Acid pH Induces Formation of Dense Cells in Sickle Erythrocytes

By Carlo Brugnara, Thuong Van Ha, and Daniel C. Tosteson

When erythrocytes from patients homozygous for hemoglobin S (SS) are swollen or exposed to pH <7.40, they lose K, Cl, and water through a volume and pH-dependent KCl cotransport system. We report that carbon monoxide-treated SS cells become progressively denser when incubated for eight to 12 hours in media with pH <7.40 (7.3 to 7.0) at constant cell 2.3-diphosphoglycerate (DPG). This phenomenon is maximal in fresh SS cells from the top and middle density fractions, and is absent in cells from the densest fraction. When AA cells are separated according to density, acid pH induces cell shrinkage in the least dense fraction of AA cells, which has considerable KCl cotransport, but produces no change in cell density in the densest fractions of AA erythrocyte, which have no KCl cotransport. These data suggest that dense cells can form in oxygenated SS erythrocytes when the KCl cotransport system is activated by acidification.

ONE OF THE HALLMARKS of sickle cell (SS) disease is the marked heterogeneity in cell volume and cation content of erythrocytes. RBCs with markedly increased density and mean corpuscular hemoglobin concentration (MCHC; 40 to 50 g/dL) and reduced water and potassium content probably contribute significantly to the vasoocclusive manifestations of the disease. These dense cells comprise most of the irreversibly sickled cells (ISC), which maintain their deformed shape even when fully oxygenated. The process of dense cells formation in SS disease is not yet well understood, but usually has been associated with deoxygenation. Deoxygenation may induce formation of dense cells through loss of K through the Ca-activated K channel or as a consequence of the increased Na entry induced by deoxygenation, which leads to activation of the Na/K pump and results in cell dehydration due to the 3Na/2K stoichiometry of this transport system. However, other investigators have reported that the deoxygenation-induced changes in cation permeability of the RBC membrane, namely the increased K efflux and Na influx, are balanced and do not produce changes in the total cation and water content. Other studies have suggested oxidative damage of the RBC membrane or shear stress as possible mechanisms of K loss and cell dehydration.

We recently described in carbon monoxide-treated SS erythrocytes the presence of a KCl cotransport system stimulated by acid pH and cell swelling and inhibited by internal Mg. The activation of this system by cell swelling induces K, Cl, and water loss. A similar system is also present in RBCs of patients homozygous for hemoglobin C (CC), in the least dense fraction of normal (AA) cells, and in LK sheep RBCs and HK sheep reticulocytes. The presence of this K transport pathway in the least dense but not in the densest fraction of normal cells suggests that cell youth is an important determinant. We have also postulated a role for positively charged hemoglobins in determining the activity of the KCl cotransport system since this pathway is not inactivated in the densest fractions of CC cells and is present in AC cells, which have a normal life span. Work by Canessa et al has confirmed and extended our findings, showing that the K movement of SS cells is chloride dependent and is markedly stimulated by NEM.

We hypothesized that one of the mechanisms by which SS cells may become denser is activation of the KCl cotransport system by acid pH. Indeed, plasma pH decreases during severe exercise and systemic or localized acidosis. Moreover, every time RBCs release oxygen in the peripheral circulation, the increased CO₂ concentration lowers plasma and cellular pH, with corresponding cell swelling. Canessa et al previously reported some evidence that activation of this KCl cotransport system in SS cells by acid pH induces cell shrinkage in 27% of the cells of the SS2 fraction. Their experiments were performed on the SS2 fraction of cells incubated in air with ouabain added. We investigated the behavior of fresh and nystatin-treated SS cells, either unseparated or separated by density. In the attempt to mimic physiologic conditions, we did not use transport inhibitors. To prove the role of KCl cotransport, we examined the effect of external K concentration in acid-induced cell shrinkage; we also used density-separated AA cells. To avoid confounding factors due to Hb S polymerization and cell sickling, we performed all experiments in SS cells saturated with carbon monoxide.

We showed that formation of dense SS cells can be accomplished in vitro, in the absence of deoxygenation, merely by incubating carbon monoxide-treated SS cells in media with pH <7.40. The dense cells formed by exposure to acid pH have high MCHC, because of loss of K, Cl, and water. When SS cells are separated according to density, acid-induced formation of dense cells is maximal in the least dense fraction and is absent in the densest fraction. These results suggest that K loss through the KCl cotransport system induced by acidification contributes to formation of dense SS cells in the absence of deoxygenation.

MATERIALS AND METHODS

Preparation of erythrocytes. After we obtained informed consent, we collected blood in heparinized Vacutainer tubes (Becton Dickinson, Rutherford, NJ) from donors homozygous for hemoglobin S (SS cells) and normal controls (AA cells). Leukocytes were removed by cotton filtration. The blood was centrifuged in a Sorvall

From the Department of Pathology, Brigham and Women's Hospital; and the Department of Cellular and Molecular Physiology, Harvard Medical School, Boston. Submitted October 6, 1988; accepted March 8, 1989. Supported by Grants No. HL-36076 and 2-P60-HL15157 from the National Heart, Lung and Blood Institute. Address reprint requests to Carlo Brugnara, MD, Department of Cellular and Molecular Physiology, Harvard Medical School, 25 Shattuck St, C1-607, Boston, MA 02115. The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

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0006-4971/89/7401-0043$3.00/0

Blood, Vol 74, No 1 (July), 1989; pp 487-495
The tubes were then spun at 25,000 rpm for 50 minutes. Three cations of Clark et al. The densities used were 1.096, 1.101, 1.110, according to density. 55 cells were layered on top of discontinuous and Na and K content, MCHC, and phthalate density distribution of AA control cells, fresh cells from normal donors were incubated at different pH for 1 2 hours at 37°C. At the end of incubation, the cells were washed with choline washing solution, and Na and K content, MCHC, and phthalate density profile were measured at the beginning and end of incubation.

**Chemicals.** NaCl and KCl were purchased from Mallinkrodt, St Louis. Tris(hydroxymethyl)amino methane (Tris), albumin (bovine, fraction V), MOPS, MES, HEPES, ouabain, sucrose, and nystatin were purchased from Sigma, St Louis. Choline chloride was purchased from Calbiochem-Behring, La Jolla, CA. MgCl₂ was purchased from Fisher Scientific, Springfield, NJ. Bumetanide was a gift from Leo Pharmaceutical, Ballerup, Denmark. All chemicals were reagent grade, and all solutions were prepared using double-distilled water.

### RESULTS

**Effect of acid pH and cell volume on K efflux from SS cells.** To study the combined effect of varying both cell pH and volume on K transport, SS cells were modified to have different MCHCs and Na and K contents, on a mmol/kg hemoglobin basis (Fig 1). K efflux was then measured at different pH, with ouabain (0.1 mmol/L) and bumetanide (0.01 mmol/L) added to inhibit the Na-K pump and the Na-K-Cl cotransport systems, respectively. As shown in Fig 1, K efflux from SS cells was pH dependent, with a pH optimum ~6.7 to 7.0 and inhibition at more alkaline or acid pH, and was also volume dependent since K efflux was increased in swollen cells and reduced in shrunken SS cells. Moreover, cell swelling induced a marked change in the pH dependence of K efflux. The bell-shaped dependence of pH noted in SS cells with normal volume was lost in swollen SS cells since, under these conditions, alkaline pH did not inhibit K efflux. In these experiments, volume was varied by the nystatin method and the osmolarity of the flux medium was 300 mosm in all cases.

In another set of experiments, K efflux was measured in cells with different volumes produced by varying the osmolarity of the flux medium, in nystatin-treated SS cells (MCHC 32 g/dL). As shown in Fig 1, K efflux had a bell-shaped dependence on pH and was further stimulated by hypotonic swelling. These two sets of experiments, together with previous research defining the nature of the K fluxes, indicate that the pH dependence of KCl cotransport decreases with decreasing cell volume but still persists in shrunken cells.

### Effect of acidification on density distribution of AA cells.

To study the effect of lowering external pH on density distribution of AA control cells, fresh cells from normal donors were incubated at different pH for 12 hours at 37°C. At the end of incubation, the cells were washed with choline washing solution at pH 7.40 and the density distribution was measured. RBCs incubated in acid media swelled because of the titration of negatively charged residues and the compensatory inward movement of chloride and water. However, since in our experiment the cells were washed at pH 7.40 after incubation, this mechanism was not responsible for the volume changes observed. As shown in Fig 2A,
Fig 1. Dependence on external pH of K efflux from CO-treated SS cells. SS cells were treated with nystatin to have different MCHC and K content (mmol/kg Hb) (left). K contents were 440, 355, 302, 255, and 227 mmol/kg Hb in cells with MCHC of 26.1, 30.4, 32.7, 35.2, and 37.4 g/dL, respectively. SS cells from another patient were treated with nystatin to have an MCHC of 32.6 g/dL (cell Na 27 mmol/kg Hb, cell K 306 mmol/kg Hb) (right) and incubated in media with different pH and osmolarities. Flux media contained 140 mmol/L NaCl (left) or 100 mmol/L NaCl and 0 to 100 mmol/L choline chloride (right). 1 mmol/L MgCl₂, 10 mmol/L glucose, 0.1 mmol/L ouabain, 0.01 mmol/L bumetanide and 10 mmol/L Tris-MES (pH 6.0 to 6.5 at 37°C) or Tris-MOPS (pH 6.75 to 8.0 at 37°C). Data are from one experiment representative of two experiments in different subjects.

Fig 2. Effect of pH on density distribution of fresh AA cells. (A) Control; (B) + adenine and inosine. Fresh AA cells (MCHC 33.6 g/dL; cell Na 21 mmol/kg Hb; cell K 268 mmol/kg Hb) were incubated for 12 hours at 37°C in a medium containing 140 mmol/L NaCl, 3 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L K-phosphate, 10 mmol/L Tris-MOPS, pH 7.40 or 7.00, 10 mmol/L glucose. (B) media also contained adenine and inosine, 1.2 and 0.9 mmol/L, respectively, at pH 7.40, and 0.6 and 0.45 mmol/L, respectively, at pH 7.40. After incubation, the erythrocytes were washed five times with choline washing solution, pH 7.40, at 4°C. Cell 2,3-DPG content was 16.7 mmol/kg Hb in fresh cells, 7.7 and 0.1 mmol/kg Hb after incubation with glucose alone at pH 7.40 and 7.0, respectively, and 22.9 and 18.4 mmol/kg Hb after incubation with glucose plus adenine and inosine at pH 7.40 and 7.0, respectively. Data are from one experiment representative of two experiments in different subjects.
AA cells swell when incubated at pH <7.40 because the 2,3-DPG content is markedly reduced by incubation in acid media.29 The decrease of cell 2,3-DPG induces a sizable inward movement of chloride and water to maintain electroneutrality. As shown in Fig 2B, when the 2,3-DPG concentration was maintained relatively constant by incubating AA cells with inosine and adenine, AA cells maintained their normal volume after being incubated in acid medium and then washed at pH 7.40.

Effect of acidification on density distribution of SS cells. We next studied the effect of lowering external pH on density distribution and cation content of SS cells. To rule out effects secondary to use of the ionophore nystatin, the influence of acid pH was first studied in fresh untreated SS blood. Figure 3 shows experiments in a patient with relatively fewer dense cells and in another patient with a sizable fraction of dense cells. Exposure to pH 7.1 induced formation of dense cells, whereas incubation at pH 7.40 had little or no effect on cell density.

Other studies were performed in SS cells swollen by the nystatin technique and then incubated for 12 hours in an isoosmotic medium. As shown in Fig 4, SS cells shrunk back toward the original volume. When the pH of the incubation medium was progressively lowered to 7.00, progressive cell shrinkage occurred. At pH 7.00, the density distribution of SS cells resembled that of fresh SS cells before swelling by the nystatin method. Thus, dense cell formation can be achieved without external Ca and without deoxygenation. This process occurs in fresh SS cells and also in nystatin-swollen SS cells, without any transport inhibitor.

The experiments described above were performed without inhibitors of the Na/K pump and Na-K-Cl cotransport. However, dense SS cells formation was not affected by ouabain (inhibitor of the Na/K pump, 0.1 mmol/L) or ouabain and bumetanide (inhibitor of the Na-K-Cl cotransport, 0.01 mmol/L). Indeed, after 12-hour incubation at pH 7.40 swollen SS cells (initial cell Na and K = 37 and 467 mmol/kg Hb) contained 20 mmol Na and 324 mmol K per kilogram of Hb without transport inhibitors and 75 mmol Na and 268 mmol K per kilogram of Hb with ouabain and
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Fig 4. Effect of external pH on density distribution of swollen SS cells. SS cells were swollen with the nystatin technique (MCHC 27.1 g/dL, Na and K content 34 and 410 mmol/kg Hb, respectively) and then incubated for 12 hours at 37°C (2% Hct) in an isosmotic medium at different pH, with composition similar to that detailed in the legend to Fig 2. At the end of incubation, the erythrocytes were washed five times with choline washing solution and the phthalate density distribution was measured. D0 and R were 1.103 and 0.025 in fresh cells, 1.090 and 0.009 in nystatin-swollen cells at zero time, 1.087 and 0.015 at pH 7.40, 1.096 and 0.016 at pH 7.30, 1.096 and 0.018 at pH 7.20, 1.098 and 0.024 at pH 7.10, and 1.097 and 0.030 at pH 7.00, respectively. Data are from one experiment representative of two experiments in different subjects.

bumetanide. After incubation at pH 7.0, the Na and K contents were 28 and 281 mmol/kg Hb, respectively, without transport inhibitors, and 79 and 230 mmol/Kg Hb, respectively, with ouabain and bumetanide.

Effect of acetic acid pH on density distribution of SS and AA cells separated according to density. Due to the marked variations in density in RBCs of patients with sickle cell disease, experiments were performed in SS cells separated according to density (Stractan II). Whole SS blood top, middle, and bottom density fractions were incubated for 12 hours at 37°C in plasmalike media at pH 7.40 and 7.00, with adenine and inosine, to avoid 2,3-DPG depletion. As shown in Fig 5, the density of whole SS blood increased at pH 7.0. Similar experiments in the three density fractions from the same patients showed that the acid pH-induced formation of dense cells was maximal in the top and middle density fractions and was absent in the bottom density fraction (Fig 5). The increase in D0 induced by acetic acid pH in the top and middle density fractions was accompanied by an increase of the middle 60% density range (R from 0.006 to 0.013 for the top fraction, and from 0.006 to 0.016 for the middle fraction), suggesting that these relatively homogeneous density fractions are heterogeneous in their response to acetic acid pH (Fig 5).

Similar results were obtained when erythrocytes from the three density fractions were first swallen with the nystatin technique and then exposed to pH 7.40 and 7.00 for 12 hours at 37°C. Cells of the top and middle density fractions shrank back toward the original volume at pH 7.40 and became denser at pH 7.10. Swollen SS cells from the bottom density fraction had a markedly different behavior, since they did not shrink at pH 7.40. However, formation of dense cells was still induced by acid pH in nystatin-swollen cells of the bottom fraction (data not shown).

These results can be explained on the basis that SS cells of the top and middle density fractions have a very active volume and pH-dependent KCl cotransport, whereas activity of this transport system is greatly reduced in SS erythrocytes of the bottom density fraction. As a consequence, cell swelling and/or acid pH can induce a large K, Cl, and water loss and cell shrinkage in SS cells of the top and middle density fractions. In cells of the bottom fraction, the stimulatory effect on K loss by cell swelling is abolished, whereas that of acid pH is diminished but not completely abolished.

If the cell shrinkage induced by acetic acid pH is mediated by KCl cotransport, it should be abolished as the outwardly directed K gradient is abolished by increasing the external K concentration. Figure 6 shows that when cells from the top density fraction were incubated at pH 7.0 in media containing 140 mmol/L KCl, the RBCs did not shrink, but actually swelled. The increased cell volume is due to an increase of the cell K content, suggesting that the KCl cotransport is activated by acetic acid pH, but mediates net K, Cl, and water gain since the K gradient is inwardly directed. Thus, the outwardly directed K gradient is the driving force for the net K loss induced by acetic acid pH.

To test further the hypothesis that the KCl cotransport system is responsible for this cell shrinkage induced by acidification, we performed experiments in AA cells separated according to density. We previously showed that the KCl cotransport system is present only in the least dense fraction of AA cells (2% to 5% of the entire RBC population) and is nonactive in the densest fraction. AA cells from the least dense and densest fractions were incubated at pH 7.40 and 7.0, with adenine and inosine, to maintain 2,3-DPG constant. As shown in Fig 7, the behavior of the least dense and densest fraction is markedly different. Erythrocytes of the least dense fraction shrink when exposed to pH <7.40.
Cells from the densest fraction swell when exposed to a similar pH. These experiments in density-separated AA cells support the hypothesis that K loss through the KCl cotransport system induced by pH < 7.40 is responsible for formation of dense SS and AA cells.

**DISCUSSION**

We showed that formation of dense cells can be induced in SS erythrocytes when hemoglobin is in the fully ligated state by incubation in media with pH < 7.40. The formation of dense cells is due to a loss of K, Cl, and water. Since net K loss through the KCl cotransport system is greatly stimulated by cell swelling or pH values < 7.40 (Fig 1), this pathway is probably responsible for formation of dense SS cells under our experimental conditions, a conclusion supported by the fact that formation of dense cells is prevented by increasing the K concentration in the medium (Fig 6). Moreover, dense cell formation induced byacidification is also found in the least dense fraction of AA erythrocytes, which possess an active KCl cotransport system, and not in the densest fraction, which does not (Fig 7).

In mature normal human RBCs containing hemoglobin A, exposure to pH < 7.40 induces cell swelling by two different mechanisms. First, by decreasing the net negative charge of nonpermeant solutes such as hemoglobin and 2,3-DPG, acidification induces a compensatory inward movement of chloride and water, with cell swelling. Second, long-term exposure to relatively acid pH induces a decrease in 2,3-DPG RBC content. To compensate for the decrease in this polyvalent nonpermeant negatively charged residue, monovalent chloride and hence water move into the cell and the cell swells. These mechanisms which promote cell swelling on acidification of mature AA cells are overcome by KCl loss and shrinkage on acidification of SS cells. SS cells shrink when deoxygenated in media with acid pH. Research by Canessa et al showed that 27% of the cells from the SS2
fraction shrink when exposed for one hour at room air to pH 7.0 with ouabain added. Our experiments, which were performed without ouabain at longer incubation times and at constant 2,3-DPG, indicate that all cells in the top and middle density fractions of SS erythrocytes become denser after being exposed to acid pH (Fig 5).

Our data indicate that the lack of cell swelling in SS cells exposed to pH <7.40 in the oxygenated state is due mainly to a marked K, Cl, and water loss through the volume- and chloride-dependent KCl cotransport system, which is very active in SS cells. Our data also suggest that fresh SS cells of the top, reticulocyte-rich, fraction have the largest K loss and cell shrinkage induced by lowering external pH as compared with cells from the other denser fractions (Fig 5). Studies on density distribution of normal RBCs after 59Fe administration have shown that at best only an approximate correspondence exists between cell density and cell age and that 59Fe can be found in the bottom densest fraction as early as ten days after labeling.32 Studies of RBC survival in SS cells appear to indicate that ISC are not the oldest cells but are formed from young cells.33,34 Theoretic work by Lew et al suggests that formation of dense ISC can occur directly from reticulocytes through activation of the Ca-dependent K channel and the KCl cotransport. Unfortunately, no studies have described survival of labeled density fractions of SS erythrocytes, so that how and when young SS cells become ISC remains to be established.

Whether shrinkage of SS cells due to activation of KCl cotransport by acidification occurs in vivo is not clear. Certainly, acidification of the magnitude used in our experiments does not occur in vivo. However, we showed (Fig 4) that formation of dense cells is not an all-or-none phenomenon, but progressively increases when the pH is <7.40. RBCs are acidified every time they pass through the capillaries of actively metabolizing tissues and hypoxic and acidic condi-
tions are normally found in the hypertensive environment of the renal medulla and in conditions of low blood flow. These small but repeated changes in cell pH may eventually produce a significant loss of potassium content in cells having an active pH, volume- and chloride-dependent KCl cotransport.

Previous in vitro studies on formation of dense SS cells have focused on changes induced by deoxygenation, either through the Ca-activated K channel or through the dehydrating effect of the Na pump. As we have shown, cell dehydration mediated by the KCI cotransport mechanism does not require external Ca, occurs whether or not the Na-K pump is inhibited by ouabain, and does not require deoxygenation, since it occurs in CO-treated cells.

Studies by Mohandas et al. and Horiuchi and Asakura showed that the magnitude of deoxygenation-induced Na and K fluxes increases with morphologic distortion of SS cells. In the latter report, external Ca and a slow rate of deoxygenation were shown to be essential requisites for formation of dense sickle cells. Other investigators have emphasized the importance of membrane oxidative damage in determining K loss and cell dehydration of sickle cells. Any one of the abovementioned mechanisms for RBC dehydration may be operative under certain conditions in sickle cells and the relative importance of each has yet to be determined.

The increased cellular concentration of 2,3-DPG observed in fresh SS cells has important pathophysiologic consequences. Due to its effect in lowering oxygen affinity of Hb, 2,3-DPG facilitates oxygen delivery to the tissues and concomitantly increases the amount of deoxyhemoglobin present at a given oxygen tension. Other consequences of the increased 2,3-DPG content of SS cells are cytoplasmic acidification and cell shrinkage, due to its effect on the equilibrium distribution of Cl, H, and water across the cell membrane. As shown by Kaperonis et al., the increased 2,3-DPG content is responsible for the lower internal pH of top-fraction SS cells as compared with top-fraction AA cells. This lower cell pH could be important in stimulating the KCl cotransport system and inducing a larger K loss and cell dehydration. Thus, another consequence of the increased 2,3-DPG content of SS cells is a reduction of cell volume and water content, either directly through the compensatory loss of chloride and water or indirectly through activation of the KCl cotransport due to the lower cell pH. Both mechanisms contribute to cell dehydration, increased Hb concentration, and increased formation of Hb S polymer.

In summary, we showed that lowering the internal pH induces loss of KCI and water in SS RBCs. This effect can be attributed to the volume-, pH-, and chloride-dependent KCl cotransport, which is active in most density fractions of SS cells and in the least dense fraction of normal AA RBCs. Since these effects take place in CO-treated SS cells and in normal least dense AA cells, they do not require deoxygenation and polymerization of S Hb. However, by inducing cell dehydration and increasing Hb S concentration, they can markedly affect Hb S polymer formation and cell sickling.

ACKNOWLEDGMENT

We are grateful to Dr Lilian McMahon of the Boston Sickle Cell Center at Boston University for providing blood samples for our study. The support, advice, and criticism of Dr H. Franklin Bunn are greatly appreciated.

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