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In vivo red blood cell sickling and mechanism of recovery in whiting, *Merlangius merlangus*

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Summary

Haemoglobin concentrations in vertebrate red blood cells are so high that in human sickle cell disease a single surface amino acid mutation can result in formation of large insoluble haemoglobin aggregates at low oxygen levels, causing peculiar cell deformations or 'sickling'. This may cause vascular occlusion and thereby severe pain, organ failure and death. Here, using light and transmission electron microscopy, we demonstrate extensive *in vivo* sickling of whiting red blood cells after capture stress without any apparent haemolysis and show its subsequent recovery. We show exceptionally high cooperative proton binding during the sickling process *in vitro* and identify the reduction of extracellular pH below resting values as the primary cause for *in vivo* sickling, although the response is modulated to a lesser extent also by oxygen tension. Using isotope tracer fluxes, we further show that β-adrenergic hormones, which are released under capture stress, activate a powerful endogenous Na/H exchanger in these fish red blood cells, which is known to elevate intracellular pH. β-adrenergic treatment further leads to a marked reduction of acid-induced *in vitro* sickling, which is impaired when Na/H exchange is inhibited by amiloride. We propose that this mechanism protects red blood cells of some fishes against the problem of haemoglobin aggregation and red blood cell sickling, except under most severe acidosis. This system offers a unique example of how, over evolutionary time, nature may have overcome what is still a deadly disease in humans.

Key words: *Merlangius merlangus*, sickle cell disease, Na/H exchanger, red blood cell, haemoglobin, Hb polymerisation, fish.

Introduction

The primary function of vertebrate haemoglobin (Hb) is the transport of oxygen from the respiratory organs to the deep tissues. This is supported by an extremely high Hb concentration inside red blood cells (RBCs), which is close to the solubility limit (Riggs, 1976). However, a single mutation in human sickle Hb (HbS) replaces a polar glutamic acid on the surface of the protein by a non-polar valine, which greatly reduces the solubility of the protein in the deoxygenated state and causes Hb polymerisation (Ingram, 1957; Perutz and Mitchison, 1950). The HbS polymers form an extremely viscous, solid-like gel, which causes peculiar cell deformations, the most obvious of which is the sickle-shape (Eaton and Hofrichter, 1987). Sickled RBCs show increased fragility and reduced deformability, causing severe anaemia and vasoocclusion. The latter can cause severe pain, organ failure and death (Serjeant, 2001).

Hb mutations leading to a reduced solubility apparently occurred in several other vertebrate groups. This is suggested by early reports of intracellular crystal formation or paracrystalline arrangements of fibres with associated RBC deformations in white-tailed deer (Undritz et al., 1960), annulated gecko (Mattei et al., 1985), common mudpuppy (Dawson, 1930) and several groups of fishes (e.g. Yoffey, 1929; Dawson, 1932; Thomas, 1971) [for reference to even earlier literature see Perutz and Mitchison (Perutz and Mitchison, 1950)]. However, most of these reports were chance observations of cells under the microscope and it is not clear to what extent observations are artefacts of RBC preparation or under which physiological conditions sickling may occur *in vivo*.

In a more recent *in vitro* study on sickling in RBCs of several fish species, Hárosi and co-workers (Hárosi et al., 1998), using polarised light microscopy techniques, were able to correlate RBC morphology with oxygenation state, spectral absorbance, linear dichroism and linear birefringence. They concluded that the large aggregates in Atlantic cod and oyster toadfish RBCs indeed consisted of Hb and that their physico-chemical characteristics were remarkably similar to those in human sickle RBCs (Hárosi et al., 1998). However, the method to induce *in vitro* sickling in these fish RBCs involved complete anoxia at very low pH and a temperature at the upper physiological limit.

Here, we report extensive *in vivo* sickling of whiting RBCs after capture stress without any apparent haemolysis and its subsequent recovery. We describe the *in vitro* effects of saline pH and P_{O_2} on sickling and show that low pH is most likely to elicit sickling *in vivo*. We further demonstrate the ameliorating effect of β-adrenergic activation of the RBC Na/H exchanger

(βNHE) on sickling induced by low pH *in vitro* and discuss the possible physiological role of the phenomenon.

Materials and methods

Animals

Whiting [*Merlangius merlangus* L.; mean mass 139.2±92.0 g and standard length (SL) 22.9±3.7 cm; *N*=18] were caught on hook and line from the Mersey estuary, Merseyside, UK, at incoming tide. 5–8 fish were kept in a 50-litre plastic container with ice-cooled, aerated artificial seawater (33 p.p.m.; Instant Ocean, Aquarium Systems, www.aquariumsystems.com) for up to 2 h while fishing and during transport, which took no more than 10–15 min. Fish were kept at the University of Liverpool in a 1200-litre tank with a biological filter and aerated, re-circulated, artificial seawater at 13–16°C for more than one week before blood sampling (exceptions see below). Fish were exposed to a 10 h:14 h light:dark regime and fed five times weekly on frozen sand eel, squid and octopus. To ensure that the possible seasonality of RBC ion transport function would not affect the results, the experiments were carried out during the winter months (December 2004–March 2005, November–December 2005 and 2006).

Blood sampling, fixation and light microscopy

The fish were killed according to a British Home Office Schedule 1 method by a sharp blow to the head and destruction of the brain, before blood sampling from the caudal blood vessels into a heparinised syringe. In eight of the fish, a 50-μl sample was fixed as described below immediately after sampling. The remainder of the RBCs were washed three times in an isotonic saline (125.5 mmol l^{-1} NaCl, 3 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 1.5 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} D-glucose and 20 mmol l^{-1} Hepes; 299 mOsm kg^{-1}; pH adjusted to 7.97 at 15°C), each time removing the buffy coat. The cells were finally suspended at a haematocrit (Hct) of 15–20%, oxygenated by contact with air and stored at 4°C for 16–24 h. Before experimentation, the RBCs were washed an additional two times and resuspended in fresh saline.

Another group of whiting (mean mass 200.0±107.0 g and SL 25.5±4.5 cm; *N*=5), rainbow trout (*Oncorhynchus mykiss* Walbaum; mean mass 913±132 g and SL 37.2±1.8 cm; *N*=8) and common carp (*Cyprinus carpio* L.; mean mass 1378±463 g and SL 36.8±4.6 cm; *N*=5) were killed as above immediately after being caught on hook and line. Reeling in whiting, rainbow trout and common carp usually took 2–4 min irrespective of species. The trout and carp were caught at the Pennine Trout Farm and Fishery, Lancashire, UK, and Llyn y gors, Anglesey, UK, respectively, during February 2007. A blood sample was taken from the caudal vessels into a heparinised syringe and fixed as described below. Another group of whiting (mean mass 132.8±26.1 g and SL 23.6±1.3 cm; *N*=4) were transported to the university and allowed to recover in the holding tank for 24 h, whereupon the animals were netted individually with a minimum of disturbance and quickly killed. Blood was sampled and fixed as described below.

All blood samples for light microscopy and transmission electron microscopy (TEM) were fixed in iso-osmotic glutaraldehyde immediately after sampling, but on several occasions freshly sampled blood was studied directly under the light microscope and confirmed the presence of sickled RBCs in unfixed samples. The glutaraldehyde fixation solution was made from a 25% stock solution (Grade I; Sigma-Aldrich Company Ltd, Gillingham, UK), diluted with distilled water to obtain an osmolality of 300 mOsm kg^{-1} and further diluted with saline (see above) to obtain a 2% glutaraldehyde solution. Samples were fixed in an equal volume of this iso-osmotic solution to yield a final glutaraldehyde concentration of 1%. They were stored at 4°C until counting or further treatment for TEM. The samples were counted on an Axiovert 135 TV microscope (Carl Zeiss Microlmaging GmbH, Göttingen, Germany; using a 100× oil immersion lens and direct interference contrast) fitted with a video camera (3-CCD Color Video Camera, KY-F55B, JVC; Alrad Instruments Ltd, Newbury, UK) and viewed with Scion Image software (Scion Corporation, Frederick, MD, USA).

Effects of pH, oxygen tension and hyperosmotic shrinkage on RBC sickling and recovery

350-μl aliquots (70 μl washed RBCs with an Hct of ~20% and 280 μl experimental saline with pH values ranging from 6.75 to 7.97) were incubated at 15°C in rotating Eschweiler tonometers (Eschweiler GmbH, Engelsdorf, Germany). Saline pH was measured with a combined pH electrode and a PHM 85 Precision pH Meter (Radiometer, Copenhagen, Denmark). Final pH values of the mixed salines were assumed to be identical to extracellular pH due to the low final Hct value and ranged from 7.03 to 7.97. The samples were gassed with humidified N_2, air or O_2, and samples were taken and fixed after 45 min.

Recovery from *in vitro* sickling by an increase in extracellular pH was studied in air-equilibrated RBCs. After 45 min, a sub-sample of RBCs suspended in saline pH 7.03 was taken and fixed, whereupon the remainder of the sample was briefly centrifuged (10 s at ~9000 ***g***; Eppendorf model 5415 D; Fisher Scientific, Loughborough, UK) and had the supernatant replaced with pH 7.97 saline. The resuspended cells were incubated as before and further samples were taken and fixed at different time points at the new, elevated saline pH.

Recovery from *in vitro* sickling by an increase in oxygen tension was studied at pH 7.61 in RBC suspensions that had been gassed with humidified nitrogen for 45 min. After a sub-sample was taken and fixed (time zero), the gas was changed to humidified oxygen. Further samples were taken and fixed after 5, 15, 30 and 45 min.

To assess the effects of intracellular Hb concentration, washed RBCs were pre-equilibrated to air in pH 7.97 saline for 45 min. Sub-samples were taken and fixed at 5 and 45 min. Appropriate volumes of sucrose stock solution (sucrose in pH 7.97 saline; osmolality of 2.5 Osm kg^{-1}) were added to change the extracellular osmolality to 350, 400, 450 or 600 mOsm kg^{-1}, causing cell shrinkage and calculated 17, 33, 50 and 100% increases in intracellular [Hb], respectively. Further samples were taken and fixed at 5, 15, 30, 60 and 120 min after shrinkage.

Effects of β-adrenergic stimulation on RBC sickling

Aliquots of 350 μl washed RBCs with an Hct of 10–20% were pre-equilibrated to humidified air in rotating Eschweiler tonometers kept at 15°C in a thermostatically controlled water bath. Samples were taken and fixed at 5 and 45 min, whereupon

sub-samples of the RBC suspension were transferred to test tubes, containing four volumes of experimental saline equilibrated with air. The cells were exposed to (1) pH 7.03 saline, (2) pH 7.03 saline and the β-adrenergic agonist isoproterenol (final concentration of 10^{-5} mol l^{-1}; saline vehicle) or (3) pH 7.03 saline and 10^{-5} mol l^{-1} isoproterenol simultaneously with the Na/H exchanger inhibitor amiloride (final concentration of 10^{-4} mol l^{-1}; 0.5% v/v DMSO vehicle). Parallel control samples were kept in the pH 7.97 saline. Sub-samples were fixed at 5, 15, 30 and 45 min after transfer to the test tubes.

Na influx studies

Aliquots of 350–700 μl washed RBCs with an Hct of 10–20% were pre-equilibrated to humidified air as described above. Ouabain (final concentration of 10^{-4} mol l^{-1}; 0.5% v/v DMSO vehicle) was added after 40 min, and duplicate samples for Hct were taken (3 min centrifugation at ~10 000 ***g***; Micro-haematocrit centrifuge; Hawksley, Lancing, UK). Sub-samples were then transferred to test tubes (defined as time zero), containing four volumes of pH 6.75 (final saline pH 7.03) or 7.97 saline equilibrated with air, ^{22}Na (~16.7 kBq ml^{-1}) and ouabain (final concentration of 10^{-4} mol l^{-1}). In addition, at both pH values, some test tubes contained isoproterenol (final concentration of 10^{-5} mol l^{-1}) and/or amiloride (final concentration of 10^{-4} mol l^{-1}). Triplicate 200-μl samples were taken from each test tube at predetermined time points. To terminate Na uptake, the RBCs were immediately washed three times in ice-cold isotonic, Hepes-containing (20 mmol l^{-1}) $MgCl_2$ solution (adjusted to pH 7.97) by centrifugation (7–8 s at ~6000 ***g***; Eppendorf Model 5410; Eppendorf GmbH, Engelsdorf, Germany) and re-suspension. The RBC pellets and extracellular samples were lysed in 0.5 ml 0.05% Triton X-100 solution and deproteinised by subsequent addition of 0.5 ml 5% trichloroacetic acid. After each experiment, a 20-μl sample was also treated with Triton X-100 and trichloroacetic acid to count the total ^{22}Na activity, which was assumed to correspond to the extracellular ^{22}Na activity at the beginning of the uptake experiment. The samples were centrifuged for 2 min (~6000 ***g***; Eppendorf Model 5410; Eppendorf GmbH, Engelsdorf, Germany), and 0.8 ml of the supernatant was counted (Tri-Carb 2100 TR; Packard Instruments Ltd, Caversham, UK) in ~3 ml scintillation cocktail (Pico-Fluor 40; Perkin-Elmer Ltd, Beaconsfield, UK).

Na uptake (mmol Na l^{-1} RBCs) was calculated as:

$$\text{Na uptake} = A_{\text{cpm}} \bullet \text{c.p.m. equiv.} / V_{\text{RBC}} \quad (1)$$

and the Na influx (mmol Na l^{-1} RBCs h^{-1}) as:

$$J_{\text{Na}} = \text{c.p.m. equiv.} \bullet \Delta\text{c.p.m.} \bullet 60 / (V_{\text{RBC}} \bullet \Delta t) , \quad (2)$$

where A_{cpm} denotes the mean activity of triplicate samples (in c.p.m.), c.p.m. equiv. denotes mmol Na equivalent to 1 c.p.m., V_{RBC} is the volume of RBCs (in litres), determined from the Hct samples taken after 45 min of pre-equilibration, Δc.p.m. denotes the difference in triplicate ^{22}Na c.p.m. between two time points, and Δt is the time (in min) between samplings.

Transmission electron microscopy

Fixed RBC samples were washed three times in pH 7.97 saline, embedded in 2% agarose and stained for 1 h in 1% OsO_4. The embedded cells were washed in 30% ethanol for 10 min and incubated for 1 h in 0.5% uranyl acetate (in 30% ethanol) followed by 10 min in each of 30, 60, 70, 80, 90 and 100% ethanol and two washes in 100% acetone to dehydrate samples. The samples were then incubated in acetone:resin (Araldite; Sil-Mid Ltd, Coleshill, UK) for 30 min in a 1:1 ratio followed by 30 min pure resin, before the final incubation in moulds for 24–48 h at 60°C in the oven. Sections were cut on an ultramicrotome (Reichert Ultracut, Leica UK Ltd, Milton Keynes, UK) and stained for 5 min each with 5% uranyl acetate and 2% Reynold's lead citrate (Sigma-Aldrich Co. Ltd, Gillingham, UK). The samples were viewed on a Tecnai electron microscope (FEI 120 kV Tecnai G 2 Spirit BioTWIN; FEI UK Ltd, Cambridge, UK; equipped with a SIS Megaview III camera; Olympus Soft Imaging Solutions Ltd, Helperby, UK). The pictures were analysed using AnalySIS Pro [SIS] software (Olympus UK Ltd, London, UK).

Data analysis and statistics

For each sample, 250–300 RBCs were studied under the light microscope and assigned as normal (Fig. 2A), sickled (Fig. 1A, Fig. 2C–F) or globular (Fig. 7A). Sickle cells were plotted as a fraction of the total cells. The saline pH value at which 50% of the RBCs were sickled (pK_{app}) was determined by non-linear curve fitting for each individual, using:

$$y = x^{n_{\text{H}}} / (x^{n_{\text{H}}} + a^{n_{\text{H}}}) , \quad (3)$$

where y is the fraction of sickle cells, x is $[H^+]$, n_{H} is the cooperativity constant and a equals $-\log pK_{\text{app}}$.

All data are presented as means ± s.d. Statistically significant differences between samples were accepted at $P<0.05$ and were tested by one- or two-way analysis of variance (ANOVA) or repeated-measures ANOVA as appropriate, followed by a Tukey test. A Kruskal–Wallis ANOVA test on ranks was carried out if requirement or normal distribution was not met. All statistical analyses were carried out with SigmaStat software (SPSS Inc., Chicago, IL, USA).

Results

Immediately after capture on hook and line, a large fraction of whiting RBCs (95.8±2.2%) consisted of irregularly shaped granular and angular RBCs, the latter often with distinct large intracellular bars that appeared to distort cell shape (Fig. 1A,B). Similar intracellular bars have previously been described *in vitro* in a number of other fish RBCs, including those of the closely related Atlantic cod, and have been identified as Hb aggregates that are notably similar to those found in human RBCs containing HbS (Hárosi et al., 1998; Perutz and Mitchison, 1950). This conclusion was based on single-cell polarized light microscopy techniques, including spectral absorbance scans in the 350–650 nm range of individual intracellular bars, which corresponded to the typical spectrum of deoxygenated Hb (Hárosi et al., 1998). Accepting that the bars consist of polymerised Hb, we refer to these cells as sickle cells. By contrast, similar samples taken from rainbow trout and common carp after capture on hook and line contained 95.9±0.03 and 97.0±0.03% RBCs with the flattened, ellipsoidal shape and smooth surface typical for nucleated RBCs of other vertebrates (e.g. Nikinmaa, 1990) (Fig. 1B). After 24 h recovery in the holding tank, the proportion of whiting sickle cells had

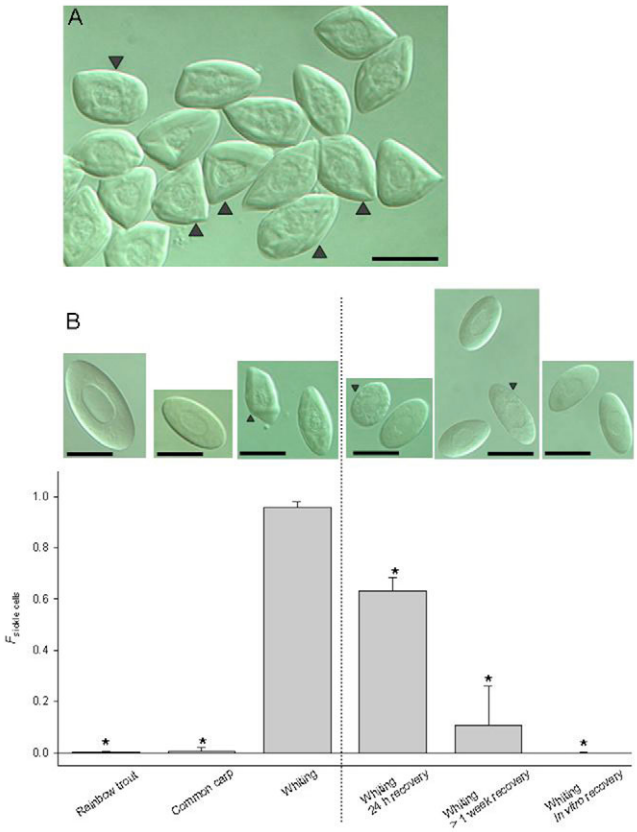

Fig. 1. (A) Light microscopy image of whiting sickle cells sampled immediately after capture of fish by hook and line. (B) Sickle cell fraction in rainbow trout (N=8), common carp (N=5) and whiting (N=5) red blood cells (RBCs) immediately after being caught by hook and line, in freshly sampled whiting RBCs after recovery in the holding tank for 24 h or more than one week (N=4 and 8, respectively) and in washed RBCs after 16–24 h storage (N=12). Small light microscopy images show the appearance of representative cells from the column immediately below. In A and B, scale bars correspond to 10 μm, ▼ shows examples of cells with granular texture and ▲ shows examples of cells with visible bars. In B, * indicates significant difference (P<0.05) from value for whiting immediately after being caught. Values are means ± s.d.

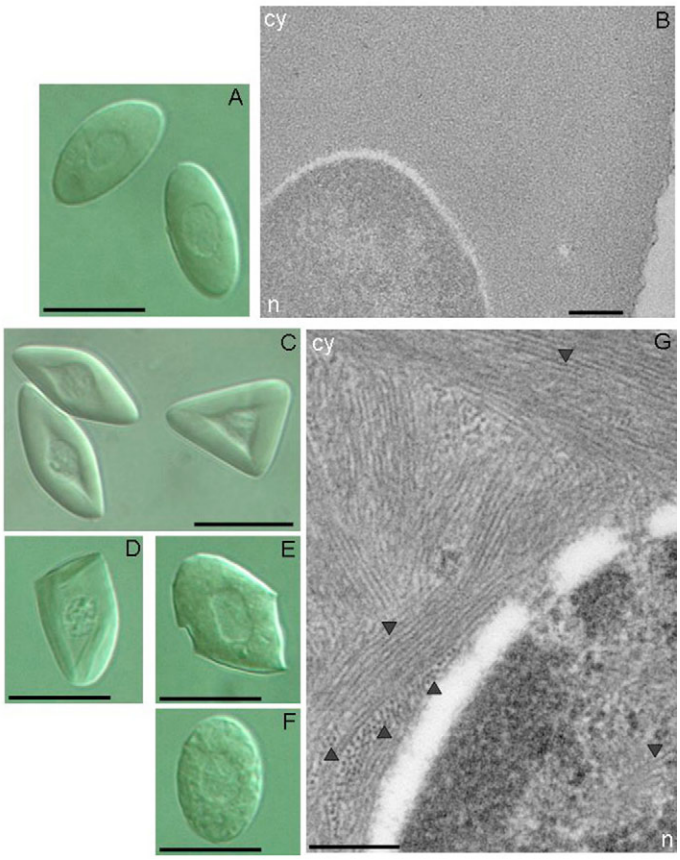

Fig. 2. Light microscopy and transmission electron microscopy (TEM) images of representative normal (A,B) and sickle (C–G) cells *in vitro* at pH 7.97 and 7.03–7.43, respectively. The scale bars correspond to 10 μm (A,C–F) or 200 nm (B,G). In the TEM images, n and cy indicate nucleus and cytosol, respectively. ▼ indicates filaments cut in longitudinal direction, and ▲ indicates filaments in cross section.

significantly declined to 63.3±5.3%, and after more than 1 week merely 10.6±15.5% of whiting RBCs were sickled (Fig. 1B). The presence of sickle RBCs even after more than one week's recovery is likely due to the stress associated with netting of the fish before blood sampling and could be avoided in catheterized fish in future studies. When, immediately after capture, whiting RBCs were washed and incubated in physiological saline for 16–24 h, virtually all cells attained a normal shape similar to that seen in trout and carp (Fig. 1B). Whiting did not seem to be adversely affected by sickling during capture stress, as they behaved as other fishes under similar conditions and resumed feeding after a few days in the holding tank, where they could be kept in apparently healthy conditions for several months. In addition, we did not observe any signs of haemolysis after blood sampling, as confirmed by clear RBC supernatants.

Exhaustive exercise in teleost fishes, such as encountered during capture stress, is associated with severe blood acidosis (pH 7.3–7.5) (Holeton et al., 1983; Jensen et al., 1983; Nikinmaa and Jensen, 1986; Milligan and Wood, 1987; Wood and Milligan, 1987; Knudsen and Jensen, 1998). Fig. 2A shows that sickling was absent in washed whiting RBCs incubated in air-equilibrated saline at 15°C and pH 7.97, a typical value for normoxic resting animals at that temperature (Butler et al., 1989; Perry et al., 1991; Ultsch and Jackson, 1996; Larsen et al., 1997; Knudsen and Jensen, 1998). The cytosol and nucleoplasm of these RBCs appeared uniform in the TEM images (Fig. 2B). At lower saline pH values, however, the appearance of the cells changed dramatically to various polygonal shapes, triangular and diamond shapes being most abundant, often with visible bars (Fig. 2C–E), and they looked very similar to whiting RBCs taken immediately after capture on hook and line (Fig. 1A). Other cells at low pH retained the usual ellipsoidal shape but acquired a granular texture (Fig. 2F). In all these cells, prominent bundles of filaments in both the longitudinal direction and in cross section were clearly visible throughout the cytosol and nucleoplasm by TEM (Fig. 2G). Similar filaments have been described in RBCs of the Baltic subspecies of Atlantic cod, *Gadus morhua callarias* (Thomas, 1971), and in human RBCs from sickle cell patients, where the filaments are known to consist of polymerised HbS

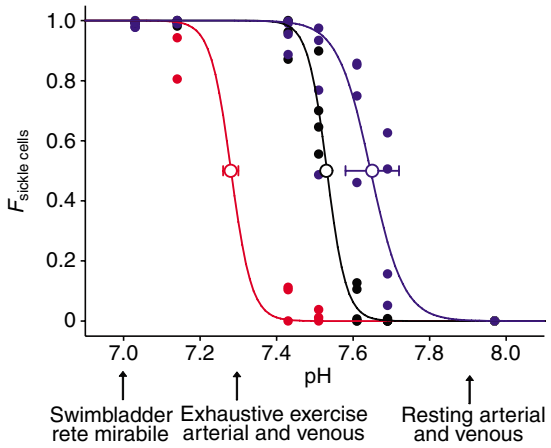

Fig. 3. Sickle cell fraction as a function of saline pH for red blood cell (RBC) suspensions incubated under humidified nitrogen (blue; P_{O_2} ~0 kPa; *N*=4), air (black; P_{O_2} ~20.8 kPa; *N*=4) or oxygen (red; P_{O_2} ~100 kPa; *N*=3) for 45 min. The filled symbols represent individual data points. Curves are fitted using the mean pK_{app} and n_H values obtained from individual animals (see Materials and methods and Table 1). The open symbols represent means ± s.d. for pK_{app} values determined from individual curve fits. In some cases at high and low sickle cell fraction, points are covered by overlaying symbols. See Results for references to *in vivo* blood pH values as indicated below the *x*-axis.

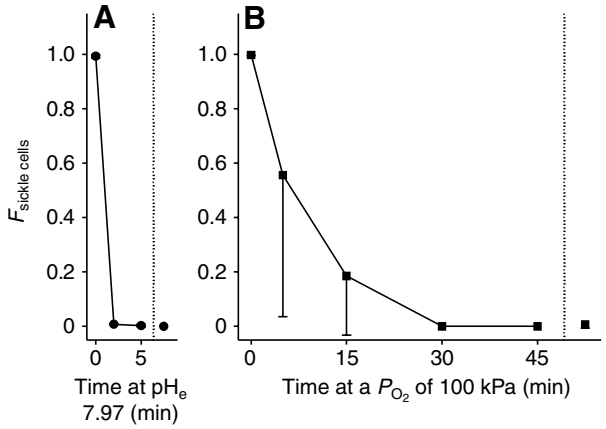

Fig. 4. Sickle cell fraction as a function of time during *in vitro* recovery by increasing (A) saline pH in air-equilibrated samples from 7.03 to 7.97 (●; *N*=3) or (B) P_{O_2} at pH 7.61 from zero to ~100 kPa (■; *N*=4). Data points on the right-hand side of the dotted line indicate values for cell suspensions kept at pH 7.97 under air (●) or pH 7.61 at a P_{O_2} of ~100 kPa (■) throughout the experiments. Values are means ± s.d. Error bars are in some cases smaller than the symbol.

(Döbler and Bertles, 1968). Sickling in whiting did not appear to increase RBC fragility as no haemolysis was observed throughout the experiments.

The transition from almost zero to ~100% sickle cells upon lowering saline pH occurred within a rather narrow interval of ~0.2 pH units in cells incubated under N_2, air and O_2 (Fig. 3). This is reflected in high apparent cooperativity constants for extracellular protons, which amounted to at least 4.7, 12.3 and 5.8 in N_2, air and O_2 equilibrated RBCs, respectively (Table 1). The pH value at half-maximal sickling, or pK_{app}, was 7.64±0.07 (mean ± s.d.) in N_2-equilibrated RBCs but decreased significantly to 7.53±0.01 and 7.28±0.02 in air- and O_2-equilibrated RBCs, respectively (Fig. 3; see Table 1 for individual data). Thus, at intermediate pH values corresponding to blood pH under severe exercise, high P_{O_2} values partly protected against RBC sickling by shifting its occurrence towards lower pH values. However, at still lower saline pH values, the protective effect of O_2 was lost and all RBCs sickled. Low pH appeared to be a necessary condition for sickling, as at high saline pH close to resting arterial pH values deoxygenation *per se* did not induce sickling (Fig. 3). Similarly, hyperosmotic shrinkage of air-equilibrated RBCs at pH 7.97, resulting in a calculated increase of intracellular Hb concentration of up to 100%, did not induce sickling, neither shortly after shrinkage (5 min) nor after prolonged incubation for up to 2 h (not shown).

Table 1. *pK_{app} and cooperativity constant (n_H) values for washed red blood cells (RBCs) incubated under nitrogen, air or oxygen*

	N_2		Air		O_2	
Animal	pK_{app}	n_H	pK_{app}	n_H	pK_{app}	n_H
1	7.69	8.8	7.51	12.8	–	–
2	7.72	7.2	7.54	12.8	7.28	35.4
3	7.63	19.8	7.54	30.9	7.26	5.8
4	7.56	4.7	7.53	12.3	7.30	7.3

Sickling was completely reversible, as re-suspension of cells from a low pH saline, where close to 100% of the cells were sickled, into a high pH saline rapidly reversed the sickling and the vast majority of cells appeared normal by the first sampling time after 2 min, i.e. the recovered cells were not different from cells kept at high pH throughout the experiment (Fig. 4A). At pH 7.61, where nearly 100% of the cells were sickled under a nitrogen atmosphere, increasing P_{O_2} from 0 to ~100 kPa resulted in a gradual decline of the sickle cell fraction towards the low values found in cells incubated under O_2 throughout the experiment at that pH (Fig. 4B).

Exhaustive exercise in teleosts leads to elevated plasma adrenalin and noradrenalin levels (Primmett et al., 1986; Berenbrink and Bridges, 1994b; Perry et al., 1996). Many teleosts possess a βNHE (Berenbrink et al., 2005), whose stimulation causes an increase in intracellular pH (Nikinmaa, 1982; Cossins and Richardson, 1985; Berenbrink and Bridges, 1994b) and thus may protect against pH-induced sickling *in vivo*. At pH 7.97, the β-adrenergic agonist isoproterenol increased the ouabain-insensitive Na influx of air-equilibrated whiting RBCs approximately 8–10-fold relative to basal influx, reaching values of around 400 mmol Na l^{-1} RBCs h^{-1} (Fig. 5A), which is by far the highest value measured in an analysis of 38 vertebrate species (Berenbrink et al., 2005). The isoproterenol-stimulated Na influx was significantly inhibited by the NHE inhibitor amiloride, which had no effect on basal Na influx. Decreasing saline pH to 7.03 did not activate any amiloride-sensitive Na influx; however, at the same pH, isoproterenol induced an amiloride-sensitive Na influx of equal magnitude as observed at pH 7.97 (Fig. 5A,B).

When endogenous catecholamines were removed by washing in saline and any potential lasting catecholamine effects were

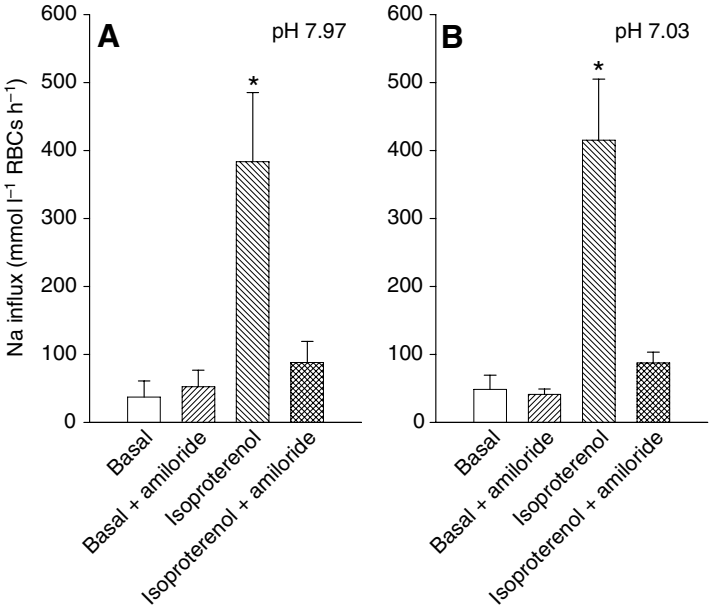

Fig. 5. The ouabain-insensitive Na influx in air-equilibrated red blood cells (RBCs) measured during the initial 5 min at a saline pH of (A) 7.97 or (B) 7.03 in unstimulated RBCs (basal) with or without 10^{-4} mol l^{-1} amiloride, or RBCs stimulated by 10^{-5} mol l^{-1} isoproterenol alone or together with 10^{-4} mol l^{-1} amiloride. Values are means ± s.d. A significant difference (P<0.05) from the respective isoproterenol-stimulated Na influx is indicated by *. N=5.

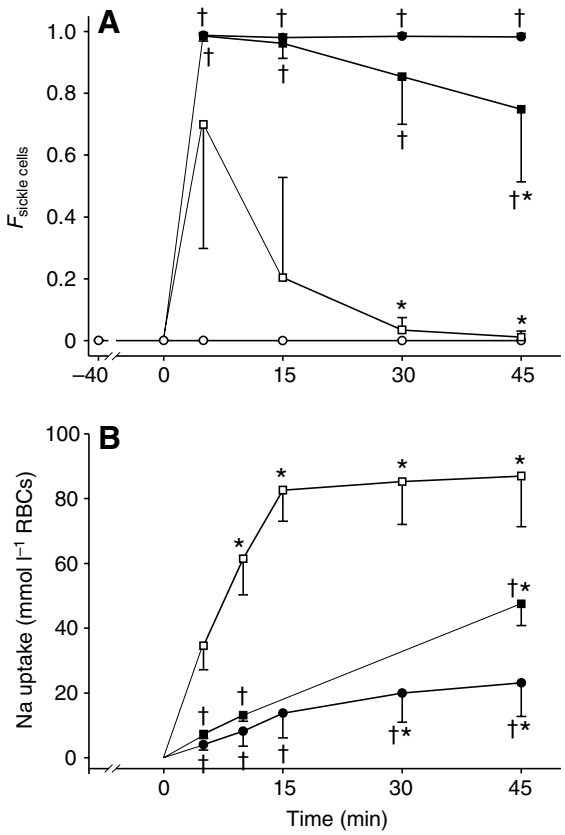

Fig. 6. The sickle cell fraction (A) and ouabain-insensitive Na uptake (B) in cells exposed to a saline pH of 7.97 (○); saline pH of 7.03 (●); saline pH of 7.03 with 10^{-5} mol l^{-1} isoproterenol alone (□) or together with 10^{-4} mol l^{-1} amiloride (■). * indicates significant difference (P<0.05) from first sampling time within same treatment, † indicates cells at pH 7.03 only that are significantly different (P<0.05) from samples at saline pH 7.03 with isoproterenol. Values are means ± s.d. N=8 (A) and 5 (B). Error bars are in some cases smaller than the symbol.

allowed to decline for 16–24 h, exposure of whiting RBCs to pH 7.03 induced rapid and persistent RBC sickling in virtually all cells, whereas no sickle cells were present at pH 7.97 (Fig. 6A). However, when these cells were exposed to low saline pH in the presence of isoproterenol, the fraction of sickle cells was reduced to ~0.7 after 5 min, and after 30–45 min practically no sickle cells were present (Fig. 6A). The cells under these conditions rather obtained a globular shape and to some extent still contained aligned Hb filaments (Fig. 7A–C). However, with time, an increasing proportion of these cells contained ordered filaments only in the nucleus, whereas the cytosolic filaments were clearly fragmented and irregular (Fig. 7D,E), showing that recovery from Hb polymerisation and sickling had taken place. The change from sickle to globular cells induced by isoproterenol was significantly inhibited by the NHE inhibitor amiloride (Fig. 6A).

Indeed, parallel experiments showed that the ouabain-insensitive Na uptake in isoproterenol-stimulated RBCs was an approximate mirror image of the reduction in sickle cell fraction in similarly treated RBCs (Fig. 6A,B). Thus, within the first 15 min, isoproterenol stimulated a profound, linear increase in intracellular Na, whereupon a distinct plateau was reached. In the absence of isoproterenol, Na uptake was significantly reduced throughout the experiment. The β-adrenergically stimulated Na uptake was significantly inhibited by amiloride (Fig. 6B).

Discussion

The present work demonstrates extensive *in vivo* RBC sickling in a lower vertebrate, the teleost whiting. As in human sickle cell disease, where the famous sickle shape is only one of several types of RBC deformations whose prevalence is determined by the speed of Hb polymer formation (Eaton and Hofrichter, 1987; Bunn, 1997; Christoph et al., 2005), formation of long, aligned intracellular filaments in whiting RBCs (Fig. 2G) results in a variety of cell shapes. These include triangular, diamond-shaped or polygonal cells with three, four or more bars, respectively (Fig. 1A, Fig. 2C–E). Yet other RBCs retain their normal oval shape but acquire a granular appearance, very similar to rapidly polymerising human sickle RBCs that contain numerous short random fibres (Fig. 2F) (Eaton and Hofrichter, 1987; Bunn, 1997). In contrast to human sickle cell disease (Lange et al., 1951), these deformations are not associated with increased cell fragility, as haemolysis of sickled whiting RBCs was not observed *in vitro*, nor *in vivo* even immediately after capture. One factor that could reduce haemolysis and alleviate other ill effects of sickling such as vasoocclusion would be an increased capillary diameter relative to RBC size. In goldfish and rainbow trout muscle, the capillary diameter of 3–4 μm is comparable to that in mammals (Egginton, 2002; Egginton et al., 2000; Vink and Duling, 1996),

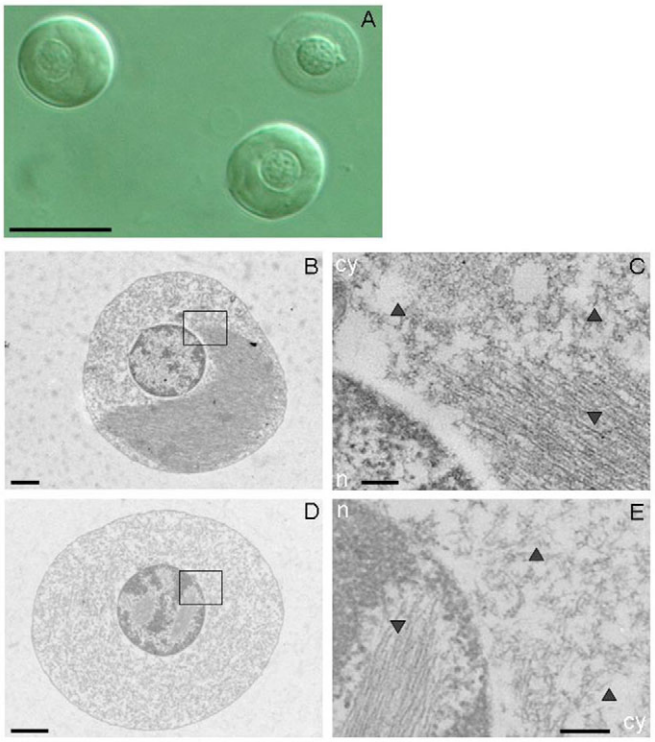

Fig. 7. Light microscopy (A) and TEM images (B–E) of globular red blood cells (RBCs). C and E are magnifications of the areas within the squares in B and D, respectively. Scale bars correspond to 10 μm (A), 1 μm (B,D) or 200 nm (C,E). In the TEM images, n and cy indicate nucleus and cytosol, respectively. ▼ indicates filaments cut in longitudinal direction, and ▲ indicates fragmented filaments.

despite the generally larger size of fish relative to mammalian RBCs (Prosser, 1950). However, capillary diameters in the choroid and swimbladder retia mirabilia of various teleosts are at least 2–3-fold larger than the above values for muscle (Krogh, 1922; Scholander, 1954; Wittenberg and Wittenberg, 1974). Unfortunately, comparable data on fishes where RBC sickling has been established are missing.

The primary cause of sickling in whiting RBCs appears to be low pH as deoxygenation alone did not induce sickling of whiting RBCs at pH values typical for arterial and venous blood in resting fishes (~7.8–7.9 at 10–15°C; see above), which contrasts with the situation in human sickle RBCs (Lange et al., 1951; Bookchin et al., 1976; Ueda and Bookchin, 1984). At the same pH, an increase in cellular Hb concentration in air-equilibrated whiting RBCs by hyperosmotic shrinkage equally failed to induce sickling, which again differs from human sickle RBCs, where Hb polymerisation and sickling are extremely dependent on intracellular [Hb] (Hofrichter et al., 1974; Bookchin et al., 1976) and where the solubility of HbS is reduced 100-fold upon deoxygenation, compared with a 2-fold reduction in deoxygenated normal human HbA (Perutz and Mitchison, 1950). It is currently not known whether high pH also prevents shrinkage-induced sickling in deoxygenated whiting RBCs.

Low P_{O_2} can, however, induce sickling in whiting RBCs under slightly more acidic conditions than in blood of resting fishes (less than or equal to approximately pH 7.7). Our *in vitro* results suggest that at these pH values, which may be regularly encountered during routine activity, P_{O_2} values close to zero in poorly perfused muscles or even mixed venous P_{O_2} values may be sufficient to induce sickling (Fig. 3), as already seen in human sickle RBCs at normal resting pH (Bookchin et al., 1976).

Dramatic changes in P_{O_2} and pH are experienced in blood perfusing the swimbladder and choroid retia mirabilia, where oxygen is delivered by diffusion to the swimbladder and poorly vascularised retina, respectively. Thus, capillary pH in the swimbladder rete mirabile of the European eel may drop to 6.5–7.0 (Steen, 1963; Kobayashi et al., 1990) (for a review, see Berenbrink, 2007), whereas capillary P_{O_2} may rise to 120 kPa or above in the swimbladder rete of the cod, as calculated on the basis of the values for gas composition in the swimbladder given by Bohr (Bohr, 1894). In the choroid rete mirabile of the Atlantic cod, P_{O_2} values of up to 157 kPa have been measured (Wittenberg and Wittenberg, 1962). In our study, 100 kPa O_2 did not protect from RBC sickling at pH values close to those measured in the swimbladder rete mirabile, which suggests that RBC sickling can take place in these capillaries. However, this may depend on the transit time of RBCs in the rete mirabile and whether a lag time before sickling is present in whiting RBCs. In human sickle RBCs, a sufficient delay time before polymer formation prevents a large fraction of cells from sickling during the time spent in the capillaries and venous system, where they are most likely to experience conditions of low P_{O_2} that induce sickling (Mozzarelli et al., 1987; Christoph et al., 2005).

Whereas the very low pH values in the retia mirabilia are localized and only experienced by RBCs during their passage, the general blood acidosis down to pH 7.3–7.5 caused by strenuous exercise in many teleosts may take several hours to recover to normal resting pH values (e.g. Holeton et al., 1983; Milligan and Wood, 1987). Under these conditions, a possible delay time before sickling in whiting RBC, even if amounting to minutes, cannot protect from sickling.

However, strenuous exercise *in vivo* is associated with the release of adrenalin and noradrenalin into the plasma, which stimulates the RBC βNHE and thereby maintains or even increases intracellular pH as well as cell volume (see above). We show that the sickle cell fraction in β-adrenergically stimulated RBCs indeed decreases as a mirror image of βNHE activation. The previously sickled cells attain a globular, swollen shape in which the cytosolic Hb filaments become very fragmented. It remains to be shown to what extent cell swelling and the increase in intracellular pH contribute to the break-up of Hb filaments and the recovery from sickling.

The mechanism of Hb polymerisation and consequent sickling in whiting RBCs is presently unknown. The apparent cooperativity constants for low-pH-induced sickling suggest highly cooperative proton binding to Hb during the sickling process. In the absence of catecholamines, protons are usually passively distributed across the RBC membrane, with changes in plasma pH causing distinctly smaller changes in intracellular pH, as also seen in the close relative of whiting, the Atlantic cod (Berenbrink and Bridges, 1994a; Berenbrink and Bridges, 1994b) (for a review, see Brauner and Berenbrink, 2007). When sickling is more appropriately related to intracellular pH

changes in the vicinity of Hb, even higher cooperativity constants can be calculated. For example, using the *in vitro* relationship between extracellular and intracellular pH of air-equilibrated Atlantic cod RBCs (Berenbrink and Bridges, 1994a), the value for the apparent cooperativity constant under air increases from 12 (Table 1) to ~30 (not shown). This indicates that about 30 proton binding sites interact during sickling and demonstrates the extreme pH-dependent nature of the process. Using the same approach, an intracellular pK_{app} value for sickling of air-equilibrated RBCs of 7.16±0.01 can be calculated. This suggests the involvement of protein moieties with p*K* values in the physiological range, such as N-terminal C-alpha amino groups or the imidazole ring of histidines (Berenbrink, 2006).

Indeed, despite the general trend towards a reduction in histidine content in teleost haemoglobins (Berenbrink et al., 2005), one of the two different β globin chains in Atlantic, Arctic and Polar cod (Verde et al., 2006) contains two extra histidine residues on the external surface of the protein. These occur in position 7 of the A-helix (His A7) and position 1 of the corner between the E and F helices (His EF1) and are absent in other teleost β chains (M.B., unpublished). In other teleosts, His A7 is usually substituted by a variety of non-ionisable amino acids and His EF1 is occupied by neutral asparagine. Interestingly, in all three species the mutation to His EF1 is accompanied by a change of the preceding amino acid in position E20 from positively charged lysine to negatively charged aspartic acid (M.B., unpublished), which may facilitate proton binding by His EF1. It is tempting to speculate that one or both of the above two histidine substitutions, which are likely to change surface charge, are responsible for the pH-dependent polymerisation of at least some Hb isoforms in codfishes. Alternatively, or in addition, the globins of codfishes are also unusually rich in cysteine, which has been implicated in the *in vitro* formation of Hb polymers in some other teleosts (Borgese et al., 1988; Borgese et al., 1992; Fago et al., 1993). Clearly, the elucidation of the mechanism behind Hb polymerisation awaits detailed molecular structure–function analyses, ideally on otherwise similar, polymerising and non-polymerising Hbs.

Hárosi and co-workers (Hárosi et al., 1998) reported that the intracellular aggregates in Atlantic cod RBCs consist of deoxygenated Hb. Many fishes possess Hbs with a highly pH-sensitive oxygen affinity, which can be deoxygenated by low pH even when equilibrated with 100 kPa O_2. This so-called Root effect has been explained as a stabilization of the low O_2 affinity T-state conformation over the high O_2 affinity R-state of Hb by the binding of protons (see Berenbrink, 2007). Our observation that fully deoxygenated cells with Hb in the T-state only sickle at low pH suggests that sickling in whiting does not depend on a conformational transition from R- to T-state Hb. Further exploration of the mechanism of Hb polymerisation awaits sequencing of whiting Hb and detailed molecular structure–function studies.

In vitro, and possibly also *in vivo*, haemoglobin polymerisation and RBC sickling appears to be widespread in codfishes, occurring in whiting as well as in poor cod and pouting (Yoffey, 1929), Atlantic cod and haddock (Hárosi et al., 1998) and pollack, saithe and five-bearded rockling (P.K. and M.B., unpublished). Curiously, RBCs from all individuals within populations appear to sickle (Hárosi et al., 1998; present study), in contrast to the balanced polymorphism for human sickle cell trait (Aluoch, 1997; Jones, 1997). In the latter case, the disadvantage to homozygote human carriers of the disease is outweighed by the benefits of heterozygote carriers against infection with malaria parasites. Our *in vitro* results suggest that activation of the βNHE *in vivo* may protect whiting RBCs to a certain degree from sickling, at least at intermediate pH values. Thus, the disadvantages of possessing a Hb with the potential of polymerising and causing sickling appear reduced in whiting when compared with homozygous human sickle cell patients. It is less clear whether RBC sickling in fishes also offers an advantage like in human heterozygote carriers. Fishes are infected by numerous parasites, including several protozoan species in the blood stream as well as in the RBCs (Hemmingsen and MacKenzie, 2001; Davies and Johnston, 2000). The intracellular RBC parasites include a virus causing viral erythrocytic necrosis (VEN), which is widespread in Atlantic cod, chum salmon and Atlantic herring but has adverse effects such as haemorrhage, haemolysis, anaemia and increased mortality only in the latter two species (Smail, 1982), whose RBCs (in contrast to Atlantic cod) are not known to sickle. Even more interestingly, VEN infection in Atlantic cod is associated with the occurrence of intracellular fibrillar material, which looks similar to the Hb filaments observed in the present work and was absent in uninfected RBCs (Appy et al., 1976). It is tempting to speculate that, in analogy to the human condition (Ayi et al., 2004), VEN-infected RBCs have a higher tendency to sickle, which may enable selective removal from the circulation or impair intracellular virus propagation.

The present study on whiting is the first to suggest a physiological mechanism that protects against RBC sickling, which remains a lethal disease in humans. We can only speculate about the selective advantages that led to the fixation and spread of the initial mutation causing sickling through populations of the ancestral species. However, the disadvantages of this mutation seem to have been sufficiently suppressed by the presence of a βNHE, such that the mutation persisted during several speciation events, which gave rise to the current diversity of codfishes. This mechanism offers a unique opportunity to study how the problem of RBC sickling has been managed successfully by nature through evolutionary time.

Abbreviations and symbols

βNHE	β-adrenergically stimulated Na/H exchanger
Hb	haemoglobin
HbA	normal haemoglobin A
HbS	sickle haemoglobin S
Hct	haematocrit
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
n_H	cooperativity constant
NHE	Na/H exchanger
pK_{app}	apparent p*K* or pH at half-maximal sickling
P_{O_2}	oxygen partial pressure
RBC	red blood cell
SL	standard length
TEM	transmission electron microscopy
VEN	viral erythrocytic necrosis

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