Oxygen-sensitive membrane transporters in vertebrate red cells

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Accepted 8 February; published on WWW 6 April 2000

Summary

Oxygen is essential for all higher forms of animal life. It is required for oxidative phosphorylation, which forms the bulk of the energy supply of most animals. In many vertebrates, transport of O2 from respiratory to other tissues, and of CO2 in the opposite direction, involves red cells. These are highly specialised, adapted for their respiratory function. Intracellular haemoglobin, carbonic anhydrase and the membrane anion exchanger (AE1) increase the effective O2- and CO2-carrying capacity of red cells by approximately 100-fold. O2 also has a pathological role. It is a very reactive species chemically, and oxidation, free radical generation and peroxide formation can be major hazards. Cells that come into contact with potentially damaging levels of O2 have a variety of systems to protect them against oxidative damage. Those in red cells include catalase, superoxide dismutase and glutathione. In this review, we focus on a third role of O2, as a regulator of membrane transport systems, a role with important consequences for the homeostasis of the red cell and also the organism as a whole. We show that regulation of red cell transporters by O2 is widespread throughout the vertebrate kingdom. The effect of O2 is selective but involves a wide range of transporters, including inorganic and organic systems, and both electroneutral and conductive pathways. Finally, we discuss what is known about the mechanism of the O2 effect and comment on its physiological and pathological roles.

Key words: oxygen, membrane transporter, red blood cell, regulation, sickle cell disease, K+/Cl- cotransport, Na+/K+/Cl- cotransport, Na+/H+ exchange, cell volume, ion channel, amino acid transport, homeostasis.

Introduction

Red cells and membrane transport

In addition to the central role of red blood cells in gas transport, historically, their simplicity, ease of procurement and relative homogeneity have resulted in their use as model systems for the study of membrane transport. In particular, red blood cells have been used to characterise volume regulatory solute transporters (Hoffmann and Dunham, 1995). Many of these volume regulatory transporters are inorganic ion co- or counter-transporters, such as Na+/H+ exchange or cation-coupled Cl- cotransporters (CCCs), for which red cells provided early evidence (Ellory et al., 1982). Besides their obvious dependence on substrate concentration (i.e. [Na+], [K+], [Cl-], etc.), many physiological and pharmacological manipulations have been shown to affect the activity of red cell membrane transporters (see Parker, 1993). These include cell volume, but also other stimuli such as H+ concentration, urea, ATP, Li+, Mg2+, β-adrenergic stimulation, macromolecular crowding, phosphorylation, protein kinase/phosphatase inhibitors; O2 tension (Po2) has an important place in this list.

Oxygen-sensitive membrane transporters

During their transit through the circulatory system, red blood cells encounter considerable variations in O2 and CO2 tension and in [HCO3-] and [H+]. Variation in Po2 provides the concentration gradient for loading and unloading of O2 to and from red cell haemoglobin. It also represents a specific signal regulating the activity of many red cell transport proteins. This is not a new observation. O2-sensitive cation transport in avian red cells has been known for over 40 years (Orskov, 1954; Tosteson and Robertson, 1956), but the significance of O2 as a transport controller has been largely disregarded, probably because of the technical difficulty, or at least inconvenience, in controlling Po2 in experiments. It has become apparent, however, that the interaction between O2 and other stimuli can be a critical determinant of red cell transporter activity.

O2-dependent transporters are found in red cells across the vertebrate spectrum, involving mainly CCCs and Na+/H+ exchangers, but also other transport systems including amino acid transporters and ion channels (Table 1). Where studied, the effect of O2 has been observed over the physiological range...
of $P_O_2$ values, with marked effects on transporters at approximately the tension required for half-maximal $O_2$ saturation of haemoglobin (Nielsen et al., 1992; Speake et al., 1997; Campbell and Gibson, 1998; Muzyamba et al., 1999). It is also selective: some transporters are stimulated, others are inhibited, whilst some, notably the $Na^+/K^+$ pump and probably AE1, are unaffected by the $O_2$ status. The effect of $O_2$ will be an important consideration for ionic homeostasis of the intracellular environment of the red cell and also for the integrative functioning of other body systems, including circulation and respiration. In addition, the abnormal control of membrane permeability by $O_2$ may be significant in pathological conditions such as sickle cell disease.

This review highlights some of the effects of $O_2$, summarises what is understood about its mechanism and indicates the possible significance of $O_2$-dependent transport systems.

### The transporters

$K^+/Cl^-$ cotransport

The obligatory coupling of $K^+$ and $Cl^-$ transport is observed in several different cell types, including epithelia and neurons as well as red cells, from a wide variety of vertebrate species (Lauf et al., 1992; Hoffmann and Dunham, 1995). Recently, the molecular identities of at least four $K^+/Cl^-$ cotransport proteins (KCC1–4) have been determined together with their relationship with other CCCs (Gillen et al., 1996; Payne et al., 1996; Mount et al., 1998, 1999; Hiki et al., 1999). $K^+/Cl^-$ cotransport in red cells is probably via KCC1 (Pellegrino et al., 1998). The transporter in red cells is stimulated by an increase in cell volume, as are KCC1 and KCC3 (Gillen and Forbush, 1999; Race et al., 1999) but not KCC2 (Payne, 1997). In normal high-$[K^+]$-containing red cells, it mediates a net efflux of $K^+$ and $Cl^-$; water will follow osmotically, resulting in cell shrinkage. $K^+/Cl^-$ cotransport has therefore been implicated in regulatory volume decrease (RVD). Given efficient osmoregulation in healthy animals, the red cell will rarely encounter anisotonic conditions, exceptions being the hypertonic environment of the renal medulla during antidiuresis and the hypotonic (or hypertonic) environment in the splanchnic circulation during water (or nutrient) absorption. Apart from the small swelling on deoxygenation, a Gibbs–Donnan effect as the pK of haemoglobin rises, cell volume will therefore be relatively constant. $K^+/Cl^-$ cotransport is also modulated by other important stimuli, notably $H^+$ and urea. Both of these will be encountered predominantly in localised regions of the circulation: low pH in active muscles beds, urea in the renal medulla during antidiuresis. Although often regarded as an RVD transporter responding to cell swelling, it has been argued that physiologically low pH is the most important stimulus to $K^+/Cl^-$ cotransport (Ellory et al., 1989).

Critically, the response of $K^+/Cl^-$ cotransport to all three potential physiological stimuli (swelling, $H^+$ and urea) depends on $P_O_2$. In the absence of a sufficiently high $P_O_2$, the transporter is inactivated and remains refractory to swelling (Fig. 1A), $H^+$ and moderate concentrations of urea (Canessa et al., 1987; Nielsen et al., 1992; Honess et al., 1996; Speake and Gibson, 1997; Speake et al., 1997). $O_2$ acts as a switch: at high $P_O_2$, the transporter is turned on and is able to respond to other stimuli; at low $P_O_2$, the transporter is turned off (Fig. 1B). The process is repeatable and reversible, but desensitisation can

### Table 1. $O_2$-sensitive transporters in vertebrate red cells

<table>
<thead>
<tr>
<th>Class</th>
<th>Species</th>
<th>Transporter</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primates</td>
<td>Human</td>
<td>KCC</td>
<td>Canessa et al. (1987); Gibson et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>AA</td>
<td>Kiessling (1997)</td>
</tr>
<tr>
<td>Carnivores</td>
<td>Ferret</td>
<td>NKCC</td>
<td>P. W. Flatman (personal communication)</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>KCC</td>
<td>Campbell and Gibson (1997)</td>
</tr>
<tr>
<td></td>
<td>Cattle</td>
<td>KCC</td>
<td>J. S. Gibson (unpublished observations)</td>
</tr>
<tr>
<td></td>
<td>Horse</td>
<td>KCC</td>
<td>Gibson et al. (1995a); Speake et al. (1997)</td>
</tr>
<tr>
<td>Birds</td>
<td>Pigeon</td>
<td>?NKCC</td>
<td>Orskov (1954)</td>
</tr>
<tr>
<td></td>
<td>Duck</td>
<td>?NKCC</td>
<td>Tosteson and Robertson (1956)</td>
</tr>
<tr>
<td></td>
<td>Duck</td>
<td>?NKCC</td>
<td>Allen and McM anus (1968)</td>
</tr>
<tr>
<td></td>
<td>Turkey</td>
<td>NKCC</td>
<td>Palfrey and Greengard (1981); Muzyamba et al. (1999)</td>
</tr>
<tr>
<td>Reptiles</td>
<td>Turtle</td>
<td>?NKCC</td>
<td>Klahr et al. (1969)</td>
</tr>
<tr>
<td>Amphibia</td>
<td>Trout</td>
<td>NHE</td>
<td>Kaloyianni and R asidaki (1996)</td>
</tr>
<tr>
<td>Teleosts</td>
<td>Trout</td>
<td>KCC</td>
<td>Borgese et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>Carpenter</td>
<td>NHE</td>
<td>Mortais et al. (1987); Nielsen et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>Carps</td>
<td>KCC</td>
<td>Jensen (1990, 1992)</td>
</tr>
<tr>
<td></td>
<td>Flounders</td>
<td>NHE</td>
<td>Salama and Nikinmaa (1988)</td>
</tr>
<tr>
<td></td>
<td>Flounders</td>
<td>KCC</td>
<td>Kiessling et al. (1998)</td>
</tr>
<tr>
<td>Agnathans</td>
<td>Lampreys</td>
<td>Cl$^-$ channel</td>
<td>Virkki et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>Lampreys</td>
<td>NHE</td>
<td>Virkki et al. (1998)</td>
</tr>
</tbody>
</table>

Transporters: KCC, $K^+/Cl^-$ cotransporter; NKCC, $Na^+/K^+/2Cl^-$ cotransporter; NHE, $Na^+/H^+$ exchanger; AA, amino acid transport; ?, transporter not identified definitively. † stimulated by oxygenation; ‡ stimulated by deoxygenation; * effect of $O_2$ is particularly marked.
occur. Thus, when cells are maintained at high $P_O$·, the transporter slowly inactivates, and exposure to low $P_O$· for some 30–60 min is required before the transporter can be reactivated by O₂ (Nielsen et al., 1992; J. S. Gibson, unpublished observations on K⁺/Cl⁻ cotransport in equine red cells). K⁺/Cl⁻ cotransport with these characteristics has been observed in red cells from many species (Table 1), in both the enucleated red cells of mammals and the nucleated red cells of lower vertebrates. It is also seen in normal haemoglobin A (HbA)-containing human red cells but, importantly, although present in human sickle cells (containing haemoglobin S, HbS), it has an abnormal O₂-dependence in these cells (Gibson et al., 1998). The control exerted by O₂ occurs over the physiological range of $P_O$ values (Fig. 1C), with maximal activity of the transporter apparent at $P_O$ values over approximately 70 mmHg (9.33 kPa), minimal activity at values below 20 mmHg (2.67 kPa); with $P_O$ for half-maximal activity ($P_{50}$) observed at approximately 30 mmHg (4 kPa) in human, horse, trout, sheep and cattle (Nielsen et al., 1992; Speake et al., 1997; Campbell and Gibson, 1998; J. S. Gibson, personal observations). Thus, the system will be activated fully in arterial blood, half-activated in mixed venous blood and inactive in areas of low $P_O$ such as active muscle beds. This behaviour will limit the regions of the circulation where H⁺ and urea can stimulate the transporter. The situation is somewhat different for urea-stimulated transport (Speake and Gibson, 1997). Stimulation by low concentrations of urea, like

Fig. 1. The responses of the K⁺/Cl⁻ cotransporter (KCC) and the Na⁺/K⁺/2Cl⁻ cotransporter (NKCC) to O₂ tension. Transporter activities are expressed as percentages of the maximal activity measured in each experiment. (A) O₂-dependence of equine red cell KCC to volume change. Values are means ± s.e.m., N=3. (B) O₂ acts as a switch controlling equine red cell KCC activity. (C) Relationship between the activities of equine red cell KCC and turkey red cell NKCC and O₂ tension. 1 mmHg=0.133 kPa. (D) Response of turkey red cell NKCC to changes in O₂ tension and to the β-adrenergic agonist isoproteronol (5 μmol l⁻¹). Values are means ± s.e.m., N=3. Experimental details can be found in Speake et al. (1997) and Muzyamba et al. (1999).
that by swelling, is fully O₂-dependent, whilst stimulation by higher urea concentrations (above 500 mmol l⁻¹) becomes progressively O₂-independent. A similar escape from O₂ control is also observed at very low (unphysiological) pH values (<6.8), when K⁺/Cl⁻ cotransport activity in equine red cells can be measured even in fully deoxygenated cells (Campbell et al., 1999).

**Na⁺/K⁺/Cl⁻ cotransport**

The Na⁺/K⁺/Cl⁻ cotransporter, like the K⁺/Cl⁻ cotransporter, is also a volume regulatory transporter with a stoichiometry of 1Na⁺:1K⁺:2Cl⁻. It has been cloned and termed NKCC, with that in red cells probably being NKCC1 (Haas, 1994; Mount et al., 1998). The transporter can have very high rates of activity mediating fluxes at more than ten times the rate of K⁺/Cl⁻ cotransport. In contrast to K⁺/Cl⁻ cotransport, it usually thought of as mediating regulatory volume increase (RVI) (Hoffmann and Dunham, 1995), with its principal stimuli being cell shrinkage and β-adrenergic agonists (McManus and Schmidt, 1978; Palfrey and Greengard, 1981). Although the cotransporter is capable of mediating very high unidirectional fluxes (measured as tracer fluxes and representing ion exchange; Duhm and Geobel, 1984), net transport, which depends on the combined chemical potential gradient of the participating ions (Na⁺, K⁺, 2Cl⁻), may be low. (Note that the term for the chemical potential of Cl⁻ is squared, because of its two participating ions, and it should dominate the overall driving force for the cotransporter. In red cells, however, the Cl⁻ ratio [Cl⁻]/[Cl⁻]₀ is small.) Alteration in ion gradients, however, by slight elevation of extracellular [K⁺] or even cell shrinkage, favours net transport (see below), and under these circumstances the transporter does have significant effects on cell volume.

In the early 1990s, Parker, Cossins and others established that a number of RVD and RVI systems had reciprocal responses to various manipulations, both physiological and pharmacological (Parker et al., 1990; Cossins, 1991; Parker, 1994; Bize and Dunham, 1994). For example, cell swelling, Li⁺, urea, N-ethylmaleimide (NEM) and Mg²⁺ depletion stimulate K⁺/Cl⁻ cotransport but inhibit Na⁺/K⁺/Cl⁻ cotransport (Palfrey and Greengard, 1981; Palfrey and Rao, 1983; Flatman, 1988; Lim et al., 1995; Mairbaurl and Herth, 1996; Muzyamba et al., 1999; Flatman and Creanor, 1999). It is therefore unsurprising that a similar complementary response is observed to changes in O₂ tension: high Pₐ₀ stimulates RVD via K⁺/Cl⁻ cotransport but inhibits RVI systems such as Na⁺/K⁺/Cl⁻ cotransport (Fig. 1C, D) and Na⁺/H⁺ exchange. Such reciprocity has been observed for K⁺/Cl⁻ cotransport and Na⁺/H⁺ exchange within red cells from a single species, the flounder (Kiessling et al., 1998), but the comparison is usually indirect, involving cells from different species. Na⁺/K⁺/Cl⁻ cotransport stimulated by deoxygenation has been observed in a number of avian species (Tosteson and Robertson, 1956; Orskov, 1956; Allen and McManus, 1968; Palfrey and Greengard, 1981). For turkey red cells, we have shown recently that the values of Pₐ₀ affecting the transporter are also over the physiological range for Pₐ₀ (Muzyamba et al., 1999). Pₐ₀ is approximately 30 mmHg (4 kPa), like that for K⁺/Cl⁻ cotransport, albeit in red cells from a different species (Fig. 1C). The interaction between O₂ and other stimuli shows some differences from that observed for K⁺/Cl⁻ cotransport.

For K⁺/Cl⁻ cotransport, O₂ activation is required before the transporter can respond to other stimuli. In oxygenated turkey red cells, Na⁺/K⁺/Cl⁻ cotransport responds markedly to the stimuli of cell shrinkage, β-adrenergic agonists and phosphatase inhibition (Muzyamba et al., 1999). In contrast, where Na⁺/K⁺/Cl⁻ cotransport activity is low, it is activated markedly by deoxygenation, after which these other stimuli have a relatively small additional effect (Fig. 1D) (Muzyamba et al., 1999). In fact, with the exception of phosphatase inhibitors, deoxygenation represents the most potent stimulus for Na⁺/K⁺/Cl⁻ cotransport, at least in turkey red cells.

**Na⁺/H⁺ exchangers**

A third electroneutral transporter showing considerable O₂-sensitivity is the Na⁺/H⁺ exchanger (NHE). At least five, possibly six, isoforms have been identified in mammals (NHE1–5: Wakabayashi et al., 1997; Orlowski and Grinstein, 1997), in addition to the βNHE of trout red cells (Borgese et al., 1992). In many cell types, Na⁺/H⁺ exchange has a role in pH regulation, acting to elevate intracellular pH when stimulated or in response to intracellular acidification. It is also stimulated by cell shrinkage and, coupled to Cl⁻/HCO₃⁻ exchange via the membrane anion exchanger AE1, can mediate NaCl uptake and thus participate in regulatory volume increase (RVI). In some cases, β-adrenergic agonists can act as a third stimulus. The relative importance of these stimuli varies among species. For example, Na⁺/H⁺ exchange in trout red cells responds mainly to β-adrenergic stimuli, modestly to cell shrinkage, whilst being insensitive to acidification; in the flounder, Na⁺/H⁺ exchange responds to all three stimuli and cell shrinkage is as important a stimulus as the β-adrenergic agonist isoproterenol; whilst in the eel, the transporter responds to shrinkage but not to β-adrenergic stimuli (Romero et al., 1996; Weaver et al., 1999). Mammalian Na⁺/H⁺ exchangers are activated by acidification, a variety of growth factors and shrinkage, but are unaffected by cyclic AMP. This difference in behaviour has been correlated with the life-style of the species under study. The molecular basis for it is under study, focusing on differences in the carboxy cytoplasmic domain (Borgese et al., 1994).

The effect of O₂ on Na⁺/H⁺ exchange activity in red cells is variable, depending on both species and stimulus. Like the Na⁺/K⁺/Cl⁻ cotransporter, the activity of the Na⁺/H⁺ exchanger is often increased by deoxygenation. In some cases, low Pₐ₀ stimulates exchange, and it may even be a prerequisite for transporter function. In other cases, O₂ is without effect. In several teleosts (e.g. trout, Motaïs et al., 1987; sea bream, Roig et al., 1997) and in frog red cells (Kaloyiannis and Rasidaki, 1996), deoxygenation amplifies the response to β-adrenergic agonists; in lamprey red cells, hypoxia increases the response to intracellular acidification (Virkki et al., 1998). In these
cases, however, deoxygenation is not a prerequisite, but instead amplifies the response. Very low $P_O$ values, however, are required before carp red cells exhibit adrenergically stimulated $Na^+/H^+$ exchange (Salama and Nikinmaa, 1988), a behaviour pattern reciprocal to that shown by $K^+/Cl^-$ cotransport, for which adequate $P_O$ is essential for its response to other stimuli and without which it remains refractory (see above). In the case of flounder red cells, $Na^+/H^+$ exchange exhibits both types of response (Weaver et al., 1999); thus, $Na^+/H^+$ exchange is only stimulated by shrinkage in deoxygenated cells, but stimulation by low pH or $\beta$-adrenergic agonists is observed regardless of $P_O$ (although it is modestly stimulated in hypoxic cells). The $Na^+/H^+$ transporter is controlled by several transduction pathways, interacting with a number of different stimuli, some of which are $O_2$-sensitive, others $O_2$-independent. The variable role of $O_2$, with species and with stimulus modality, may have arisen because of the multiple functions in which $Na^+/H^+$ exchange participates. It may also depend on structural changes in the transporter (or its regulatory proteins), for example the identity of the isoform present.

**Ion channels**

The existence of $O_2$-sensitive ion channels in excitable cells is now well established; for example, in the carotid body type 1 cells, $K^+$ channels close at low $P_O$ values, causing depolarisation. There is much less information on $O_2$-sensitive conductive pathways in red cells. The $Cl^-$-independent $K^+$ permeability pathway of trout red cells (termed KX), like $K^+/Cl^-$ cotransport, is also stimulated by swelling, but it is not $O_2$-sensitive (Nielsen et al., 1992). The molecular identity of this pathway remains uncertain. It may represent conductive ion fluxes through the anion exchanger (Motais et al., 1997). It has also been suggested that $K^+/Cl^-$cotransport and KX are mediated by the same protein, with modified anion-dependence under different conditions (Motais et al., 1991), although this view has been challenged (Berenbrink et al., 1997). If the same transporter is responsible for both modes of $K^+$ flux, then its $O_2$-sensitivity must also change. In contrast to trout, there is evidence for $O_2$-sensitive conductive channels in lamprey red cells. Red cells from these primitive fish lack a swelling-induced $K^+/Cl^-$ cotransporter, although one can be revealed by treatment with NEM, but have separate $K^+$ and $Cl^-$ channels (Kirk, 1991a,b). It appears that at least the $Cl^-$ channels are inhibited by deoxygenation (Virkki et al., 1998). Other $O_2$-dependent channels have been observed in red cells. In human sickle cells, a cation-selective channel is induced on deoxygenation, possibly following mechanical distortion of the membrane by HbS polymerisation (Joiner, 1993; Lew et al., 1997). Finally, patch-clamp studies on trout (Egee et al., 1998) and human (Kaestner et al., 1999) red cells have also identified cation channels that may be stretch-sensitive; their $O_2$-dependence has not yet been established.

**Amino acid and glucose transport**

The systems discussed above all transport inorganic ions. Much less is known about transporters of organic solutes; however, $O_2$-sensitive amino acid transporters have been observed in carp and human red cells, and there are early reports of $O_2$-sensitive glucose transport (Ellory and Lew, 1976). In human red cells, oxygenation stimulates systems ASC (a $Na^+$-dependent transport system for small neutral amino acids such as alanine, serine and cysteine; hence the acronym) and gly (a $Na^+$- and $Cl^-$-dependent transporter for glycine), whilst other amino acid transporters, $y^+$ ($Na^+$-independent transporter for cationic amino acids, e.g. lysine, ornithine and arginine) and $y^+$L (a $Na^+$-dependent transporter of neutral amino acids such as leucine, methionine and glutamine, in addition to $Na^+$-independent transport of cationic ones), are unaffected (Kiesling, 1997). The kinetic details are complicated. The affinity for serine through ASC was increased on oxygenation (a reduction in $K_m$), whilst for system gly, glycine transport was stimulated by oxygenation, with an increase in capacity (higher $V_{max}$) but no change in affinity. The situation in carp is not well defined. Deoxygenation increased amino acid efflux in the first 5 min following swelling, compared with oxygenated red cells, with little effect on sustained release (Jensen, 1995). Transport was inhibited by DIDS, furosemide and $Cl^-$ substitution (with nitrate), but otherwise the transporters involved have not been characterised.

**Mechanism of oxygen-dependent membrane transporters**

The $O_2$ sensor

In several systems, well known for their $O_2$ sensitivity (e.g. erythropoietin-secreting cells, type 1 cells of the carotid body, $N_2$-fixing bacterium), one of a number of heme-containing proteins has been suggested as the $O_2$ sensor (Bunn and Poyton, 1996; Wang and Semenza, 1996), although definitive evidence is often lacking. Similarly, results from various experimental manipulations that affect heme groups suggest a role for such proteins in the response of vertebrate red cells to $O_2$. CO can mimic $O_2$ in the control of $Na^+/H^+$ exchange in trout red cells (Motais et al., 1987) and in the control of $K^+/Cl^-$cotransport and amino acid transporters in both trout and mammalian red cells (Borgese et al., 1991; horse, human, J. S. Gibson, unpublished results; Kiesling, 1997). The oxidant $NO_2^-$ prevents inactivation of $K^+/Cl^-$cotransport by reduction in $O_2$ tension (carp, Jensen, 1990, 1992; horse and human, J. S. Gibson, unpublished results) and inhibits deoxygenation-stimulated $Na^+/H^+$ exchange (Nikonmaa and Jensen, 1992). Oxygenation of haemoglobin by $O_2$, oxidation to methaemoglobin by $NO_2^-$ and reaction with CO all favour the oxy-or relaxed conformation of haemoglobin. Deoxygenated haemoglobin is in the tense form. This conformational change has been proposed as mediating the effects of $O_2$ (Motais et al., 1987; Jensen, 1990).

Other indirect evidence also suggests that haemoglobin is involved. $K^+/Cl^-$ cotransport regulated by $O_2$ is not present in untransformed K562 cells or in hepatocytes, lacking haemoglobin (although it has not been established whether
transformed K562 cells show O2-sensitive K+/Cl− cotransport. Further, the relationships between O2 saturation, horse red cell K+/Cl− cotransport (Fig. 1C) and turkey red cell Na+/K+/Cl− cotransport activity and PO2 are all sigmoidal, with similar, although not identical, P50 values for haemoglobin saturation and transporter control (Speake et al., 1997; Muzyamba et al., 1999). In trout red cells, however, activation of K+/Cl− cotransport only becomes significant at haemoglobin O2 saturations above approximately 70 % (Nielsen and Lykkeboe, 1992a). Species differences may occur, but the likelihood of red cell heterogeneity and haemoglobin isofoms complicates this type of correlation. Third, whilst oxygenation-induced changes in haemoglobin pK do not appear to mediate the effects of O2 on the transporter (Motais et al., 1987), pH manipulations can affect transport activity consistent with mediation via the Root effect, through alterations in O2 affinity and, hence, haemoglobin conformation (Jensen, 1992). However, although haemoglobin is the most prevalent heme-containing protein in red cells, there are many others and they cannot be excluded at present. Red cell ghosts, haemoglobin-free or refilled with different haemoglobins, have been used to investigate volume and H+ responses of K+/Cl− cotransport (Sachs and Martin, 1993; Vitoux et al., 1999); they represent an obvious way of investigating a role for haemoglobin in O2-sensitive transport.

If haemoglobin does participate, it is unlikely to be bulk cytoplasmic haemoglobin. This is supported by experiments with the substituted benzaldehyde compound 12C79. This reagent is able to form Schiff bases and hydrogen bonds with amino groups, some of which will be between the N termini of the two alpha chains of haemoglobin (Beddell et al., 1984). 12C79 thereby increases the O2 affinity of haemoglobin; it was designed as an anti-sickling agent, to prevent deoxygenation of HbS and hence its polymerisation and the formation of sickled red cells. It also has profound effects on the O2 response of K+/Cl− cotransport (Gibson et al., 1999; Speake et al., 1999). When added to human or horse red cells, 5 mmol l−1 12C79 prevents the inactivation of transport seen at low PO2 values. This is not because haemoglobin remains saturated with O2. At these low PO2 values, O2 saturation is also minimal (<10%), so that most red cell haemoglobin will be in the deoxy-configuration, whilst the transporter behaves as though O2-activated (see Fig. 2 in Gibson et al., 1999).

Role of protein phosphorylation

There is evidence for a signalling cascade in red cells, coupling changes in O2 tension to transporter activity, involving both protein phosphorylation and alterations in redox state. The role of cellular protein kinases and phosphatases in the regulation of the activity of electroneutral transporters, such as the K+/Cl− and Na+/K+/Cl− cotransporters, has received considerable attention, particularly in the context of volume regulation (Palfrey, 1994; Hoffmann and Dunham, 1995). The situation is complex, with the participation of a cascade of phosphatase/protein kinase enzymes acting on residues of both the serine–threonine and tyrosine amino acids (Cossins et al., 1994; Flatman et al., 1996). Most evidence relies on the effect of more-or-less specific pharmacological agents. Thus, K+/Cl− cotransport is inhibited by the protein phosphatase inhibitors calyculin A, okadaic acid and fluoride (Jennings and Al-Rohil, 1990; Jennings and Schulz, 1991; Kaji and Tsukitani, 1991); it is stimulated by protein kinase inhibitors, such as staurosporine and probably NEM (Jennings and Schulz, 1991; Bize and Dunham, 1994), and by Mg2+ or ATP depletion (Delpire and Lauf, 1991). These findings imply that protein phosphorylation inhibits K+/Cl− cotransport and that protein dephosphorylation stimulates it. The opposite pertains for Na+/K+/Cl− cotransport (Pewitt et al., 1990; Palfrey and Pewitt, 1993), an example of reciprocal control of RVD and RVI systems first established by Parker et al. (1990). Neither the enzymes, nor in most cases the proteins involved, have been identified. In the case of K+/Cl− cotransport, it is not clear whether the transporter per se represents one of the substrates for the phosphorylation cascade or whether some other unidentified regulatory protein modulates its activity. The Na+/K+/Cl− cotransporter is certainly phosphorylated (Lytle, 1997, 1998), however, and the K+/Cl− cotransporter does have consensus sites for phosphorylation (Gillen et al., 1996), making the existence of phosphosides a probability.

Recently, we have shown that treatment of red cells with NEM alone will stimulate K+/Cl− cotransport activity markedly, treatment with calyculin A causes complete inhibition, whilst combinations of these inhibitors with calyculin A added during NEM stimulation will lock transporter activity at some intermediate level (Cossins et al., 1994; Honess et al., 1995). A similar ‘phosphorylation clamp’ can be carried out on Na+/K+/Cl− cotransport; in this case, NEM inhibits, calyculin A stimulates and clamping requires NEM to be added during calyculin A stimulation (Muzyamba et al., 1999). These manipulations prevent the transporter responding to a number of stimuli, including PO2, implying that a functional phosphorylation system is required for its normal operation. Using monoclonal antibodies to immunoprecipitate the Na+/K+/Cl− cotransporter, phosphorylation of the transporter was studied in response to a variety of agonists (shrinkage, cyclic AMP, fluoride, calyculin A), but not PO2 (Lytle, 1997). A similar approach would allow correlation of the phosphorylation of Na+/K+/Cl− cotransport (and K+/Cl− cotransport if the relevant antibodies to it were available) to changes in O2 tension. Changes in phosphosides of membrane proteins in human red cells have been observed in response to deoxygenation (Fathallah et al., 1995), but those involved in transporter regulation have not been identified. It is possible that yeast two-hybrid experiments (using known oligopeptides encompassing the transporter phosphosides as baits) or newer advances in proteomics could be used to identify the relevant phosphatase/protein kinases and other proteins. In any case, an obvious way for O2 to interact with a phosphorylation cascade in nucleated red cells of lower vertebrates is through an alteration in ATP levels via changes in oxidative phosphorylation. This is not the situation, however. Transporter activity does not correlate with ATP
levels and metabolic inhibitors such as cyanide are unable to mimic the effects of deoxygenation (Allen and McManus, 1968; Motais et al., 1987).

**Role of redox systems**

The role of redox reactions also remains unclear. A number of oxidants deplete reduced glutathione (GSH) levels and stimulate K⁺/Cl⁻ cotransport in a reciprocal manner (Lauf et al., 1995; Olivieri et al., 1993). They also make the transporter less responsive to changes in PO₂ (J. S. Gibson, unpublished observations). Under these conditions, the transporter remains sensitive to phosphatase inhibition by calyculin A, implying that GSH depletion inhibits protein phosphorylation but without abolishing the activity of the relevant protein kinase(s) (Lauf et al., 1995). Low GSH levels have been correlated with elevated K⁺/Cl⁻ cotransport activity in dog red cells (Fujise et al., 1997) and may also be responsible for the high K⁺/Cl⁻ cotransport activity of sickle cells (Olivieri et al., 1993; Adragna and Lauf, 1998). GSH depletion will cause oxidation of protein thiol groups, but again the relevant proteins responsible for its effects on membrane transport are unknown. Other components of the anti-oxidant system of the red cell may also be involved, such as NADH or NADPH, rather than GSH per se. Further, H₂O₂ stimulates K⁺/Cl⁻ cotransport, possibly via stimulation of the regulatory phosphatase (Bize et al., 1998). We speculate that changes in PO₂ could act via red cell redox systems to generate a chemical signal (e.g. a rise in H₂O₂ level, a fall in NADPH level) acting on the phosphatase/protein kinases involved in transporter regulation. A possible role for nitric oxide has not yet been investigated.

**Role of intracellular [Mg²⁺]**

A central role for changes in free intracellular [Mg²⁺] for control of O₂-sensitive transporters has been suggested but is not supported by our recent work. In red cells from many vertebrate species, organic phosphates, notably 2,3-diphosphoglycerate (DPG), ATP and inositol pentaphosphate (IPP), bind with greater affinity to deoxyhaemoglobin than to diphosphoglycerate (DPG), ATP and inositol pentaphosphate. In red cells from many vertebrates, organic phosphates, notably 2,3-diphosphoglycerate (DPG), ATP and inositol pentaphosphate (IPP), bind with greater affinity to deoxyhaemoglobin than to diphosphoglycerate (DPG), ATP and inositol pentaphosphate. In vertebrate species, organic phosphates, notably 2,3-diphosphoglycerate (DPG), ATP and inositol pentaphosphate (IPP), bind with greater affinity to deoxyhaemoglobin than to diphosphoglycerate (DPG), ATP and inositol pentaphosphate. In vertebrate species, organic phosphates, notably 2,3-diphosphoglycerate (DPG), ATP and inositol pentaphosphate (IPP), bind with greater affinity to deoxyhaemoglobin than to diphosphoglycerate (DPG), ATP and inositol pentaphosphate.

Role of membrane-bound haemoglobin

A fraction of red cell haemoglobin is membrane-bound, much of it associating with the N terminus of the cytoplasmic domain of AE1/band 3 (sometimes termed cdb3). This domain is rich in acidic amino acid residues and is highly negatively charged (Low, 1986). Haemoglobin has a higher affinity for this site when deoxygenated than when oxygenated (Walder et al., 1984; Chetrite and Cassoly, 1985). It may also compete for binding with a complex of glycolytic and other enzymes, including phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, catalase and perhaps hexokinase (Low, 1986). The N terminus has several tyrosine residues and these can be phosphorylated, thereby reducing its affinity for haemoglobin and glycolytic enzymes (Low et al., 1986). Oxygenation of red cells is associated with an increased flux of glucose through the pentose phosphate pathway and, hence, increased production of NADPH; conversely, deoxygenation stimulates its flow through the glycolytic path (Messana et al., 1996). Antibodies to cdb3 stimulate glycolysis (Low et al., 1993). These effects have been attributed to deoxyhaemoglobin (or anti-cdb3) displacing the glycolytic enzymes and thus stimulating glycolysis. This site, therefore, has many attributes associated with modulation of membrane transport: protein phosphorylation, redox components, O₂ effects. It is not surprising that this fraction of haemoglobin has been implicated in the regulation of transport by O₂ (Motais et al., 1987; Jensen, 1992; Ellory et al., 1998). The details, however, and, in particular, how the various proteins are arranged are critical and remain to be elucidated. It has been suggested that the links between AE1 and the rest of the red cell cytoskeleton allow events at cdb3 to be transmitted to more distal sites (Motais et al., 1987). Again, lamprey red cells provide a caveat. Red cells from lamprey and hagfish, both primitive vertebrates, lack a functional anion exchanger (Ohnishi and Asai, 1984; Ellory et al., 1987), although there is some evidence for the presence of band 3 precursors from immunoprecipitation and polymerase chain reaction studies (Kay et al., 1995; Cameron et al., 1996). If AE1 is normally required for O₂ modulation of membrane transport, either the lamprey represents an exception or its red cells may have another protein, perhaps a precursor of band 3, that fulfils this role.
Physiological and pathological role of oxygen-dependent membrane transporters

The prevalence of O₂-sensitive membrane transport systems in red cells from many vertebrate species, together with the reciprocal response of RVI and RVD systems to O₂, suggests an important physiological role for these systems. In many cases, however, this role is unclear and we can only speculate. In some cases, such systems may be functional only in immature cells during red cell development but not in the fully mature erythrocyte; in others, they may be non-functional vestiges retained from their evolutionary history. Finally, in some situations, the O₂ control of the transporters may be abnormal, resulting in a pathological condition.

Extrarenal regulation of plasma [K⁺]

Control of plasma [K⁺] is essential for the normal function of excitable cells. Dysfunction of the cardiac muscle and/or nerves concerned with respiratory function will be fatal. The high transport rates for K⁺ in the nucleated cells of lower vertebrates mean that they can mediate rapid alterations in plasma K⁺ concentration. In the case of rainbow trout, red cells sequester K⁺ when held at low P,O₂, as a result of inhibition of K⁺/Cl⁻ cotransport, whilst the Na⁺/K⁺ pump remains active; conversely, at high P,O₂ values, the cells lose K⁺ rapidly via the cotransporter overcoming influx via the pump. By these means, O₂ tension has marked effects on plasma [K⁺] in vivo (Nielsen and Lykkeboe, 1992a,b). A similar situation occurs in carp (Jensen, 1995).

In birds, the Na⁺/K⁺/Cl⁻ cotransporter represents the most powerful K⁺ transport system. This transporter is activated by β-adrenergic stimuli and by deoxygenation. At normal plasma K⁺ concentrations of approximately 4 mmol l⁻¹, Na⁺/K⁺/Cl⁻ cotransport is almost at electrochemical equilibrium. At higher K⁺ concentrations, there is a net inward gradient for transport of ions (McManus and Schmidt, 1978). The rate will be greatly increased in cells stimulated by deoxygenation or adrenaline, conditions that apply in anaerobic white muscle of birds during the stress of flight. One can estimate that K⁺ flux through the Na⁺/K⁺/Cl⁻ cotransporter can raise or lower plasma [K⁺] at the rate of several mmol l⁻¹ min⁻¹, but a role for these cotransporters in modulation of avian plasma [K⁺] has not yet been demonstrated in vivo. Flight at high altitudes at low ambient P,O₂, or over long distances during migration, may well represent situations in which significant modulation of Na⁺/H⁺ exchange, K⁺/Cl⁻ cotransport and Na⁺/K⁺/Cl⁻ cotransport occur, but the magnitude and direction of these changes are difficult to predict.

Although, in mammals, K⁺/Cl⁻ cotransport and Na⁺/K⁺/Cl⁻ cotransport are much less powerful, they may still exert a significant effect on plasma [K⁺]. In the horse, for example, during strenuous exercise, haemocrit can almost double, from 35 to 70 %, as a result of the release of stored red cells from the spleen (Jeffcott, 1977). The high cell/plasma ratio, with an O₂-dependent K⁺/Cl⁻ cotransporter but an O₂-independent Na⁺/K⁺ pump, may regulate plasma K⁺ concentration (Speake et al., 1997). Thus, at low P,O₂ values, with K⁺/Cl⁻ cotransport inactivated but an ongoing Na⁺/K⁺ pump, red cells might sequester plasma K⁺; at high P,O₂, rapid efflux via the cotransporter might elevate plasma [K⁺].

In many cases, these examples represent animals with very energetic lifestyles. The O₂-dependence of red cell transport systems may be necessary to limit efflux of K⁺. It will also provide a mechanism to limit fluctuations in plasma [K⁺] resulting from loss from muscle and other tissues, over a much shorter time scale than possible by renal compensation.

Carriage of blood gases

As discussed above, red cells from trout and certain other species of teleost are unusual in having a Na⁺/H⁺ exchanger stimulated by β-adrenergic stimuli. It has been proposed that this system contributes to the physiological regulation of intracellular pH necessary for the efficient carriage of blood gases (for reviews, see Nikinmaa, 1992, 1997). The stress of hypoxia, due to low ambient P,O₂ or high levels of exercise, causes the release of adrenaline (Fievet et al., 1990), which will act on red cell adrenoceptors. The resulting stimulation of red cell βNHE will be enhanced by low P,O₂ values, allowing entry of Na⁺ and loss of H⁺ to occur at high rates; meanwhile, the low P,O₂ will also inhibit K⁺/Cl⁻ cotransport. Stimulation of Na⁺ influx, with inhibition of K⁺/Cl⁻ cotransport, promotes cell swelling (Nielsen, 1997), diluting intracellular haemoglobin and thereby raising its O₂ affinity. In addition, H⁺ efflux, together with the low availability of extracellular carbonic anhydrase and the low H⁺-buffering capacity of haemoglobin, uncouples intra- and extracellular pH (Motaïs et al., 1989; Nikinmaa, 1997). The cytoplasm becomes more alkaline, whilst plasma pH falls. A fall in haemoglobin concentration and elevation of pH (Root effect) both increase haemoglobin O₂-affinity, enabling it to become more fully saturated with O₂ in the gills. O₂-sensitive Na⁺/H⁺ exchange may also contribute to O₂ unloading in the swimbladder and retina (Pelster and Weber, 1991). In these tissues, extracellular acidification, caused by lactate secretion, acidifies the red cell cytoplasm via the Jacob–Stewart cycle, and the reduction in haemoglobin O₂-affinity promotes unloading of O₂ (e.g. Scheid et al., 1990). The high P,O₂ values generated by this shift in affinity will inhibit Na⁺/H⁺ exchange activity, preventing it from removing the cellular acid load and thereby attenuating the reduction in intracellular pH.

Protection from oxidant damage

In human red cells, O₂ stimulation of the amino acid transporters, systems gly and ASC, would facilitate entry of two of the three precursors of reduced glutathione (GSH), glycine and cysteine (Kiesling, 1997). Entry of the third, glutamate, has not been ascribed to a specific amino acid transporter in red cells, and it may enter via AE1. Glucose consumption via the pentose phosphate pathway may also be elevated at high P,O₂ values, allowing increased generation of NADPH (Messana et al., 1996). Both GSH and NADPH...
are important components of the anti-oxidant capacity of the red cell. They are also intimately associated, NADPH can reduce oxidised glutathione (GSSG) regenerating GSH, and mechanisms to elevate levels of both together may be synergistic in increasing the anti-oxidant capacity of the red cell cytoplasm. Regulation of these aspects of red cell physiology by O₂ itself may be a major factor in protection against oxidative damage, as well as representing part of the transduction system regulating membrane transporters.

**Volume decrease during early red cell development**

In red cells from human and other mammalian species, K⁺/Cl⁻ cotransport activity is low or silent in mature cells, but its activity is markedly elevated in young red cells (Lauf et al., 1992). It has been suggested that K⁺/Cl⁻ efflux via this transporter mediates the volume decrease that occurs as erythroblasts mature into erythrocytes (Lauf and Bauer, 1987), with the system then becoming largely quiescent in the mature red cell. In mature human red cells, the transporter is activatable by pharmacological means (NEM, staurosporine) or using high hydrostatic pressure. The transporters therefore appear to remain in the cell membrane, with inactivity attributable to modification of the requisite intracellular signalling pathway (e.g. phosphatase/protein kinases) or components of it. The O₂-sensitivity of membrane transport has not been examined in detail in erythrocyte precursors, although we have shown that full O₂-dependence of K⁺/Cl⁻ cotransport is present in the low-density fraction of separated human red cells (A. Kahn, P. F. Specke, J. C. Ellory, and J. S. Gibson, unpublished observations), which are presumably young and contain a small proportion of reticulocytes. In early erythroid precursors, the O₂ response may be absent. The response may develop with expression of haemoglobin or some elements of the red cell cytoskeleton. In this context, it is interesting to note that K562 cells, an erythroid line, do not show O₂-dependent K⁺/Cl⁻ cotransport activity, although KCC1 has been identified by reverse transcription/polymerase chain reaction (RT-PCR) (Golding et al., 1997).

**Pathology of O₂-sensitive red cell transporters**

Regulation of red cell membrane transporters by O₂ may contribute to their physiological functions. Failure of such control may also be important in the aetiology of disease. The best-known example of inappropriate K⁺/Cl⁻ cotransport activity occurs in sickle cell disease (Ellory et al., 1998). In this disorder, HbS replaces HbA. The substitution of a single amino acid, valine for glutamic acid at position 6 of the β chain, is significant because it enables adjacent HbS molecules to associate following deoxygenation, forming polymers that distort the red cell shape into the characteristic sickled appearance. Cell shrinkage is deleterious because elevation of [HbS] reduces the lag time to HbS polymerisation upon deoxygenation (Eaton and Hofrichter, 1987). Shrunken sickle cells are therefore more likely to sickle, become trapped in the microvasculature and cause painful ischaemic crises (Stuart and Ellory, 1988). K⁺/Cl⁻ cotransport activity in sickle cells is some 10-fold higher than in HbA-containing red cells and contributes to cell shrinkage via inappropriate loss of KCl. The O₂-dependence of K⁺/Cl⁻ cotransport, observed in normal HbA-containing red cells, is perturbed in sickle cells (Gibson et al., 1998). In these cells, H⁺, urea and swelling are all able to stimulate K⁺/Cl⁻ cotransport at low P₀₂ values (Gibson et al., 1998). This response may be important because these stimuli will usually be encountered only in hypoxic areas of the circulation. They will have a maximal effect on K⁺/Cl⁻ cotransport activity and promote solute loss in situations in which the transporter would be inactive in normal red cells.

Horses, like sickle cell patients, are unusual amongst mammals in having mature, circulating HK red cells with a high capacity for K⁺/Cl⁻ cotransport. Many factors regulate equine red cell K⁺/Cl⁻ cotransport, including some as yet unidentified plasma factor (Speake et al., 1997). By analogy with sickle cell disease, we have suggested that certain ischaemic disorders of the horse, for example laminitis and rhabdomyolysis, may be caused by an inability to control red cell K⁺/Cl⁻ cotransport adequately, thereby causing an elevation of plasma [K⁺] at critical regions of the circulation.

**Concluding remarks**

We have described the characteristics of a number of O₂-sensitive membrane transport systems in red cells. Their appearance across the whole vertebrate spectrum implies an early phylogenetic appearance. Most transport inorganic ions, and there is little information concerning O₂ effects on transporters of organic solutes. O₂ tension per se represents a critical determinant in the activity of a variety of transport systems, but it also affects their response to other stimuli, such as cell volume and H⁺. The effect of O₂ is selective: not all transporters are affected, while some are stimulated, and others inhibited. Details of the underlying mechanism, although it appears to involve protein phosphorylation and redox events, remain obscure. A role for haemoglobin, or at least some other cooperative heme-containing protein, is suggested. The fraction of haemoglobin bound to the cytoplasmic tail of AE1 (cdb3) may play a pivotal role. How haemoglobin interacts with O₂, with cdb3, with enzymes of the glycolytic pathway, of the pentose phosphate shunt and of protein phosphorylation, and also with the membrane transporters, represent important areas for future research. Modern methods for investigating protein–protein interaction will be particularly useful for this. Comparison of O₂-sensitive transporters in red cells across the vertebrate kingdom has been invaluable in elucidating many aspects of their behaviour; nevertheless, their physiological and pathological roles warrant further definition.

This work is supported by The Wellcome Trust.


