealed ghosts were labeled with DIDS as described (Salhany et al., 1991). Because of the presence of tris in the pH 8 buffer, these cells were labeled with DIDS prior to making resealed ghosts. At pH 7, cells were labeled after resealing. Previous studies have shown that osmotic hemolysis and resealing does not change the final percent inhibition caused by DIDS labeling of intact cells (Salhany et al., 1987). Control ghosts (A) show the typical pH-dependent biphasic or apparent negative cooperative behavior seen previously (Salhany & Swanson, 1978; Salhany & Gaines, 1981), while 85% DIDS labeling of the band 3 monomers (B) eliminates the negative cooperativity (note the difference in the y-axis scale between panels A and B).

The substrate dependence of k_R is shown in Figure 5A. Over the initial substrate concentration range between zero and 25 mM, k_R decreases with increasing dithionite. At higher substrate concentration, k_R clearly increases with increasing dithionite. Thus, a minimum is defined for the substrate dependence of k_R for dithionite transport into resealed ghosts.

The amplitude of the transients is also substrate dependent (Figure 5B). It appears to show non-Michaelian behavior in that a fit of the data to a hyperbolic function resulted in large systematic deviations in a plot of the residuals (not shown). Furthermore, an apparent nonhyperbolic dithionite dependence for the amplitude factor is expected on the basis of eq 2. At values above about 25 mM, the final steady-state velocity begins to saturate (Figure 3B). Thus, $V_0 - V_s$ is expected to remain nearly constant over that concentration range. Under these circumstances, the amplitude factor P in eq 3 will vary inversely with variation in k_R. Since k_R increases at dithionite concentrations above 25 mM (Figure 5A), the amplitude

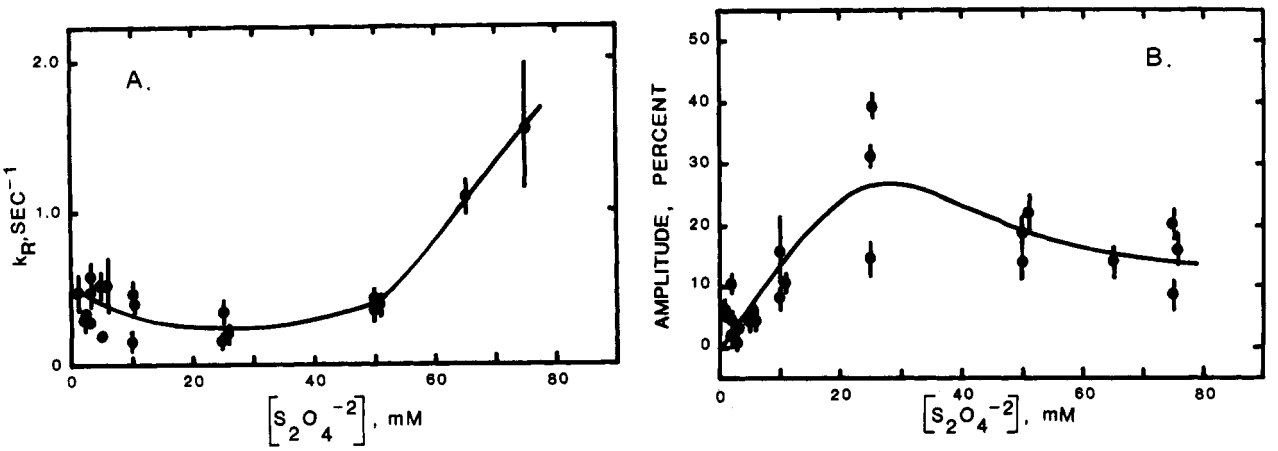

FIGURE 5: Concentration dependence of the relaxation constant k_R and of the amplitude factor for the transient lag phase in dithionite uptake by resealed ghosts. Conditions were the same as in Figure 3. The values of k_R and the amplitude factor P were determined based on fitting eq 3 to the time course data. (A) shows the dithionite dependence of k_R. The k_R values follow a decreasing function between 0 and 25 mM dithionite, while above 25 mM, k_R increases. Thus, a minimum is defined in this plot. (B) is the corresponding plot for the amplitude factor P from eq 3, expressed as a percent absorbance change for the transient phase compared to the total change in absorbance for a given reaction. These data are suggestive of an apparent maximum, since a hyperbolic function did not give a good fit to the data based on an analysis of a plot of the residuals.

can be expected to decrease above 25 mM, as is observed (Figure 5B). The amplitude reaches an apparent maximum of about 25% of the total reaction at 25 mM dithionite. This large size further supports our calculations given above, suggesting that the transient phase involves multiple turnovers of the transport cycle.

C. Evidence Suggesting That Subunit Interactions Contribute Significantly to the Transient Phase and to the Steady-State Velocity of Dithionite Uptake. In order to demonstrate the existence of subunit interactions, we partially labeled the band 3 monomer population covalently with DIDS and then studied the kinetic properties of the remaining active monomers. DIDS is an apparent competitive inhibitor of anion exchange which binds to band 3 monomer with a 1:1 stoichiometry (Jennings & Passow, 1979). We have shown previously [see Figure 2 of Salhany et al. (1987)], and confirm here (data not shown), that covalent binding of DIDS to all copies of band 3 inhibits >99% of dithionite transport. Inhibition is directly proportional to labeling of the band 3 monomers. Thus, one may use the percent inhibition of the steady-state velocity as a measure of the fraction of the band 3 monomer population bound by DIDS (Salhany, 1990). The logical basis for our experimental approach is the premise that if the transient phase has no contribution from subunit interactions, inhibition of one monomer by DIDS should not alter the value of k_R for the remaining monomers, nor should the dithionite dependence of k_R change. Finally, the apparent negative cooperative kinetic pattern should also remain unchanged if that characteristic does not involve interactions betwen subunits.

Figure 6 shows typical time courses for dithionite uptake by control ghosts, and by ghosts covalently labeled with sufficient DIDS to inhibit about 85% of the steady-state velocity. It is obvious from visual inspection that it takes longer to reach steady state for DIDS-labeled cells than for control. Curve fitting of eq 3 to the data confirms that partial covalent labeling of the band 3 population greatly decreases the value of k_R for the remaining monomers (k_R for control 0.46 s^{-1}; k_R for 85% inhibited cells 0.035 s^{-1}).

Figure 7, shows correlation plots of k_R vs fractional inhibition of band 3 anion exchange by DIDS at various dithionite concentrations. A similar plot was constructed for the amplitude of the transient phase (Figure 8). At dithionite concentrations of 10 mM or less, there is a linear decrease in

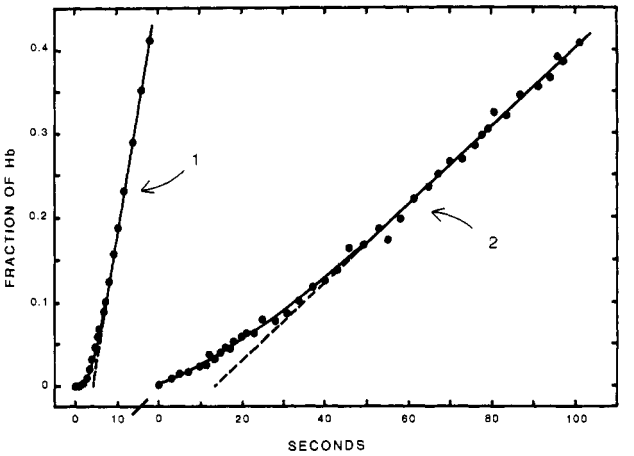

FIGURE 6: Time course of dithionite uptake for control ghosts and for ghosts partially labeled with DIDS to inhibit 85% of the transport activity. Ghosts were in buffer B, the temperature was 32 °C, and the concentration of dithionite was 10 mM. The curves are drawn based on fits of eq 3 to the data using the following parameters: (line 1, control) $P = -0.11 \pm 0.01$, $k_R = 0.46 \pm 0.07$ s^{-1}, $V_s = 0.06$ mM/s; (line 2, DIDS treated) $P = -0.15 \pm 0.002$, $k_R = 0.035 \pm 0.001$ s^{-1}, $V_s = 0.009$ mM/s.

k_R with increasing DIDS inhibition (Figure 7). Above 10 mM dithionite, there is a tendency for k_R to decrease nonlinearly (Figure 7). The reduction in k_R by partial inhibition of cells with DIDS (Figure 7) suggests a role for subunit interactions in the kinetic properties of band 3.

The amplitude factor remains nearly constant below 25 mM dithionite, while above that value it also decreases nonlinearly (Figure 8). Based on the definition of the amplitude factor in eq 3, the lack of significant change below 25 mM dithionite with increasing binding of DIDS [despite significant decreases in k_R (Figure 7) and V_s (Salhany et al., 1987)] implies that $V_0 - V_s$ is decreasing in proportion to the decrease in k_R. Thus, the amplitude factor P should remain constant, as is observed. However, above 25 mM dithionite, $V_0 - V_s$ must be decreasing faster than k_R because the amplitude factor is decreasing (Figure 8 and eq 2). Since the incremental decrease in V_s with increasing binding of DIDS is the same at all dithionite concentrations used here (data not shown), the decrease in the amplitude factor must be reflecting the slower, nonlinear decrease in k_R seen at high dithionite (Figure 7). These general comparisons demonstrate that our quan-

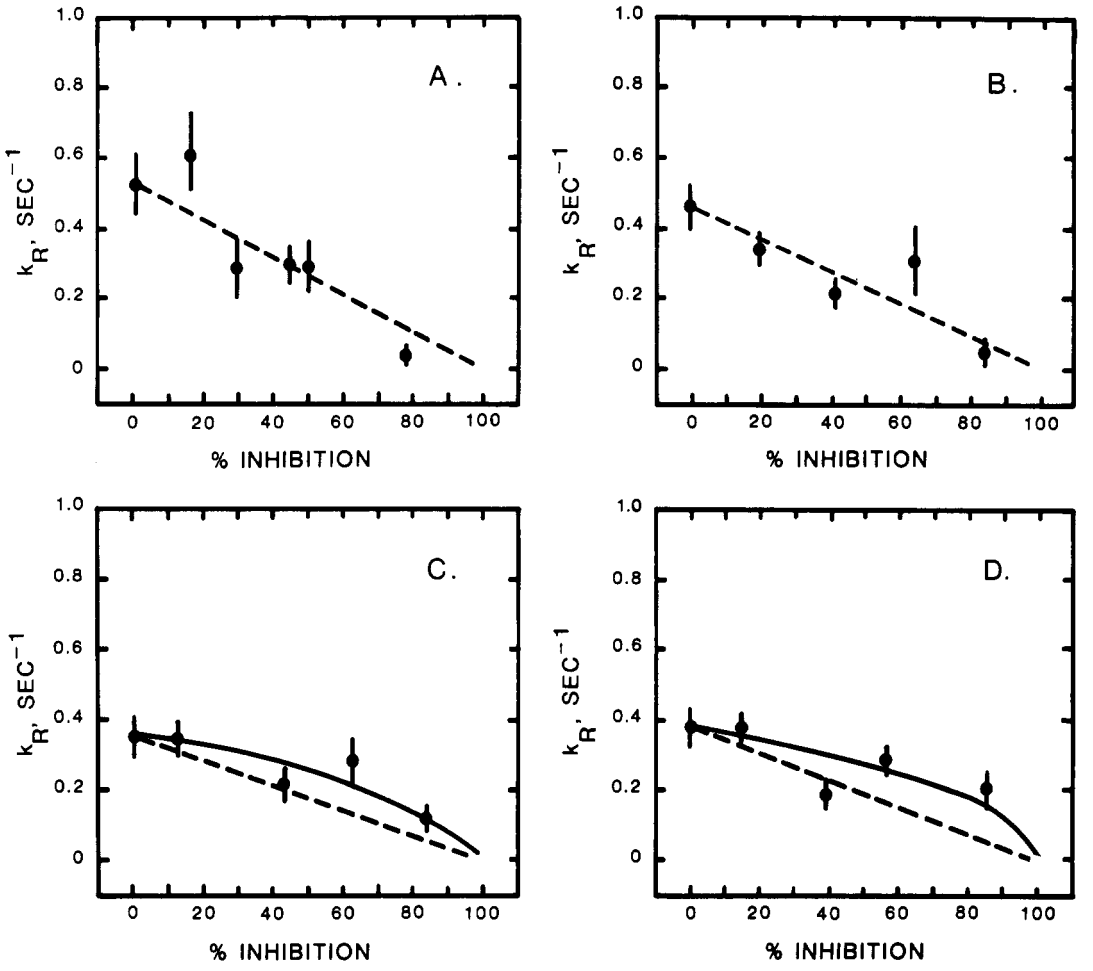

FIGURE 7: Correlation between k_R and the fractional inhibition of the steady-state velocity of band 3 anion exchange by DIDS measured at various dithionite concentrations. These experiments were performed under the conditions described in Figure 6. Equation 3 was used to fit the time courses. Percent inhibition was calculated from the steady-state velocity and was linearly related to the fraction of DIDS bound to the band 3 monomer population (Salhany, 1990; Salhany et al., 1987). The dashed lines are 1:1 correlation lines. The solid lines are drawn by hand and have no theoretical significance. Concentration of dithionite for each panel: (A) 5, (B) 10, (C) 25, and (D) 50 mM.

titative analysis of the various progress curves forms a self-consistent data set.

Additional evidence that subunit interactions are involved in band 3-mediated dithionite transport includes experiments on the dithionite dependence of V_s and k_R for the remaining active monomers, after inhibiting 85% of the monomer population by covalent binding of DIDS. Figure 4B shows Lineweaver–Burk plots for dithionite uptake by cells whose transport activity was 85% inhibited by DIDS (note the 10-fold difference in the *y*-axis scale between DIDS-treated and control). Whereas control ghosts show apparent negative cooperativity at both pH 7 and pH 8 (Figure 4A), no cooperativity is detectable when 85% of the monomers are inhibited by DIDS. The conversion of the substrate dependence of the steady-state velocity from apparent negative cooperative to Michaelian patterns (Figure 4) suggests that a change in the mechanism of anion exchange has occurred with partial labeling of cells with DIDS. These results extend our previous findings with SITS (Salhany & Swanson, 1978), and they show that the steady-state velocity is also influenced by some type of interaction between the subunits of band 3.

Figure 9 illustrates the dithionite dependence of k_R for cells with about 85% of the band 3 monomers inhibited by DIDS. Comparison of Figure 9A with Figure 6 shows that the characteristics of the transient phase depend both on the fraction of DIDS inhibition (Figure 6) and on the concentration of dithionite (Figure 9A). As dithionite concentration increases, the transient phase for DIDS-labeled cells becomes smaller in amplitude (Figure 9A). This is opposite to observations using control cells where raising dithionite promoted the lag (Figure 3A). Also different is the substrate dependence of k_R (Figure 9B). Instead of showing a minimum as in control cells (Figure 5A), DIDS-labeled cells show a net increase in k_R with increasing dithionite (Figure 9B). The line drawn in Figure 9B has a nonzero intercept, suggesting that the relaxation process is reversible (Halford, 1972). While saturation of k_R is expected at high dithionite, we cannot tell if k_R saturates because the amplitude of the transient phase decreases to zero (Figure 9A).

DISCUSSION

We have examined thoroughly a pre-steady-state transient phase which occurs during dithionite transport in resealed ghosts. The characteristics of the transient phase depend on pH and on the type of co-anion present during the assay (Figures 1 and 2). The initial half of the progress curve can be characterized quantitatively using an equation describing a single exponential plus a linear steady-state term when transport is measured in sulfate medium. Under these conditions, we saw a high enthalpy of activation for k_R, thus ruling out diffusion-limited processes as an explanation for the transient phase. We propose that the transient phase is due to some type of slow, dithionite-induced change in the system leading to hysteresis in the transport velocity. The amplitude of the transient was too large to be catalytic (Figures 1 and 5B), while the relaxation constant k_R showed a quadratic

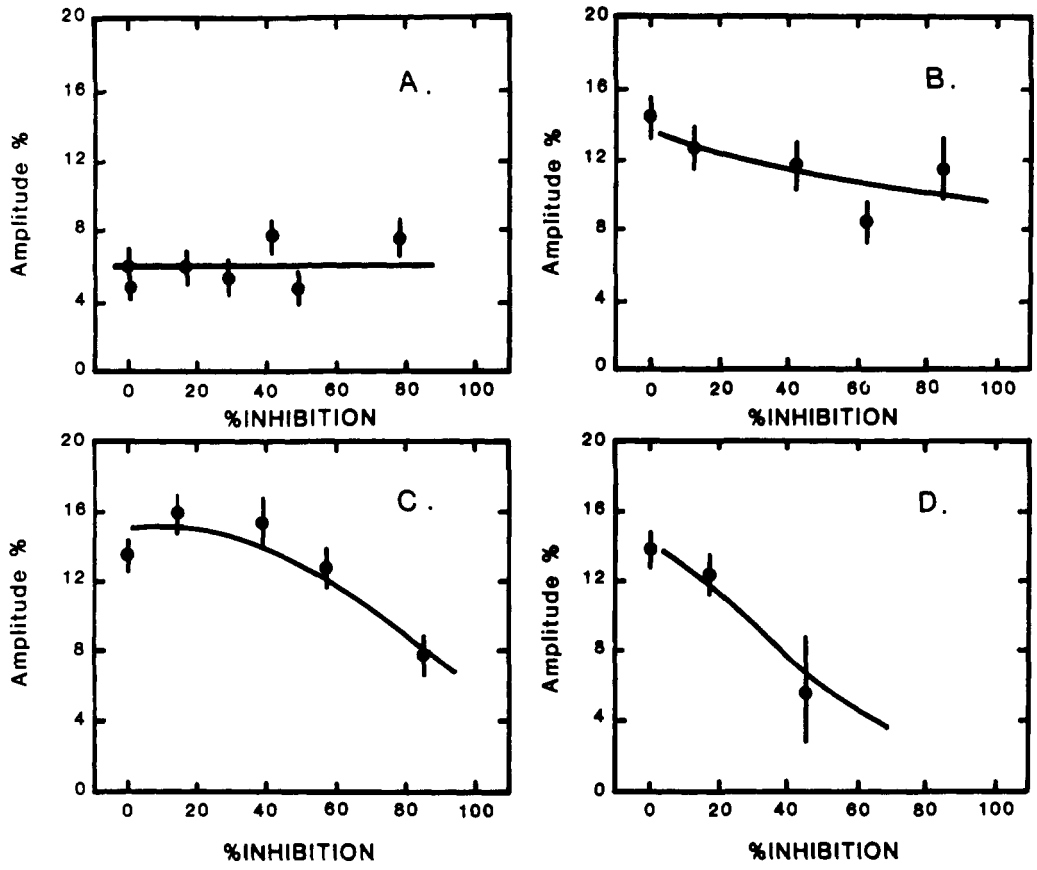

FIGURE 8: Plots of the amplitude of the transient phase in dithionite uptake vs the percent inhibition of the steady-state velocity by DIDS. The amplitude is expressed as the absolute value of P from eq 3 times 100 (i.e., as percent of the total absorbance change). Experimental conditions were the same as in Figure 6. Concentration of dithionite for each panel: (A) 5, (B) 25, (C) 50, and (D) 65 mM.

substrate dependence (Figure 5A). These are expected characteristics for an hysteretic system (Frieden, 1979; Neet & Ainslie, 1980).

What is the physical basis for hysteresis in the dithionite transport velocity? Several factors can potentially explain a hysteretic response of a system to the rapid addition of a substrate (Frieden, 1979). The possibility of slow anion binding or release steps on band 3 seems unlikely as an explanation since anion binding to band 3 is too rapid to explain our transient kinetics (Falke et al., 1984; Dix et al., 1986; Verkman et al., 1983). Band 3 association/dissociation as a possible explanation for hysteresis also seems unlikely. The concentration of the band 3 monomer is so high in situ (Salhany, 1990) that porter tetramers predominate (Salhany et al., 1990; Casey & Reithmeier, 1991; Schubert, 1988). Furthermore, there is evidence suggesting that interactions with cytoskeletal and cytosolic proteins (Mulzer et al., 1990; Thevenin & Low, 1990; Schuck and Schubert, 1991) restrict the lateral movement of band 3 (Peters et al., 1974; Golan & Veatch, 1980). In contrast to these possibilities, solid evidence exists for a slow conformational change in band 3 in response to the rapid binding of stilbenedisulfonates (Verkman et al., 1983; Dix et al., 1986). It is interesting to note that the stilbenedisulfonate-induced conformational change in band 3 (Dix et al., 1986) has an apparent relaxation constant which is quite comparable to the measured values of k_R we observe. Thus, we favor a conformationally based argument as the most likely explanation for the hysteresis observed in the dithionite transport progress curve.

What type of conformational change accounts for hysteresis in the dithionite transport velocity? While an hysteretic transition can be assigned as a property of functionally independent protein monomers, our experiments with DIDS suggest that subunit interactions contribute significantly to band 3 hysteresis. First, k_R decreased significantly with partial DIDS labeling of the band 3 population (Figure 7). Second, the dithionite dependence of k_R changed completely with partial labeling (compare Figures 5A and 9B). Finally, the Lineweaver–Burk plots for the substrate dependence of V_s lost all detectable indication of negative cooperativity with partial labeling (Figure 4). Thus, the kinetic properties of the remaining monomers change dramatically when a portion of the monomer population in the membrane is inhibited by covalent binding of DIDS. We interpret these findings to mean that intersubunit interactions contribute significantly to dithionite transport by band 3. In this regard, it is worth noting that pressure-dependent studies of sulfate transport show a very large activation volume for band 3 anion exchange (Canfield & Macey, 1984). This result seems consistent with a change in the quaternary conformational state of the porter (Heremans, 1982).

It is interesting to consider intersubunit allosteric models which might best explain our current results and other related data in the band 3 kinetic literature. In this discussion, k_R is viewed as a measure of the band 3 allosteric conformational change. The two general classes of allosteric models are the concerted transition model of Monod, Wyman, and Changeux (1965; the MWC model) and the "induced-fit" or sequential model of Koshland, Nemethy, and Filmer (1966; KNF model). Loudon and Koshland (1972) have examined the relaxation kinetic properties of dimeric allosteric models. The dimeric KNF model can generate k_R vs substrate plots showing a net decrease or a net increase in k_R, but it cannot generate a minimum when the assumptions of excess substrate and fast ligand binding/slow conformational changes are made (Loudon & Koshland, 1972). Furthermore, the dimeric version of

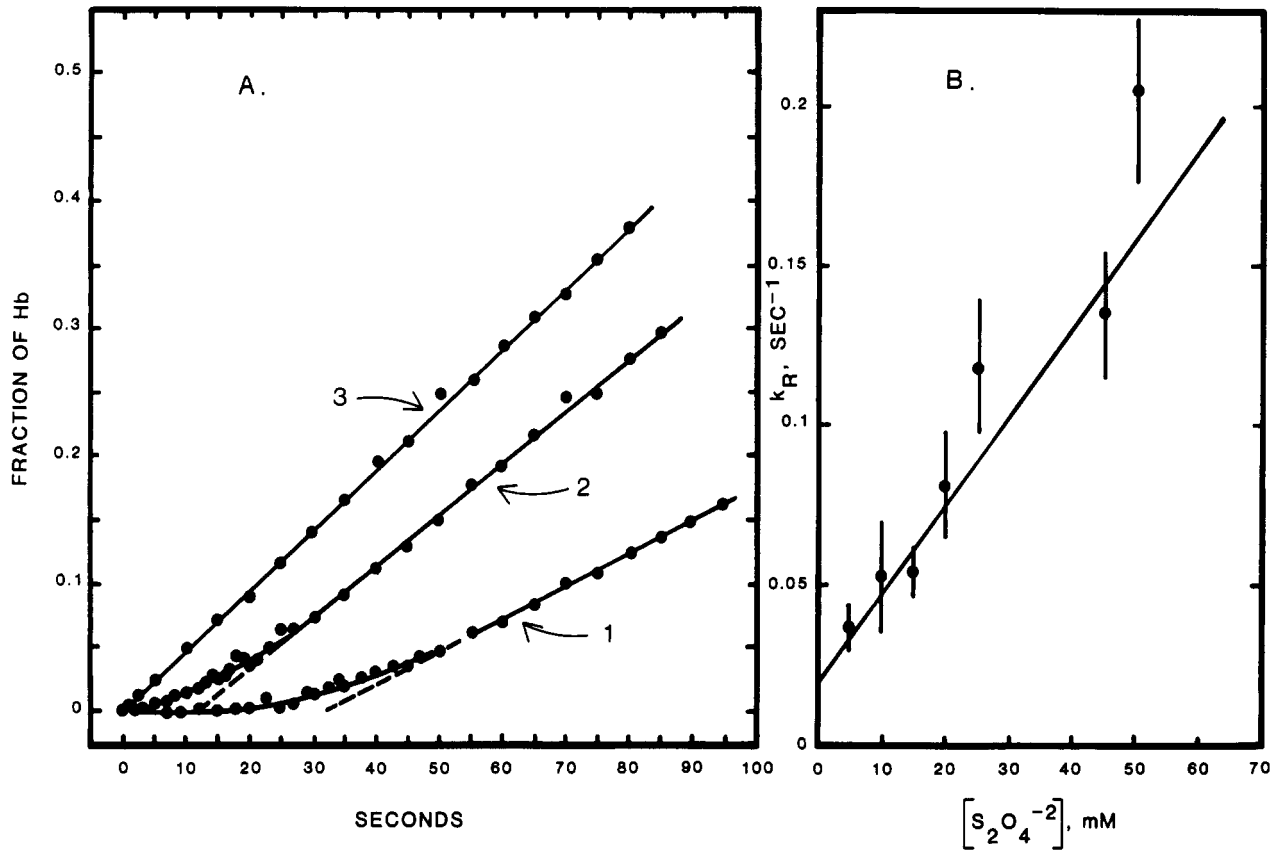

FIGURE 9: Dithionite dependence of the transient phase for cells whose steady-state velocity was ~85% inhibited by covalent binding of DIDS. Cells were prepared and labeled with DIDS as described in Figure 4. Transport was measured and eq 3 used to fit the data (except for time course line 3). The concentrations of dithionite for each time course in (A) are as follows: line 1, 5 mM; line 2, 20 mM; and line 3, 60 mM. The line drawn in (B) is based on a weighted least squares fit of a linear function to the data with slope 0.003 ± 0.0005 mM^{-1} s^{-1} and intercept 0.019 ± 0.008 s^{-1}.

the MWC model, with either exclusive or nonexclusive ligand binding, also cannot generate a minimum in plots of k_R vs substrate (Loudon & Koshland, 1972). Thus, dimeric allosteric models do not seem to be an adequate explanation for band 3 allosteric hysteresis measured in sulfate medium, since the data show a minimum in the k_R vs dithionite plot (Figure 5A). Nevertheless, dimeric models can explain our results if a structural intermediate exists which contains a third monomeric conformational state (Loudon & Koshland, 1972). At present, there is no direct evidence for the existence of three monomeric conformations of band 3. Only two conformational states of the band 3 monomer have been suggested based on measurements at the intramonomeric stilbenedisulfonate site (Kampmann et al., 1982; Salhany et al., 1990, 1991; Salhany, 1992).

In contrast to dimeric two-state allosteric models, the tetrameric nonexclusive ligand binding MWC model is capable of generating a minimum in the substrate dependence of k_R (Kirshner et al., 1966; Eigen, 1968; Janin, 1971; Kurganov et al., 1976; Frieden, 1979; Neet & Ainslie, 1980). A general concerted transition model for dithionite transport is shown in Figure 10, along with an expression for the substrate dependence of k_R and some plots of simulated k_R vs substrate concentration. With the rapid ligand binding assumption and the other assumptions given in the legend to the figure, it is possible to generate a k_R vs substrate plot which has a minimum (Kurganov et al., 1976). This occurs when $k_{-0} < k_{-i}$ and $k_{+0} > k_{+i}$ as the sites on the protein oligomer become liganded. A tetrameric MWC model can also explain the change in the substrate dependence of k_R consequent to partial labeling of cells with DIDS (Figure 5A vs 9B). If we allow that, for band 3 populations 85% inhibited by DIDS, $k_{-0} < k_{-i}$ and $k_{+0} = k_{+i}$ as sites on the protein oligomer become liganded, then k_R will show a net increase with increasing substrate (Figure 10).

A tetrameric concerted transition model is expected to yield positive cooperativity in functional properties measured at equilibrium (Monod et al., 1965; Segel, 1975). On the basis of the extensive kinetic work of Schnell and co-workers (Schnell & Besl, 1984; Stadler & Schnell, 1990), it is now apparent that sulfate and phosphate both show positive cooperativity in the equilibrium exchange experiment.[2] Our demonstration of allosteric hysteresis in a rapid mixing experiment can potentially explain why the equilibrium exchange experiment for divalent anion shows positive cooperativity (Schnell & Besl, 1984) while the influx exchange type of kinetic experiment shows apparent negative cooperativity (Salhany & Swanson, 1978; Salhany & Gaines, 1981; Schnell & Besl, 1984). If cooperativity from a kinetic assay is different from cooperativity when the system is at dynamic equilibrium, one may infer that a kinetic component accounts for the differences (Neet & Ainslie, 1980). This is supported by the fact that partial DIDS labeling both slows k_R (Figure 7) and converts the substrate dependence of the steady-state velocities from negative cooperative to hyperbolic (Figure 4). Kurganov et al. (1976) have shown that hysteretic effects can generate apparent negative cooperativity even within an MWC model

[2] It should be noted that chloride equilibrium exchange kinetics show a hyperbolic dependence over the low substrate concentration range, with partial substrate inhibition occurring at higher concentration (Gunn & Frohlich, 1979; Hautmann & Schnell, 1985; Gasbjerg & Brahm, 1991). There may be a fundamental difference in transport mechanism between monovalent and divalent anions related to the cotransport of a proton with divalents (Jennings, 1976; Milanick & Gunn, 1982).

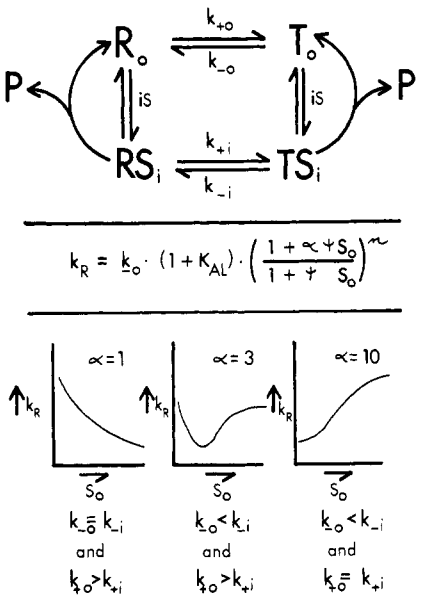

FIGURE 10: Allosteric concerted transition model for band 3 anion exchange. In this model, each monomer is considered to be a transport unit within an oligomeric structure. Monomers can exist in two alternate conformations modulated by a concerted change in quaternary conformational state. That is, all monomers are either in one conformational state (the R state) or the other conformational state (the T state). It is assumed that ligand binding to both states is much faster than the kinetics for conformational interconversion between states (k_{+i}/k_{-i} and k_{+o}/k_{-o}). The equation for k_R is shown as derived by Kurganov et al. (1976). $[K_{AL}] = [T]/[R]$; α is the denominator of the progression for the series of constants k_{-i}, such that $k_{-i}/k_{-(i-1)} = \alpha$, and $k_{-i}/k_{-0} = \alpha^i$. The denominator of progression for the sequence of constants k_{+i} is $\alpha\psi$, such that $k_{+i}/k_{+(i-1)} = \alpha\psi$ and $k_{+i}/k_{+0} = (\alpha\psi)^i$, where $\psi = K_R/K_T$. K_R and K_T are the microscopic substrate dissociation constants for the R and T states. $s_0 = [S]/K_R$. The value n is the number of monomers per oligomer, assuming one active site per monomer. To generate the equation for k_R, the additional assumption that the sequence of constants k_{+i} and k_{-i} produces a geometric progression was used (Kurganov et al., 1976). The following constants were used to generate the simulated plots for $\alpha = 1$, 3, and 10: $n = 4$; $K_{AL} = 100$; $\psi = 0.1$. These values imply that the resting state has more T-state species present than R state and that the affinity of the T state for substrate is lower than that for the R state. See Kurganov et al. (1976) and the text of this paper for further discussion.

which is otherwise incapable of generating such a pattern at equilibrium (Segel, 1975). Thus, it may be the rapid imposition of an inwardly directed anion gradient which induces the slow conformational response in band 3 leading to the observed negative cooperativity in the steady-state velocity (Neet & Ainslie, 1980; Kurganov et al., 1976). At equilibrium, positive cooperativity followed by partial substrate inhibition is observed for divalent anion exchange (Schnell & Besl, 1984; Stadler & Schnell, 1990).

While our discussion of the kinetics in terms of a tetrameric concerted transition model seems consistent with the band 3 divalent anion exchange literature, further analysis will be required to test the generality of this or any other model for band 3 function. For example, it will be necessary to explain the appearance of multiple transients in the dithionite/chloride heteroexchange experiment (Figure 1). Such a complexity could indicate the presence of more than one hysteretic step during divalent/monovalent anion exchange (Frieden, 1979). Such a change in mechanism with substitution of co-anion could be related to the demonstrated need to transport a proton during divalent/monovalent exchange (see footnote 2).

In summary, we have shown that dithionite uptake by band 3 involves an intersubunit allosteric hysteresis. The exact model which best describes all aspects of band 3 allosterism and the significance of such a regulatory mechanism for band 3 function both remain to be established.

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