Comparative analysis of nitrite uptake and hemoglobin-nitrite reactions in erythrocytes: sorting out uptake mechanisms and oxygenation dependencies

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Running title:
Nitrite uptake and reactions with hemoglobin in RBCs
Abstract
Nitrite uptake into red blood cells (RBCs) precedes its intracellular reactions with hemoglobin (Hb) that forms nitric oxide (NO) during hypoxia. We investigated the uptake of nitrite and its reactions with Hb at different oxygen saturations (So2), using RBCs with (carp and rabbit) and without (hagfish and lamprey) anion exchanger-1 (AE1) in the membrane, with the aim to unravel the mechanisms and oxygenation dependencies of nitrite transport. Added nitrite rapidly diffused into the RBCs until equilibrium. The distribution ratio of nitrite across the membrane agreed with that expected from HNO2 diffusion and AE1-mediated facilitated NO2⁻ diffusion. Participation of HNO2 diffusion was emphasized by rapid transmembrane nitrite equilibration also in the natural AE1 knockouts. Following the equilibration, nitrite was consumed by reacting with Hb, which created a continued inward diffusion controlled by intracellular reaction rates. Changes in nitrite uptake with So2, pH or species were accordingly explained by corresponding changes in reaction rates. In carp, nitrite uptake rates increased linearly with decreasing So2 over the entire So2 range. In rabbit, nitrite uptake rates were highest at intermediate So2, producing a bell-shaped relationship with So2. Nitrite consumption increased approximately 10 fold with a one unit decrease in pH, as expected from the involvement of protons in the reactions with Hb. The reaction of nitrite with deoxyhemoglobin was favored over that with oxyhemoglobin at intermediate So2. We propose a model for RBC nitrite uptake that involves both HNO2 diffusion and AE1-mediated transport and which explains both the present and previous (sometimes puzzling) results.

Key words: anion exchanger-1; facilitated diffusion; HNO2 diffusion; nitric oxide

Introduction
Endogenous nitrite has emerged as a physiologically important reservoir of NO activity that can be activated by a number of cellular proteins under hypoxic and/or acidic conditions (1, 7, 8, 13, 29,
Nitrite-derived NO has been implicated in functions such as blood flow regulation (8, 14) and cytoprotection during hypoxic/anoxic insults (17, 46). Among the different NO generating mechanisms, the nitrite reductase activity of deoxygenated hemoglobin (Hb, deoxyHb) has attracted considerable attention, as this mechanism allows red blood cells (RBCs) to produce a vasodilator (NO) in a deoxygenation-dependent manner that may help increase blood flow in hypoxic tissues (8, 9, 33).

Extracellular nitrite that permeates the RBC membrane reacts with deoxygenated Hb (deoxyHb), whereby ferrous hemes are oxidized to ferric heme (methemoglobin; metHb) while nitrite is reduced to NO (8):
\[
\text{Hb}(\text{Fe}^{2+}) + \text{NO}_2^- + \text{H}^+ \rightarrow \text{Hb}(\text{Fe}^{3+}) + \text{NO} + \text{OH}^- \quad \text{(Equation 1)}
\]
The NO can subsequently bind to an adjacent ferrous heme to form nitrosylhemoglobin (HbNO):
\[
\text{Hb}(\text{Fe}^{2+}) + \text{NO} \rightarrow \text{Hb}(\text{Fe}^{2+})\text{NO} \quad \text{(Equation 2)}
\]
Nitrite may also react with oxygenated Hb (oxyHb) to form nitrate and metHb:
\[
4\text{Hb}(\text{Fe}^{2+})\text{O}_2 + 4 \text{NO}_2^- + 4\text{H}^+ \rightarrow 4\text{Hb}(\text{Fe}^{3+}) + 4 \text{NO}_3^- + \text{O}_2 + 2 \text{H}_2\text{O} \quad \text{(Equation 3)}
\]
The oxyHb reaction involves several intermediate reactions, and its reaction kinetics show an initial slow lag phase that is followed by an autocatalytic increase in reaction rate (27, 28). The oxyHb reaction does, however, not enter the autocatalytic phase at the low nitrite concentrations occurring in vivo (28). Recent scrutiny of the reactions of nitrite in Hb solutions has revealed that the deoxyHb reaction (eq. 1) is allosterically controlled and that it is favored over the oxyHb reaction at intermediate oxygen saturation (So2), as appropriate for a role of deoxyHb-derived NO in vasodilation (15, 19, 23).

Uptake of nitrite across cell membranes is an essential step preceding its intracellular conversion to NO. In pig RBC suspension, added nitrite rapidly permeates and equilibrates across the RBC membrane, and then continues to enter the cells as result of the intracellular consumption of nitrite in its reactions with Hb, which set up a continual diffusion gradient (21). Nitrite uptake is strongly
oxygenation-dependent in some fish species, like carp, where nitrite only marginally permeates oxygenated RBCs, whereas it extensively enters deoxygenated RBCs (20, 24). While this appears favorable for deoxyHb-mediated NO generation, a similar difference between fully oxygenated and deoxygenated RBCs is not observed in mammalian (pig and sheep) RBCs (3, 21). Human and sheep RBCs, in contrast, show maximal nitrite uptake around 50% So2 (3, 45), which coincides with a maximal nitrite reduction rate around 50% So2 (19). There accordingly appears to be considerable species differences in the oxygenation dependency of RBC nitrite uptake, the origin of which remains to be uncovered.

The mechanism of cellular nitrite uptake has not been finally resolved. It has been discussed that nitrite mainly enters RBCs via HNO2 diffusion through the lipid bilayer or via NO2⁻ anion diffusion through a channel (conductive transport) or the RBC anion exchanger AE1 (facilitated diffusion) (20, 21, 31, 41). AE1 would seem a likely candidate to mediate nitrite transport in RBCs, because it is the most abundant membrane protein in erythrocytes (1 million copies per cell) and known to transport various different anions (30). However, application of DIDS (an AE1 inhibitor) and other inhibitors of facilitated diffusion have not been able to provide conclusive answers. While an absent influence of DIDS on nitrite uptake in carp (20), human (31) and pig (21) RBCs seems to argue against the involvement of AE1, the finding of DIDS inhibition of NO2⁻/HCO3⁻ exchange in horse RBC ghosts (41) favors the AE1 route. In the light that DIDS binds to a different site than the AE1 transport site (38) and has a different potency among species (26), an AE1 knockout model would seem ideal for evaluating the role of AE1 in RBC nitrite transport. Hagfish and lampreys are special among all chordates/vertebrates by being functionally devoid of AE1 in their RBCs (6, 11, 34, 35, 43) and they thus provide natural knockout models for such an investigation.

The present study uses a comparative approach to unravel the mechanisms and oxygenation dependency of nitrite uptake and consumption in RBCs. A primary purpose was to test the hypothesis that AE1 is involved in RBC nitrite transport, by comparing nitrite uptake in two AE1
knockout species (Atlantic hagfish and sea lamprey) with nitrite uptake in a teleost fish (carp) and a mammal (rabbit) that have AE1 in the RBC membrane. This tactic was predicted to allow discrimination between the roles of AE1-mediated NO2⁻ transport and HNO2 diffusion. A second aim was to improve knowledge on the oxygenation dependency of nitrite uptake and differences between species. Notably, we wanted to establish the complete So₂ dependency of nitrite uptake in carp (i.e. over the full range of So₂ values) and to compare this with a mammalian species (rabbit) examined under the same experimental conditions. Finally, we wished to explore the reactions of nitrite with Hb at different So₂ values in intact RBCs, and to evaluate the importance of the intracellular reaction rates in the So₂ and pH dependencies of RBC nitrite transport. Our analysis leads to a model for RBC nitrite uptake that complies with available evidence and explains conflicting results reported in the literature.

Materials and methods

Animals, blood sampling, and preparation of erythrocyte suspensions

Common carp (Cyprinus carpio) were maintained in 500 l tanks in an aquaria facility at Odense campus with recirculated/filtered freshwater at 25 °C. The fish were subjected to a 12 h/12 h light/dark cycle and fed commercial fish pellets twice a week. Sea lamprey (Petromyzon marinus) were caught by a local fisherman at Hvide Sande, Jutland, Denmark and transported to Odense, where they were kept in 300 l tanks with seawater of 18 ‰ salinity and a temperature of 15 °C. Atlantic hagfish (Myxine glutinosa) were caught in Gullmarsund and maintained in full strengt seawater at Sven Lovén Centre for Marine Sciences, Kristineberg, Sweden before blood sampling. Some of the hagfish were transported to Odense and kept in a 300 l seawater (32 ‰) tank for 3-4 days before blood sampling. The fish were anaesthetized in MS-222 (ethyl 3-aminobenzoate methanesulfonate, Sigma) and blood was sampled from the caudal vessels (carp and lamprey) or sinus (hagfish) into heparinized syringes. Freshly drawn blood from rabbit (Oryctolagus cuniculus)
was obtained from the Biomedical Laboratory, University of Southern Denmark, Odense. Use of animals was approved by the Danish Ministry of Justice (permission no. 2008/561-1470). Authorizations to catch hagfish in Sweden and sample their blood were obtained from the Swedish Board of Fisheries and Göteborg Ethical Committee on Animal Research, and permission to import some of the hagfish to Denmark was obtained from the Danish Ministry of Food, Agriculture and Fisheries.

Blood was centrifuged and plasma and buffy coat were removed. The red blood cells (RBCs) were washed twice in species-specific albumin-containing physiological saline (APS) that agreed with the different plasma osmolality/ionic composition in the four species (Table 1). Albumin (albumin from bovine serum, Sigma-Aldrich, Steinheim, Germany) was present at 14 mg/ml to minimize/avoid RBC hemolysis (25). The RBCs were suspended in the species-specific APS to a hematocrit of approximately 20%.

**Experiments**

An accurate amount of RBC suspension was pipetted into shaking Eschweiler (Kiel, Germany) tonometers and equilibrated for one hour with a continuous flow of humidified gas with constant Po2 and Pco2 values at constant temperature. Gas mixtures were delivered from Wösthoff (Bochum, Germany) gas mixing pumps, and the desired Po2 and Pco2 values were obtained by appropriate mixing of air, CO2 and N2. Different Po2 values were used in separate experiments in order to obtain different hemoglobin (Hb) O2 saturation (So2) values. The pH of the RBC suspensions was adjusted by the gas CO2 level. Due to the different physiological Pco2 values in water breathing fish and air breathing mammals, the reference setting of CO2 was 0.4% CO2 (Pco2 = 3 mmHg) in the three fish species and 5% CO2 (Pco2 = 37 mmHg) in rabbit.

Following one hour of equilibration, a known amount of RBC suspension was sampled and used to measure hematocrit (Hct), pH and hemoglobin derivatives (cf. below). The Hct and volume of
RBC suspension remaining in the tonometer were used to calculate the volume of a 140 mM NaNO₂ stock solution that should be added to elevate extracellular [NO₂⁻] to either a high (3 mM) or low (0.1 mM) concentration. Following addition of nitrite (defined as time zero), further samples were withdrawn from the tonometer at specified times (typically 0.5, 20, 40, 60, 90, 120, 140, 170 and 200 min). Due to the time spent on processing a sample and separating RBC from plasma by centrifugation, results were given a time assignment 1 min above the sampling times (e.g. results from a 0.5 min sample were plotted at 1.5 min). The gas supply to the tonometer was maintained constant throughout each experiment. The experimental temperature was 25 °C for carp and rabbit, and 15 °C for hagfish and sea lamprey.

The oxygenation-dependency of RBC nitrite uptake and the reactions of nitrite with intraerythrocytic Hb were evaluated by conducting experiments on fully oxygenated RBCs, fully deoxygenated RBCs, and RBCs with various intermediate So₂ values. The influence of pH was investigated by performing experiments at different pH values (obtained by equilibrating to different Pco₂ values). Furthermore, experiments were conducted at both high and low extracellular [NO₂⁻] to evaluate the influence of different nitrite concentrations.

**Measurements and calculations**

Nitrite was measured spectrophotometrically, using the Griess reaction (5). Uptake of nitrite into RBCs was assessed from the time-dependent decrease in extracellular [NO₂⁻]. The initial nitrite consumption rate (Jₐ) was determined by fitting the measured extracellular [NO₂⁻] (time interval 1.5 – 221 min) to exponential equations and calculating Jₐ for t approaching zero. The intraerythrocytic reactions between nitrite and hemoglobin were evaluated by recording absorption spectra of sample hemolysates in the wavelength interval 480-700 nm and assessing the concentrations of oxyHb, metHb, HbNO and deoxyHb by spectral deconvolution (22). The reference spectra of oxyHb, metHb, HbNO and deoxyHb required in this analysis were made for
each individual species (22). Hct was determined by centrifugation (2 min at 12000 rpm) in glass capillaries. Mean cellular Hb concentration was assessed from [Hb]/Hct. Extracellular pH was measured with the capillary pH electrode of a Radiometer (Copenhagen, Denmark) BMS 3 electrode set-up connected to a PHM 73 monitor. The So2 of RBC suspensions was evaluated from the equilibration Po2 and the relationship between So2 and Po2 (i.e. O2 equilibrium curve) obtained in parallel experiments, using the mixing technique (16).

Statistics
Results are presented as means ± SE unless otherwise stated. Statistical analysis was done with a two-factor (time and treatment) analysis of variance (ANOVA) for repeated measurements. Subsequently, the treatments were compared pairwise with a two-factor ANOVA for repeated measurements, using a Bonferroni correction. Differences were considered significant at \( P<0.05 \). Statistical analyses were performed with SAS version 9.1.

Results
RBC nitrite uptake and its dependencies on species, oxygenation degree and pH
Nitrite was added to RBC suspensions (Hct ~ 20%) to acutely elevate the extracellular nitrite concentration and create a diffusion gradient for nitrite into the RBCs. This allowed nitrite uptake into the RBCs to be assessed from the time-dependent decrease in extracellular \([\text{NO}_2^-]\) (Fig. 1). Control experiments in which nitrite was added to oxygenated and deoxygenated physiological saline (Hct = 0) revealed that the extracellular \([\text{NO}_2^-]\) remained constant in the absence of RBCs, and that it assumed the expected calculated value (e.g. Fig. 1D), confirming that extracellular nitrite disappearance in RBC suspensions was due to RBC nitrite uptake and consumption. When nitrite was added to an extracellular concentration of 3 mM (time zero), the first subsequent measured sample (1.5 min) had an extracellular \([\text{NO}_2^-]\) below that value (Fig. 1A,B,C,D), reflecting that
nitrite rapidly equilibrates across the RBC membrane (21). This rapid equilibration was generally followed by an exponential decrease in extracellular \([\text{NO}_2^-]\) (Fig. 1), revealing a continued RBC nitrite uptake that can be ascribed to the ongoing removal of intracellular nitrite via its reactions with intracellular Hb, which continuously reestablishes an inward diffusion gradient (21).

The uptake of nitrite into RBCs differed between species and with RBC oxygenation degree. In Atlantic hagfish, the initial equilibration of nitrite across the membrane caused a drop in extracellular \([\text{NO}_2^-]\) from 3 to 2.7 mM, where after extracellular \([\text{NO}_2^-]\) stayed constant in oxygenated RBC suspensions and only showed a marginal decrease in deoxygenated RBC suspensions (Fig. 1A). In sea lamprey, nitrite uptake was similarly small in oxygenated RBCs but somewhat larger in deoxygenated RBCs (Fig. 1B). Further, in RBCs with 50% So2, the uptake was in-between that observed for oxygenated and deoxygenated RBCs (Fig. 1B). The extracellular nitrite decay profiles were significantly different for all three So2 values (Fig. 1B), and a similar dependence on So2 was found at low (0.1 mM) extracellular nitrite load (not illustrated).

In carp, the decrease in extracellular \([\text{NO}_2^-]\) with time was larger than observed in hagfish and sea lamprey and it showed a marked oxygenation dependency. Nitrite uptake into oxygenated RBCs was moderate at pH 7.8, whereas nitrite uptake/consumption was extensive in deoxygenated RBCs. In order to gain insight into how this major difference between fully oxygenated (So2 = 100%) and fully deoxygenated (So2 = 0%) RBCs develops, experiments were conducted at several intermediate oxygen saturations (So2 values of 95%, 90%, 75%, 50% and 35%). Most nitrite decay curves were significantly different from each other (Fig. 1C), and it is evident from the many curves that nitrite uptake and consumption in carp RBCs was gradually increased as So2 was gradually decreased (Fig. 1C). The initial nitrite consumption rates of the RBCs were calculated for each individual experiment, and a plot of these values as function of So2 showed that the increased nitrite consumption with decreasing So2 was practically linear (Fig. 2A). It is important to note that the initial nitrite consumption rate refers to the situation immediately after the rapid equilibration of
nitrite across the membrane, because it was calculated from fits of the measured data from 1.5 min onwards.

Rabbit RBC suspensions exposed to a high extracellular [NO$_2^-$] (3 mM) at pH 7.4 showed a response that differed from that observed in the fish species. There was an extensive decline in extracellular [NO$_2^-$] in oxygenated RBC suspensions within the first 20 min, whereas the [NO$_2^-$] decay was slower and smaller in deoxygenated suspensions (Fig. 1D). It appears that the initial nitrite equilibration across the membrane raised the intracellular nitrite concentration to a level that caused the nitrite reaction with oxyHb inside oxygenated rabbit RBCs to enter the autocatalytic phase. The ensuing high reaction rates quickly consumed intracellular nitrite (promoting its further entry from the extracellular space) and raised metHb to very high levels within 20 min (cf. below). In order to circumvent activation of autocatalysis in oxygenated rabbit RBCs we also examined rabbit RBCs at a lower nitrite load (0.1 mM). At low extracellular [NO$_2^-$], the nitrite uptake was the same in fully oxygenated and fully deoxygenated RBCs, but it was increased at an intermediate So$_2$ of 60% (Fig 1F). Calculation of the initial nitrite consumption rates of the RBCs therefore suggested a bell-shaped relationship with So$_2$ with maximal nitrite consumption around 50% So$_2$ (Fig. 3A). We also evaluated the influence of pH on nitrite uptake in rabbit RBCs at low [NO$_2^-$]. These data revealed that nitrite consumption increased with decreasing pH, but that it was comparable in oxygenated and deoxygenated RBCs at any constant pH within the pH interval from 7 to 8 (Fig. 3B).

Even though an autocatalytic reaction between nitrite and oxyHb was not observed in any of the fish species, we also investigated nitrite uptake at low [NO$_2^-$] in carp RBCs to rule out any influence of nitrite concentration on the observed oxygenation dependency. Indeed, the large difference in nitrite uptake between oxygenated and deoxygenated RBCs at high [NO$_2^-$] (Fig. 1C) was likewise present at low [NO$_2^-$] (Fig. 1E). Furthermore, even though initial nitrite consumption rates were lower at low than at high extracellular [NO$_2^-$], the consumption rates increased linearly with
decreasing So₂ in both conditions (Fig. 2). Thus, overall results and conclusions were the same at high and low nitrite load.

_Intracellular reactions between nitrite and hemoglobin_

Given that nitrite disappearing from the extracellular (ex) fluid entered the RBCs, it is possible to calculate the increase in intracellular (RBC) nitrite between two consecutive measurements according to (21):

\[
\Delta [\text{NO}_2^-]_{\text{RBC}} = \Delta [\text{NO}_2^-]_{\text{ex}} \times \frac{(100-\text{Hct})}{\text{Hct}}.
\]

By summing up the change in \([\text{NO}_2^-]_{\text{RBC}}\) during an experiment, one obtains the accumulated intracellular \([\text{NO}_2^-]\). This does not give the real \([\text{NO}_2^-]_{\text{RBC}}\), because nitrite is consumed in reactions with Hb, but the accumulated \([\text{NO}_2^-]_{\text{RBC}}\) provides a measure of the reactant nitrite that can be directly compared with the measured cellular concentrations of the reaction products metHb and HbNO.

In fully deoxygenated carp RBC suspension exposed to 3 mM nitrite at a pH<sub>ex</sub> of 7.8, \([\text{NO}_2^-]_{\text{RBC}}\) rose to 1.8 mM within the first 1.5 min (Fig. 4A). At this time [metHb] and [HbNO] were indifferent from control values, suggesting that the intracellular reaction between nitrite and Hb had not begun and that the concentration of un-reacted intracellular nitrite was 1.8 mM (Fig. 4A). The extracellular concentration was 2.5 mM at 1.5 min (Fig. 1C), whereby the distribution ratio \([\text{NO}_2^-]_{\text{RBC}}/[\text{NO}_2^-]_{\text{ex}}\) was 0.72. Nitrite started to react with cellular deoxyHb (17 mmol heme l⁻¹ RBC) after the initial nitrite equilibration across the membrane. Cellular [HbNO] rose with time in parallel with the curve for accumulated [nitrite] (Fig 4A), which shows that nitrite uptake was guided by the intracellular reaction with Hb and that nitrite was consumed to form HbNO in a 1:1 relationship (as expected from equations 1 and 2). Furthermore, even though the cellular [HbNO] was lower than \(\Sigma[\text{nitrite}]\), the difference was relative constant during the experiment (Fig. 3A), suggesting that the intracellular concentration of un-reacted free nitrite that was attained at 1.5 min remained relatively stable. The reaction rate calculated from the initial intracellular [HbNO] increase (first 21 min) was 0.116 mmol l⁻¹RBC min⁻¹, which compares well with the initial nitrite consumption rate of ~0.11
mmol l⁻¹RBC min⁻¹ at So₂ = 0 (Fig. 2A). The reaction between nitrite and deoxyHb produced HbNO and metHb in approximately equal amounts during the first 60 min (as predicted by equations 1 and 2), where after [metHb] began to decrease (Fig. 4A). The latter can be ascribed to the presence of metHb reductase in intact RBCs that reduced metHb to functional Hb.

At intermediate So₂, nitrite reacted with both deoxyHb and oxyHb, and the amount of HbNO produced was lower than in fully deoxygenated cells (Fig. 4). HbNO was present in significant amounts at low So₂ values, but levels decreased with increasing So₂ to vanish at the highest So₂ (Fig. 4). MetHb, in contrast, increased with Σ[nitrite] at all intermediate So₂ values (Fig. 4). The reactions of nitrite with deoxyHb (eq. 1) and oxyHb (eq. 3) both form 1 mol metHb per mol nitrite consumed. However, at intermediate So₂, some of the NO formed by the deoxyHb reaction will react with oxyHb to form metHb and nitrate rather than combine with vacant ferrous heme groups to form stable HbNO. The molar ratio between metHb production and nitrite consumption will therefore exceed 1 at intermediate So₂. MetHb levels are further compounded by being a balance between metHb production and metHb removal via metHb reductase activity. The distance between Σ[nitrite] and [metHb] therefore cannot be used as a measure of the intracellular nitrite concentration during the reaction as was the case with the distance between Σ[nitrite] and [HbNO] in fully deoxygenated RBCs.

In fully oxygenated carp RBCs, the [NO₂⁻]RBC rose to 0.7 mM at 1.5 min (Fig. 4F), while extracellular [NO₂⁻] decreased from 3 mM to 2.76 mM (Fig. 1C). Accordingly, oxygenated carp RBCs seemed to have a [NO₂⁻]RBC of 0.7 mM and a [NO₂⁻]RBC/[NO₂⁻]ex distribution ratio of 0.25, which were both lower than corresponding values in deoxygenated RBCs.

In hagfish RBCs, [NO₂⁻]RBC rose to about 1 mM at 1.5 min and then stayed constant in oxygenated RBCs, while Σ[nitrite] increased slightly in deoxygenated RBCs (Fig. 5A,B). The [NO₂⁻]RBC/[NO₂⁻]ex ratio at 1.5 min was 0.4 for both oxygenated and deoxygenated RBCs. [HbNO] increased slowly in parallel with Σ[nitrite] in deoxygenated RBCs, reflecting a significant but
modest nitrite reduction to NO (Fig. 5B). [MetHb] was kept below [HbNO] by metHb reductase activity.

Oxygenated sea lamprey RBCs took up nitrite to an intracellular concentration of 1 mM at 1.5 min, which rose further to 1.9 mM at 20 min, where after $\Sigma$[nitrite] was rather constant (Fig. 5C). In deoxygenated RBCs, [NO$_2^-$]$\text{_{RBC}}$ increased to 0.9 mM at 1.5 min and further to 2.3 mM at 20 min, where after $\Sigma$[nitrite] showed a slow linear increase with time between 40 and 200 min (Fig. 5D). HbNO started to increase in deoxygenated lamprey RBCs after a delay, but then rose in parallel with $\Sigma$[nitrite] (Fig. 5D). Again, the more or less constant difference between $\Sigma$[nitrite] and [HbNO] suggested a relatively steady concentration of free nitrite inside the RBCs. However, the time taken before nitrite uptake resulted in a steady intracellular [nitrite] in sea lamprey appeared longer than in the other species. Taking this into account, the apparent [NO$_2^-$]$\text{_{RBC}}$/[NO$_2^-$]$\text{_{ex}}$ ratio at 20 min calculates as 0.7 in oxygenated RBCs and 0.97 in deoxygenated RBCs. [MetHb] stayed well below [HbNO] in deoxygenated sea lamprey RBCs (Fig. 5D), pointing at efficient metHb reductase activity.

Oxygenated rabbit RBCs rapidly entered the autocatalytic phase of nitrite-induced oxidation of oxyHb when treated with 3 mM extracellular nitrite at pHe$_{ex}$ 7.4, as evidenced by the rapid and extensive increases in intracellular $\Sigma$[nitrite] and [metHb] (Fig. 5E). Following a maximum cellular [metHb] of 7.2 mM (corresponding to 42.5% of total Hb) at 21 min, metHb decreased slowly due to metHb reductase activity, while $\Sigma$[nitrite] continued to increase, but at a much slower rate than during the initial nitrite uptake (Fig. 5E). [NO$_2^-$]$\text{_{RBC}}$ in deoxygenated rabbit cells rose acutely to 2.4 mM at 1.5 min and then continued to increase at a slower pace (Fig. 5F). [HbNO] was indifferent from control at 1.5 min but subsequently started to increase at a rate that was comparable to that for the increase in $\Sigma$[nitrite] (Fig. 5F). The relative stable difference between $\Sigma$[nitrite] and [HbNO] suggested a steady intracellular concentration of un-reacted nitrite around the initial 2.4 mM. As
extracellular $[\text{NO}_2^-]$ was 2.3 mM at 1.5 min, the initial $[\text{NO}_2^-]_{\text{RBC}}/[\text{NO}_2^-]_{\text{ex}}$ distribution ratio appeared to be around 1 in deoxygenated rabbit RBCs.

**Discussion**

Our data shows that nitrite diffuses across the RBC membrane at a rate that is faster than the reaction rates between nitrite and hemoglobin. Thus, after the initial fast (<1.5 min) equilibration of nitrite across the membrane, it is the intracellular reactions between nitrite and Hb that becomes rate limiting for subsequent nitrite entry.

**Nitrite uptake mechanisms**

The initial diffusion was completed within the time needed to take and process the first sample (<1.5 min) and caused a decrease in extracellular $[\text{NO}_2^-]$ (below the added 3 mM) and rise in intracellular $[\text{NO}_2^-]$ until equilibrium for the diffusing species. The transport mechanism could be simple diffusion of HNO$_2$ through the lipid bilayer and/or diffusion of the nitrite anion through a protein channel (conductive transport) or the anion exchanger AE1 (facilitated diffusion). Our results support that diffusion of HNO$_2$ is a main transport mechanism, because the initial fast diffusion/equilibration of nitrite across the membrane occurred in all four species, including Atlantic hagfish and sea lamprey that are functionally devoid of AE1 in their RBCs (6, 35, 43). Nitrous acid has a pK$_a$ around 3.3 and will only be present at very low concentrations at physiological pH (mass law considerations reveal a HNO$_2$ concentration of 95 nM at pH 7.8 and 239 nM at pH 7.4 if NO$_2^-$ is 3 mM). This does, however, not preclude its relative rapid diffusion across the membrane, as documented with phospholipid bilayer of liposomes (40). HNO$_2$ diffusing into the RBCs will dissociate to H$^+$ and NO$_2^-$ inside the RBCs, and the diffusion will continue until [HNO$_2$] is the same inside and outside the cells (40). By writing the law of mass action for both compartments and equating [HNO$_2$] in the two compartments, the equilibrium condition implies:
\([\text{NO}_2^-]_{\text{RBC}} / [\text{NO}_2^-]_{\text{ex}} = [\text{H}^+]_{\text{ex}} / [\text{H}^+]_{\text{RBC}} = 10^{(\text{pH}_{\text{ex}} - \text{pH}_{\text{RBC}})}\). Thus, with \(\text{HNO}_2\) diffusion as the transport mode, the nitrite distribution ratio will be determined by intracellular and extracellular pH. At an extracellular pH of 7.8, oxygenated carp RBCs have a pH of 7.2 and deoxygenated RBCs have a pH of 7.5 (2), predicting \([\text{NO}_2^-]_{\text{RBC}} / [\text{NO}_2^-]_{\text{ex}}\) values of 0.25 and 0.5, respectively. The actually determined nitrite distribution ratios were 0.25 in oxygenated RBCs and 0.72 in deoxygenated RBCs, so values seem to compare fairly well. However, a direct comparison is complicated, because the estimated \([\text{NO}_2^-]_{\text{RBC}}\) refers to packed cell volume, and values become higher if correctly referred to cell water. This may, however, be offset by the fact that intracellular nitrite to some extent is metabolized to S-nitroso compounds (4, 8), which should be subtracted from the estimated \([\text{NO}_2^-]_{\text{RBC}}\). Also, some of the anionic intracellular nitrite may bind to deoxygenated Hb in a manner like organic phosphates and chloride, which would also lower the free intracellular \([\text{NO}_2^-]_{\text{RBC}}\).

These uncertainties can be circumvented by performing a correlation test that includes all four species. Thus, by using literature values for \(\text{pH}_{\text{RBC}}\) at \(\text{pH}_{\text{ex}}\) 7.8 in oxygenated and deoxygenated RBCs of sea lamprey (12) and Atlantic hagfish (44), and by using the human value (\(\text{pH}_{\text{RBC}}\) 7.27) at \(\text{pH}_{\text{ex}}\) 7.4 (10) for deoxygenated rabbit RBCs, it is possible to probe for an overall correlation between \([\text{NO}_2^-]_{\text{RBC}} / [\text{NO}_2^-]_{\text{ex}}\) and \(10^{(\text{pH}_{\text{ex}} - \text{pH}_{\text{RBC}})}\). Indeed, this analysis reveals a significant positive correlation between the two parameters (Fig. 6). Notably, the large increase in \(\text{pH}_{\text{RBC}}\) upon deoxygenation in both carp (2) and sea lamprey (12) is paralleled by increased \([\text{NO}_2^-]_{\text{RBC}} / [\text{NO}_2^-]_{\text{ex}}\) in both cases, whereas the similar \(\text{pH}_{\text{RBC}}\) in oxygenated and deoxygenated RBCs of Atlantic hagfish (44) results in the same \([\text{NO}_2^-]_{\text{RBC}} / [\text{NO}_2^-]_{\text{ex}}\) for the two oxygenation states (Fig. 6). Accordingly, the observed distribution ratios suggest that nitrite is passively distributed across the RBC membrane, but they cannot be used to distinguish between \(\text{HNO}_2\) diffusion and AE1-mediated nitrite transport. If nitrite is transported by the anion exchanger and assumes a Donnan-like distribution across the membrane, then the distribution ratio will also be given by \(10^{(\text{pH}_{\text{ex}} - \text{pH}_{\text{RBC}})}\) (18).
We propose that nitrite transport across the RBC membrane involves both HNO₂ diffusion and AE1-mediated NO₂⁻ transport as the main mechanisms (Fig. 7). Indeed, all available data are compatible with this idea. In studies that use an experimental design comparable to ours, nitrite transport is not inhibited by the AE1 inhibitor DIDS or other inhibitors of facilitated diffusion (20, 21, 31). This lack of influence of DIDS can be explained if both transport mechanisms are at play, because inhibition of the AE1 pathway will still leave the HNO₂ route open, and nitrite would continue to equilibrate relatively fast across the membrane. The transport velocity would be reduced when AE1 is inhibited by DIDS, but it would require an experimental approach with a time-resolution of milliseconds/seconds (rather than ~1 min) to pick up this effect before the equilibrium distribution is reached via HNO₂ diffusion. In support of this argument, a study on horse erythrocyte ghosts with millisecond/second resolution showed that nitrite is transported by AE1-mediated anion exchange and that the transport can be inhibited by DIDS (41). In most vertebrate RBCs, the AE1-mediated nitrite transport is likely to dominate over HNO₂ diffusion, because NO₂⁻ is present in much higher concentrations than HNO₂ (i.e. lower inward diffusion gradient for the latter), and because AE1 is the most abundant membrane protein and accomplish very fast equilibration of monovalent anions (26, 30). Both mechanisms are, however, capable of transporting nitrite to equilibrium. In hagfish and sea lamprey that both lack AE1 in the RBC membrane, the nitrite equilibration appeared completed within 1.5 min in hagfish, whereas it seemed delayed in sea lamprey. The latter lend some support to a prolonged equilibration time when the AE1 route is absent. The difference between the two natural AE1-knockout species may be explained by different membrane permeability to non-electrolytes (i.e. HNO₂), resulting from a different membrane composition (e.g. cholesterol content) in the two species.

HNO₂ diffusion and NO₂⁻ transport via AE1 can both be expected to elevate extracellular pH during the initial equilibration phase. HNO₂ diffusion will increase extracellular pH, because H⁺ required to form HNO₂ from NO₂⁻ is taken from the extracellular space, whereas facilitated
diffusion of NO$_2^-$ via AE1 in exchange for HCO$_3^-$ will elevate extracellular [HCO$_3^-$] (Fig. 7). In line with this we typically observed a small increase in extracellular pH at 1.5 min (not illustrated), as previously documented with pig RBC suspensions (21).

In the present study, the intracellular concentrations of un-reacted nitrite appeared relatively stable during the reactions between nitrite and Hb, which relates to the instantaneous inward diffusion of new nitrite when nitrite is consumed in the cells. A recent study on nitrite uptake in human RBCs also reported stable intracellular nitrite concentrations and found the levels to increase in the presence of DIDS; but absolute values were very low ([NO$_2^-$]$_{\text{RBC}}$ = 1.5 μM at [NO$_2^-$]$_{\text{ex}}$ ~ 100 μM), translating into extremely low [NO$_2^-$]$_{\text{RBC}}$/[NO$_2^-$]$_{\text{ex}}$ distribution ratios (45). At first sight, these results seem puzzling, but on second sight they actually provide supplementary evidence for the rapid transport of nitrite via both HNO$_2$ diffusion and AE1 here advocated for, because the RBCs were washed in saline before intracellular nitrite was measured (45). The low [NO$_2^-$]$_{\text{RBC}}$ can therefore be explained by rapid outward diffusion of nitrite from the cells to the nitrite-free saline during the wash, while the slightly higher [NO$_2^-$]$_{\text{RBC}}$ in presence of DIDS reflects the somewhat longer equilibration time when only HNO$_2$ diffusion is working.

Although available evidence supports the thesis that nitrite is transported by AE1 and by HNO$_2$ diffusion, this does not rule out that other minor pathways are also involved. Chloride channels and other chloride transport pathways (e.g. Na$^+$/K$^+$/2Cl$^-$ cotransport; K$^+$/Cl$^-$ cotransport) might also transport nitrite, but these proteins are present at much lower concentration than AE1 in the RBC membrane, and will contribute little in the overall picture.

Nitrite only starts to react with intracellular Hb after the initial nitrite equilibration across the membrane is completed (Fig. 4 and 5) and the subsequent reaction rates are lower than the rates by which nitrite at first diffuses into the cells. This implies that any nitrite consumed inside the RBCs will be instantaneously replenished by new nitrite diffusing in. The reaction rates are accordingly controlling nitrite uptake rates after the initial equilibration (i.e. nitrite uptake rates will equal nitrite
consumption rates that again equal reaction rates). Any differences in nitrite uptake with oxygenation degree and between species will therefore be a consequence of differences in reaction rates.

**Intracellular hemoglobin-nitrite reactions**

While fully oxygenated carp RBCs showed minor nitrite consumption and metHb formation (Fig. 4F), a reduction in So2 from 100% to 90% significantly increased metHb production, showing that presence of deoxyHb (10%) speeds up the reaction between nitrite and Hb (Fig. 4E). A further lowering of So2 resulted in further acceleration of the reaction (Fig. 4D,C,B,A). This extends two findings from the molecular level in Hb solutions (23) to the cellular level: namely that the deoxyHb reaction is favored over the oxyHb reaction at intermediate So2, and that the reaction rate for the carp deoxyHb reaction increases with a decrease in So2. The increased reaction rate with lowered So2 can therefore explain the practically linear increase in nitrite uptake with decreasing So2 in carp (Fig. 2). It has earlier been argued that a membrane permeability increase upon deoxygenation contributes to the oxygenation dependency of nitrite uptake in carp RBCs (20, 24). This cannot be excluded, but it seems that changes in intracellular reaction rates suffice to explain the So2 dependency.

Oxygenated rabbit RBCs entered an autocatalytic reaction with excessive nitrite consumption and metHb formation when treated with 3 mM extracellular nitrite (Fig. 1D, Fig. 5E). As activation of autocatalysis for the oxyHb reaction is not occurring at physiological nitrite concentrations (28), we focus on the rabbit results from low (0.1 mM) nitrite load. Here nitrite uptake was similar in oxygenated and deoxygenated RBCs (Fig. 1F), as earlier reported in pig and lamb (3, 21). Interestingly, nitrite consumption was increased at intermediate So2 (Fig. 1F), as also observed in lamb and human RBCs (3, 45). This provides a bell-shaped relationship between nitrite uptake/consumption and So2 (Fig. 3A) as extensively documented with human RBCs (45), which
contrasts with the linear relationship in carp (Fig. 2). Thus, there appears to be a fundamental difference between fish and mammalian RBCs. When studied in Hb solutions, the reaction between nitrite and human deoxyHb shows a sigmoid decay of [deoxyHb] with time (19). As the reaction starts with Hb in the deoxygenated T structure the reaction rate is relatively low, but the rate speeds up during the reaction due to the formation of HbNO and metHb, which promotes a shift to the R structure that has lower redox potential (better ability to reduce nitrite) than the T structure (19). This behavior predicts that the initial reaction rate is highest when Hb and RBCs are half-saturated with O₂ (9, 19), which accordingly accounts for the increased nitrite uptake around half-saturation in mammalian RBCs (Fig. 3A) (3, 45). In fish Hbs (carp and rainbow trout), [deoxyHb] comes more close to an exponential decay with time (and thus pseudo first-order kinetics), when the deoxyHb reaction is studied under the same conditions where mammalian Hbs (rabbit and harbor porpoise) show a clear sigmoid decay in [deoxyHb] (24). This may relate to a less pronounced T → R shift during the reaction in the fish Hbs, because carp Hb has a high tendency to assume the R conformation (as reflected by its very high O₂ affinity), whereas rainbow trout Hb has a high tendency to assume the T structure (as reflected by its very low O₂ affinity) (24). In carp, the deoxyHb reaction rate increases with decreasing Sₒ₂ in Hb solutions (23) and RBCs (Fig. 4), which, as mentioned above, results in a practically linear increase in nitrite consumption with decreasing Sₒ₂ (Fig. 2).

The increased nitrite consumption with pH decrease in both oxygenated and deoxygenated rabbit RBCs (Fig. 3B) is likewise the outcome of increased reaction rates. The reactions of nitrite with Hb require protons (eqs. 1 and 3) and speed up with decreasing pH (19). From the proton requirement of the reaction, one would expect that a pH decrease of 1 pH unit should increase the initial reaction rate by a factor of 10 (19). The initial nitrite consumption rate increased by a factor 7.76 when extracellular pH decreased from 8 to 7 (Fig. 3B), which (by division with a ΔpH_RBC/ΔpH_ex of 0.8
inferred from ref. 10) translates into a 9.7 times increase for a one unit decrease in intracellular pH, underpinning that the pH influence on nitrite uptake is guided by the intracellular reaction rates.

The rate of HbNO formation in deoxygenated RBCs was higher in carp (Fig. 4A) than in rabbit (Fig. 5F), supporting that carp Hb is a more effective nitrite reductase than rabbit Hb (23). HbNO formation was lower in deoxygenated sea lamprey (Fig. 5D) and hagfish RBCs (Fig. 5B). While this result indicates a reduced nitrite reductase capability compared to carp, it may also be a consequence of lower intracellular nitrite concentrations in hagfish and lamprey RBCs than in carp RBCs. Indeed, the intracellular reaction rates depend on the RBC nitrite and Hb concentrations, as reflected by the lower RBC nitrite consumption rates at low than high nitrite concentration (Fig. 2).

Conclusions and perspectives

Our results lead to the overall conclusions that nitrite uptake in RBCs involves both HNO$_2$ diffusion and facilitated diffusion of NO$_2^-$ via AE1, and that the variations in nitrite uptake rates with So$_2$, pH and species are explained by differences in the intracellular reaction rates between nitrite and Hb (Fig. 7). RBCs are special compared to other tissue cells by having a large number of AE1 in their membrane. Nitrite uptake in other tissue cells may therefore be dominated by HNO$_2$ diffusion, and this transport route may be responsible for the rapid uptake and distribution of nitrite among tissues after an acute nitrite load (4).

It has been suggested that NO formed by the nitrite reductase activity of deoxyHb is involved in hypoxic vasodilation (8, 9). One dilemma with this hypothesis is that NO formed inside RBCs will be either tightly bound to ferrous hemes as HbNO or react with oxygenated hemes to form nitrate and metHb. However, the escape of only a minute amount of NO activity may suffice to produce vasodilation in the microcirculation. Such escape of NO activity could be eased via a localized reaction between deoxyHb and nitrite at the membrane (14). DeoxyHb binds to the cytoplasmic domain of AE1, where it would be ideally positioned to reduce nitrite entering via AE1 and liberate
NO at the membrane. AE1-bound Hb furthermore reacts faster with nitrite than Hb in solution (39). The membrane metabolon associated with AE1 also involves carbonic anhydrase (37). This enzyme furnishes HCO\textsubscript{3}\textsuperscript{-} for exchange with NO\textsubscript{2}\textsuperscript{-} via AE1, and it provides H\textsuperscript{+} for the reaction between nitrite and deoxyHb (Fig. 7). Carbonic anhydrase is, however, also itself capable of generating NO activity from nitrite by functioning as a nitrous anhydrase that dehydrates 2HNO\textsubscript{2} to N\textsubscript{2}O\textsubscript{3}, which can diffuse out of the RBCs to form NO (and NO\textsubscript{2}) and thereby contribute to local vasodilation in metabolic active tissues (1). The relative importance of these alternative mechanisms for blood flow regulation must await further study.

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References


39. **Salhany JM.** Kinetics of reaction of nitrite with deoxy hemoglobin after rapid deoxygenation or predeoxygenation by dithionite measured in solution and bound to the cytoplasmic domain of band 3 (SLC4A1). *Biochemistry* 47: 6059-6072, 2008.


Figure legends

Fig. 1. Time-dependent changes in extracellular [NO$_2^-$] following addition of nitrite to red blood cell (RBC) suspensions from Atlantic hagfish (A), sea lamprey (B), carp (C, E) and rabbit (D, F). Nitrite was added at time zero to an extracellular concentration of 3 mM (‘high [NO$_2^-$]’; A, B, C, D) or 0.1 mM (‘low [NO$_2^-$]’; E, F). The initial RBC oxygen saturation (So$_2$) ranged from 0% (filled symbols, deoxygenated RBCs) over various intermediate values (partially filled symbols) to 100% (open symbols, oxygenated RBCs). Hct was ~20% for all species. Extracellular pH was 7.8 for the three fish species and 7.4 for rabbit. Data are means ± SE (N = 3-4). Different letters at curves indicate a significant (P<0.05) difference.

Fig. 2. Oxygenation dependency of nitrite consumption in carp red blood cells exposed to (A) 3 mM extracellular nitrite and (B) 0.1 mM extracellular nitrite. The initial RBC nitrite uptake rates obtained in individual experiments are plotted as a function of RBC oxygen saturation.

Fig. 3. A: Oxygenation dependency of nitrite consumption in rabbit red blood cells exposed to 0.1 mM extracellular nitrite. The initial RBC nitrite consumption rate is plotted as function of oxygen saturation (means ± SE, N = 3). The drawn curve represents a 2$^{nd}$ order polynomial fit to the data. B: pH dependency of nitrite consumption in rabbit RBCs exposed to 0.1 mM extracellular nitrite. The initial nitrite consumption rate (note logarithmic scale) is plotted versus extracellular pH for fully oxygenated (open circles) and fully deoxygenated (filled circles) RBCs. The drawn line represents linear regression on all data: log(nitrite consumption) = -0.89×pH + 3.81 (R = -0.98; P < 0.0001; N = 18).

Fig. 4. Changes in the intracellular concentration of total accumulated nitrite ($\Sigma$[nitrite]), methemoglobin (metHb) and nitrosylhemoglobin (HbNO) in carp RBC suspensions exposed to 3
mM extracellular nitrite at time zero. The RBC oxygen saturation (So2) ranged from 0% (A) over various intermediate values (B, C, D, E) to 100% (F). Total mean cellular Hb concentration was ~17 mmol heme l⁻¹. Data are means ± SE (N = 3-4).

Fig. 5. Changes in the intracellular concentration of total accumulated nitrite (Σ[nitrite]), methemoglobin (metHb) and nitrosylhemoglobin (HbNO) in oxygenated (open symbols) and deoxygenated (filled symbols) RBC suspensions from hagfish (A, B), sea lamprey (C, D) and rabbit (E, F). The RBCs were exposed to 3 mM extracellular nitrite at time zero. Total mean cellular Hb concentrations (mmol heme l⁻¹) were ~12 for hagfish, ~15 for sea lamprey and ~17 for rabbit. Data are means ± SE (N = 3).

Fig. 6. Relationship between the nitrite distribution ratio [NO₂⁻]_{RBC}/[NO₂⁻]_{ex} and 10^{pH_{RBC}−pH_{ex}} in oxygenated (open symbols) and deoxygenated (filled symbols) red blood cell suspensions from the four experimental species. The dashed curves depict a linear fit of the data (R = 0.89, P = 0.0075, N = 7) and the 95% confidence bands. See text for further details.

Fig. 7. Model of nitrite uptake across the RBC membrane. Nitrite added to the extracellular space (left) enters the RBC cytosol (right) both via HNO₂ diffusion through the lipid bilayer and facilitated diffusion of NO₂⁻ via the anion exchanger (AE1). Both mechanisms rapidly establish an equilibrium distribution of nitrite across the membrane given by [NO₂⁻]_{RBC}/[NO₂⁻]_{ex} = 10^{pH_{RBC}−pH_{ex}}. The AE1 route involves exchange of NO₂⁻ for HCO₃⁻. Intracellular HCO₃⁻ is quickly re-formed via CO₂ hydration catalyzed by carbonic anhydrase (CA), and the Jacobs-Stewart cycle establishes its equilibrium distribution. Following the rapid equilibration of nitrite across the membrane, nitrite starts to react with intracellular hemoglobin, and the consumed intracellular nitrite is promptly replenished by new nitrite diffusing in from the extracellular space. In this phase, nitrite uptake
rates are determined by the reaction rates. Increased nitrite uptake rates with decreased oxygen saturation or decreased pH are accordingly a consequence of increased reaction rates between nitrite and hemoglobin.
A Hagfish high [NO$_2$]

B Sea lamprey high [NO$_2$]

C Carp high [NO$_2$]

D Rabbit high [NO$_2$]

E Carp low [NO$_2$]

F Rabbit low [NO$_2$]
Initial rate of NO₂ consumption (mmol l⁻¹ RBC min⁻¹)

**A**
- Carp high [NO₂⁻]

**B**
- Carp low [NO₂⁻]

Oxygen saturation (%)
A) Rabbit low [NO₂⁻]

B) Initial rate of NO₂⁻ consumption (mmol l⁻¹ RBC min⁻¹)

Initial rate of NO₂⁻ consumption (mmol l⁻¹ RBC min⁻¹)

Oxygen saturation (%)  

7.0  7.2  7.4  7.6  7.8  8.0

Rabbit low [NO₂⁻]
\[\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3 \rightarrow \text{HCO}_3^- + \text{H}^+ \]

\[\text{NO}_2^- + \text{H}^+ \rightarrow \text{NO}_3^- \]

\[\text{HNO}_2 \rightarrow \text{NO} + \text{metHb} \]

\[\text{deoxyHb} \rightarrow \text{oxylHb} \]

\[\text{NO} + \text{metHb} \rightarrow \text{NO}_2^- + \text{metHb} \]
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