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# Oxygen dependence of $K^+$–$Cl^-$ cotransport in human red cell ghosts and sickle cells

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**Abstract**

KCC activity in normal human red cells (containing haemoglobin A, HbA, and termed HbA cells) is $O_2$-dependent, being active in oxygenated cells but inactive in deoxygenated ones. The mechanism for $O_2$ dependence is unknown but a role for Hb has been suggested. In this paper, we address two main questions. First, do membrane ghosts prepared from HbA cells retain an $O_2$-sensitive KCC activity? Second, how is the response of KCC to changes in $O_2$ tension altered in sickle cell patients heterozygous for HbS and HbC? We found that substantial $Cl^-$-dependent $K^+$ influx, indicative of KCC activity, was present in both pink (5–10% normal Hb complement) and white (no measurable Hb) ghosts when equilibrated with air. KCC responded to deoxygenation in pink ghosts only (86 ± 10% inhibition, mean ± S.E.M., $n = 3$), whilst KCC activity in white ghosts remained high (23 ± 8% inhibition). Results indicate that pink ghosts retain an $O_2$-dependent KCC activity but that this is lost in white ghosts. Second, HbSC-containing red cells showed sickling (88 ± 3%) when deoxygenated, together with activation of the deoxygenation-induced cation pathway ($P_{sickle}$) and the Gardos channel. KCC activity, however, was elevated in oxygenated HbSC cells, but inhibited by deoxygenation. Thus Hb polymerisation and sickling could be dissociated from the abnormal response of KCC to deoxygenation observed in HbS-containing red cells. These preparations provide a useful system with which to study the components involved in $O_2$-sensitive membrane transport and why it is perturbed in certain pathological conditions (such as sickle cell disease and oxidant toxicity).
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*Keywords:* $O_2$; KCl cotransport; Ghost; Haemoglobinopathy; Red cell

## 1. Introduction

Oxygen-sensitive membrane transport systems have been observed in red cells from a number of different vertebrates [1]. Changes in transporter activity consequent upon those in $O_2$ tension are specific, selective and highly conserved. The effect can be profound, markedly affecting both transporter activity and its ability to respond to other stimuli (e.g. Ref. [2]). A number of possible physiological roles for $O_2$-sensitive transporters have been hypothesised. They may also contribute to various pathological conditions should regulation by $O_2$ become abnormal [3,4], as observed in HbS-containing red cells from sickle cell patients.

The signal transduction pathway linking changes in $O_2$ tension to the activity of membrane transporters remains to be elucidated, although evidence suggests that protein phosphorylation is often involved [5]. Nor has the sensor been unequivocally identified. Haemoglobin is the major protein of red cells and also reacts reversibly with $O_2$ making it an obvious candidate [6,7]. A number of observations support a role for Hb, such as the sigmoidal response of some transporters to $O_2$ tension and the inability of deoxygenation to influence transporter activity following carbon monoxide or nitrite treatment [6,8,9]. In addition, it has been suggested that in sickle cell disease,

*Abbreviations:* ATP, adenosine triphosphate; DIOA, [dihydroindenyloxy] alkanoic acid); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hb, haemoglobin; KCC, $K^+$–$Cl^-$ cotransporter; MBS, MOPS-buffered saline; MOPS, (3-[*N*-morpholino] propanesulphonic acid; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulphonic acid].

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HbS polymerisation at low $O_2$ tensions subverts the normal response of transporters such as the $K^+$–$Cl^-$ cotransporter (KCC), e.g. Ref. [10]. It has been suggested, however, that only a fraction of Hb is involved, in particular that associated with the plasma membrane [7]. Furthermore, recent observations with trout red cells, in which KCC cotransport appears to respond to changes in $O_2$ tension (up to 0.1 MPa) under conditions when Hb $O_2$ saturation is maximal and cannot change, raise the possibility that Hb may not be the sensor [11].

In this paper, we have approached the possible role of Hb in two ways. First, we use red cell ghosts, which are red cell membrane preparations, usually formed by hypotonic lysis followed by isotonic resealing, and which lack some, or all, of the normal intracellular components. To investigate the requirement for Hb, we made pink ghosts (with 5–10% normal Hb concentration) and white ghosts (with no detectable Hb). Second, we compared the behaviour of red cells from sickle trait individuals (HbAS) and sickle cell patients, either homozygous HbSS or heterozygous HbSC. Red cells from both groups of sickle patients undergo the sickling shape change, but in the case of HbSC-containing red cells, the ensuing disease is less severe. For both, we measured the response of KCC (probably the KCC1 isoform) to changes in $O_2$ tension.

## 2. Materials and methods

### 2.1. Blood

Samples of HbA-containing red cells (HbA cells) were obtained by venepuncture of normal individuals (homozygous HbAA), sickle cell patients of the HbSS and HbSC genotype, and a limited number of sickle trait (HbAS) individuals—all from consenting volunteers and with ethical consent. Samples were taken in heparin-containing syringes and stored at 4 °C until required, when they were processed as described previously [12].

### 2.2. Chemicals

Bumetanide, [dihydroindenyloxy] alkanoic acid (DIOA), (3-[*N*-morpholino] propanesulphonic acid (MOPS), ouabain and inorganic salts were all purchased from the Sigma, Poole, Dorset, UK; $^{86}Rb^+$ from NEN Du Pont, Stevenage, UK.

### 2.3. Solutions

The standard medium for ghost experiments was MOPS-buffered saline (MBS) comprising, in mM: NaCl 145, MOPS 10, glucose 5, pH 7.4 at 37 °C, 290 ± 5 mosM $kg^{-1}$. HbA cells, HbSS-containing red cells (HbS cells), HbSC-containing red cells (HbSC cells) were suspended in saline containing, in mM: KCl 80, NaCl 70, $MgCl_2$ 0.15, $CaCl_2$ 2.5, inosine 10, and MOPS 10, pH 7.4 at 37 °C. Where required, NaCl was replaced by equimolar $NaNO_3$ ($Cl^-$-free MBS); anion substitution was achieved by washing the cells 5 × in the appropriate medium. Stock solutions of ouabain (10 mM) and bumetanide (1 mM) were prepared in water and 100 mM Tris base, respectively. Lysing solution (LS) contained, in mM: ethylenediaminetetraacetic acid (EDTA) 0.1, piperazine-*N*,*N*′-bis[2-ethanesulphonic acid] (PIPES) 15, pH 6.5 at 0 °C. Resealing solution (RS) contained, mM: NaCl 10, KCl 140, MOPS 10, $MgCl_2$ 0.15, ethylene glycol-bis(β),*N*,*N*′,*N*′-tetraacetic acid (EGTA) 0.1, Na phosphocreatine 5, $K_3ATP$ 3, and creatine kinase 10 units $ml^{-1}$, pH 7.4 at 37 °C.

### 2.4. Preparation of ghosts

Pink ghosts were prepared by adding packed red cells to four volumes of LS at 0–2 °C. The haemolysate was placed on ice for 5 min, centrifuged (5 min, 40 000 × *g*, 0 °C) and the supernatant was removed. Cooled RS (five times the initial red cell volume) was added (producing a 10-fold dilution of cytoplasm). The suspension was incubated on ice for 10 min, and then for 1 h at 37 °C. White ghosts were prepared by gel filtration chromatography using a method based on that of Wood and Passow [13,14]. Briefly, red cells were layered onto a Pharmacia column (length 40 cm, diameter 2.5 cm) containing Biogel A50 m (50–100 mesh, 50 mDa exclusion limit), equilibrated with LS at 0–2 °C. Membranes were eluted at a rate of 0.55 ml $min^{-1}$ and collected on ice. Membranes appeared after 60–70 ml elutant had been collected, during which the Hb band had traveled about half the length of the column. The suspension of eluted membranes was centrifuged at 40 000 × *g*, 10 min, 0 °C, and the supernatant removed. The membranes were then suspended in RS, for 10 min on ice and then for 1 h at 37 °C. After resealing, both sets of ghosts were washed three times in $Cl^-$-free MBS by successive centrifugations and resuspensions (40 000 × *g*, 5 min).

### 2.5. Tonometry

$O_2$ tension was controlled by incubating cells in Eschweiler tonometers coupled to a Wösthoff gas mixing pump as described previously [8]. Ghosts (about 20% ghosts to saline in $Cl^-$-free MBS) or HbSC red cells (40% haematocrit, Hct) were pre-incubated for 30 min in air to allow ATP synthesis. This and all subsequent procedures were carried out at 37 °C. They were then placed in tonometers at the requisite $O_2$ tension for 15 min. Samples were then simultaneously (i) diluted 10-fold into MBS ± $Cl^-$, pH 7, 245 mosM $kg^{-1}$, for measurement of the acid- and swelling-stimulated $Cl^-$-dependent $K^+$ influx (taken as representing the activity of KCC) or (ii) diluted in MBS ± 0.1 mM ouabain for measurement of the ouabain-sensitive $K^+$ influx (taken as $Na^+/K^+$ pump activity).

### 2.6. Flux measurements

Potassium fluxes were determined at 37 °C using $^{86}Rb$ as a congener, added in $KNO_3$ to give a final $K^+$ concentration of 7.5 mM (see Ref. [12] for details). Ouabain (0.1 mM) and bumetanide (0.01 mM) were present unless stated, to inhibit the $Na^+/K^+$ pump and $Na^+–K^+–2Cl^-$ cotransporter. Packed ghost volume (around 20%) or Hct of intact red cells were determined by microhaematocrit centrifugation before the 10-fold dilution. $K^+$ influxes are expressed in standard units of mmol $K^+$ (l ghosts h)$^{-1}$ and given as means ± S.D. of *n* measurements. $Cl^-$-dependent $K^+$ influx was taken as a measure of KCC activity; $Cl^-$-independent $K^+$ influx at low $O_2$ tension as a measure of the deoxygenation-induced cation pathway, $P_{sickle}$ [15]; and clotrimazole (5 μM)-sensitive $K^+$ influx as a measure of Gardos channel activity.

### 2.7. Sickling assay

Red cell samples, taken under the identical conditions (particularly $O_2$ tension) used for influx assays, were fixed in saline containing 1% glutaraldehyde for analysis of cell shape. See Gibson et al. [16] for full methodology.

### 2.8. Statistics

Data are presented as means ± S.E.M. for three separate determinations on samples from different individuals, unless otherwise stated. Comparisons were made using Student's paired *t*-test.

## 3. Results

### 3.1. $K^+$ transport pathways in pink and white ghosts

All ghosting procedures interfere to some extent with normal membrane permeability. In particular, in some cases, cation permeability remains elevated after resealing and makes it unfeasible to measure the relatively small component of $K^+$ transport occurring via specific pathways. In the first series of experiments, therefore, we determined the integrity of the pink and white ghosts to $K^+$ under oxygenated conditions. Ouabain-sensitive or $Cl^-$-dependent $K^+$ influxes were measured to assess the activities of $Na^+/K^+$ pump and $K^+–Cl^-$ cotransporter (KCC), respectively. Results are shown in Fig. 1. Although the "basal" $K^+$ permeability (i.e. resistant to ouabain and bumetanide, as well as being $Cl^-$-independent) was higher than in intact cells, being about 1–1.5 mmol (l cells h)$^{-1}$, both pink and white ghosts had considerable $K^+$ influx through these specific pathways. Ouabain-sensitive $K^+$ transport was 0.59 ± 0.13 mmol (l cells h)$^{-1}$ and 0.66 ± 0.03 in pink and white ghosts, respectively; $Cl^-$-dependent $K^+$ influxes were 0.93 ± 0.16 in pink ghosts and 0.69 ± 0.07 in white ghosts. Ouabain-sensitive $K^+$ transport was decreased compared to intact HbA cells, but was of comparable magnitude to that observed in human red cell ghosts reported in the literature [17]. By contrast, KCC, which is normally quiescent or of very low capacity in mature intact red cells, was elevated, and again this is a normal feature of ghost preparations [17]. Both $Na^+/K^+$ pump activity and KCC activity had a similar magnitude in the two types of ghosts. Regulation of KCC is complex, involving a cascade of protein kinases and phosphatases, and this similarity in magnitude may indicate that both types of ghosts retained similar levels of these regulatory proteins; $Na^+/K^+$ pump activity usually reflects substrate, and especially ATP, concentrations. It was also noticeable, however, that absolute values of KCC activity were variable from individual to individual, range 0.5–0.8 mmol (l cells h)$^{-1}$. Finally, DIOA-sensitive $K^+$ influxes, another measure of KCC activity, were also determined and found to be similar to $Cl^-$-dependent ones, for example, 1.00 ± 0.07 mmol (l cells h)$^{-1}$ in pink ghosts.

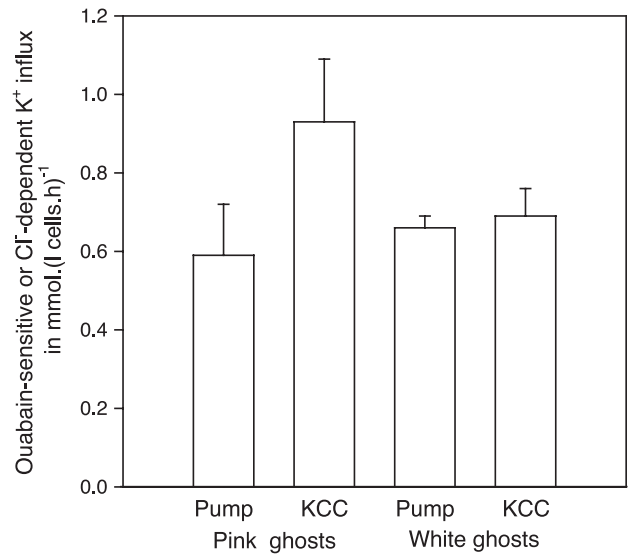


Fig. 1. Ouabain-sensitive and $Cl^-$-dependent $K^+$ influxes in pink and white ghosts from HbA cells. $K^+$ influx was measured in the presence and absence of ouabain (100 μM), and ± $Cl^-$, to determine the activity of the $Na^+/K^+$ pump and the $K^+–Cl^-$ cotransporter (KCC). Bumetanide (10 μM) was present in all cases; ouabain (100 μM) was present for determination of KCC activity. Histograms represent means ± S.E.M. ($n = 3$).

### 3.2. Effect of changes in oxygen tension on $K^+–Cl^-$ cotransport

Having ascertained the integrity of our ghost and their suitability for measuring KCC activity, we went on to investigate the $O_2$ dependence of $K^+$ transport. Fig. 2 shows $Cl^-$-dependent $K^+$ influx in both pink and white ghosts in fully oxygenated or fully deoxygenated conditions. As noted above, this measure of KCC activity was high in both ghost preparations in air. When pink ghosts were deoxygenated, there was a marked reduction

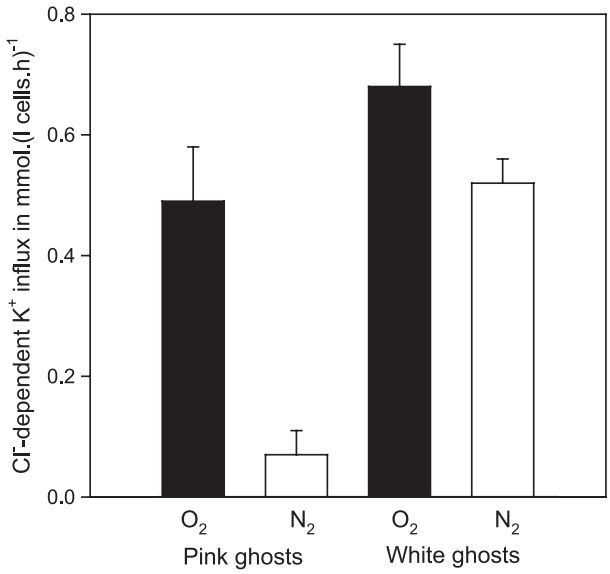


Fig. 2. $O_2$ dependence of $Cl^-$-dependent $K^+$ influx in pink and white ghosts from HbA cells. $K^+$ influxes were measured $\pm Cl^-$, to determine the activity of the $K^+$–$Cl^-$ cotransporter in fully oxygenated and fully deoxygenated ghosts. Bumetanide (10 μM) and ouabain (100 μM) were present in all cases. Histograms represent means ± S.E.M. ($n = 3$).

in this component of $K^+$ influx (86 ± 10% inhibition, $p < 0.05$). By contrast, although white ghosts had a comparable level of $Cl^-$-dependent $K^+$ influx in oxygenated ghosts, there was no significant change upon deoxygenation (23 ± 8% inhibition, N.S.). We conclude that pink ghosts, with about 5–10% normal total Hb concentrations, show an $O_2$-dependent KCC activity but that white ghosts (with no measurable Hb content) do not.

### 3.3. Membrane transport and sickling in sickle cell disease

The activity of KCC, Gardos channel and $P_{sickle}$ in oxygenated and deoxygenated HbSS and HbSC cells are presented in Table 1, and for HbSC cells in Fig. 3. Red cells from both genotypes of sickle cell disease patients showed high levels of KCC activity (about 10- to 20-fold greater than the normal HbA cells). There were differences, however, in the $O_2$ sensitivity of KCC. Thus, as published previously, KCC in HbSS cells remained at similar magnitudes in fully oxygenated and deoxygenated cells, whilst, by contrast, in the case of HbSC cells, the transporter was inhibited by deoxygenation. In this respect, its behaviour was more similar to that in HbA cells. Typical KCC activities for the four different phenotypes show the following order: HbAA = HbSA ≪ HbSC = HbSS; whilst inhibition of KCC by deoxygenation was 87 ± 4%, 96 ± 8% and 90 ± 3% for HbAA, HbSA and HbSC-containing RBCs, respectively. As expected, both HbSS and HbSC cells sickled upon deoxygenation (about 90% cells). Finally, elevation of clotrimazole-sensitive and deoxygenation-induced $Cl^-$-independent $K^+$ influxes showed, in both cases, activation at low $O_2$ tensions of the Gardos channel and $P_{sickle}$.

Table 1
Comparison of $K^+$ transport and sickling in red cells from HbSS and HbSC sickle cell patients

| | HbSS | | HbSC | |
|---|---|---|---|---|
| | $O_2$ | $N_2$ | $O_2$ | $N_2$ |
| KCC | 16.3 ± 6.5 | 15.5 ± 6.3 | 14.5 ± 1.9 | 1.4 ± 1.1 |
| Gardos | 0.5 ± 0.5 | 5.8 ± 1.4 | 0.0 ± 0.4 | 6.1 ± 2.0 |
| $P_{sickle}$ | 4.3 ± 1.5 | 15.3 ± 3.5 | 4.8 ± 0.6 | 14.3 ± 3.1 |
| Sickling (%) | N.A. | 76 ± 8 | N.A. | 88 ± 3 |

Activities, measured as $K^+$ influx in mmol (l cells h)$^{-1}$, of the different transport systems are shown for fully oxygenated cells ($O_2$) and fully deoxygenated cells ($N_2$). Sickling gives the percent sickled cells in $N_2$. KCC: $K^+$–$Cl^-$ cotransport; Gardos: Gardos channel or $Ca^{2+}$-activated $K^+$ channel (or IK1/SK4); $P_{sickle}$: deoxygenation-induced cation selective pathway.

## 4. Discussion

Three important findings arise from this work. First, red cell ghosts with only a small fraction of their normal total Hb content retain an $O_2$-dependent KCC activity. Second, white ghosts, stripped of all Hb, whilst showing considerable KCC activity in oxygenated conditions, have lost the ability to respond to changes in $O_2$ tension. Third, polymerisation of HbS/HbSC and the sickling-shape change can be dissociated from activation of KCC in sickle cells at low

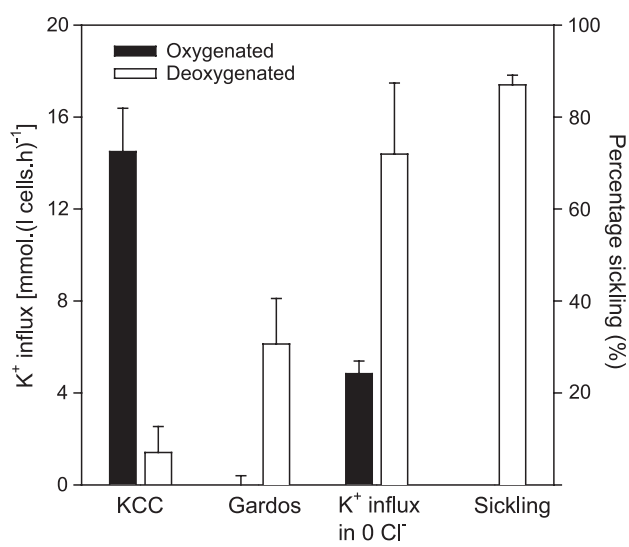


Fig. 3. $O_2$ dependence of cation transport in HbSC cells. $K^+$ transport through KCC, the Gardos channel and the $Cl^-$-independent $K^+$ pathway (as a measure of flux through the $P_{sickle}$ pathway) is shown in fully oxygenated and fully deoxygenated HbSC cells. Percentage sickling is also indicated at each $O_2$ tension. HbSC cells sickled upon deoxygenation and developed a $P_{sickle}$-type pathway. Activation of $P_{sickle}$ correlated with sickling. The Gardos channel was also activated in deoxygenated cells. However, KCC was substantially inactivated on deoxygenation (by 90%), a characteristic similar to that shown by normal (HbAA) RBCs.

$O_2$ tensions. These results are significant because they imply that only a subfraction of Hb, or some other cytoplasmic regulator, is required for $O_2$ dependence. They also show that more rigorous removal of Hb during white ghost preparation removes the $O_2$ sensitivity, at least of KCC. The red cell ghost preparation therefore represents a very useful system with which to study the detailed mechanisms of $O_2$ dependence of membrane transport. Finally, investigating the response of KCC in different genotypes showed that Hb polymerisation and red cell shape change per se are not sufficient to subvert the normal $O_2$ dependence of KCC, but always correlate with the activation of the deoxygenation-induced cation pathway ($P_{sickle}$).

Red cell ghosts have been used for a number of years to study various membrane transport mechanisms whilst controlling the cytoplasmic solute concentrations [18]. Several techniques exist for this purpose, including ion substitution, ionophores and electroporation, in addition to ghosting. Ghosting is particularly useful in that it allows complete control of the intracellular environment, including levels of proteins such as Hb. It also allows for introduction of specific antibodies against possible components of the signal transduction apparatus controlling the transporters. Its disadvantage is that membrane integrity is never completely restored. In addition, the activity of some transporters becomes higher or lower than in intact cells. Several different methods for making ghosts exist and it is important to select the one which is optimum for the intended study, here the $O_2$ dependence of KCC.

Pink ghosts were produced by hypotonic lysis. By dilution of the intracellular contents during lysis and resealing, they contained about 5–10% total Hb of intact red cells. This represents about $10^7$ molecules of Hb per ghost. There would be a similar dilution of other cytoplasmic constituents, but not membrane-bound components unless the conditions of lysis caused their disassociation from the membrane. The pink ghosts exhibited considerable $Na^+/K^+$ pump and KCC activity showing that these two membrane transporters were able to function under the conditions used. KCC activity was also found to be $O_2$-dependent, largely inhibited at low $O_2$ tensions. If Hb is the sensor for $O_2$-dependent membrane transporters, 90–95% of the total Hb does not appear to be necessary. Of the remainder, a large proportion is membrane-bound supporting the hypothesis that it is this subfraction of Hb that is responsible for the $O_2$ dependence. Much (about $10^6$ molecules or 1/10 of the remaining ghost Hb) is probably associated with the cytoplasmic tail of band 3 (AE1), a domain (termed cdb3) with considerable number of acid residues and a high negative charge [19]. Furthermore, it has been suggested that fluctuations in free intracellular $Mg^{2+}$ ($[Mg^{2+}]_i$) consequent to changes in organic phosphate binding to Hb upon deoxygenation (which results in a rise in free $[Mg^{2+}]_i$ from about 0.4 to 0.6 mM) may be involved in $O_2$-dependent KCC activity [20]. This mechanism would require a large proportion of the total Hb for its operation and the presence of $O_2$-dependent KCC in pink ghosts makes it unlikely.

White ghosts were obtained using a gel filtration method [13,14]. This method strips most, if not all, the Hb from the membrane preparations. White ghosts showed similar levels of KCC activity to pink ghosts when oxygenated. However, they lacked $O_2$ sensitivity, and activity was not significantly diminished upon complete deoxygenation. It is tempting to conclude that some Hb is required for $O_2$ sensitivity. In a similar way, it has been shown that Hb is required for volume dependence and pH sensitivity [21]. Unfortunately, white ghosts, as well as lacking Hb, will also lose more of the other intracellular constituents when compared to pink ghosts and one cannot unambiuously claim a role for Hb. Notwithstanding, the presence of KCC activity in oxygenated white ghosts of a similar magnitude to that in pink ones implies that they have some regulatory components remaining.

Finally, we compared $O_2$ dependence of KCC in red cells from sickle cell patients. In some respects, HbS polymerisation, which occurs in deoxygenated sickle cells and which reduces the soluble [Hb], is analogous to the reduction in total [Hb] in ghost preparations. The abnormal response of KCC in red cells from homozygous (HbSS) patients has been documented previously [3] and was confirmed here. Hitherto, the behaviour of red cells from heterozygous HbSC individuals has not been considered in detail. We show that activity of KCC in oxygenated HbSC cells is similar in magnitude to that in HbSS cells, 10- to 20-fold greater than in normal HbA red cells. On deoxygenation, however, differences were apparent between HbSS and HbSC cells. Both sickled, and showed the deoxygenation-induced pathway characteristic of HbSS cells [10,22], which may result from membrane damage from Hb polymers. Gardos channel activation was also observed for both. In the case of KCC, however, deoxygenation inhibited KCC in HbSC cells markedly, whilst that in HbSS cells was little affected. It is thus possible to dissociate Hb polymerisation, sickling and abnormal deoxy activity of KCC. We speculate that sufficient unpolymerised HbC remains on deoxygenation, notwithstanding polymerisation of HbS, to allow normal control of membrane transport by deoxyHb.

In summary, the present results show that pink, but not white ghosts, and HbSC but not HbSS cells, have $O_2$-dependent KCC activity. These preparations allow one to explore further the regulatory elements. For example, specific components can be reintroduced to the white ghosts during resealing (i.e. pure Hb, regulatory protein kinases and phosphatases, ATP, etc). It is possible to introduce antibodies to the signalling pathways (protein kinases and phosphatases) or cytoskeletal elements (e.g. to cdb3) in both pink and white ghosts. In addition, possible differences in protein phosphorylation, as seen between HbSS and HbA cells, remain to be elucidated. We are currently investigating some of these possibilities.

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