Evidence for a Second Binding/Transport Site for Chloride in Erythrocyte Anion Transporter AE1 Modified at Glutamate 681

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ABSTRACT Transport kinetics have been examined in erythrocyte anion transporter AE1 that has been chemically modified to convert glutamate 681 to an alcohol (E681OH AE1). Outward conductive Cl^- flux in E681OH AE1 is inhibited by removal of extracellular Cl^-; this effect is the opposite of that in native AE1 and is consistent with coupled electrogenic 2:1 Cl^-/Cl^- exchange. A second Cl^- binding/transport site is also suggested by the characteristics of $^{35}SO_4^{2-}$ flux in E681OH AE1: bilateral and *cis* Cl^-, which are normally inhibitory, accelerate $^{35}SO_4^{2-}$ flux. These effects would be expected if Cl^- binds to a second transport site on SO_4^{2-}-loaded E681OH AE1, thereby allowing Cl^-/SO_4^{2-} cotransport. Alternatively, the data can be explained without proposing Cl^-/SO_4^{2-} cotransport if the rate-limiting event for $^{35}SO_4^{2-}/SO_4^{2-}$ exchange is external SO_4^{2-} release, and the binding of external Cl^- accelerates SO_4^{2-} release. With either interpretation, these data indicate that E681OH AE1 has a binding/transport site for Cl^- that is distinct from the main transport site. The effects of graded modification of E681 or inhibition by H_2DIDS are consistent with the idea that the new Cl^- binding site is on the same E681OH-modified subunit of the AE1 dimer as the normal transport site.

INTRODUCTION

The AE1 protein (Band 3) of the erythrocyte membrane mediates the exchange of Cl^- for HCO_3^- as part of the process of CO_2 transport in the blood (Wieth et al., 1982; Alper et al., 2002). The catalytic cycle for anion exchange is believed to be ''ping-pong'', in which there are distinct inward-facing and outward-facing conformations of the protein and the anions cross the membrane one at a time (Knauf, 1979; Fröhlich and Gunn, 1986; Passow, 1986). In addition to 1:1 monovalent anion exchange, there are other modes of AE1-mediated transport, including anion conductance (Knauf et al., 1977), H^+/Cl^- cotransport (Jennings, 1978; Lepke et al., 2003), and H^+/SO_4^{2-} cotransport (Jennings, 1976; Milanick and Gunn, 1984). Although the fluxes via these transport modes are much smaller than the Cl^-/HCO_3^- exchange flux, these fluxes are of interest because an understanding of alternative transport modes can potentially provide insights regarding the normal catalytic mechanism of AE1.

The H^+/SO_4^{2-} cotransport mode of AE1 is believed to depend on protonation of a specific glutamate residue, E681 (Jennings and Smith, 1992). When E681 is protonated, AE1 is converted from the normal monovalent anion transporter into a form that can transport SO_4^{2-} and other divalent anions (Fig. 1). Several years ago, we found that treatment of intact human red blood cells with Woodward's reagent K, followed by reductive cleavage of the active ester adduct with BH_4^-, causes selective conversion of the side chain of E681 to an alcohol (Jennings and Anderson, 1987; Jennings and Smith, 1992); AE1 modified in this manner is designated here as E681OH AE1. The modification causes several major changes in AE1 function: 1), Monovalent anion exchange is inhibited (Jennings and Al-Rhaiyel, 1988); 2), divalent anion transport is accelerated and is much less dependent on pH than in native AE1 (Jennings and Al-Rhaiyel, 1988); and 3), the exchange of Cl^- for SO_4^{2-} is an electrogenic 1:1 exchange, with no H^+ cotransport, in E681OH AE1 (Jennings, 1995). Chernova et al. (1997) extended these studies by showing that mutagenesis of mouse AE1 E699 (equivalent to human E681) to glutamine stimulates Cl^-/SO_4^{2-} exchange and converts the process from electroneutral to electrogenic. All these findings are consistent with the idea that E681 of human AE1 binds the H^+ that is cotransported with SO_4^{2-}. In keeping with this idea, E681 is believed to be located in the interior of the membrane at a position that is near the permeability barrier (Tang et al., 1998).

In addition to cotransporting H^+ with SO_4^{2-}, AE1 also mediates H^+/Cl^- cotransport (Jennings, 1978; Lepke et al., 2003). One possible mechanism of H^+/Cl^- cotransport is that at low pH the E681-protonated form of AE1 can bind and transport two Cl^- ions (Fig. 1, *lower left*), resulting in the exchange of two Cl^- + one H^+ for one Cl^-. H^+/Cl^- cotransport is inhibited in E681OH AE1 (Lepke et al., 2003), as would be expected if E681 normally participates in H^+/Cl^- cotransport. The binding and transport of 2 Cl^- ions by the low-pH (E681-protonated) form of AE1 was proposed many years ago as a potential mechanism of H^+/Cl^- cotransport (Jennings, 1978). At that time it was known that there is at least one binding site for Cl^- in addition to the main transport site (Dalmark, 1976). However, this binding site is an inhibitory ''modifier'' site (Knauf, 1979; Knauf and

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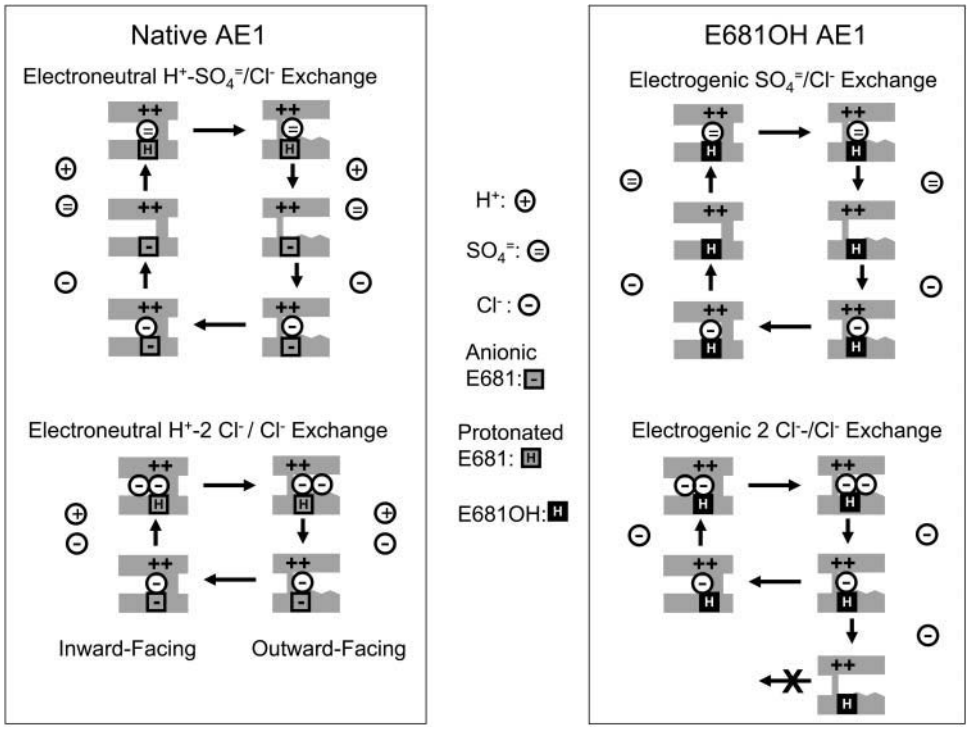

FIGURE 1 (*Upper left*) Cycle for exchange of Cl^- for $H^+ + SO_4^{2-}$ in normal AE1. SO_4^{2-} and H^+ bind to the transporter in either order (Milanick and Gunn, 1982). Two protein-bound positive charges are depicted to emphasize the idea that the translocation events for both Cl^- and SO_4^{2-} appear to be electroneutral (Jennings et al., 1990; Jennings, 1995). (*Lower left*) Cycle for the exchange of two $Cl^- + H^+$ for Cl^- (Jennings, 1978; Lepke et al., 2003) in native AE1. This cycle is similar to that for H^+-SO_4^{2-}/Cl^- exchange, except that two Cl^- ions instead of a single SO_4^{2-} are translocated by the low pH (E681-protonated) form of AE1. The net result is electroneutral cotransport of $H^+ + Cl^-$. (*Upper right*) Ping-pong cycle for SO_4^{2-}/Cl^- exchange in E681OH AE1. The charge state of E681OH band 3 at pH 7.4 is the same as that of native AE1 at low pH, i.e., with E681 protonated. The main charge-carrying event appears to be the Cl^- limb of the cycle (Jennings, 1995). (*Lower right*) Hypothetical catalytic cycle for $2Cl^-$/Cl^- exchange through E681OH AE1. By analogy to H^+-Cl^- cotransport in normal AE1, two Cl^- are translocated through E681OH AE1, and the transport of a single Cl^- in the opposite direction completes the cycle. There is no proton cotransport because E681OH is no longer reversibly protonated.

Mann, 1986), and there was no reason to propose that Cl^- bound to this site could be transported.

The possibility that two anions can be transported in the same direction by AE1 was not given much further attention until a recent study by Passow and coworkers (Lepke et al., 2003), which showed that the kinetics of AE1-mediated H^+/Cl^- cotransport are consistent with a model in which Cl^- can bind with low affinity to a second transport site, and two Cl^- ions are cotransported with H^+ in exchange for a single Cl^- ion, as depicted in Fig. 1. Moreover, Salhany et al. (2003) have recently presented evidence that in E681OH AE1 there is a new moderate-affinity Cl^- binding site that modulates the displacement of stilbenedisulfonate inhibitors from AE1.

This article examines the kinetics of Cl^- and SO_4^{2-} transport in E681OH AE1. We find several results that are consistent with the idea that removal of the negative charge on E681 causes the appearance of a new Cl^- binding/transport site:

1. Outward Cl^- conductance is inhibited by removal of extracellular Cl^-, as expected if the conductance consists in part of electrogenic 2:1 Cl^-/Cl^- exchange.
2. Extracellular Cl^- accelerates SO_4^{2-} efflux by a mechanism other than recruitment of transporters from the outward to the inward state.
3. Bilateral Cl^- stimulates $^{35}SO_4^{2-}/SO_4^{2-}$ exchange, and extracellular Cl^- stimulates unidirectional $^{35}SO_4^{2-}$ influx.

These results can be explained by a model in which E681OH AE1 has a site at which extracellular Cl^- can bind and either be cotransported with or facilitate the extracellular release of SO_4^{2-} bound to the main transport site. Finally, the possibility that the anomalous kinetics of anion transport in E681OH AE1 are a consequence of altered subunit interactions in the AE1 dimer (Salhany et al., 2003) was tested by graded chemical modification; the data are completely consistent with the idea that the second Cl− binding site and the main anion transport site are on the same subunit of the E681OH AE1 dimer.

MATERIALS AND METHODS

Materials

Human blood was drawn into heparin by venipuncture from healthy adults and was stored as whole blood at 4°C for up to 1 week before use. Gramicidin (87% gramicidin A) was purchased from Calbiochem (San Diego, CA). H_2DIDS (4,4′-diisothiocyanatodihydrosilbene-2,2′-disulfonate) was synthesized from DADS (4,4′-diaminostilbene-2,2′-disulfonate) as described previously (Jennings et al., 1984). Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3′-sulfonate) was purchased from Sigma (St. Louis, MO). All other salts and buffers were obtained from either Sigma or Fisher Scientific (Pittsburgh, PA). Radionuclides ($Na_2^{35}SO_4$, $Na^{36}Cl$, and $^{86}RbCl$) were from DuPont NEN (Boston, MA).

Treatment of cells with Woodward's reagent K

Cells were washed and modified with 2 mM Woodward's reagent K (WRK) and $NaBH_4$ at 0°C as described previously (Jennings, 1995). This procedure converts ~75% of the copies of AE1 to E681OH. The remainder of the

copies of AE1 are either unmodified or contain uncleaved WRK adduct and are functionally silent in the transport assays used here. In experiments involving Cl^- gradients, in which it was important to inhibit Cl^-/HCO_3^- exchange as much as possible, two successive exposures to WRK at 0°C were made before BH_4^- addition; this method results in the modification of ~95% of the copies of AE1 (Jennings, 1995).

Cl^- conductive efflux

The conductive Cl^- permeability was estimated from the $^{86}Rb^+$ efflux mediated by gramicidin. The method was a variation on those used in other laboratories (Knauf et al., 1977; Hunter, 1977; Fröhlich et al., 1983). Cells were loaded with $^{86}Rb^+$ by incubating for 1 h at 37°C in HEPES-buffered physiological saline (140 mM NaCl, 5 mM KCl, 1 mM Na-phosphate, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4, 10 mM glucose). Cells were then treated with WRK/BH_4^- at 0°C in 150 mM KCl/MOPS, pH 7.0, as described above. The gramicidin-mediated efflux of $^{86}Rb^+$ was measured in a medium in which all Na^+ and K^+ were replaced by impermeant *N*-methylglucamine (NMG). The medium consisted of mixtures of 150 mM NMG-glutamate and 150 mM NMG-Cl, buffered at pH 7.0 with 10 mM NMG-MOPS. Gramicidin A was added to a final concentration of 20 nM, and the efflux of $^{86}Rb^+$ was measured at 20°C by centrifuging aliquots and measuring radioactivity in the supernatant. The rate constant (min^{-1}) for $^{86}Rb^+$ efflux was calculated as described previously (Jennings, 1995).

The gramicidin-mediated efflux of $^{86}Rb^+$ was assumed to follow the constant field equation (Goldman, 1943; Hodgkin and Katz, 1949):

$$J_{Rb} = P_{Rb}[Rb^+]_{in}[FV_m/RT]/[1 - \exp(-FV_m/RT)], \quad (1)$$

where J_{Rb} is the efflux (nmol/ml cells/min) of $^{86}Rb^+$, P_{Rb} is the permeability coefficient for Rb^+ (min^{-1}), $[Rb^+]$ is the intracellular Rb^+ concentration (nmol/ml cells), V_m is the membrane potential, F is Faraday's constant, R is the gas constant, and T is the absolute temperature. The efflux of $^{86}Rb^+$ into a 150 mM KCl medium provided a good estimate of P_{Rb} in the same cell preparation, same hematocrit (2%), and same gramicidin concentration, because in the high-K^+ medium the membrane potential is ~0 and Eq. 1 is reduced to $J_{Rb} = P_{Rb}[Rb^+]_{in}$.

In the absence of extracellular permeant cations, the membrane potential depends mainly on the concentrations and permeability coefficients of intracellular K^+, intracellular Cl^-, and extracellular Cl^-. (Rb^+ is present in trace amounts and does not itself affect the membrane potential; at neutral pH, H^+ conductance through gramicidin is not significant, as indicated by the very low $^{86}Rb^+$ efflux from SO_4^{2-}-loaded cells in an NMG-SO_4^{2-} medium.) Therefore, the membrane potential in NMG medium is given by

$$FV_m/RT = -\ln\{(P_K[K^*]_{in} + P_{Cl}[Cl^-]_o)/P_{Cl}[Cl^-_{in}\}. \quad (2)$$

In these experiments, $[K^*]_{in}$ and $[Cl^-]_{in}$ are both ~140 mM, and the only variable is $[Cl^-]_o$. The Cl^- conductive permeability coefficient P_{Cl} was determined at each value of $[Cl^-]_o$ from the measured rates of $^{86}Rb^+$ efflux, the measured P_{Rb}, and Eqs. 1 and 2.

$^{35}SO_4^{2-}$ and $^{36}Cl^-$ Efflux

Cells were loaded with SO_4^{2-} by washing three times in at least 20 cell volumes of 80 mM K_2SO_4, 10 mM HEPES, pH 7.4, with a 10-min incubation at 37°C before each centrifugation to allow Cl^- efflux and SO_4^{2-} influx (Jennings, 1995). Cells were loaded with $^{35}SO_4^{2-}$ by incubating at 30% hematocrit in 80 mM K_2SO_4, 10 mM HEPES, pH 7.4, plus 10 μCi/ml $^{35}SO_4^{2-}$. Efflux was performed as described previously (Jennings, 1995) in media specified in the figure legends. The efflux of $^{36}Cl^-$ was measured by the method of Ku et al. (1979) at 0°C in cells at Donnan equilibrium in 150 mM KCl, 10 MOPS, pH 7.0.

Preparation of low-SO_4^{2-} cells

The following method was used to prepare intact cells containing a low concentration (~0.5 mM) of SO_4^{2-} and no other permeant anion. Cells were treated with 2 mM WRK/BH_4^- as usual, and then incubated 30 min, 37°C, with 10-μg/ml cells of gramicidin A in at least 20 volumes of HEPES-buffered 150 mM K-gluconate. In this medium there is net loss of KCl driven by the outward Cl^- gradient; there is also cellular alkalinization caused by Cl^- exchange with traces of HCO_3^- in the medium. After the 30-min depletion of Cl^-, cells were centrifuged, resuspended in 50 mM K-gluconate, 0.5 mM K_2SO_4, 10 mM HEPES, pH 7.4, and incubated 30 min further at room temperature. Cells were washed once more in this medium and then incubated at least 20 min further in the same medium plus 1 μCi/ml $^{35}SO_4^{2-}$. The intracellular SO_4^{2-} concentration (measured as the distribution of $^{35}SO_4^{2-}$) was ~1.1 times the extracellular concentration. The Donnan ratio in these cells was therefore near unity despite the low concentration of permeant anion, because the negative charge on 50 mM extracellular gluconate balances the impermeant intracellular negative charge from hemoglobin and organic phosphates. The cells were also slightly shrunken (0.68 g H_2O/ml cells versus normal of 0.71 g H_2O/ml cells).

Preparation of resealed ghosts

Ghosts were prepared from control or WRK/BH_4^--treated cells by the method of Schwoch and Passow (1973). Lysis was at 0°C in 20 volumes of 4 mM $MgSO_4$, 1.2 mM acetic acid, followed by addition at 0°C of concentrated stock solutions to produce final concentrations of 40 mM K_2SO_4, 10 mM HEPES, pH 7.4, and 0–40 mM Cl^-. Ghosts were incubated in these media 45 min at 37°C for resealing, and then washed and loaded with 1 μCi/ml $^{35}SO_4^{2-}$ in the same media as was used for resealing. Finally, ghosts were washed twice at 0°C to remove external radioactivity, and the efflux of $^{35}SO_4^{2-}$ was measured in the same medium at 20°C.

RESULTS

Test of the hypothesis that E681OH AE1 can mediate 2:1 Cl^-/Cl^- exchange

It is well established that E681OH AE1 (or mouse E699Q AE1) can carry out electrogenic 1:1 SO_4^{2-}/Cl^- exchange (Jennings, 1995; Chernova et al., 1997). Therefore, E681OH AE1 is capable of binding and transporting either a single SO_4^{2-} or a single Cl^- ion. However, the fact that SO_4^{2-}/Cl^- exchange is electrogenic does not rule out the possibility that there are translocation events involving two Cl^- ions in E681OH AE1. Such events must be less frequent than single Cl^- transport events, because SO_4^{2-}/Cl^- exchange is electrogenic.

If E681OH AE1 mediates translocation events in which two Cl^- ions are cotransported (with no proton cotransport), the resultant exchange of two Cl^- for one Cl^- would be electrogenic and may account for the large (~8-fold higher than normal) H_2DIDS-sensitive Cl^- conductive flux that is observed in E681OH (Jennings, 1995). A conductive outward Cl^- flux resulting from 2:1 exchange should be inhibited by removal of extracellular Cl^-, because in the absence of extracellular substrate the catalytic cycle should be arrested by the formation of empty outward-facing transporters (Fig. 1, *lower right*). To test this idea, the Cl^- conductance was estimated by measuring the gramicidin-mediated efflux

of $^{86}Rb^+$ in media containing impermeant *N*-methyl glucamine as the only cation other than H^+.

Fig. 2 depicts the results of four experiments in which P_{Cl} was estimated in WRK/BH_4^--treated red cells in the presence of varying concentrations of extracellular Cl^- (glutamate substitute). In all cases the initial intracellular Cl^- concentration was 120–140 mM (Donnan equilibrium, pH 7). The P_{Cl} at each extracellular Cl^- concentration is plotted relative to the P_{Cl} measured in the same cells in the absence of extracellular Cl^-. In all cases the addition of extracellular Cl^- causes an increase in P_{Cl}. In contrast, P_{Cl} in control cells is inhibited ~50% by addition of a relatively low concentration of Cl^- (10 mM) to a Cl^--free medium (*open symbols*), in excellent agreement with the data of Fröhlich et al. (1983). The dependence of P_{Cl} on extracellular Cl^- in E681OH AE1 is not absolute; there is a significant conductive Cl^- efflux even in the absence of extracellular Cl^-. However, there is a clear acceleration of the conductive Cl^- efflux by the addition of Cl^- to the extracellular medium, as expected if a component of the outward conductive flux takes place as 2:1 Cl^-/Cl^- exchange.

Trans acceleration of SO_4^{2-} efflux by Cl^- in E681OH AE1

The idea that E681OH AE1 has two possible transport sites for Cl^- raises the possibility that the second Cl^- binding/transport site may be responsible for some of the effects of Cl^- on SO_4^{2-} in E681OH AE1 that were reported previously

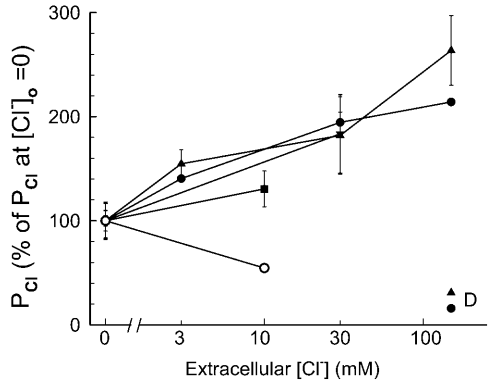

FIGURE 2 Stimulation by extracellular Cl^- of outward Cl^- conductance in E681OH AE1. Cells were loaded with $^{86}Rb^+$ in HEPES-buffered physiological saline and then treated with 2 mM WRK (two exposures) and finally 2 mM BH_4^- (Jennings, 1995). The efflux of $^{86}Rb^+$ was measured in 150 mM NMG glutamate, 10 mM NMG-MOPS, pH 7.0, 20°C, after the addition of 20 nM gramicidin A. The extracellular Cl^- concentration was varied by substituting the indicated concentration of NMG-Cl for NMG-glutamate. The conductive Cl^- permeability coefficient was calculated as described in Methods and is plotted as percent of P_{Cl} measured in the same cell preparation in a Cl^--free medium. The figure shows the results of duplicate flux measurements at the indicated Cl^- concentration for four separate preparations of E681OH red cells (*solid symbols*) and one preparation of untreated cells (*open symbols*). The conductive Cl^- flux is inhibited almost entirely by 20 μM H_2DIDS (*solid symbols* marked *D*).

(Jennings, 1995). One such effect is a remarkably large *trans* accelerating effect of extracellular Cl^- on SO_4^{2-} efflux; replacement of 80 mM extracellular SO_4^{2-} with 120 mM Cl^- causes a 20-fold acceleration of the $^{35}SO_4^{2-}$ efflux. This *trans*-acceleration of SO_4^{2-} efflux by extracellular Cl^- is not a consequence of an effect of the Cl^- gradient on the membrane potential, because a large *trans*-acceleration is observed even if the membrane potential is clamped near zero with gramidicin (Jennings, 1995).

A ping-pong anion exchange mechanism can, in principle, explain *trans*-acceleration on the basis of ''recruitment'' by the inward Cl^- gradient of transporters from the outward-facing to the inward-facing state (Knauf, 1979; Gunn and Fröhlich, 1979; Jennings, 1980). For example, if the transporters are symmetrically distributed in the presence of saturating concentrations of SO_4^{2-} on both sides of the membrane, then Cl^- should cause a twofold *trans* acceleration in each direction. This is very nearly what is observed in normal cells for SO_4^{2-} at neutral pH (Jennings, 1980). In E681OH cells, however, the asymmetry of the *trans* acceleration is quite different from normal cells. At pH 7.4, replacement of extracellular SO_4^{2-} with Cl^- causes an ~20-fold acceleration of $^{35}SO_4^{2-}$ efflux; replacement of intracellular SO_4^{2-} with Cl^- accelerates $^{35}SO_4^{2-}$ influx by a factor of ~3 (Jennings, 1995). These magnitudes of *trans* acceleration are too large to be explained by a ping-pong/recruitment mechanism.

To examine further the mechanism of the large stimulation of SO_4^{2-} efflux by extracellular Cl^- in E681OH AE1, we used conditions in which single turnovers of the catalytic cycle should be detectable. Because of the large number of copies (~10^6/cell) of AE1 in red cells (Fairbanks et al., 1971), it is possible to prepare intact cells in which SO_4^{2-} is the only permeant anion, and the initial amount of intracellular SO_4^{2-} is not much higher than the number of copies of AE1. Cells containing 0.5 mM $^{35}SO_4^{2-}$ and much lower concentrations (<0.01 mM) of Cl^- and HCO_3^- were prepared by using gramicidin to lower the total cellular ion contents following treatment with WRK/BH_4^- (see Methods). These cells were suspended at 0°C in Cl^--free, SO_4^{2-}-free 50 mM K-gluconate, 10 mM HEPES, pH 7.4, and the time course of $^{35}SO_4^{2-}$ efflux was measured. The efflux is initially slow because of the absence of an exchangeable extracellular anion (Fig. 3). Addition of 25 mM Cl^- to the medium causes an immediate increase in the efflux, reflecting the rapid rate of Cl^-/SO_4^{2-} exchange in E681OH AE1, even at 0°C. At the arrow, the suspension was diluted by a factor of 10 into Cl^--free 50 mM K-gluconate/HEPES medium, thereby lowering the extracellular Cl^- concentration to 2.5 mM, and further time points were taken.

Within the time resolution of our measurements, we find that the rate of SO_4^{2-} efflux is reduced immediately upon lowering the extracellular Cl^- concentration. The initial amount of $^{35}SO_4^{2-}$ in these cells (0.5 mM) was only ~20 times the amount of AE1 polypeptide, assuming 1.2×10^6 copies

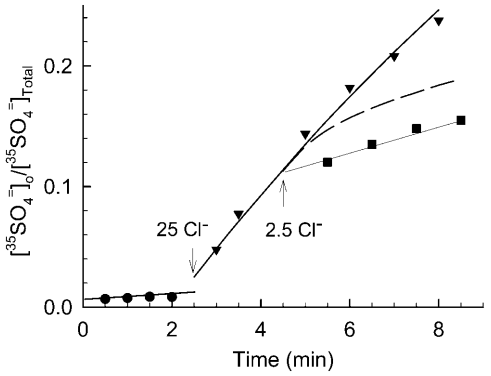

FIGURE 3 Efflux of $^{35}SO_4^{2-}$ from E681OH erythrocytes that had been treated with gramicidin and equilibrated with a medium consisting of 50 mM K-gluconate, 0.5 mM [^{35}S]K_2SO_4, 10 mM HEPES, pH 7.4. Efflux was initiated by suspending cells in a medium containing 50 mM K-gluconate, 10 mM HEPES, pH 7.4, and no permeant anion. At the first arrow, KCl was added to a final concentration of 25 mM to initiate rapid efflux of $^{35}SO_4^{2-}$. The inverted triangles represent the time course of efflux in the presence of 25 mM Cl^-. At the second arrow, an aliquot of the suspension was diluted 10-fold into 50 mM K-gluconate/10 mM HEPES, pH 7.4; the squares represent the time course of efflux of SO_4^{2-} from the suspension after reduction of extracellular Cl^- to 2.5 mM. The dashed curve represents the expected time course of extracellular $^{35}SO_4^{2-}$ if AE1 were initially (in the high Cl^- medium) in the inward-facing conformation, and one efflux event per copy of AE1 took place after reduction of extracellular Cl^-.

per cell (Fairbanks et al., 1971; Passow, 1986). If 25 mM Cl^- accelerates SO_4^{2-} efflux by recruiting most of the transporters to the inward-facing state, then there should be a continuing rapid efflux of ~5% of the initial cellular $^{35}SO_4^{2-}$ after reduction of the extracellular Cl^- concentration, because the inward-facing transporters should be able to perform one more SO_4^{2-} efflux event before the cycle slows down in the low-Cl^- medium. Experimentally, we detected no delay in the reduction of SO_4^{2-} efflux after a reduction of extracellular [Cl^-] (eight effluxes on four cell preparations). There is no electrical constraint on the efflux because the cells had been treated with gramicidin to raise the K^+ conductance. This finding indicates that extracellular Cl^- accelerates SO_4^{2-} efflux in E681OH AE1 by a mechanism other than recruitment of transporters to the inward-facing state.

Acceleration of SO_4^{2-} equilibrium exchange by bilateral Cl^-

Further evidence of anomalous transport kinetics in E681OH AE1 comes from the effects of bilateral Cl^- on $^{35}SO_4^{2-}$ fluxes. We had previously reported the preliminary finding that bilateral 10–20 mM Cl^- can accelerate the rate constant for SO_4^{2-}/SO_4^{2-} exchange in E681OH AE1 by a factor of nearly 2 (Jennings, 1995). These earlier studies used intact cells, in which the intracellular SO_4^{2-} concentration was not well controlled when Cl^- was varied. We have subsequently used resealed ghosts to examine this issue in a more rigorous way. Ghosts from control or E681OH cells were resealed in media containing 40 mM K_2SO_4, 10 mM HEPES, pH 7.4, plus 0–40 mM KCl, and then loaded with $^{35}SO_4^{2-}$ in the resealing medium. The efflux of $^{35}SO_4^{2-}$ was measured in the same medium, i.e., under conditions of no net ion flux. The presence of Cl^- on both sides of the membrane causes a clear acceleration of the $^{35}SO_4^{2-}$ efflux (Fig. 4). A similar acceleration is observed if ghosts are first resealed in a Cl^--free 40 mM K_2SO_4 medium and Cl^- is subsequently introduced by incubating the ghosts with varying concentrations of NH_4Cl, which can enter the ghosts as NH_3 influx followed by Cl^-/HCO_3^- exchange with CO_2 recycling to result in net NH_4Cl influx (Jacobs and Stewart, 1942). This acceleration of SO_4^{2-}/SO_4^{2-} exchange by bilateral Cl^- was observed only in E681OH AE1; in native human red cells, we find that bilateral Cl^- always inhibits SO_4^{2-} transport, as is well known (Passow, 1986).

Acceleration of SO_4^{2-} influx by extracellular Cl^-

The data in Fig. 5 show that extracellular Cl^-, in the initial absence of intracellular Cl^-, stimulates unidirectional SO_4^{2-} influx in E681OH AE1. This *cis* acceleration of a tracer anion flux by another anion is, to our knowledge, unprecedented in the literature on AE1. In control cells, 10 mM *cis* Cl^- strongly inhibits unidirectional SO_4^{2-} influx, and the inhibition is progressively relieved at later times as the inward Cl^- gradient is dissipated, exactly as observed previously (Jennings, 1980). The acceleration of SO_4^{2-} influx by extracellular Cl^- in E681OH AE1 is therefore in very sharp contrast to the strong inhibition in native AE1.

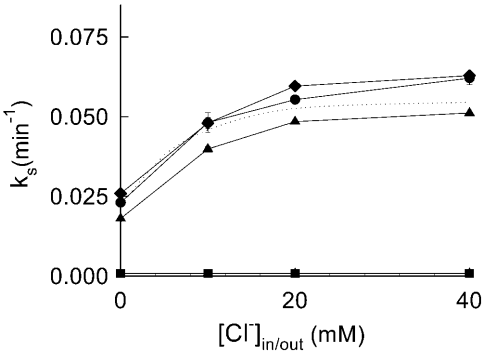

FIGURE 4 Stimulation of $^{35}SO_4^{2-}/SO_4^{2-}$ exchange through E681OH AE1 by bilateral Cl^- in resealed ghosts. Cells were treated with 2 mM WRK followed by 2 mM BH_4^-. Ghosts were prepared and resealed in a medium containing 40 mM K_2SO_4, 10 mM HEPES, pH 7.4, and 0–40 mM Cl^-, added either as KCl at 0°C before resealing (▲, ♦, ■) or as NH_4Cl after resealing and before $^{35}SO_4^{2-}$ loading (●). Flux was measured at 20°C in media of the same composition as the resealing medium. Data represent mean and range of duplicate measurements at each Cl^- concentration in three separate ghost preparations. Squares represent flux measured in the presence of 20 μM H_2DIDS. The dotted curve represents the prediction of the model described in the Discussion.

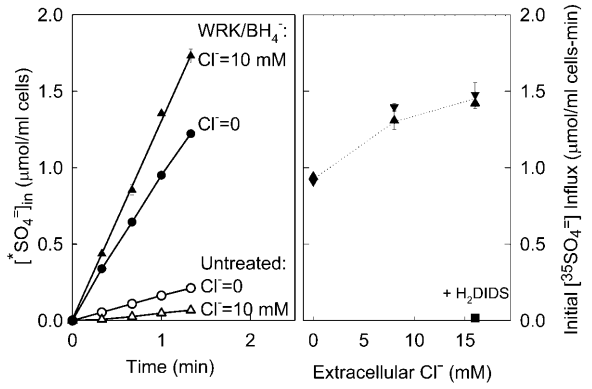

FIGURE 5 Effect of extracellular Cl^- on unidirectional $^{35}SO_4^{2-}$ influx in control and E681OH AE1. (*Left*) Cells were treated with (●, ▲) and without (○, △) 2 mM WRK/BH_4^- and then washed and equilibrated with Cl^--free, low-HCO_3^- (N_2-purged) medium consisting of 80 K_2SO_4, 10 HEPES, pH 7.4. Cells were then centrifuged under N_2 and resuspended in the same medium containing 10 μCi $^{35}SO_4^{2-}$ plus either 0 (●, ○) or 10 mM (▲, △) KCl. The influx of $^{35}SO_4^{2-}$ over the first 80 s was measured at 20°C. The experiments were carried out under N_2 to minimize Cl^- influx via Cl^-/HCO_3^- exchange. (*Right*) Initial SO_4^{2-} influx in two preparations of WRK/BH_4^--treated cells, performed as in the left side of the figure, except 0, 8, or 16 mM KCl replaced 0, 4, or 8 mM K_2SO_4 in the extracellular medium. Different symbols represent different cell preparations. The square represents the influx in the presence of 20 μM H_2DIDS. Data represent mean and range of duplicate determinations. The dotted line represents the prediction of the model described in Discussion.

Increased apparent affinity for extracellular SO_4^{2-} in E681OH AE1

In addition to accelerating SO_4^{2-}/SO_4^{2-} exchange, modification of E681 causes a large increase in the apparent affinity of AE1 for SO_4^{2-} at outward-facing transport sites. Fig. 6 shows the SO_4^{2-} influx into Cl^--free, SO_4^{2-}-loaded cells as a function of the extracellular SO_4^{2-} concentration in E681OH AE1. The flux is a saturable function of extracellular SO_4^{2-}, with $K_{1/2}$ of 0.25–0.30 mM. The same $K_{1/2}$ was measured for

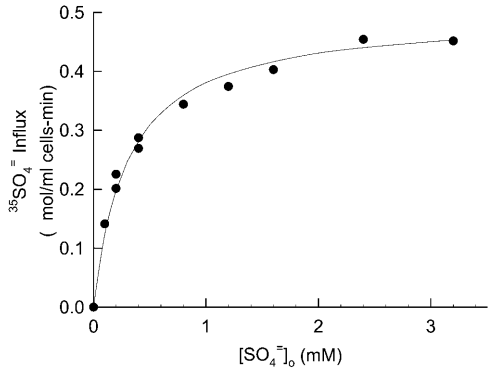

FIGURE 6 Influx of $^{35}SO_4^{2-}$ through E681OH AE1. Cells were pretreated with WRK/BH_4. and then washed in Cl-free 100 mM K_2SO_4 medium to replace all intracellular Cl^- with SO_4^{2-}. Initial influx of $^{35}SO_4^{2-}$ was measured at 20°C in 10 mM HEPES, pH 7.4, 250 mM sucrose, plus the indicated concentration of K_2SO_4. The curve through the data is derived from the model described in the Discussion.

extracellular SO_4^{2-} stimulation of $^{35}SO_4^{2-}$ efflux from all-SO_4^{2-} cells into Cl^--free sucrose or gluconate media (two experiments, not shown). This $K_{1/2}$ is much lower than that of control cells at pH 7.4 (Milanick and Gunn, 1982, 1984). Although the $K_{1/2}$ for transport is not identical with the dissociation constant for substrate binding to transport sites, the low $K_{1/2}$ suggests that the affinity of transport sites for extracellular SO_4^{2-} is considerably higher in E681OH AE1 than in normal protein. This finding is in agreement with previous work (Milanick and Gunn, 1982, 1984; Jennings, 1989), which showed that protonation of an acid-titratable group (probably E681) on AE1 causes a 10-fold increase in the apparent affinity for extracellular SO_4^{2-}.

Acceleration of SO_4^{2-} flux by WRK/BH_4^- following partial H_2DIDS inhibition

Conversion of native AE1 to E681OH AE1 by treatment with WRK and BH_4^- inhibits Cl^-/Cl^- exchange by over 90% and accelerates SO_4^{2-}/SO_4^{2-} exchange (measured at pH 7.4 in an all-SO_4^{2-} medium) by ~7-fold (Jennings and Al-Rhaiyel, 1988; Jennings, 1995). It is well established that AE1 is a dimer, and Salhany et al. (2003) have recently found that the kinetics of inhibitor dissociation from E681OH AE1 can be explained by a model in which conversion of one subunit in the dimer to E681OH affects the kinetics of inhibitor release from the other subunit. In light of this finding, it is of interest to determine whether transport kinetics in AE1 during graded conversion to E681OH are consistent with independently functioning subunits.

Fig. 7 shows the effects of graded WRK/BH_4^- treatment on $^{36}Cl^-/Cl^-$ exchange and $^{35}SO_4^{2-}/SO_4^{2-}$ exchange. Cells were washed and treated at 0°C with 0–1.6 mM WRK followed by 1 mM BH_4^-. Cells were then washed and split in half. One half was loaded with $^{36}Cl^-$, and the equilibrium exchange flux was measured at 0°C in 150 mM KCl/MOPS, pH 7.0. The other half was washed and loaded with $^{35}SO_4^{2-}$ in 80 mM K_2SO_4, 10 mM HEPES, pH 7.4, and the efflux was measured at electrochemical equilibrium in the same medium at 20°C. Over a wide range of WRK concentrations, there is a linear relationship between the inhibition of Cl^- exchange and acceleration of SO_4^{2-} exchange. The linear relationship between acceleration of SO_4^{2-} and inhibition of Cl^- transport is consistent with the idea that modification of the same site is responsible for both the acceleration of SO_4^{2-} flux and inhibition of Cl^- flux and that the presence of a modified subunit has no effect on either Cl^- or SO_4^{2-} transport through the adjacent unmodified subunit in the dimer. At the highest levels of inhibition of Cl^- flux and acceleration of SO_4^{2-} flux, there is a slight deviation from a linear relationship between the two fluxes. This slight deviation from linearity is very likely caused by inhibitory effects of secondary reactions (with residues other than E681) at high levels of modification (Jennings, 1995).

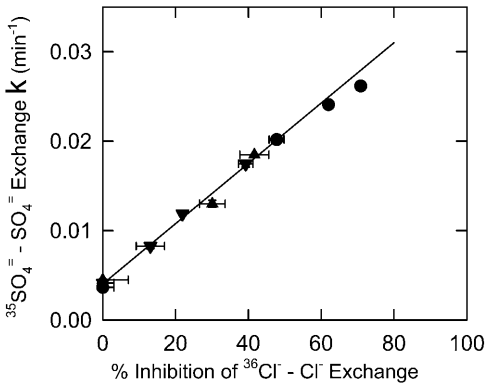

FIGURE 7 Inhibition of $^{36}Cl^-/Cl^-$ exchange and acceleration of $^{35}SO_4^{2-}/SO_4^{2-}$ exchange by graded treatment with WRK/BH_4^-. Cells were washed in 150 mM KCl/10 mM MOPS, pH 7.0, and treated at 0°C with 0, 0.4, 0.6, 1.6 mM (●); 0, 0.08, 0.16, 0.32 mM (▼); or 0, 0.2, 0.4 mM (▲) WRK followed by two additions of 1 mM BH_4^-. Each suspension was then washed in KCl/MOPS and then split in half. One half was loaded with $^{35}SO_4^{2-}$, and the rate constant for equilibrium exchange of $^{35}SO_4^{2-}$ was measured in 80 mM K_2SO_4, 10 mM HEPES, pH 7.4. The other half was loaded with $^{36}Cl^-$ in 150 KCl, 10 mM MOPS, pH 7.0, and the efflux of $^{36}Cl^-$ was measured in the same medium at 0°C. For each concentration of WRK, the rate constant for $^{35}SO_4^{2-}$ efflux is plotted on the vertical axis, and percent inhibition of $^{36}Cl^-$ efflux is plotted on the horizontal axis. Error bars denote the range of two determinations.

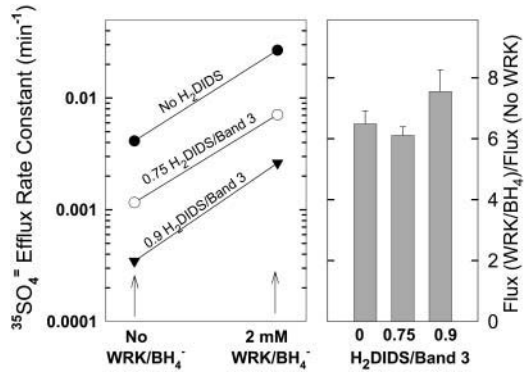

FIGURE 8 Acceleration by WRK/BH_4^- of SO_4^{2-} transport in cells pretreated with H_2DIDS. Cells were washed in HEPES-buffered saline and incubated 1 h at 37°C with 0, 0.75, or 0.9 mol H_2DIDS/mol AE1. Cells were then washed three times in 150 mM KCl, 10 mM MOPS, chilled, and treated with or without WRK/BH_4^- as in the previous figures. Cells were then washed and loaded with $^{35}SO_4^{2-}$ in all-SO_4^{2-} medium as previously, and the efflux of $^{35}SO_4^{2-}$ was measured in 80 mM K_2SO_4, 10 mM HEPES, pH 7.4, at 20°C. (*Left*) The rate constants for $^{35}SO_4^{2-}$ efflux. (*Right*) The factor by which WRK/BH_4^- accelerates efflux for each preparation. Irrespective of whether transport is initially inhibited by 0, 75%, or 90% by H_2DIDS, the acceleration by 2 mM WRK/BH_4^- is six- to sevenfold.

As an additional approach to studying the role of subunit interactions in anion transport through E681OH AE1, cells were pretreated with enough H_2DIDS to inhibit SO_4^{2-} transport irreversibly by 0, 75%, or 90%. After treatment with H_2DIDS, cells were treated with 2 mM WRK/BH_4^-, washed, and loaded with SO_4^{2-} in a HEPES-buffered 80 mM SO_4^{2-} medium, and the efflux of $^{35}SO_4^{2-}$ was measured under equilibrium conditions in the same medium. In excellent agreement with previous work (Jennings and Al-Rhaiyel, 1988; Jennings, 1995), 2 mM WRK/BH_4^- accelerates SO_4^{2-}/SO_4^{2-} exchange at pH 7.4 by six- to sevenfold (Fig. 8). The same six- to sevenfold acceleration is observed if 75% or 90% of the AE1 subunits are irreversibly inhibited by H_2DIDS before treatment with WRK/BH_4^-. In cells treated with these concentrations of H_2DIDS, the majority of the AE1 dimers will have at least one subunit occupied with H_2DIDS. Accordingly, the stimulation of SO_4^{2-} transport by WRK/BH_4^- in a given subunit does not depend on having two functioning subunits of the AE1 dimer.

DISCUSSION

Second Cl^- binding site in E681OH AE1

The data presented here provide two kinds of evidence that E681OH AE1 has a second Cl^- binding/transport site that is distinct from the Cl^- transport site in the native protein. The first evidence is that the H_2DIDS-sensitive conductive efflux of Cl^- is inhibited by removal of extracellular Cl^-. This effect is the opposite of that found in the native protein (Fröhlich et al., 1983; Fröhlich, 1984) and is consistent with the idea that an electrogenic 2:1 Cl^-/Cl^- exchange represents part of the conductive Cl^- efflux in E681OH AE1 (Fig. 2). The other evidence for a second Cl^- binding site is that extracellular Cl^- accelerates SO_4^{2-} transport by a mechanism other than recruitment of transporters to inward-facing states (Figs. 3–5). This acceleration implies that there must be a Cl^- binding site in E681OH AE1 that is distinct from the SO_4^{2-} transport site, and that Cl^- binding to this site accelerates the catalytic cycle for SO_4^{2-}/SO_4^{2-} exchange.

Although we refer to the new Cl^- site in E681OH AE1 as a binding/transport site, it is possible that Cl^- bound to this site is not actually transported; instead, Cl^- may exert its stimulatory effects as a cofactor rather than a transported substrate.

Comparison with recent structural work on *E. coli* ClC

The creation of a Cl^- binding site by removing the charge on a glutamate side chain was recently demonstrated in a prokaryotic member of the ClC family of chloride channels. Interestingly, *Escherichia coli* ClC does not function as a Cl^- channel, but rather as a coupled exchanger of Cl^- for H^+ (Accardi and Miller, 2004). A critical glutamate residue in *E. coli* ClC, E148, is clearly involved in this exchange (Accardi and Miller, 2004). Replacement of E148 in *E. coli* ClC with alanine or glutamine results in the appearance of an additional bound Cl^- ion in the interior of the protein in the crystal structure (Dutzler et al., 2002,

2003). The reason for the appearance of the additional Cl^- binding site is that the negative charge on E148 normally provides electrostatic repulsion that prevents Cl^- binding.

In comparing *E. coli* ClC with AE1, it is worth pointing out that native erythrocyte AE1 also can carry out coupled exchange of Cl^- for H^+, but only if a SO_4^{2-} ion moves in the same direction as H^+ (Jennings, 1976). This is of course a major difference between AE1 and *E. coli* ClC, but it is conceivable that there are similarities between the catalytic cycle for Cl^-/H^+ exchange in *E. coli* ClC and that for Cl^-/H^+-SO_4^{2-} exchange in AE1. The structure of the membrane domain of AE1 is known only at low resolution (Wang et al., 1994), and there is no significant sequence homology between AE1 and the ClC family. Accordingly, there may actually be no mechanistic connection between AE1 and *E. coli* ClC at all, other than blockage by stilbenedisulfonates. Nonetheless, it is intriguing that both proteins can exchange H^+ for Cl^- under some conditions (with SO_4^{2-} accompanying H^+ in AE1) and that neutralization of a critical glutamate residue in both proteins may reduce electrostatic barriers to anion binding and create an additional Cl^- binding site.

Attempts to detect 2:1 Cl^-/Cl^- tracer exchange

The above evidence for 2:1 Cl^-/Cl^- exchange (Fig. 2) is indirect because it relies on Cl^- conductance estimates derived from gramicidin-mediated $^{86}Rb^+$ fluxes. It is possible in principle, using $^{36}Cl^-$ flux measurements, to test more directly the idea that E681OH AE1 has two transport sites for Cl^- and can carry out 2:1 Cl^-/Cl^- exchange. The effects of membrane potential on SO_4^{2-}-Cl^- exchange catalyzed by E681OH AE1 indicate that most of the charge carried in a complete catalytic cycle is positive charge moving with Cl^- rather than negative charge moving with SO_4^{2-} (Jennings, 1995). If the same idea applies to 2:1 Cl^-/Cl^- exchange, then the translocation step with a single bound Cl^- ion should be the main current-carrying event. We made several attempts to detect an effect of membrane potential on $^{36}Cl^-$ influx (3 mM extracellular Cl^-, 140 mM intracellular Cl^-) in E681OH AE1 and could not detect any significant effect (data not shown). Unfortunately, this kind of experiment is technically much more difficult than measuring SO_4^{2-}/Cl^- exchange or Cl^- conductance, because the Cl^-/Cl^- exchange flux in E681OH is ~100-fold smaller than in native AE1 (Jennings, 1995). Therefore, even if only 1–2% of AE1 is unmodified, the unmodified copies of the protein will make a sizable contribution to the measured $^{36}Cl^-/Cl^-$ exchange flux. The fact that we did not observe an effect of potential could be related to interference from native AE1.

Ping-pong model with bimolecular displacement event can explain exchange kinetics

The conventional ping-pong model predicts that *cis* or bilateral Cl^- should inhibit, not accelerate, the flux of $^{35}SO_4^{2-}$ (Knauf, 1979; Fröhlich and Gunn, 1986; Passow, 1986). Therefore, the findings in Figs. 4 and 5 are the opposite of the prediction of the ping-pong model. If there are forms of E681OH AE1 that can bind and cotransport two Cl^- ions, it is possible that the transporter can bind and cotransport Cl^- and SO_4^{2-} when both ions are present on the same side of the membrane. Such a Cl^-/SO_4^{2-} cotransport event could easily explain the acceleration of $^{35}SO_4^{2-}$ flux by bilateral or *cis* Cl^- (Figs. 4 and 5), but only if the Cl^-/SO_4^{2-} cotranslocation event were more rapid than simple SO_4^{2-} translocation. Although this is possible in principle, it seems unlikely that a two-anion translocation event would be more rapid than a single-ion event, even in modified protein. Nonetheless, the cotransport of Cl^- with SO_4^{2-} is formally a possible explanation of the acceleration of $^{35}SO_4^{2-}$ flux by bilateral or *cis* Cl^-.

Another potential explanation for the accelerating effects of Cl^- on $^{35}SO_4^{2-}$ transport would be if Cl^- can displace SO_4^{2-} from a self-inhibitory site and if Cl^- bound to that site were less inhibitory than SO_4^{2-}. We did not do a thorough study of possible self-inhibition of SO_4^{2-} transport in E681OH AE1. However, the $^{35}SO_4^{2-}$ efflux into an 80 mM SO_4^{2-} medium is indistinguishable from that into a 40 mM SO_4^{2-} medium, indicating that, in this range of extracellular SO_4^{2-} concentrations, there is not a strong self-inhibitory effect of extracellular SO_4^{2-} on $^{35}SO_4^{2-}$ efflux. Therefore, possible relief of self-inhibition is not a likely explanation of the acceleration of $^{35}SO_4^{2-}$ flux by Cl^-.

We examined other possible variations of the ping-pong model to attempt to explain the effects of Cl^- on SO_4^{2-} transport in E681OH AE1. One such variation is derived from a model proposed by Salhany and Rauenbuhler (1983) and is shown in Fig. 9. As in the original ping-pong model, the transporter has distinct inward-facing and outward-facing states. The difference between the original ping-pong model and that shown in Fig. 9 is that external release of SO_4^{2-}, in the absence of external Cl^-, is proposed to be rate-limiting for SO_4^{2-}/SO_4^{2-} exchange in E681OH AE1. In this model, extracellular Cl^- can accelerate SO_4^{2-} release by binding to the outward-facing SO_4^{2-}-bound form of AE1, resulting in a ternary complex, from which SO_4^{2-} is released rapidly into the extracellular medium.

The catalytic cycle shown in Fig. 9 has been simulated using Model Maker 4 software (Cherwell Scientific, Cambridge, UK; http://www.cherwell.com/). The outward translocation rate constant for SO_4^{2-} was assumed to be 10-fold higher than the inward translocation rate constant, in keeping with the observed asymmetry of SO_4^{2-}/Cl^- exchange through E681OH (Jennings, 1995). The Cl^- translocation rate constants that gave the best fit to the data were less asymmetric, but there is no reason to expect that the asymmetry in the translocation rates of the two ions would be the same. It is known, for example, that Cl^- and HCO_3^- translocation events in native AE1 have completely different asymmetries (Knauf et al., 2002).

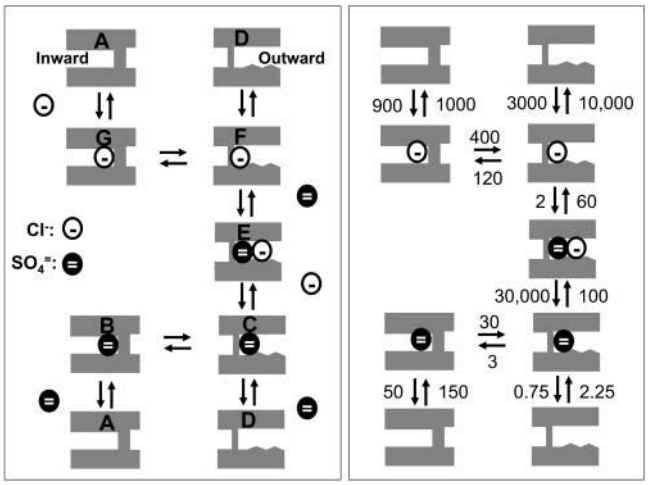

FIGURE 9 (*Left*) Model for SO_4^{2-}/SO_4^{2-} and SO_4^{2-}/Cl^- exchange in E681OH AE1. Inward-facing and outward-facing states are respectively labeled *A* and *D* (empty); *B* and *C* (SO_4^{2-}-loaded); and *F* and *G* (Cl^--loaded). State *E* is the outward-facing state with SO_4^{2-} bound to the transport site and Cl^- bound to a second site. The catalytic cycle for $^{35}SO_4^{2-}/SO_4^{2-}$ exchange is the series of transitions $A \rightarrow *B \rightarrow *C \rightarrow D \rightarrow C \rightarrow B \rightarrow A$, where **B* and **C* represent the protein loaded with $^{35}SO_4^{2-}$; the other states are either empty or loaded with nonradioactive SO_4^{2-}. According to this model, the extracellular $^{35}SO_4^{2-}$ release step $*C \rightarrow D$ is slow in the absence of extracellular Cl^-. Once Cl^- is bound (state *E*), $^{35}SO_4^{2-}$ is released rapidly, Cl^- then replaces SO_4^{2-} at the main transport site, and Cl^- is transported inward. The catalytic cycle for $^{35}SO_4^{2-}$ efflux into a Cl^--containing medium is therefore $A \rightarrow *B \rightarrow *C \rightarrow *E \rightarrow F \rightarrow G \rightarrow A$. (*Right*) Rate constants (relative) for each transition for fitting the model to the data in Figs. 4–6. The units of the translocation rates and dissociation rates are s^{-1}, and the units of the association rates are $mM^{-1}\ s^{-1}$. The translocation events are represented by the horizontal arrows. The vertical arrows represent association or dissociation events.

Fig. 9 (*right*) depicts a set of rate constants that can account semiquantitatively for many different functional aspects of anion transport in E681OH AE1, including 1), the accelerating effect of bilateral Cl^- on $^{35}SO_4^{2-}$ flux (Fig. 4); 2), the accelerating effect of *cis* Cl^- on $^{35}SO_4^{2-}$ influx (Fig. 5); 3), the high apparent affinity of the transporter for extracellular SO_4^{2-} (Fig. 6); and 4), the ~20-fold acceleration of $^{35}SO_4^{2-}$ efflux by extracellular Cl^- relative to extracellular SO_4^{2-} (Jennings, 1995). The model of course includes a large number of adjustable parameters, and we certainly do not claim that the rate constants shown in Fig. 9 represent a unique explanation of the data. Nonetheless, the modeling demonstrates that the kinetics of anion exchange in E681OH AE1 can be explained by a second outward-facing site, to which the binding of Cl^- causes the rapid release of $^{35}SO_4^{2-}$ into the extracellular medium. It is possible that the Cl^- binding event that leads to rapid extracellular release of stilbenedisulfonate in E681OH AE1 (Salhany et al., 2003) is related to the Cl^- binding event that may facilitate the rapid SO_4^{2-} release we are proposing here. It is also possible that negatively charged E681 has a role in facilitating substrate anion release in normal AE1.

Regulatory effect of Cl^- is not a likely explanation of anomalous kinetics

In mammalian erythrocytes AE1 is constitutively active as an anion exchanger, as would be expected from its physiological function. For anion exchange to contribute optimally to CO_2 transport, the exchanger must respond to anion gradients when the cell arrives in the capillary, without a regulatory activation step (Wieth et al., 1982). There are effects of ATP depletion on AE1-mediated anion exchange (Bursaux et al., 1984), and AE1 is clearly a substrate for protein kinases (Low et al., 1987; Harrison et al., 1994; Brunati et al., 2000). However, phosphorylation does not appear to have major effects on anion transport through AE1 (Jennings and Adame, 1996). In addition, we do not observe any time lags in the activation of SO_4^{2-} flux by Cl^- (Fig. 3, and Jennings, 1995). Therefore, slow conformational transitions between different functional states (Salhany and Cordes, 1992; Salhany, 2004) do not appear to be involved in the effects of Cl^- on SO_4^{2-} transport in E681OH AE1.

Lack of role of subunit interactions

It is well established that AE1 is a stable dimer (Wang et al., 1994; Casey and Reithmeier, 1991). The possible role of subunit interactions in anion transport has been the subject of debate. Each subunit of the dimer has one high-affinity binding site for H_2DIDS (Jennings and Passow, 1979), and there is a linear relationship between stilbenedisulfonate binding and transport inhibition (Cabantchik and Rothstein, 1974; Passow, 1986). The simplest interpretation of this finding is that the presence of H_2DIDS on one subunit does not prevent the other subunit from transporting anions. However, although the H_2DIDS data indicate that two functioning subunits of the dimer are not required for transport, there are considerable data in favor of allosteric interactions in the functioning of AE1 (see Salhany, 1996).

Our results add to the evidence that the subunits of the AE1 dimer catalyze anion exchange without major effects of one subunit on transport through the other. We find exactly the same fractional acceleration of SO_4^{2-} self-exchange by WRK/BH_4^- irrespective of whether most of the dimers have a subunit that has been irreversibly inhibited by H_2DIDS (Fig. 8). Moreover, graded treatment with WRK/BH_4^- causes acceleration of SO_4^{2-} self-exchange that parallels the inhibition of Cl^- self-exchange (Fig. 7), indicating that modification of the same amino acid residue (E681) causes both acceleration of SO_4^{2-} flux and inhibition of Cl^- flux and that there is no evidence that the presence of a modified subunit affects transport through an unmodified subunit.

Salhany et al. (2003) recently performed an extensive study of the effects of modification of erythrocyte AE1 with WRK and BH_4^- on anion transport and inhibitor binding/release kinetics. In agreement with the results presented here, Salhany et al. (2003) conclude that modification with

WRK/BH_4^- causes the appearance of a new binding site for Cl^- on AE1. Binding of Cl^- to this site was detected on the basis of the effects of Cl^- on stilbenedisulfonate binding/displacement kinetics. It is tempting to compare the apparent affinities for the effects of Cl^- on SO_4^{2-} exchange, Cl^- conductance, and stilbenedisulfonate displacement (Salhany et al., 2003) in E681OH AE1. Unfortunately, the data on the effect of Cl^- on Cl^- conductance (Fig. 2), though they show a clear stimulation, are not of sufficient accuracy to estimate an apparent affinity. The data on Cl^- stimulation of SO_4^{2-} exchange (Fig. 4) indicate a half-maximal effect at slightly <10 mM, which is close to the estimated dissociation constant for Cl^- binding to the site in E681OH AE1 that is responsible for altering the kinetics of DBDS (4,4′-dibenzamidostilbene-2,2′-disulfonate) displacement by DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonate) (Salhany et al., 2003). However, the conditions (e.g., concentrations of potentially competing SO_4^{2-} ion) were sufficiently different in the two studies to make it impossible to say whether the same Cl^- binding event is responsible for both the transport acceleration observed here and the alterations in stilbenedisulfonate displacement kinetics.

Our data disagree with one major aspect of the Salhany et al. (2003) study. The authors conclude that band 3 dimers in which both subunits have been modified with WRK/BH_4^- do not bind the stilbenedisulfonate DBDS. Our data indicate that, even at high degrees of modification (double exposure to WRK before reductive cleavage with BH_4^-), anion transport is strongly inhibited by low concentrations of H_2DIDS (Figs. 2, 4, and 5). Under these conditions, a large fraction of dimers are modified on both subunits by WRK/BH_4^-, as indicated by the fact that monovalent anion exchange is inhibited and divalent anion transport accelerated maximally (Jennings, 1995). The WRK/BH_4^--stimulated SO_4^{2-} exchange and Cl^- conductance are nonetheless inhibited by H_2DIDS, indicating that dimers in which both subunits are WRK/BH_4^--modified can still bind stilbenedisulfonate derivatives.

In comparing our data with those of Salhany et al. (2003), it is worth pointing out that the experiments here involve modes of transport (Cl^- conductance or $^{35}SO_4^{2-}$ exchange), that are stimulated by modification of E681 by WRK/BH_4^-. Therefore, copies of the protein in which WRK is bound but not reductively cleaved, or copies of the protein with adducts at sites other than E681, are invisible in these transport assays. At high levels of modification, especially under conditions of two successive treatments with WRK/BH_4^-, secondary reactions become much more important (Jennings, 1995). The finding by Salhany et al. (2003) that WRK/BH_4^--modified AE1 does not bind stilbenedisulfonate could be the result of WRK modifications (uncleaved adduct at E681 or adducts with other residues) in addition to conversion of E681 to an alcohol. These modifications may prevent stilbenedisulfonate binding and lead to the conclusion that dimer modified at both subunits cannot bind DBDS. In any case, we are very confident that, at levels of WRK/BH_4^- modification that produce maximal stimulation of Cl^- conductance and $^{35}SO_4^{2-}$ exchange, the resultant E681OH AE1 binds H_2DIDS with high affinity. Some of the difference between our findings and the work of Salhany et al. (2003) could be related to the fact that DBDS is a much more bulky compound than H_2DIDS.

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