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KINETICS OF CHLORIDE TRANSPORT ACROSS FISH RED BLOOD CELL MEMBRANES

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Summary

The continuous flow tube method was used to investigate the kinetics of chloride transport, and its potential oxygenation-dependency, in red blood cells (RBCs) from four teleost fish species and man. A significant interspecific variation in Cl^- transport kinetics was found. At 15 °C, the rate constant *k* for unidirectional $^{36}Cl^-$ efflux was significantly lower in RBCs from eel and carp than in RBCs from rainbow trout and Atlantic cod. The values of *k* of cod RBCs at 15 °C and of human RBCs at 37 °C were not significantly different. The volume and surface area of the RBCs were evaluated and used to calculate the apparent membrane permeability to Cl^- (P_{Cl}). The magnitude of P_{Cl} followed the sequence: eel<carp<trout≤cod. P_{Cl} values in trout and cod at 15 °C were similar to human values at 37 °C. An extrapolation of human values to 15 °C revealed that the Cl^- shift at this temperature was considerable faster in all four teleosts than in man. This illustrates appropriate adaption of band-3-mediated anion transport to the different temperature regimes encountered by fish and mammals. The Cl^- transport kinetics did not differ significantly between oxygenated and deoxygenated RBCs in any of the species examined. The apparent absence of any effect of a change in haemoglobin oxygen-saturation may be related to the presence of a flexible link which results in minimal interaction between the membrane domain (mediating Cl^- transport) and the cytoplasmic domain (to which oxygenation-dependent haemoglobin binding occurs) of band 3. In carp, Cl^- transport kinetics were not influenced by pH over the extracellular pH (pHe) range 7.6–8.36, which spans the *in vivo* pHe range. The data are discussed in relation to the rate-limiting role of red blood cell HCO_3^-/Cl^- exchange for CO_2 excretion.

Key words: Cl^- transport, chloride shift, anion exchange, band 3, red blood cells, haemoglobin, interspecific differences.

Introduction

Two processes increase the CO_2-carrying capacity of blood by driving the catalysed red cell CO_2 hydration reaction in tissue capillaries towards bicarbonate formation: (i) binding of the hydrogen ions produced to haemoglobin (Hb), and (ii) an exchange of bicarbonate with chloride across the red blood cell (RBC) membrane *via* the 'band 3' anion exchanger. In humans at rest, the relative contributions of oxygenation-linked H^+ binding (the Haldane effect) and of anion exchange to HCO_3^- formation are about equal (Wieth *et al.* 1982). In many teleost fishes, the buffer values of the oxygenated and deoxygenated Hb conformations are lower and the oxygenation-linked H^+ binding is much higher than seen in other vertebrates (Jensen, 1989). It was suggested that the Haldane effect plays a larger role for blood CO_2 transport in teleosts than in mammals, and that the large-oxygenation-linked H^+ binding might compensate for a relatively slow HCO_3^-/Cl^- exchange (Jensen, 1988, 1989). This latter hypothesis implies a significant species variation in anion exchange capacity. Indirect support for this idea was obtained from measurements of the rate of pH equilibration across fish RBC membranes following an acid load (Jensen, 1988; F. B. Jensen and H. Malte, unpublished data). In addition, sulphate exchange rates have been reported to differ between carp and rainbow trout RBCs (Pasternack and Nikinmaa, 1988). The kinetics of Cl^- or HCO_3^- transport across fish RBC membranes have only been measured in a few studies, and the available data may not reflect physiological transport rates because of the limited time resolution of the techniques applied or the use of pH and temperature values outside the physiological range in order to produce measurable rates (e.g. Cameron, 1978; Romano and Passow, 1984).

The purpose of the present study was to use the continuous flow tube method (Brahm, 1977) to measure the kinetics of Cl^- transport across the RBC membrane in four teleost species (carp, rainbow trout, eel and Atlantic cod) and in humans. A further aim was to investigate whether band 3 Cl^- transport is influenced by the degree of oxygenation of Hb. Band 3 appears to be an allosteric transporter with both homotropic and

heterotropic allosteric interactions (Salhany, 1990). Haemoglobin in the T ('deoxy') structure binds to the cytoplasmic fragment of band 3 with higher affinity than does haemoglobin in the R ('oxy') structure (Walder *et al.* 1984; Chétrite and Cassoly, 1985; Tsuneshige *et al.* 1987), and this binding may induce a conformational change in band 3 that influences its anion transport functions (Salhany, 1990). Fish RBCs provide a good model for testing this hypothesis, since many transport processes in these cells, including adrenergic Na^+/H^+ exchange (Motais *et al.* 1987) and K^+/Cl^- cotransport (Jensen, 1990, 1992; Borgese *et al.* 1991; Nielsen *et al.* 1992), show a strong oxygenation-dependency that may be related to the differential binding of oxyhaemoglobin and deoxyhaemoglobin to band 3.

Materials and methods

Carp (*Cyprinus carpio*, 1–2 kg in mass), rainbow trout (*Oncorhynchus mykiss*, 0.5–1.5 kg) and eel (*Anguilla anguilla*, 0.2–0.4 kg) were obtained from local suppliers in Denmark and acclimated in 400 l tanks to aerated, running Odense tap water at 15 °C for 2 weeks prior to use. Atlantic cod (*Gadus morhua*, 0.6–1.4 kg) were caught in sea water with a salinity of 32 ‰ close to Kristineberg Marine Biological Station, Sweden, and kept in 300 l tanks with running sea water (32 ‰) for 1 week prior to experimental use at the station.

Fish blood was drawn from the caudal blood vessels into heparinized syringes. Human blood was drawn from an arm vein of a non-smoking volunteer. Freshly drawn blood was centrifuged, and the plasma osmolality was measured. The RBCs were then washed three times in iso-osmotic saline, which was prepared by appropriate mixing of 'high'- and 'low'-osmolality saline. The 'high' saline had the following composition (in $mmol\,l^{-1}$): NaCl, 200; $NaHCO_3$, 6; KH_2PO_4, 2.9; $CaCl_2$, 2; $MgSO_4$, 1; glucose, 3.9; Hepes buffer, 10. The 'low' saline had the same composition except that [NaCl] was reduced to $100\,mmol\,l^{-1}$.

Following the washing procedure, the RBCs were suspended to a haematocrit of approximately 30 %. Samples of 5 ml were equilibrated for 45–60 min in an Eschweiler (Kiel, Germany) tonometer with either humidified air (to oxygenate the cells) or N_2 (to deoxygenate the cells) at 15 °C (fish RBCs) or 37 °C (human RBCs). Some 25 min prior to the end of this equilibration period, 75 μl of isotope ($^{36}Cl^-$ in $0.1\,mol\,l^{-1}$ HCl, $18.5\times10^9\,Bq\,mol^{-1}$) was added to load the RBCs with radioactive Cl^-. In some experiments with carp RBCs, 200 μl of $0.155\,mol\,l^{-1}$ NaOH was added simultaneously to obtain a higher pH. In some experiments, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS; Aldrich Chemical Co.) was added to the RBC suspension (5 min before the end of the equilibration period) and to the saline used in the $^{36}Cl^-$ efflux experiments to give a final concentration of $0.2\,mmol\,l^{-1}$. Following equilibration, pH was measured, and the RBC suspension was centrifuged for 10 min at 3850 ***g*** to obtain packed RBCs for Cl^- flux measurements.

$^{36}Cl^-$ efflux experiments were carried out at 15 °C (fish RBCs) or 37 °C (human RBCs), using the continuous flow tube method (Brahm, 1977, 1989). Briefly, 0.5 ml of radioactively labelled RBCs, packed to a haematocrit of approximately 85–90 %, was continuously mixed with 270 ml of an isotope-free saline solution in a mixing chamber, and the dilute suspension was forced down a tube at constant velocity. At predetermined distances, the tube wall was replaced by micropore filters that allow the fluid, but not the cells, to pass. The cell-free filtrates contained increasing amounts of radioactivity with increasing distance from the mixing chamber, where the release of tracer from the cells was initiated. Hence, the radioactivity can be related to the distance from the mixing chamber and, thus, to time. The saline used was air-equilibrated in experiments with oxygenated RBCs and N_2-equilibrated in experiments with deoxygenated RBCs.

Inhibition of Cl^- efflux was determined in a few experiments on human RBCs by means of the Millipore–Swinnex filtering technique. The principle of the kinetics is the same as above, but the cell-free filtrates are collected over much longer time intervals (see Dalmark and Wieth, 1972).

The radioactivity of the samples was determined by liquid β-scintillation spectrometry and was used to calculate the tracer efflux rate according to:

$$a_t = a_\infty(1 - e^{-kt}) + a_0\,, \qquad (1)$$

where a_t, a_∞ and a_0 denote the extracellular radioactivity at time t, at equilibrium and at time zero, respectively. a_0 represents the extracellular radioactivity trapped between the cells during the incubation and packing procedure. This fraction of radioactivity does not contribute to the efflux process (the efflux curves have the same slope, and thus the same rate constant k, at variable fractions of trapped extracellular radioactivity; see Brahm, 1989). The rate constant for the unidirectional efflux of $^{36}Cl^-$, k (s^{-1}), is related to the apparent chloride permeability, P_{Cl}, by:

$$P_{Cl} = kV_w/A_m\,, \qquad (2)$$

where V_w/A_m is the ratio of cell water volume to cell membrane area.

In a separate series of experiments, RBC volume and water content, and extra- and intracellular pH, were measured on fish RBC suspensions that had been equilibrated in the tonometer as described above. Extracellular pH was measured with a Radiometer (Copenhagen, Denmark) BMS3 electrode system thermostatted at the experimental temperature and connected to a PHM 73 monitor. Intracellular pH was measured after twice freezing (in liquid N_2) and thawing the packed RBCs. Haematocrit (Hct) was determined by centrifugation. The number of RBCs (N_{RBC}) was assessed using a Bürger–Türk counting chamber and a microscope. Mean cellular volume (MCV) was calculated from Hct/N_{RBC}. The RBC water content was determined from the wet and dry (24 h at 90 °C) masses of packed RBCs and was not corrected for trapped extracellular water. The morphological dimensions of carp RBCs were evaluated by videomicroscopy and digital image processing

(GIPS, Image House, Copenhagen) of phase-contrast photomicrographs (Leitz Ortholux II) of the cells.

The data are presented as means ± S.E.M. and were analysed by two-factor analysis of variance (ANOVA) followed by the Tukey multiple comparison test.

Results

Under the experimental conditions used in the interspecific comparison, the mean cellular volume (MCV) of oxygenated RBCs was (means ± S.E.M.): 185±5 μm^3 (N=8) in carp, 225±12 μm^3 (N=6) in rainbow trout, 171±7 μm^3 (N=9) in eel and 172±2 μm^3 (N=3) in cod. Thus, the fish RBCs had a volume 2–2.6 times larger than the volume of human RBCs (87 μm^3, Wieth *et al.* 1974). Deoxygenation of the cells increased MCV by 6–10 %, and the fractional RBC water content varied between 0.67 and 0.69, depending on pH and degree of oxygenation (not illustrated).

The extracellular and intracellular pH values (pHe and pHi) were of comparable magnitude among fish species. pHe of oxygenated RBC suspensions was 7.6–7.7 and pHi was 7.1–7.24 (Fig. 1). These pH values are slightly below values in resting fish, but correspond to pH values seen in arterial blood of fish after exercise stress, which is a situation in which the rate of anion exchange is thought to be limiting for CO_2 excretion. Deoxygenation caused an elevation of pHe to 7.7–8.0 and of pHi to about 7.5 (Fig. 1). The relatively large increase in pH upon deoxygenation is a consequence of the large Haldane effect and the low buffer values of the oxy and deoxy conformations of teleost haemoglobins (Jensen, 1986, 1989).

Typical examples of $^{36}Cl^-$ efflux experiments are shown in Fig. 2. In general, efflux curves were linear. Occasionally, however, non-linearity was observed. In these cases, the initial part of the curve was used to calculate the rate constant k.

The presence of DIDS at 0.2 mmol l^{-1} significantly inhibited Cl^- transport (decreased k) in trout and human RBCs (Fig. 2). With carp RBCs, an unexpected apparent lack of effect of DIDS was found (Fig. 2).

Analysis of the data on the rate constant for unidirectional $^{36}Cl^-$ efflux (Fig. 3) by a two-factor (i.e. species and oxygenation degree) ANOVA revealed a significant (P<0.0001) interspecific variation in Cl^- transport kinetics, whereas there was no significant influence of the degree of oxygenation nor an interaction between species and degree of oxygenation. The rate constants for Cl^- efflux from oxygenated and deoxygenated RBCs were significantly lower in both carp and eel RBCs than in trout (P<0.05) and cod (P<0.01) RBCs (Fig. 3), whereas k values did not differ significantly between carp and eel nor between trout and cod. The k values of human RBCs at 37 °C were significantly higher than in carp (P<0.01), eel (P<0.01) and trout (P<0.05) RBCs at 15 °C. The rate constants did not differ significantly between cod and man.

The time required for 63 % equilibration of Cl^- fluxes is given by 1/k and is of interest for evaluating the degree of completion of the Cl^- shift within RBC transit times in capillaries. Mean values ranged from 81 ms in deoxygenated human RBCs to 382 ms in deoxygenated eel RBCs (Fig. 3).

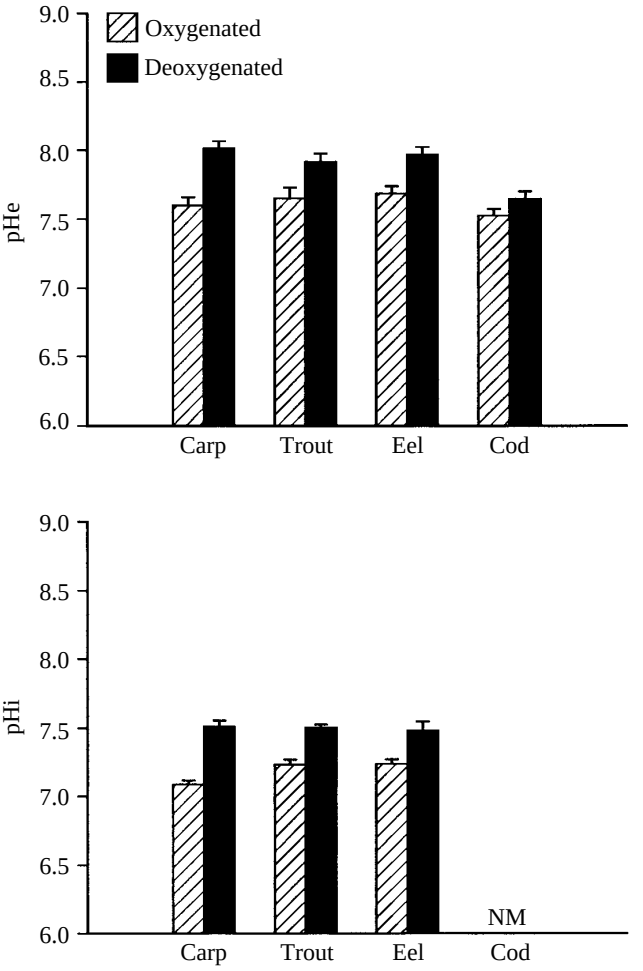

Fig. 1. Extracellular pH (pHe) and intracellular pH (pHi) in suspensions of oxygenated (hatched bars) and deoxygenated (filled bars) RBCs from carp, rainbow trout, eel and cod. Means + S.E.M., N_{oxy}/N_{deoxy}: carp, 8/8; trout, 6/6; eel, 9/9; cod, 4/3. NM, not measured.

The possible influence on k of a rise in pHe to values seen in fish at rest was investigated using carp RBCs. The rate constants determined for oxygenated RBCs at pH 7.6 and pH 8.03 and for deoxygenated RBCs at pH 8.02 and pH 8.36 were not significantly different (Fig. 4).

In order to calculate the apparent membrane permeability to chloride, the membrane area (A_m) was evaluated. Under the microscope, fish RBCs appear elliptical. By computer image analysis, the length (l) and width (w) of oxygenated carp RBCs were found to be 14.1±0.4 and 8.4±0.8 μm (means ± S.D., N=10), respectively. If the cell is considered to be an ellipsoid, the third axis (cell thickness h) can be calculated from the mean cellular volume of 185 μm^3 (MCV=4πabc/3, where a, b and c are the three semi-axes), giving a value for h (h=2c) of 3 μm. The surface area of an ellipsoid cannot be evaluated from a simple formula. We computed the surface area integral numerically as a double integral using Simpson's rule, obtaining a value of 218 μm^2 for oxygenated carp RBCs. An

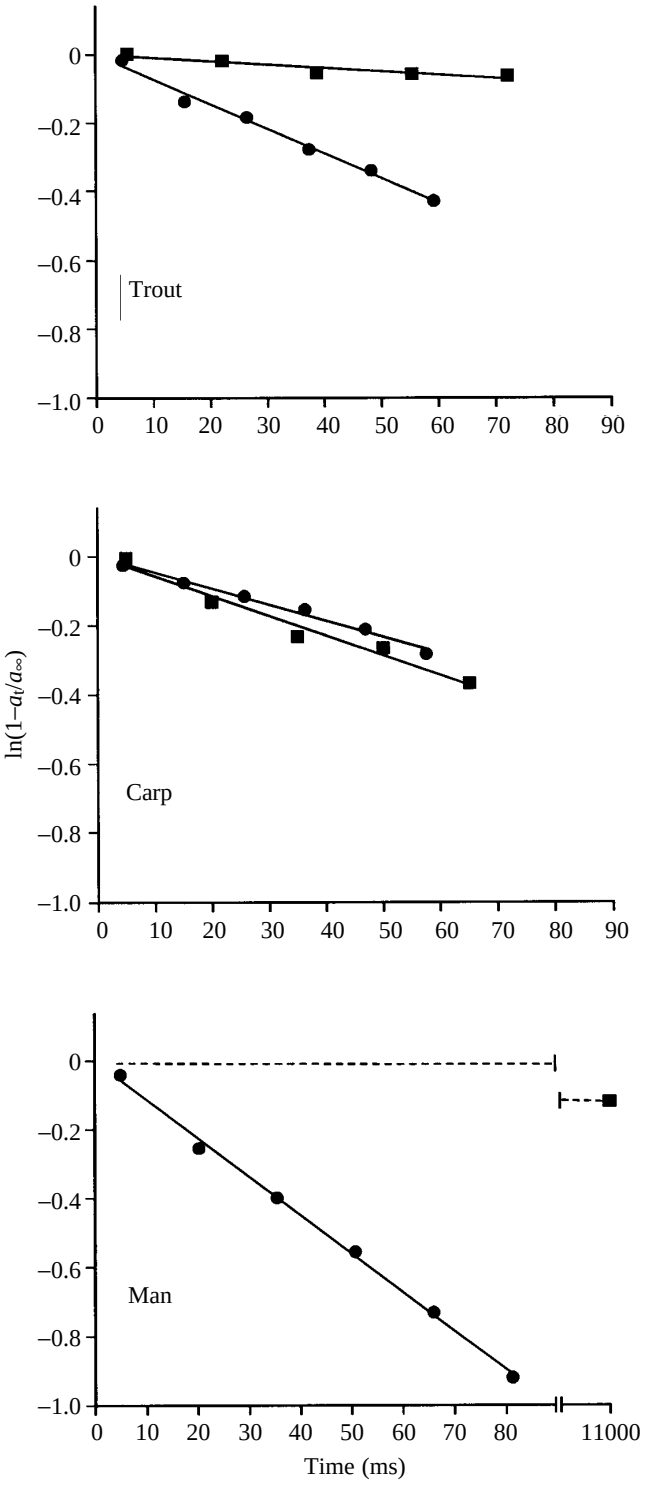

Fig. 2. Typical examples of $^{36}Cl^-$ efflux experiments with oxygenated rainbow trout, carp and human RBCs in the absence (●) and presence (■) of DIDS (at 0.2 mmol l^{-1}). The slope of the curves, determined by linear regression analysis, represents the negative value of the rate coefficient k for the transport process. The curves were corrected for the y-intercept, which does not affect the slopes (cf. Brahm, 1977, 1989). Note the different time axis in the experiments with human RBCs. To illustrate the pronounced inhibition of Cl^- transport by DIDS in human RBCs (dashed curve), the inhibition experiments were performed by means of the Millipore–Swinnex filtering technique (Dalmark and Wieth, 1972), which has poorer time resolution than the continuous flow tube method. Temperature: 15 °C for trout and carp RBCs and 37 °C for human RBCs.

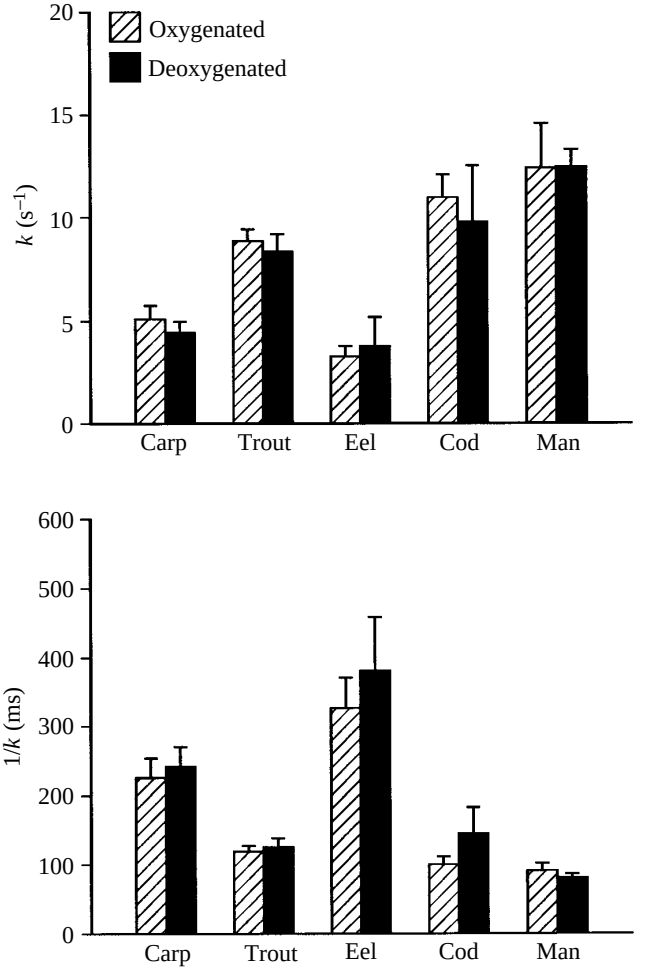

Fig. 3. The rate constant for unidirectional $^{36}Cl^-$ efflux (k), and the time required for 63 % equilibration of Cl^- fluxes ($1/k$), in oxygenated (hatched bars) and deoxygenated (filled bars) RBCs from carp, rainbow trout, eel, cod and man at 15 °C (the four teleosts) or 37 °C (man). Means + S.E.M., N_{oxy}/N_{deoxy}: carp, 10/7; trout, 15/8; eel, 4/6; cod, 9/6; man, 7/5.

alternative, and simpler, model is to consider the cell as two elliptical surfaces separated by a marginal band with height h. With this model, the volume equals πabh, and the equivalent value of h producing an MCV of 185 μm^3 is 2 μm. The surface area according to this model is:

$$A_m = 2\pi ab + 2\pi h \sqrt{\frac{a^2 + b^2}{2}}\ , \qquad (3)$$

giving a value of 259 μm^2 for oxygenated carp RBCs. The ellipsoid model gives a minimal, but presumably the best, estimate for A_m, whereas the latter model gives a maximal estimate.

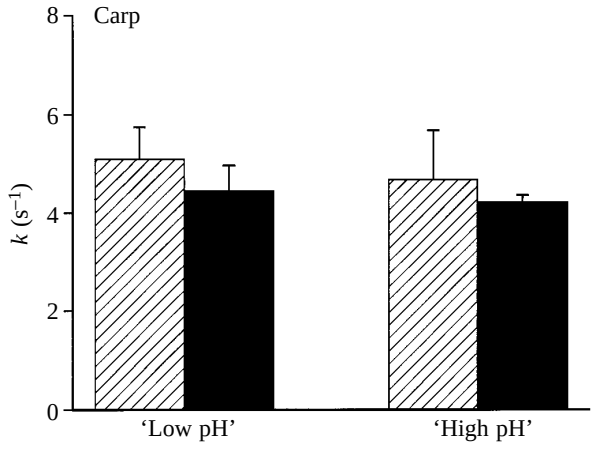

Fig. 4. The rate constant for $^{36}Cl^-$ efflux (k) in oxygenated (hatched bars) carp RBCs at pHe 7.6 ('low pH', N=10) and 8.03 ('high pH', N=5) and in deoxygenated (filled bars) carp RBCs at pHe 8.02 ('low pH', N=7) and 8.36 ('high pH', N=6). Means + S.E.M. Temperature: 15 °C.

Since the areas (A) and volumes (V) of geometrically similar RBCs from two different species are related according to:

$$A_1/A_2 = (V_1/V_2)^{2/3}, \quad (4)$$

the A_m values for the other fish RBCs could be calculated from their measured volume and the values of V and A for carp. Estimates were made both on the basis of the ellipsoid model and on the basis of the elliptical-surface/marginal-band model (the results of the latter are given in parentheses). This resulted in an A_m of 248 μm^2 (295 μm^2) for trout [which compares well with the value of 267 μm^2 estimated by Romano and Passow (1984) by alternative means], an A_m of 207 μm^2 (246 μm^2) for eel and an A_m of 208 μm^2 (247 μm^2) for cod.

The membrane chloride permeability (P_{Cl}) was evaluated from the rate constant k, the membrane area (A_m) and the intracellular water volume (V_w=MCV×F_w, where F_w is the RBC fractional water content) according to:

$$P_{Cl} = kV_w/A_m. \quad (5)$$

With a value for F_w of 0.67, the P_{Cl} values for oxygenated carp, trout, eel and cod RBCs at 15 °C were calculated as 2.9 $\mu m\,s^{-1}$ (2.4 $\mu m\,s^{-1}$), 5.4 $\mu m\,s^{-1}$ (4.5 $\mu m\,s^{-1}$), 1.8 $\mu m\,s^{-1}$ (1.5 $\mu m\,s^{-1}$) and 6.0 $\mu m\,s^{-1}$ (5.1 $\mu m\,s^{-1}$), respectively. For human RBCs at 37 °C, P_{Cl} was 5.2 $\mu m\,s^{-1}$, using an MCV of 87 μm^3 and an A_m of 142 μm^2 (Wieth *et al.* 1974; Brahm, 1982).

Discussion

The chloride permeability of RBCs is very high owing to the presence of a large number of band 3 molecules in the membrane. By using the continuous flow tube method to measure the fast fluxes of chloride across the RBC membrane directly under physiologically relevant conditions, we obtained evidence for a significant species variation in the band-3-mediated Cl^- shift.

Absolute values of Cl⁻ transport parameters

The k values for human RBCs were close to 12 s^{-1}, which compares well with earlier reported values (e.g. Wieth *et al.* 1982). Among the four teleosts investigated, previous data are only available for rainbow trout. Cameron (1978) used a Ag/AgCl electrode system to study the Cl^- shift in a thin film of trout blood upon a near-instantaneous change in CO_2 tension. He reported a time constant for 63 % response (i.e. 1/k) of about 400 ms at 14 °C. This value should be considered as a maximum estimate for the time constant, since it incorporated the electrode response time, diffusive CO_2 equilibria and carbonic-anhydrase-catalysed CO_2 hydration (Cameron, 1978). Romano and Passow (1984) used the inhibitor stop and filtration techniques to measure Cl^- transport in trout RBCs and reported a rate constant k of 0.2 s^{-1} (1/k=5000 ms) at 0 °C and pH 7.4 and a value for 1/k of 1170 ms at 15 °C. At pH values above pH 7.4, the Cl^- fluxes were too fast to measure with their techniques (Romano and Passow, 1984). Taking advantage of the better time resolution offered by the continuous flow tube method, we assessed Cl^- transport parameters in trout RBCs at 15 °C and at pHe values of 7.65 (oxygenated RBCs) and 7.92 (deoxygenated RBCs). We found k values of about 8.5 s^{-1}, corresponding to values of 1/k of about 120 ms (Fig. 3). Thus, the Cl^- shift in trout RBCs is faster than earlier anticipated.

Species variation in Cl⁻ transport

The four teleost species showed a significant interspecific variation in Cl^- transport kinetics. Both the rate constants for unidirectional Cl^- efflux and the Cl^- permeabilities were significantly lower in carp and eel than in trout and cod RBCs. This difference suggests that the number of band 3 molecules is lower in carp and eel than in trout and cod RBC membranes.

It is of considerable interest that the k values for Cl^- transport of trout and cod RBCs at 15 °C were similar to the human values at 37 °C. The Cl^- permeability, which takes into account the different sizes and shapes of fish and human RBCs, was also of comparable magnitude in cod, trout and human RBCs at their respective physiological temperatures. From the strong temperature-dependence of the Cl^- shift in human RBCs (Brahm, 1977), the human values of k, 1/k and P_{Cl} at 15 °C can be calculated to be about 0.92 s^{-1}, 1088 ms and 0.4 $\mu m\,s^{-1}$, respectively. Thus, when compared at 15 °C, the Cl^- shift is considerably faster in all the four teleosts species than it is in human RBCs. This illustrates appropriate adaptation of band 3 function to the different temperature regimes encountered by fish and mammals. The temperature sensitivity of anion transport is lower in fish than in human RBCs (Obaid *et al.* 1979; Romano and Passow, 1984), which may allow appropriate anion exchange rates to be achieved at the lower and more variable temperatures experienced by fish.

Even though anion exchange is fast, it is believed to be rate-limiting for blood CO_2 uptake and elimination. The HCO_3^-/Cl^- exchange requires some 400 ms to reach 90 % equilibration in humans (Wieth *et al.* 1982; Wieth and Brahm, 1985). Other events, which are faster but in series with anion

exchange, such as CO_2 diffusion, catalysed CO_2 hydration/dehydration and oxygenation-linked H^+ exchange with haemoglobin, may further delay equilibration time. Thus, CO_2 equilibration will not be completed in the pulmonary capillaries during physical exercise, during which the RBC capillary transit time can be reduced from 0.7 to 0.3 s. The equilibration continues in the closed arterial system, producing time-dependent changes in arterial pH and P_{CO_2} (Crandall and Bidani, 1981).

Anion exchange is also thought to be rate-limiting for CO_2 excretion in fishes (e.g. Perry, 1986). The present results show that, under physiological conditions, the fish RBC chloride permeability is either comparable to or lower than that in man. Furthermore, fish have lower haematocrits than mammals, which tends to reduce the number of band 3 molecules per unit volume of blood. With a typical blood O_2 capacity in fish of about 5 mmol l^{-1}, a blood O_2 extraction that may reach 80–90 % and a respiratory quotient of 1, the turnover of CO_2 per unit volume of blood approaches 4.5 mmol l^{-1} during near-maximal exercise. If full equilibration is to be achieved during capillary transit, then the flux *via* the anion exchanger should be very high. Even with a capillary transit time of about 1 s in fish during exercise (Randall, 1982), it is conceivable that the blood CO_2 system does not reach equilibrium during capillary transit. This is supported by a theoretical analysis suggesting that the finite blood gas equilibration rate imposes a larger inefficiency on gas exchange in the fish gill than in the mammalian lung and that the gill transit time should exceed the reaction time for 63 % equilibration by a factor of about 50 for disequilibria to be negligible (H. Malte, in preparation). Thus, the finite Cl^- shift rates (Fig. 3) may significantly influence the efficiency of CO_2 exchange not only during exercise but also at rest.

In a situation where the rate of HCO_3^-/Cl^- exchange is limiting for CO_2 excretion, it is an advantage to have a large Haldane effect. In view of the significantly slower Cl^- shift in carp than in rainbow trout RBCs (Fig. 3), it is interesting that the Haldane effect in car p is very large, whereas the Haldane effect in trout is in the lower range among teleosts (but still comparable to or larger than that in mammals) (Jensen, 1989). The large amount of H^+ binding to carp haemoglobin upon deoxygenation increases pHi and improves HCO_3^- formation in the RBC when CO_2 is added in the tissues, just as it effects a large increase in the RBC/plasma distribution ratio of HCO_3^-. Aided by a blood [HCO_3^-] that is relatively high among teleosts, this enables a proportionately higher HCO_3^- concentration to be achieved in the RBCs when the blood enters the gills. Upon oxygenation of the Hb in the gills, the massive release of oxygenation-linked H^+ mediates an extensive conversion of red cell HCO_3^- to CO_2 (catalysed by carbonic anhydrase), compensating for a somewhat delayed entry of plasma HCO_3^- *via* band 3. This strategy finds its extreme manifestation in lampreys, which are devoid of band 3 and rely exclusively on dehydration of erythrocytic HCO_3^- for CO_2 excretion (Tufts and Boutilier, 1989; Nikinmaa and Mattsoff, 1992).

Disequilibria in the CO_2 system in arteries and veins are not avoided by having a large Haldane effect to compensate for rate limitations in anion exchange, but the build-up of venous P_{CO_2} otherwise needed to secure an adequate CO_2 excretion is limited. According to the analysis of Lapennas (1983), the large Bohr effect (which is equivalent to the Haldane effect) in carp can be viewed as an adaptation that mainly serves pH and CO_2 homeostasis, whereas blood O_2-affinity does not change significantly upon CO_2 addition/removal in the arterio-venous cycle (Jensen, 1988).

Potential influence of pH

In carp, the rate coefficient for unidirectional Cl^- effux was unaffected by an increase in pHe from 7.6 to 8.03 with oxygenated RBCs and by an increase in pHe from 8.02 to 8.36 with deoxygenated RBCs (Fig. 4). These pH values span a narrow pH range on an absolute pH scale but, in the case of oxygenated RBCs, correspond to the arterial pHe values measured in cyprinid fish after exercise stress and at rest, respectively (Jensen, 1988). Thus, the data suggest that physiological pH changes have an insignificant effect on Cl^- transport, but do not preclude the possibility that band 3 function is influenced by an increase or decrease in pH outside the pH range examined. In human RBCs, Cl^- exchange fluxes are rather constant in the pH range considered here, but they become strongly influenced when pHe decreases below 7 or increases above 10 (Wieth *et al.* 1982). In rainbow trout RBCs, Cl^- equilibrium exchange at 0 °C was reported to decrease as pHe decreased from 7.4 to 6.0 (Romano and Passow, 1984).

Potential influence of the degree of haemoglobin oxygenation

Band 3 consists of a membrane domain, mediating anion transport, and a cytoplasmic domain, to which cytoskeletal proteins, haemoglobin and glycolytic enzymes bind (reviewed by Salhany, 1990). Recent evidence suggests that band 3 exists as a functional dimer with allosteric interactions between the subunits (Wang, 1994; van Dort *et al.* 1994). The potential role of both homotropic and heterotropic allosteric interactions for band 3 function was emphasised by Salhany (1990). In particular, he hypothesised that a compensatory mechanism could exist to speed up anion exchange rates during severe exercise, and that this mechanism could be mediated by the binding of deoxyhaemoglobin (the amount of which increases in venous blood during exercise) to the N-terminal cytoplasmic fragment of band 3. This would require that a conformational change induced in the cytoplasmic domain upon Hb binding/release be transmitted to the anion exchange domain. Evidence for a linkage between binding sites was provided by the finding that DIDS binding to its exofacial site on band 3 lowers Hb binding to its cytoplasmic site (Salhany *et al.* 1980; Salhany, 1990).

To our knowledge, the present study is the first to test whether there is a link between the degree of oxygenation of Hb and band-3-mediated Cl^- transport. In neither human nor fish RBCs did we observe a significant influence of the degree of Hb oxygenation on Cl^- fluxes (Fig. 3). This ‘negative’

result necessitates a closer examination and evaluation of experimental conditions and assumptions. Our study primarily concerns fish RBCs (though measurements were also performed on human RBCs). A search for an effect of Hb on Cl^- fluxes in fish RBCs assumes that the cytoplasmic fragment of band 3 in fish binds to the organic phosphate binding site between the β chains of deoxyhaemoglobin, as in mammals (Walder *et al.* 1984). This assumption seems justified by the primary structure of trout band 3 which, like mammalian band 3, has a cluster of negatively charged amino acid residues at the N terminus that presumably fits the organic phosphate binding site of the Hb (Hübner *et al.* 1992). At the pH values used in the interspecific comparison (Fig. 3), the allosteric equilibrium between R and T structure Hb is moderately shifted towards the T state. This, in principle, could have reduced a potential effect of a change in Hb oxygenation on band 3 Cl^- transport. This argument is, however, countered by the absence of an effect of oxygenation in carp RBCs when the stabilising effect of a lowered pH on the T structure was overcome by an elevation in pH (Fig. 4). Since fish RBCs have an aerobic metabolism, it should also be considered whether storage of the packed RBCs in syringes for some minutes prior to use in Cl^- flux experiments could have lowered the O_2 saturation of oxygenated RBCs. The oxygenated RBCs had a tetrameric Hb concentration of about 4.3 mmol l^{-1} RBCs, corresponding to an O_2-carrying capacity of 17.2 mmol l^{-1} RBCs. With an RBC O_2 consumption rate of about 30 μmol l^{-1} RBCs min^{-1} (Wang *et al.* 1994), the oxygen should theoretically last for 573 min, arguing against a significant influence of RBC O_2 consumption on Hb O_2-saturation. Thus, in conclusion, if Hb oxygenation status has an influence on band 3 anion transport, then it is either small and hidden in the data variability or its expression requires conditions other than those used in our experiments.

The absence of a significant effect of the degree of Hb oxygenation on band 3 Cl^- transport is puzzling, since Hb conformation has a large influence on other transport mechanisms in fish RBCs. The effect of the degree of oxygenation of Hb on adrenergic Na^+/H^+ exchange was suggested to be mediated *via* the differential binding of oxyhaemoglobin and deoxyhaemoglobin to band 3 and a conformational change transmitted *via* the cytoskeleton to other transport sites (Motais *et al.* 1987). A similar mechanism may apply to oxygenation-activated K^+/Cl^- cotransport, where an involvement of band 3 is further indicated by the inhibition of the transport by DIDS (Jensen, 1990, 1992; Borgese *et al.* 1991; Nielsen *et al.* 1992). One possible explanation for the apparent lack of effect of oxyhaemoglobin/deoxyhaemoglobin transitions on band 3 Cl^- transport would be that the link between the cytoplasmic domain (where Hb and the cytoskeleton binds) and the membrane domain (mediating anion transport) of band 3 is flexible, with minimal interactions between the domains (Wang, 1994). Thus, a conformational change in the cytoplasmic domain upon Hb binding could affect other transport functions *via* the cytoskeleton but need not necessarily be transmitted to the anion transport site in the membrane domain. The influence of DIDS on cytosolic Hb binding and on K^+/Cl^- cotransport referred to above suggests, however, that DIDS binding to band 3 is capable of eliciting an interaction between the membrane and cytoplasmic domains. In view of this, it may be rewarding to explore further the possible interaction between Hb and band 3 Cl^- transport under alternative experimental conditions.

One other puzzling observation was the apparently minor influence of DIDS on rapid Cl^- transport in carp RBCs (Fig. 2). This contrasts with the significant inhibition of Cl^- fluxes in trout and human RBCs (Fig. 2) as well as with the potent inhibition of oxygenation-activated and volume-activated K^+/Cl^- cotransport by DIDS in carp RBCs (Jensen, 1990, 1992, 1995). The small influence of DIDS on rapid Cl^- fluxes in carp RBCs need not imply that this transport is not mediated by band 3 (no other known transport route would produce the observed high transport rates) or that DIDS does not bind to carp band 3 molecules. Binding of Cl^- and DIDS may involve separate but interacting sites (Salhany, 1990), and the degree of interaction (i.e. inhibition of Cl^- transport by DIDS) may vary with species, depending on differences in band 3 primary and spatial structures. Thus, in the case of carp, DIDS binding could produce a conformational change that leads to inhibition of K^+/Cl^- cotransport but has only a minor influence on band-3-mediated Cl^- exchange fluxes. An evaluation of this idea calls for more information on the structure and function relationships in teleost band 3 molecules.

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