Anion Transport in Sickle Red Blood Cells

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ABSTRACT. Anion transport in sickle cells (SS RBC) mediated by the band 3 membrane protein was evaluated by three different measures in both oxygenated and deoxygenated conditions and compared to normal red cells. First, Cl^- self-exchange measured as $^{36}\text{Cl}^-$ efflux at 0°C was normal in SS RBC in both $V_{\max}$ and dependence on extracellular Cl^- concentration. There was no effect of deoxygenation on either parameter. Second, stilbene-sensitive $^{35}\text{SO}_4^{2-}:\text{SO}_4^{2-}$ exchange, measured at 37°C where morphologic sickling occurred, was also unaffected by deoxygenation and was normal compared to normal red cells. Third, conductive Cl^- flux was assessed by measuring the rates of Cl^- -limited K^+ efflux in valinomycin-treated cells at 37°C . Both the stilbene-sensitive and insensitive components of net Cl^- flux were similar in SS RBC and normal red cells, and were unaltered by morphologic sickling. Thus, despite dramatic alterations in cation transport in SS RBC and the demonstration of interaction between band 3 protein and sickle cell, anion transport functions appear to be normal in SS RBC and are unaffected by deoxygenation. These data suggest that the majority of the anion exchangers in SS RBC are functionally normal. (*Pediatr Res* 28: 587–590, 1990)

Abbreviations

HbS, sickle cell hemoglobin
SS, sickle
AA, normal
HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate
DNDS, 4,4'-dinitrostilbene-2,2'-disulfonate
 $\text{K}_{1/2}$, concentration at one-half maximal velocity
RBC, red blood cell

MATERIALS AND METHODS

RBC. Blood was drawn from patients homozygous for HbS and normal subjects by venipuncture, using heparin or EDTA as anticoagulant. Blood was washed three times with the appropriate buffer, and the buffy coat was carefully aspirated after each centrifugation. Cells were generally used the day of venipuncture, but washed cells that were resuspended in a HEPES-buffered solution containing 135 mM KCl, 15 mM NaCl and stored at 10°C overnight gave similar results and are also reported.

$\text{Cl}^-:\text{Cl}^-$ exchange. Washed, unfractionated RBC were suspended in glycylglycine buffered saline (140 mM NaCl, 27 mM glycylglycine, pH 7.8) and exposed to humidified air or N_2 in a rotary evaporator flask for at least 1 h at 22°C . We then used previously published methods to measure the $\text{Cl}^-:\text{Cl}^-$ exchange flux (29). Briefly, cells were loaded with $^{36}\text{Cl}^-$, packed by centrifugation at 4°C and injected into solutions (at 0°C) with different external Cl^- concentrations ($[\text{Cl}]_o = 1\text{--}150\text{ mM}$) made by mixing glycylglycine buffered saline with a solution containing 25 mM $\text{Na}_3\text{ citrate}$, 27 mM glycylglycine, 200 mM sucrose, pH 7.8. These solutions with different $[\text{Cl}]_o$ all have equal osmolalities and equal ionic strengths (29). The time course of appearance of $^{36}\text{Cl}^-$ in the medium, corrected for hemolysis, was followed and used together with the chloride content of duplicate cell samples to calculate the flux [$\text{mmol chloride (kg Hb)}^{-1}\cdot\text{min}^{-1}$]. These flux values were analyzed using nonlinear least squares analysis

Sickle cell anemia is associated with numerous perturbations of the red cell membrane. Oxygenated SS cells exhibit abnormalities in the membrane skeleton (1, 2), oxidation of membrane lipid (3) and proteins (4, 5), and membrane mechanical properties (6). The activities of the volume-sensitive and pH-sensitive KCl cotransport system (7, 8) and the Na^+/H^+ exchange mechanism (9) are increased, whereas that of the Na^+/K^+ pump is apparently reduced in certain populations of SS cells (10). Deoxygenation of SS cells induces additional changes in transbilayer

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assuming Michaelis-Menten kinetics to calculate a $V_{\max}$ and Cl_0^- at $1/2$ maximal velocity ($K_{1/2}$) (29).

$^{35}\text{SO}_4^-:\text{SO}_4^-$ exchange. SO_4^- exchange was measured as $^{35}\text{SO}_4^-$ uptake in cells suspended in 140 mM NaCl, 20 mM HEPES (pH 7.4 at 37°C), 5 mM Na_2SO_4 , 10 mM glucose. After 1 h preincubation at 37°C to equilibrate nonradioactive SO_4^- , cells were gassed with air or N_2 for 15 min using vacuum and gas flushing, and then $^{35}\text{SO}_4^-$ (5 $\mu\text{Ci}/\text{mL}$; 185 kBq/mL) was added. Samples were taken at 4 and 19 min for control and 4 and 64 min for cells treated with 45 μM DIDS and spun through dibutyl phthalate, as described previously for cellular cation determinations (30). SO_4^- uptake was calculated from medium sp act measured in each suspension and normalized to sample Hb.

Although $^{35}\text{SO}_4^-$ uptake in the presence of DIDS was subtracted from total uptake to calculate the DIDS-sensitive component, the DIDS-insensitive component was too small and variable to analyze quantitatively. A portion of DIDS-insensitive $^{35}\text{SO}_4^-$ uptake was neither directly proportional to $^{35}\text{SO}_4^-$ sp act nor linearly dependent on uptake times. These nonspecific, non-band 3 mediated, DIDS-insensitive SO_4^- influx rates are not reported.

Table 1. Effect of deoxygenation on the kinetics of $\text{Cl}^-:\text{Cl}^-$ exchange at 0°C in SS and AA red cells*

	SS		AA	
	O_2	N_2	O_2	N_2
$V_{\max}$ [mmol·(kgHb) $^{-1}$ ·min $^{-1}$]	710 (40)	610 (20)	730 (50)	880 (60)
$K_{1/2}$ for Cl_0^- (mM)	660 (50)	710 (70)	790 (30)	950 (30)
	4.5 (0.1)	2.1 (0.2)	3.5 (0.8)	3.7 (0.7)
	3.3 (0.5)	2.1 (0.6)	2.1 (0.3)	3.0 (0.3)

* In each experiment, $^{36}\text{Cl}^-$ efflux was measured at five to eight different Cl_0^- concentrations, and the curve fitted by nonlinear regression analysis to a rectangular hyperbola to calculate $V_{\max}$ and apparent affinity for Cl_0^- ($K_{1/2}$). The data represent the values in oxy and deoxy AA and SS RBC in eight separate experiments. Numbers in parentheses are standard errors of estimate of the given parameter derived from the regression analysis of the fluxes for that experiment. Although these standard errors are small, in our experience $K_{1/2}$ values vary from 2 to 4 mM in experiments from normal RBC, even with the same donor (29). Thus, the standard errors given are more reflective of the correlation coefficient of the regression curve for a given experiment than the variance among experiments or individuals. Formal statistical analysis, therefore, is not warranted, and $K_{1/2}$ values between 2 and 5 mM are considered normal. The values for $V_{\max}$ and $K_{1/2}$ for a given experiment are in corresponding positions in the table.

Fig. 1. Stilbene sensitive SO_4^- uptake in SS and AA RBC. Cells were first pre-equilibrated with 5 mM SO_4^- , then to half of the cells DIDS was added (45 μM). Cells were subsequently equilibrated with air or N_2 and $^{35}\text{SO}_4^-$ added. Samples were taken and processed as described for assay of radioactivity and Hb.

Table 2. Effect of deoxygenation on anion transport functions at 37°C in SS and AA red cells

	SS			AA		
	O_2	N_2	n	O_2	N_2	n
DIDS-sensitive $\text{SO}_4^-:\text{SO}_4^-$ exchange*	6.1 (0.9)	6.2 (1.0)	7	7.3 (1.6)	7.0 (1.8)	5
Cl^- -limited K efflux in valinomycin-treated cells†						
Total	21.4 (5.2)	24.3 (7.2)	4	24.8 (6.0)	24.4 (6.0)	3
DNDS-insensitive	4.5 (0.9)	5.2 (1.4)	4	6.0 (1.2)	5.5 (0.5)	3
DNDS-sensitive	16.9 (5.2)	19.0 (8.1)		18.7 (4.7)	18.9 (5.6)	

* mmol (kg Hb) $^{-1}$ ·h $^{-1}$. Data are means (SD) of the paired O_2/N_2 determinations depicted in Figure 1.

† mmol (kg Hb) $^{-1}$ ·min $^{-1}$. Data are means (SD) of the paired O_2/N_2 determinations depicted in Figure 2. DNDS-sensitive flux was calculated for each O_2/N_2 pair and the results averaged.

Fig. 2. Chloride-limited K^+ efflux in valinomycin-treated SS and AA RBC. K^+ efflux was measured as described in Materials and Methods. Each data point represents the mean of three flux determinations. Standard error bars for each data point are omitted for the sake of clarity; the average coefficient of variation of triplicate fluxes for these experiments was 7%. Oxy and deoxy pairs are connected.

Conductive Cl^- permeability. Conductive Cl^- permeability was measured as the rate of K^+ loss in valinomycin-treated cells, in which K^+ permeability exceeds Cl^- permeability and the latter therefore limits the rate of net KCl efflux (26). Cells were washed in HEPES buffered saline (140 mM NaCl, 20 mM HEPES, pH 7.4) and resuspended at 2°C hematocrit in HEPES buffered saline

containing 4 mM KCl. After warming to 37°C and equilibration with air or N₂, valinomycin was added to give a final nominal concentration of 5 μ M (31). DNDS, a reversible stilbene inhibitor of band 3, when present, was added (50 μ M) before valinomycin. Triplicate samples were taken at four or five time points over 6–10 min for centrifugation through phthalate. Cellular K⁺ was measured by atomic absorption spectrophotometry, and normalized to sample Hb. Flux rates were calculated from least square regression slopes of cellular K⁺ versus time.

RESULTS

Cl⁻:Cl⁻ exchange in deoxygenated SS cells: The kinetic parameters for Cl⁻:Cl⁻ exchange obtained from the analysis of 118 separate flux measurements are summarized in Table 1. Values of K_{1/2} for external Cl⁻ fell within the normal range (2–5 mM) for both SS and AA cells, regardless of oxygenation state. The degree of variation in K_{1/2} among these experiments is normally seen among different experimental determinations, even with RBC from the same donor (29). Similarly, there was no substantial difference in V_{max} values comparing AA and SS cells or oxygenated versus deoxygenated cells. The V_{max} determinations in oxygenated AA and SS cells fall into the range of values found in independent experiments to be 600 to 1100 mmol (kg Hb)⁻¹·min⁻¹ (32).

SO₄²⁻:SO₄²⁻ exchange in AA cells. Because morphologic sickling is inhibited by the low temperatures required for measurement of Cl⁻:Cl⁻ exchange (33), SO₄²⁻:SO₄²⁻ exchange was assayed at 37°C in oxygenated and deoxygenated SS cells under conditions where morphologic sickling occurred. These data are depicted in Figure 1 for both SS and AA erythrocytes. There was no consistent effect of deoxygenation on anion exchange in either cell type. Mean values in oxygenated and deoxygenated cells were virtually identical in both SS and AA cells (Table 2), and the differences in SO₄²⁻:SO₄²⁻ exchange between oxygenated AA and SS cells were not statistically significant by *t* test. Under these conditions, DIDS inhibited 97 to 99% of SO₄²⁻ exchange, with no effect of deoxygenation or Hb type on inhibition (34).

Conductive anion permeability in SS cells. Cl⁻-limited K⁺ efflux in valinomycin-treated cells was used to assay the conductive anion permeability. Cells were deoxygenated (or exposed to air), then valinomycin was added to initiate the K⁺ efflux in the presence or absence of 50 μ M DNDS. This concentration of DNDS was insufficient to affect deoxygenation-induced cation movements, with 500 μ M DNDS inhibiting these cation fluxes by only 51% (± 11 SEM, *n* = 5) (34). Thus, under the conditions of the Cl⁻-limited K⁺ efflux experiments, the deoxygenation-induced cation pathway was not inhibited by DNDS. Figure 2 shows the effect of deoxygenation on total Cl⁻-limited K efflux and the DNDS-sensitive component in valinomycin-treated cells. In Table 2, the average values of these fluxes are given, along with the DNDS-sensitive component calculated from them. As with anion exchange, there was no consistent effect of deoxygenation on any of these components of conductive anion movement in AA or SS cells. Net anion movements in SS and AA cells were not substantially different.

DISCUSSION

Our study has characterized anion transport in SS red cells with respect to the kinetic parameters of Cl⁻ exchange at 0°C, sulfate exchange at 37°C, conductive Cl⁻ movements at 37°C, and the effects of deoxygenation. No substantial differences were found between SS and AA cells in oxygenated, unfractionated cells. Our study, therefore, did not confirm the preliminary reports that conductive Cl⁻ permeability (estimated from membrane potential measurements) was either increased (35) or decreased (36) in SS cells compared to AA cells, or that SO₄²⁻ exchange flux was increased by 30% in SS cells (35). Our finding of normal anion transport function in SS cells suggests that the

anion exchanger is normal despite the membrane abnormalities described in oxygenated SS cells—increased Hb binding to the membrane (37, 38), lipid and sulfhydryl oxidation (3–5), and abnormal cytoskeletal interactions (1, 2)—and is unaffected by the processes that produce these pathologic changes. Because these experiments used unfractionated red cell populations, subtle abnormalities in anion transport in a small subpopulation of SS cells might have gone undetected. We did not explore this possibility further.

We found no effect of sickling on either the exchange or conductive functions of the anion transport mechanism in SS cells as assessed by SO₄²⁻ influx and DNDS-sensitive, Cl⁻-limited net K⁺ efflux. This indicates that the majority of anion exchange molecules are not affected by deoxygenation of HbS. The lack of effect of deoxygenation on DIDS-sensitive SO₄²⁻ efflux, recently reported in an abstract by Clark and Rossi (39), supports this interpretation. The experimental techniques used in our study, as well as that of Clark and Rossi (39), would not permit the detection of changes in anion transport localized to the membrane spicules of deoxygenated SS cells, inasmuch as only 1 to 3% of the membrane surface is involved in spicule formation (11). Thus, no inferences can be made about the effect on local anion transport function of those deoxygenation-induced processes that are thought to be localized to membrane spicules, such as uncoupling of the lipid bilayer from the cytoskeleton and changes in lipid dynamics (11, 12).

The question of whether Cl⁻ may transverse the deoxygenation-induced pathway that mediates the increased cation permeability of deoxygenated SS cells is of interest, particularly in view of the observation that DIDS inhibits these cation movements by 80–90% (34). Our finding that deoxygenation was without effect on DNDS-insensitive Cl⁻-limited K efflux indicates clearly that Cl⁻ permeability is not increased by deoxygenation proportionally to the increase in cation permeability, *i.e.* Cl⁻ permeability is clearly not increased 3- to 10-fold as is cation permeability. On the other hand, a deoxygenation-induced Cl⁻ flux equivalent in magnitude to the cation flux [0.3–0.5 mmol (kg Hb)⁻¹·min⁻¹] would not be detectable on top of the large baseline net Cl⁻ flux of 4–5 mmol (kg Hb)⁻¹·min⁻¹ (see Table 2). Clark and Rossi (39) have reported that DIDS-insensitive SO₄²⁻ efflux was unchanged by deoxygenation of SS cells but those experiments were performed at 50- μ M DIDS concentrations, where the deoxygenation-induced cation pathway is substantially inhibited (34, 39). Considered together, the high basal Cl⁻ permeability of the red cell relative to cations, the even higher anion exchange rates, and the sensitivity of the deoxygenation-induced pathway to DIDS represent substantial experimental impediments to assessing the capacity of the deoxygenation-induced membrane lesion to mediate Cl⁻ movements. Thus, although it is clear from our experiments that anion transport via band 3 in SS red cells is not changed by deoxygenation, it will require a different experimental approach to determine if anions can traverse the deoxygenation-induced pathway that increases cation permeability in these cells.

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