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The zebrafish Cytochrome b_5 /Cytochrome b_5 reductase/NADH system efficiently reduces Cytoglobins 1 and 2 – Conserved activity of Cytochrome b_5 /Cytochrome b_5 reductases during vertebrate evolution.

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Abstract

Cytoglobin is a heme protein evolutionarily related to hemoglobin and myoglobin. Cytoglobin is expressed ubiquitously in mammalian tissues; however its physiological functions are yet unclear. Phylogenetic analyses indicate that the cytoglobin gene is highly conserved in vertebrate clades, from fish to reptiles, amphibians, birds, and mammals. Most proposed roles for cytoglobin require the maintenance of a pool of reduced cytoglobin (Fe^{II}). We have shown previously that the human cytochrome b_5 / cytochrome b_5 reductase system, considered a quintessential hemoglobin/myoglobin reductant, can reduce human and zebrafish cytoglobins up to 250-fold faster than human hemoglobin or myoglobin. It was unclear whether this reduction of zebrafish cytoglobins by mammalian proteins indicates a conserved pathway through vertebrate evolution. Here we

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SEQUENCE INFORMATION

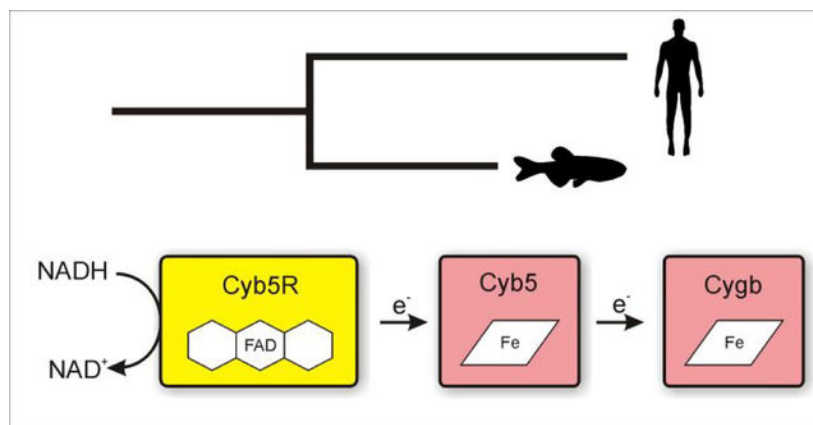
Zebrafish Cytoglobin 1, Uniprot KB Q8UUR3; zebrafish Cytoglobin 2, Uniprot KB Q575S8; zebrafish Cytochrome b_5a , Uniprot KB Q7T341; zebrafish Cytochrome b_5b , Uniprot KB Q6NY41; zebrafish Cytochrome b_5 reductase 3, Uniprot KB Q6NYE6.

CONFLICT OF INTEREST STATEMENT

A.W.D., J.J.R., M.T.G. and J.T. are coinventors of provisional and pending patents for the use of recombinant cytoglobin, neuroglobin and other heme-based molecules as antidotes for carbon monoxide poisoning. Globin Solutions, Inc. has licensed this technology. J.J.R., M.T.G. and J.T. are shareholders and officers in Globin Solutions, Inc. J.J.R. is an officer and director of Globin Solutions, Inc. M.T.G. is a director and advisor of Globin Solutions, Inc. J.T. is an officer of Globin Solutions, Inc. The remaining authors have disclosed that they do not have any potential conflicts of interest.

report the reduction of zebrafish cytoglobins 1 and 2 by the zebrafish cytochrome *b*₅ reductase and the two zebrafish cytochrome *b*₅ isoforms. In addition, the reducing system also supports reduction of Globin X, a conserved globin in fish and amphibians. Indeed, the zebrafish reducing system can maintain a fully reduced pool for both cytoglobins, and both cytochrome *b*₅ isoforms can support this process. We determined the P₅₀ for oxygen being 0.5 torr for cytoglobin-1 and 4.4 torr for cytoglobin-2 at 25 °C. Thus, even at low oxygen tension, the reduced cytoglobins may exist in a predominant oxygen-bound form. In these conditions, the cytochrome *b*₅/cytochrome *b*₅ reductase system can support a conserved role for cytoglobins through evolution, providing electrons for redox signaling reactions such as nitric oxide dioxygenation, nitrite reduction or phospholipid oxidation.

Graphical Abstract



Keywords

Cytoglobin; Cytochrome b5; Cytochrome b5 reductase; nitric oxide; nitric oxide dioxygenation

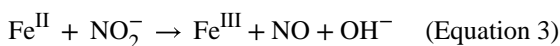
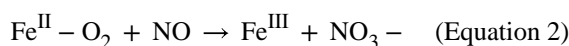
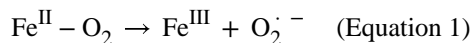
Introduction

Vertebrate organisms have retained at least eight globin genes in their genome,¹ with five globin genes identified in mammals to date.² These proteins are usually associated with oxygen transport functions, following the roles of the most conspicuous hemoglobin (Hb) and myoglobin (Mb). However, newly discovered globins have been associated with functions that defy this paradigm. Neuroglobin, androglobin and cytoglobin (Cygb) show cellular levels nowhere near the high concentrations of hemoglobin and myoglobin and appear to be involved in other processes not necessarily related to oxygen transport and storage. Proposed functions include the detoxification of reactive oxygen species, regulation of nitric oxide (NO) levels, signaling reactions and lipid peroxidation.^{3–8}

Cygb is a heme protein ubiquitously expressed in mammalian tissues.^{9–11} Unlike hemoglobin and myoglobin, but similar to neuroglobin and androglobin, the heme in Cygb is coordinated by two histidine side chains, resulting in a six-coordinate conformation. The presence of a distal ligand to the heme iron causes important changes in heme properties.

4–6, 11–14 Mammals carry one copy of the *Cygb* gene, however two copies of the gene have been identified in teleost fish (*Cygb1* and *Cygb2*), probably arising from an ancient whole genome duplication event.^{15, 16} Studies in zebrafish indicate that both genes are expressed.¹⁷ The in vitro characterization of the two proteins suggests that *Cygb1* may be involved in oxygen transport, whereas *Cygb2* has biophysical properties more alike the mammalian *Cygb* protein.^{17, 18}

As for other globins, the *Cygb* reactivity is largely dependent on the oxidation state of its heme group. As most biological reactions will oxidize the heme iron to the ferric (Fe^{III}) form, any catalytic cycle requires a source of electrons to reduce *Cygb* (equations 1–3).



To that purpose, it appears that ascorbic acid can fulfill this role in certain conditions.¹⁹ New data suggests that the cytochrome *b*₅ (CYB5) and cytochrome *b*₅ reductase 3 (CYB5R) system is a feasible candidate for the physiological reduction of *Cygb*.^{20, 21} To our surprise, we observed that the human CYB5/CYB5R system catalyzes human and zebrafish *Cygb* reduction at rates up to 250-fold higher than those for their *bona fide* substrates, Hb and Mb.²⁰

Globin X (GbX) is an ancestral globin protein recently discovered in fish and amphibians.²² The function of this globin is yet unknown. It has been noted that it can be lipidated and associated with membranes²³ and it is also present in fish thrombocytes (the equivalent to mammalian erythrocytes), where it may be involved in oxygen or nitric oxide related functions.²⁴ In our previous work we have observed that human CYB5b/CYB5R has some ability to reduce GbX heme, whereas 5mM Ascorbate shows almost no effect.²⁰

One caveat in our previous study is that we used human CYB5/CYB5R proteins to monitor the reduction of the zebrafish *Cygb*s and GbX.²⁰ In order to establish unequivocally whether this reaction is functional in zebrafish, a complete system of zebrafish proteins should be used. Moreover, we only tested the ability of human CYB5b to support *Cygb* and GbX reduction. As the properties of the CYB5a and CYB5b isoforms may differ notably,^{25, 26} and two CYB5 isoforms are present in zebrafish, as in mammalian genomes, the ability of both proteins to reduce *Cygb* and GbX may differ and hint towards tissue- or cell-specific capabilities.

Homologous genes for the 4 CYB5R isoforms found in mammals have been identified in the zebrafish genome. It has been reported that a membrane-bound CYB5R protein can reduce fish Hb and maintain proper Hb reduction levels.^{27, 28} The NADH diaphorase activity of fish red blood cell (RBC) extracts, a surrogate for CYB5R activity, correlates with the resistance of fish to methemoglobinemia-inducing conditions.^{27, 28} Although the presence of a CYB5R3 homolog – the isoform found in mammalian RBCs and mitochondria – in fish has been described,^{29, 30} and the protein shows $\approx 65\%$ identity with the characterized mammalian enzymes, the properties of the fish CYB5Rs are unknown. Thus we also characterized the properties of the zebrafish CYB5R3 protein.

Here we study the ability of the reconstituted zebrafish CYB5/CYB5R system to reduce zebrafish Cygbs and GbX. Our results indicate that these zebrafish proteins provide a viable system for the reduction of the Cygbs, with both CYB5 proteins supporting the fast reduction of Cygb1 and Cygb2 at physiological temperatures. The complete zebrafish electron transfer system also catalyzes the reduction of GbX faster than reported for the human CYB5/CYB5R system or ascorbate, and may also provide a physiological source of electrons for GbX in vivo. These observations suggest a conserved role of CYB5/CYB5R supporting Cygb function that possibly predates its role as hemoglobin and myoglobin reductases.

Materials and Methods

Reagents –

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Expression and Purification of Recombinant Globins –

Zebrafish GbX, Cygb1 and Cygb2 were cloned into the pET-28 plasmid (Novagen) and expressed in *E. coli* cells and purified as previously described.^{20, 24} The cDNA clones for zebrafish CYB5a, CYB5b and CYB5R (cytochrome *b*₅ reductase 3) were obtained from the IMAGE consortium through Thermo Fisher/Open Biosystems. The accession numbers and IMAGE identifiers are as follows: CYB5R, BC_066624 (IMAGE 6960174); CYB5a, BC_154824 (IMAGE 9002123); CYB5b, BC_066748 (IMAGE 6525311). CYB5R was cloned into the pET28a plasmid (Novagen) using the NdeI/HindIII restriction sites. The residues 1–20 necessary for membrane insertion were removed, and the 21–298 sequence, coding the soluble protein, was cloned (Figure 1). The protein sequence includes an N-terminal 6xHis tag. CYB5a and CYB5b were cloned into pET11a plasmid using the NdeI and NcoI restriction sites. The C-terminal residues 101–137 of CYB5a that form a membrane insertion helix were removed. For CYB5b, the N-terminal residues 1–17 and the C-terminal residues 115–153 were removed to produce the soluble protein as reported for mammalian proteins (Figure 1).^{25, 26} Purification of zebrafish CYB5 proteins and CYB5R was carried out as described for the human proteins.^{20, 31} Spectral data were recorded using either a Cary 50 spectrophotometer (Agilent) or an Agilent HP8453 diode array spectrophotometer. Proteins were studied in 100 mM sodium phosphate buffer, pH 7.4.

Sequence analysis –

CYB5 sequences were analyzed for possible posttranslational modifications using publicly available software. Acetylation motifs were scanned using Terminus³² and NetAcet³³ servers. Palmitoylation sequences were screened using the CSS-Palm 4.0 software.³⁴

Steady-state kinetics of CYB5R with CYB5a and CYB5b –

Steady state parameters for the reaction of CYB5R with NADH and CYB5a/CYB5b were determined as follows: NADH diaphorase activity was assayed using dichlorophenol-indophenol (DCPIP) or potassium ferricyanide as electron acceptors. In the experiments with DCPIP, zebrafish CYB5R (0.028 μM) was incubated with 95 μM DCPIP and the reaction was initiated by adding variable amounts of a 1 mM NADH stock solution for final NADH concentrations between 0 and 22 μM . The reduction of DCPIP was monitored at 600 nm. In the experiments with potassium ferricyanide, zebrafish CYB5R (0.84 nM) was incubated with 1 mM potassium ferricyanide and the reaction was initiated by adding variable amounts of a 1 mM NADH stock solution for final NADH concentrations between 0 and 50 μM . The reduction of potassium ferricyanide was monitored via the decay of NADH absorbance at 340 nm. The initial reduction rates were plotted versus the concentration of NADH and the data was fitted to the Michaelis-Menten equation to determine the k_{cat} and K_{M} parameters. To determine the apparent K_{M} for CYB5a and CYB5b, the reaction samples included CYB5R (7.3 nM), NADH (60 μM) and variable amounts of CYB5a or CYB5b (0 to 140 μM). Reduction of CYB5a or CYB5b was monitored at 555 nm. The initial rates were fitted as a function of the initial CYB5 concentrations and the data was fitted to the Michaelis-Menten equation to determine the kinetic parameters as above. Experiments were carried out in 50 mM Bis-Tris propane buffer, pH 7.4, at 25 °C.

Reduction of Cyoglobins and Globin X by CYB5/CYB5R –

Steady-state reduction of zebrafish Cygbs and GbX by the CYB5/CYB5R was studied as previously reported.²⁰ The reduction was performed in anaerobic conditions and monitored in an Agilent HP8453 spectrophotometer housed in an anaerobic glovebox (Coy Laboratories, Grass Lake, MI). Oxidized globins were prepared by treatment with excess potassium ferricyanide and then the excess ferricyanide was removed by passing the sample through a Sephadex G25 column (PD10, GE healthcare). The globin solution was diluted to a 20 μM final concentration and zebrafish CYB5R (0.2 μM) and either CYB5a or CYB5b (2 μM) was added. The reaction was initiated by addition of 100 μM NADH. The fraction reduced was determined by monitoring the absorbance changes in the characteristic peak for the ferrous form of the globins (\approx 560 nm) as reported.²⁰

Redox potentials –

Oxidation-reduction potentials for the zebrafish CYB5R and CYB5 proteins were determined in anaerobic conditions at 25 °C in 100 mM sodium phosphate buffer, pH 7.0. Spectral data were recorded using an Agilent HP8453 spectrophotometer and redox potential was collected via an Accumet 15 connected to a MI-480 electrode (Microelectrodes, Inc). Proteins (\approx 10 μM) were titrated with either sodium dithionite (reductive titrations) or

potassium ferricyanide (oxidative titrations) in the presence of redox mediators. The redox mediators (1–5 μM) used were phenosafranine ($E_m = -252$ mV) for CYB5R. In the case of CYB5a and CYB5b a modified version of the method described by Efimov et al³⁵, using sodium dithionite as reductant instead of the xanthine/xanthine oxidase system and an anaerobic setup that circumvents the use of the glucose/glucose oxidase system to remove oxygen. Indigo tetrasulfonate ($E_m = -46$ mV) was used as mediator. Spectral and redox potential readings were analyzed using the Nernst equation to calculate the midpoint potentials as described.^{13, 17, 35}

Determination of melting temperatures –

Thermal denaturation of CYB5a and CYB5b proteins in the ferric heme state was monitored by UV-Vis spectroscopy using a Cary50 spectrophotometer. Experiments were carried out in 10 mM phosphate buffered saline, pH 7.4. The temperature was increased from 20 °C to 100 °C; for each step the temperature was maintained for ~5 minutes and the spectral changes were then recorded. Changes in the Soret peak were fitted to the Santoro-Bolen equation³⁶ to determine the melting temperature (T_m) of each protein.

Determination of oxygen binding affinities –

In order to determine the oxygen partial pressures at half-saturation of the ferrous-oxygen complex (P_{50}) of Cygb1 and Cygb2, we measured oxygen equilibrium curves using a thin-layer modified diffusion chamber described elsewhere^{37–39} with some modifications.¹⁴ Samples (5 μl) contained 200 μM heme Cygb1 or Cygb2. As a reducing system, 40 μM human CYB5b, 4 μM human CYB5R and 600 μM NADH were added. 20 mM sodium formate and 10 mU of formate dehydrogenase were added to regenerate the consumed NADH. Samples were equilibrated for 5 minutes at room temperature under N_2 atmosphere to allow heme reduction before transfer to the modified diffusion chamber. Experiments were conducted at either 25°C or 37°C in 100 mM potassium phosphate buffer at pH 6.8 or 7.4. Fit of the oxygen saturation data to the Hill equation also provided the Hill coefficient measuring the degree of cooperativity in oxygen binding.

Results

Protein expression and spectral properties –

Zebrafish CYB5R was expressed in soluble form by removing the N-terminus sequence (amino acids 1–20, Figure 1A) to prevent membrane binding. In mammalian CYB5R, Gly2 has been found to be myristoylated to direct the protein to mitochondria and other membrane fractions; the initial fragment (amino acids 1–23 in mammalian proteins) is not present in the soluble isoform expressed in mammalian erythrocytes.⁴⁰ The sequence of the zebrafish CYB5R lacks the glycine in position 2. Nevertheless, the Protein in fish erythrocytes appears to be membrane-bound²⁸. Although the post translational modification responsible has not been identified, sequence analysis software predicts a possible acetylation site in Ser2 (Terminus³², NetAcet³³) and palmitoylation of Cys12 (CSS-Palm³⁴). Zebrafish CYB5a (microsomal isoform) was expressed in its soluble form without its C-terminal membrane binding-domain (amino acids 101–137, Figure 1B). Zebrafish CYB5b (mitochondrial outer membrane isoform) was also expressed in a soluble form, omitting its

N-terminal amino acids (amino acids 1–17, Figure 1B) and C-terminal transmembrane domain (amino acids 115–153, Figure 1B).

The zebrafish CYB5R and cytochromes CYB5a and CYB5b were expressed in *E. coli* and purified following similar protocols to those used previously for the mammalian proteins.^{20, 31} We did not observe notable differences in the expression and purification as compared to the human proteins. The three proteins show spectral properties similar to their mammalian counterparts.^{25, 41} Spectra for the fully oxidized and fully reduced species are shown in Figure 2. Oxidized CYB5R shows the characteristic peaks of the FAD cofactor, with maxima at 388 nm and 460 nm (Figure 2A). CYB5a and CYB5b showed similar wavelengths for their Soret peaks in the oxidized state (413 nm) and an additional peak at 531 nm for CYB5a or 528 nm for CYB5b, consistent with the spectra of other six-coordinated heme proteins in the ferric form. CYB5a and CYB5b in the reduced state show spectra characteristic of a bis-His, six-coordinate heme, with two peaks around 520 nm and 560 nm. The observed maxima were 423 nm, 527 nm and 555 nm (CYB5a) and 423 nm, 527 nm and 556 nm (CYB5b) (Figures 2B, 2C).

Steady-state kinetics –

In order to characterize the ability of CYB5R to oxidize NADH and catalyze electron transfer reactions to its electron transfer partner proteins CYB5a and CYB5b, we studied the kinetics of CYB5R with different electron acceptors in steady-state conditions.

We determined the K_M of zebrafish CYB5R for NADH in the presence of DCPIP and ferricyanide as electron acceptors. The observed rates for the reaction of CYB5R with DCPIP are shown in Figure 3A. The values were fitted to a Michaelis-Menten equation yielding a k_{cat} of 26 s^{-1} and a K_M of $0.6 \text{ }\mu\text{M}$ (Figure 3A, Table 1). This activity is comparable to that reported for the other CYB5R proteins (Table 1). When potassium ferricyanide was used as electron acceptor, we observed rates also consistent with a Michaelis-Menten fit. Calculated values were k_{cat} of 235 s^{-1} and a K_M of $3.7 \text{ }\mu\text{M}$ (Figure 3B, Table 1).

The k_{cat} values are comparable to those of the fly CYB5R (Table 1); although 3–4-fold lower than those of mammalian enzymes ($\sim 600\text{--}800 \text{ s}^{-1}$, Table 1). The K_M value is comparable to the reported K_M values for other CYB5R enzymes ($\sim 0.6\text{--}6.0 \text{ }\mu\text{M}$, Table 1) indicating a high affinity towards NADH. We then determined the activity of CYB5R with its partner proteins CYB5a and CYB5b. These experiments were conducted in saturating concentrations of NADH ($60 \text{ }\mu\text{M}$).

Experiments on the absence of CYB5R showed that NADH can also reduce directly CYB5a and CYB5b at a significant rate; background activity in the absence of CYB5R was subtracted from the rates in the presence of CYB5R to determine reduction rate due to CYB5R (Figure 3B,C). The fit of the initial rates yielded values of $k_{cat} = 165 \text{ s}^{-1}$ and $K_M = 36 \text{ }\mu\text{M}$ for CYB5a and $k_{cat} = 121 \text{ s}^{-1}$ $K_M = 40 \text{ }\mu\text{M}$ for CYB5b (Table 1). Thus, CYB5R reduces both substrates with very similar kinetic parameters, though at slightly faster rates with CYB5a. The K_M values are higher than the expected physiological CYB5

concentrations, suggesting a linear dependence of the reduction rates with CYB5 concentrations *in vivo*.

Redox potentials –

In order to further characterize the properties of the zebrafish CYB5R/CYB5 system, we determined the redox potential of the three zebrafish proteins (Figure 4). The titration of CYB5R shows a single transition, fitted to a slope of ~29.5 mV, consistent with a two-electron step (Figure 1A). Similar observations have been reported for other CYB5R proteins, where the one-electron reduced, semiquinone state is not stable and does not accumulate during the reduction step.^{43, 47–49} Thus, the two one-electron transitions show a very similar midpoint potential and the two steps are merged in a single apparent E_m value. We observe a midpoint potential of 264 mV, in the range of the values reported for mammalian proteins (Table 2).

Previous work has indicated notable differences in the redox potential of CYB5a and CYB5b in mammalian systems. Reported CYB5a midpoint potential values are around 0 mV, whereas CYB5b proteins show more negative values. In particular, human and rat proteins show values of –40 and –102 mV, respectively (Table 2).^{25, 26} We observe values in a similar range for the zebrafish proteins (Table 1). The potential for CYB5a is –28 mV, slightly more negative than the value observed for mammalian proteins. In the case of CYB5b, the observed value of –62 mV is between the observed values for rat and human CYB5b proteins. Notably, the observed values for the zebrafish Cygb1 and Cygb2, –58 mV and –26 mV respectively¹⁷, are in a similar range to those of the zebrafish CYB5 proteins (Table 2).

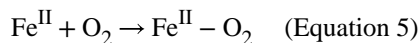
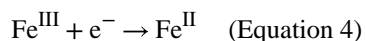
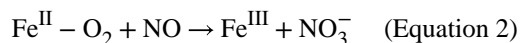
Thermal denaturation of zebrafish cytochromes b_{5a} and b_{5b} –

As noted above we observe a 35 mV difference in redox midpoint values between zebrafish CYB5a and CYB5b. This is not unlike the situation for mammalian CYB5 proteins, where differences of up to 95 mV have been reported^{52–54}. Given the high identity and similarity of the CYB5 sequences, the source of these differences is not evident (Figure 1). The changes within CYB5 sequences are limited to structural elements away from the heme binding regions, with nearly 100% identity in the regions close to the heme within CYB5a or CYB5b sequences, and limited changes between the two CYB5s. Given the high variability between mammalian CYB5a and CYB5b redox potentials (Table 3), it has been speculated that the redox potential is in part modulated by the strength of the bond between the Fe^{III}-heme and the proximal histidine.²⁵ Although the heme exposure to the solvent is one of the main contributors to the regulation on the heme redox potential in proteins,⁵⁵ the differences in heme binding in the ferrous and ferric states can also cause large variations in the redox potential, even in conditions where the heme solvent accessibility is unchanged.⁵⁶ As a proxy for the Fe^{III}-His bond strength we studied the thermal denaturation of the zebrafish CYB5a and CYB5b proteins. Our experimental determinations of the melting temperatures (T_m) are shown in Figure 5 and Table 3. Our results indicate that the two zebrafish proteins have similar T_m values (Table 3). The value for CYB5a is in line with mammalian CYB5a proteins, however the T_m for zebrafish CYB5b appears much lower than the reported values for mammalian CYB5b (Table 3). The spectral changes also indicate

clear differences between the thermal denaturation processes for CYB5a and CYB5b. The denaturation of CYB5a shows a shift from the spectra of the bis-His hexacoordinated globin towards a spectrum similar to that of free heme, with a Soret peak around 385 nm (Figure 5A). A similar increase in the 350–400nm area, indicating heme dissociation, is observed for CYB5b, but in this case the heme release correlates with visible light scattering as noted by the increase of the signal at 700nm and throughout the spectral range (Figure 5B). Without further structural data, the nature of the changes cannot be unequivocally assigned, but we can speculate that the denaturation of the zebrafish CYB5a leads to the formation of a stable apoprotein, whereas the zebrafish CYB5b aggregates when the heme is dissociated from the protein, suggesting a less stable apoprotein in our experimental conditions.

Oxygen affinity of zebrafish cytoglobins –

We have shown that zebrafish Cygb1 and Cygb2 can be reduced efficiently by the mammalian CYB5R/CYB5/NADH system.²⁰ This allows for a catalytic cycle of NO dioxygenation with continuous regeneration of the deoxy (Fe^{II}) species, and in the presence of oxygen, the subsequent fast formation of the oxygen-bound species (Fe^{II}-O₂), as shown in the following equations:



Where equation 4 indicates the regeneration of the Cygb deoxy species by reaction with the pool of reduced CYB5 and the net sum of the three reactions yields the catalytic dioxygenation of NO (Equation 6).

In order for zebrafish Cygbs to support NO dioxygenation reactions, the reduced form of the protein should be able to bind oxygen in physiological conditions, and therefore it is important to determine the oxygen affinity of these Cygbs. We used a met-reducing enzymatic system⁵⁷ to determine P₅₀ values for Cygb1 and Cygb2. We have used a similar system in the determination of the P₅₀ for other heme proteins, including several mutant Cygbs and GbX.^{14,23} The system uses ferredoxin NADP⁺-reductase, ferredoxin and NADPH to regenerate the pool of globin oxidized due to endogenous autoxidation. However this system was unable to maintain zebrafish Cygb2 in the ferrous reduced state due to its

very fast autoxidation rate.¹⁷ As we previously observed the efficient reduction by the human CYB5R/CYB5 system,²⁰ we used these proteins to keep the zebrafish Cygbs in the reduced state. Formate and formate dehydrogenase were included to regenerate the NADH consumed.

Our experimental results for the determination of the P₅₀ values at pH 6.8 and 7.4 at either 25 °C or 37 °C are shown in Figure 6 and Table 4. We observe that Cygb1 has a higher oxygen affinity than Cygb2, with values between 0.4–2.5 torr for Cygb1 compared to 3.6–6.1 torr for Cygb 2. The P₅₀ values for Cygb2 appear higher than those reported for mammalian Cygbs at pH 7.0 (0.2–2.8 torr)^{11, 14, 58, 59} which is unexpected given that most properties of Cygb2 are otherwise more similar to the mammalian protein than Cygb1.¹⁷ On the other hand, Cygb1 shows very high affinity towards oxygen with similar or lower P₅₀ values than those of the mammalian Cygbs. Comparing the data at different pH values, neither Cygb shows a significant Bohr effect, in agreement with previous data on mammalian Cygbs.¹⁴

Analysis of the Hill coefficients (*n* values in Table 4) indicates that both zebrafish proteins bind oxygen cooperatively. In particular, Cygb2 has very high *n* values (3.0–4.3), as also evident from the highly sigmoidal oxygen equilibrium curves (Fig. 6), suggesting formation of complexes larger than tetramers. It is difficult to speculate further from these data on the nature of these complexes. It has been shown that oligomerization of mammalian Cygbs, with formation of tetramers and octamers, may occur at high concentrations, probably at supraphysiological concentrations.⁵⁹ It is thus unclear if the oligomers observed in our study are physiologically relevant or are due to the high protein concentrations used and do not necessarily reflect the cellular conditions. Zebrafish GbX binds O₂ cooperatively with a overall lower affinity (P₅₀ 1.3–12.5 torr at 20 °C)²³ compared to zebrafish Cygbs, and thus may not be significantly oxygenated in vivo.

Reduction of zebrafish cytoglobins by the native zebrafish Cytochrome b₅ reductase/ Cytochrome b₅ systems–

In previous work we have shown that human cytochrome b₅ reductase, in combination with human CYB5b, can efficiently reduce zebrafish Cygb1 and Cygb2. We were thus interested in evaluating if the native zebrafish systems, using CYB5R in combination with either zebrafish CYB5 isoform, could also reduce the Cygbs, and if so how they perform as compared to the human reducing system.

In order to test the reduction of the Cygbs by CYB5/CYB5R/NADH we monitored the reduction of zebrafish Cygbs 1 or 2 (20 μM) in the presence of zebrafish CYB5a or CYB5b (2 μM, consistent with reported physiological levels,^{60, 61} either zebrafish or human CYB5R (0.2 μM), and 100 μM NADH to ensure CYB5R saturating conditions.

Our results are shown in Figure 7. As in the case of the human Cygb in the presence of human CYB5/CYB5R,²⁰ the zebrafish CYB5/CYB5R system is able to completely reduce Cygb1 or Cygb2 in around 100 s. Both CYB5 isoforms can support fast Cygb1 or Cygb2 reduction, with the CYB5b performing slightly faster than CYB5a (Figure 7). Increasing the temperature from 25 °C to 37 °C shows a modest effect on the reduction rates. In order to

compare the reduction of zebrafish Cygb by the zebrafish CYB5/CYB5R system, traces for previous experiments using the human CYB5b/CYB5R are included (Figure 7 D–E).²⁰ In the case of Cygb1, the human proteins can reduce the zebrafish Cygb1 at a comparable rate (Figure 7D). However in the case of Cygb2, the zebrafish proteins clearly outperform the human CYB5/CYB5R system, and can support full Cygb2 reduction in ≈ 50 s as compared to ≈ 200 s for complete reduction with the human proteins (Figure 7E).

We have previously hypothesized that GbX could be reduced by the CYB5/CYB5R system in zebrafish.²⁰ The traces for the reaction of the zebrafish CYB5/CYB5R system with GbX are shown in Figure 7 (Panels 7C and 7F). Our results indicate that this reduction system, although not as efficient as for Cygb, can reduce GbX at an appreciable rate. In particular CYB5b is efficient in this reduction even at 25 °C-consistent with zebrafish physiological conditions-catalyzing GbX reduction at a faster rate than the human system at 37 °C (Figure 7F). Thus, we conclude that CYB5/CYB5R can provide a pathway for GbX reduction in zebrafish.

Discussion

Our previous work strongly supported the role of the CYB5/CYB5R/NADH system as physiological reductant of Cygb in human cells.²⁰ It also noted the ability of the human proteins to reduced fish Cygb1 and Cygb2 as well; this observation suggested that this could be a pathway not limited to mammals but conserved in vertebrates.

To test this hypothesis we expressed and purified the components of the CYB5 reducing system from zebrafish in *E. coli*. This is to our knowledge the first characterization of recombinant CYB5R and CYB5 proteins from fish. Of note, the enzymatic activity of CYB5R in fish is particularly relevant to aquaculture, where the accumulation of nitrites in water can cause methemoglobinemia—accumulation of oxidized hemoglobin in the erythrocytes—^{63, 64}. Fish can recycle methemoglobin to its reduced form through several pathways, including reaction with glutathione, ascorbic acid, or NADPH-dependent methemoglobin reductase; however the main contributor is CYB5R. Indeed, the differences in CYB5R activity can partly explain fish species sensitivity to methemoglobinemia.²⁷

The CYB5R/CYB5R proteins in fish showed comparable properties to the mammalian proteins. The main differences were the more negative midpoint potential for zebrafish CYB5a and the lower thermal stability of CYB5b as compared with the more stable mammalian CYB5b paralogues. However there are differences may be partly related to the lower body temperature of the fish and do not appear to indicate large differences in their physiological properties. As the Fe^{III}-His interaction seems to be less strong in CYB5b, contrary to what is expected given its more negative redox potential,⁵⁶ it suggests that other factors such as heme exposure⁵³ may be more relevant to zebrafish CYB5b redox potential. The cause for the reduced stability is yet unknown but we notice that important residues from the hydrophobic core (Ala18, Ile32, Leu36, Leu47; rat CYB5b numbering) identified in rat CYB5s^{25, 26} are not conserved in zebrafish CYB5b; notably, Ala18 and Leu36 correspond to Gly41 and Met59, respectively, in zebrafish CYB5b.

Our experiments of Cygb reduction show that CYB5 and CYB5R are able to reduce Cygb1 and Cygb2 in the reduced state. This reduction is particularly important for Cygb2, as this protein shows autoxidation rates 5-fold faster than human Cygb.¹⁷

A limitation of our previous study²⁰ is the use of the CYB5b isoform, which is mostly present in the mitochondrial outer membrane, thus probably isolated from cytoplasmic cytoglobins. However, the CYB5a isoform is attached to the endoplasmic reticulum, with the heme domain remaining in the cytoplasmic side and thus available for interactions with cytoplasmic molecules.^{65–67} CYB5R3 is located in the mitochondrial outer membrane and also attached to the endoplasmic reticulum⁶⁷ and can thus provide electrons to cytosolic cytoglobins through CYB5a. GbX has been found to associate with the plasma membrane when expressed in mammalian cells²³. In these conditions a cytosolic, soluble CYB5 could provide electrons to GbX. The presence of soluble CYB5 proteins in zebrafish is not established. However, large scale protein expression studies in human tissues indicate that human CYB5a and CYB5b can be found in the cytosol-dependent or independent of membrane binding-^{68, 69}. Thus, we hypothesize that membrane-bound GbX could be reduced *in vivo* by the NADH/CYB5/CYB5R system either via CYB5 soluble isoforms present in zebrafish cells – as will be the case in thrombocytes, where GBX has been detected²⁴, or by localizing to the endoplasmic reticulum membrane, where CYB5a and CYB5R are available.

Mammalian Cygb may play an important role in the regulation of NO levels in the vascular wall.^{19, 21, 70} It is unclear if Cygbs are present in fish blood vessels, but given the important physiological differences between NO metabolism and vascular biology of the fish and mammals, it is conceivable that Cygb in the fish accomplish other unrelated functions. However, we have shown that fish, like mammals, have a very effective Cygb reduction system, suggesting that NO detoxification or other roles requiring the reduced Cygb can be maintained. We observe very low P_{50} for both Cygbs, indicating that these proteins would be largely saturated with oxygen *in vivo* and that their biological roles may specifically involve the ferrous-oxy species, at least for Cygb1. Alternatively, Cygb2 can be involved in O₂ transport but its P_{50} may be optimal for oxygen sensing functions. Experiments in different fish species have shown increases of Cygb1 mRNA transcripts under hypoxia – but usually not Cygb2 – which is consistent with this hypothesis^{71–73}. The deoxy Cygb2 species can be formed in hypoxic tissues, where it could fulfill other functions not related to oxygen binding.

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ABBREVIATIONS

CYB5	cytochrome <i>b</i> ₅
CYB5a	cytochrome <i>b</i> _{5a}
CYB5b	cytochrome <i>b</i> _{5b} CYB5R, cytochrome <i>b</i> ₅ reductase 3
Cygb	cytoglobin
Cygb1	zebrafish cytoglobin-1
Cygb2	zebrafish cytoglobin-2
GbX	Globin X
NADH	nicotinamide adenine dinucleotide hydrate

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A

Cyb5r3 Zebrafish	1	<i>MSFIFNLTGSCQFVLD</i> FRRLFGFKSR PAITLEDPDVKYPLRLV KREI ISHDTRRF 57
Cyb5r3 Human	1	<i>MGAQLSTLGHMVLFPVWF</i> LYSLMKLFQRST PAITLES PD IKYPLRLID REI ISHDTRRF 60
Cyb5r3 Rat	1	<i>MGAQLSTLSRVVLSFVWF</i> YSLFMKLFQRSS PAITLEN PD IKYPLRLID KREI ISHDTRRF 60
Cyb5r3 Zebrafish	58	RFAL KSP Q HL L GL P T G QH I Y L S A K I D G N L V V R P Y T F V S S D D D K G F V D L V K I Y K N I H P K 117
Cyb5r3 Human	61	RFAL PS Q HL L GL P T G QH I Y L S A R I D G N L V V R P Y T F V S S D D D K G F V D L V K I Y K D T H P K 120
Cyb5r3 Rat	61	RFAL PS Q HL L GL P T G QH I Y L S T R I D G N L V V R P Y T F V S S D D D K G F V D L V K I Y K D T H P K 120
Cyb5r3 Zebrafish	118	F P A G G K M S Q Y L E S L R I G D T I D F R G P S G L L V Y K G K G S F A I R P D K K S D A V I K T A K H V G M I A G 177
Cyb5r3 Human	121	F P A G G K M S Q Y L E S M O I G D T I E F R G P S G L L V Y Q G K G F A I R P D K K S N P I I R T V K S V G M I A G 180
Cyb5r3 Rat	121	F P A G G K M S Q Y L E N M N I G D T I E F R G P N G L L V Y Q G K G F A I R P D K K S N P V V R T V K S V G M I A G 180
Cyb5r3 Zebrafish	178	G T G I T P M L Q V I R A V L K D P D H D H V C H L L F A N C T E K D I L L R P E L E L I M A N H P T R F K L W Y T V D 237
Cyb5r3 Human	181	G T G I T P M L Q V I R A V L K D P D H D H V C H L L F A N C T E K D I L L R P E L E L R N K H S A R F K L W Y T L D 240
Cyb5r3 Rat	181	G T G I T P M L Q V I R A V L K D P D H D H V C H L L F A N C S E K D I L L R P E L E L R N E H S S R F K L W Y T V D 240
Cyb5r3 Zebrafish	238	R A P D G M E Y S Q G F I N E D M V R K H L F S E G N E T L V L M C G P P P M I Q F A C N P S L D K V G H S N D R R F M F 298
Cyb5r3 Human	241	R A P E A D W Y G Q G F V N E M I R D H L P P E E E P L V L M C G P P P M I Q M A C L P N L D H V G H P T E R C F V F 301
Cyb5r3 Rat	241	K A P D A D W Y S Q G F V N E M I R D H L P P E E E T L L M C G P P P M I Q F A C P N L E R V G H P E K E R C F T F 301

B

Cb5a Zebrafish	1	<i>M</i> ANN G T D G N G V K Y R L S E V E E R N S F R S T W L I I H N K V Y D V T K F L E 44
Cb5a Human	1	<i>M</i> AE Q S D E A V K Y T L E I T Q K H N H S K S T W L I L H H K V D L T K F L E 42
Cb5a Rat	1	<i>M</i> AE Q S D K D V K Y T L E I T Q H K D S R S T W I L H H K V D L T K F L E 42
Cb5b Zebrafish	1	<i>M</i> G E E T G S S V G R E N Q N T K N E S T Q E D S G V K Y Y T R K E V Q V H N M G R D T W L I I H D K V Y D I T S E M E 60
Cb5b Human	1	<i>M</i> A T A E A S G S D G K Q E V E T S V T Y R L E V A K R N S L K E L N L V I H G R V Y D V T R F L N 53
Cb5b Rat	1	<i>M</i> A T P E A S G S G R N Q G S D P A V T Y R L E V A K R N T A E T W M V I H G R V Y D I T R F L S 53
Cb5a Zebrafish	45	E PG G E V L R EQ A G G D A T E S F D V G S T D A R E M A S S M L I G E V H P D R D K I A K P E S L V T T 104
Cb5a Human	43	E PG G E V L R EQ A G G D A T E N F D V G S T D A R E M S K T F I G E L H P D R P K N K P E T L I T T 102
Cb5a Rat	43	E PG G E V L R EQ A G G D A T E N F D V G S T D A R E L S K T Y I I G E L H P D R S K I A K P S E T L I T T 102
Cb5b Zebrafish	61	E PG G E V L L EQ A G A D A T E S F D V G S T D A R E M L Q Q Y I G E L H M D R R K E S -- K K E V Y I T 118
Cb5b Human	54	E PG G E V L L EQ A G V D A S E S F D V G S S D A R E M L K Q Y Y I G D I H P S D L K P E S -- G S K D P S K 111
Cb5b Rat	54	E PG G E V L L EQ A G A D A T E S F D V G S P D A R E M L K Q Y Y I G D V H P N D L K P K D -- G D K D P S K 111
Cb5a Zebrafish	105	<i>V</i> Q E T T S M A S N L I FAVAVIVTLMYRIYTAEA 137
Cb5a Human	103	<i>I</i> D S S S S M W T N V I F A I S A V A V A L M Y R L Y M A E D 134
Cb5a Rat	103	<i>V</i> E S N S S M W T N V I F A I S A L V V A L M Y R L Y M A E D 134
Cb5b Zebrafish	119	<i>T</i> S K D S R S M S T W F I F A I A V L V G I M Y R Y T L E H K S S 153
Cb5b Human	112	<i>N</i> D T C K S Q W A Y I L F I C A V L L G F L Y R Y T S E S K S S 146
Cb5b Rat	112	<i>N</i> N S C Q S W A Y I V I V G A L L I G F L Y R H F W A D S K S S 146

Transmembrane helix

Figure 1. Alignment of cytochrome b_5 reductase and cytochromes b_5a and b_5b protein sequences for zebrafish, human, and rat.

A; alignment of CYB5R3 sequences (Uniprot accession codes: zebrafish, Q6NYE6; human P00387; rat, P20070). Initial portions in italics indicate the missing residues in the soluble isoform (CYB5R3–2) of the mammalian proteins and the homologous residues in zebrafish protein; the first amino acid in the recombinant protein used in this work and the initial amino acid in the mammalian isoform 2 are marked in bold and yellow background. B; alignment of CYB5a (microsomal CYB5) and CYB5b (outer membrane CYB5) sequences (Uniprot accession codes: zebrafish b_5a , Q7T341; human b_5a , P00167; rat b_5a , P00173; zebrafish b_5b , Q6NY41; human b_5b , O43169; rat b_5b , P04166). Conserved heme-binding histidines are indicated in blue background. Amino acids in italics indicate sequences removed in the N-termini of CYB5b and the membrane binding C-terminal regions of CYB5a and CYB5b. The putative membrane intercalating helix region is shown in a rectangle. The first and last amino acids in the recombinant proteins used in this work, along

with the splicing sites for the soluble isoform of mammalian cytochrome *b*₅a proteins are marked in bold and yellow background; the first and last amino acids in the recombinant proteins used in other works^{25, 26} are indicated in bold with green background.

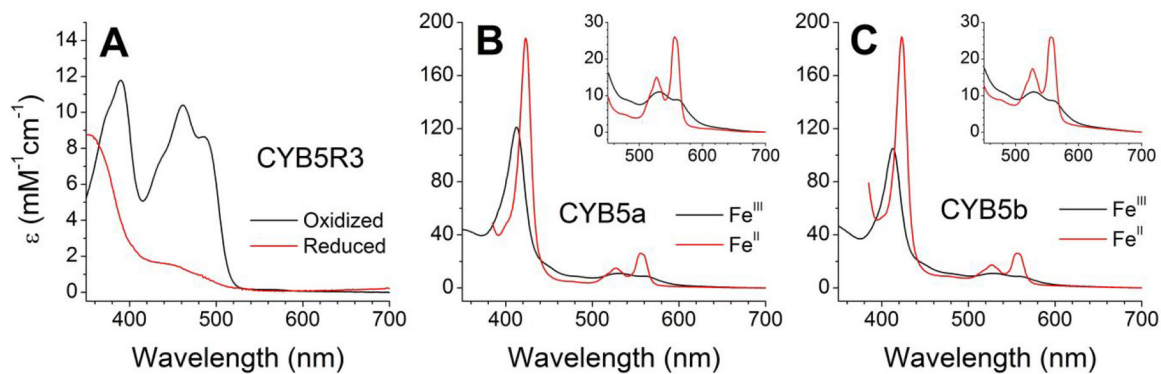


Figure 2. Spectral properties of zebrafish cytochrome b_5 reductase and cytochromes b_{5a} and b_{5b} . Panel A, CYB5R; Panel B, CYB5a; Panel C, CYB5b. The red traces represent the reduced state (FADH_2 for CYB5R or ferrous (Fe^{II}) heme for CYB5a and CYB5b) and the black traces represent the oxidized proteins (FADH_2 for CYB5R or ferric (Fe^{III}) heme for CYB5a and CYB5b). Insets in Panels B and C show the 450–700nm range

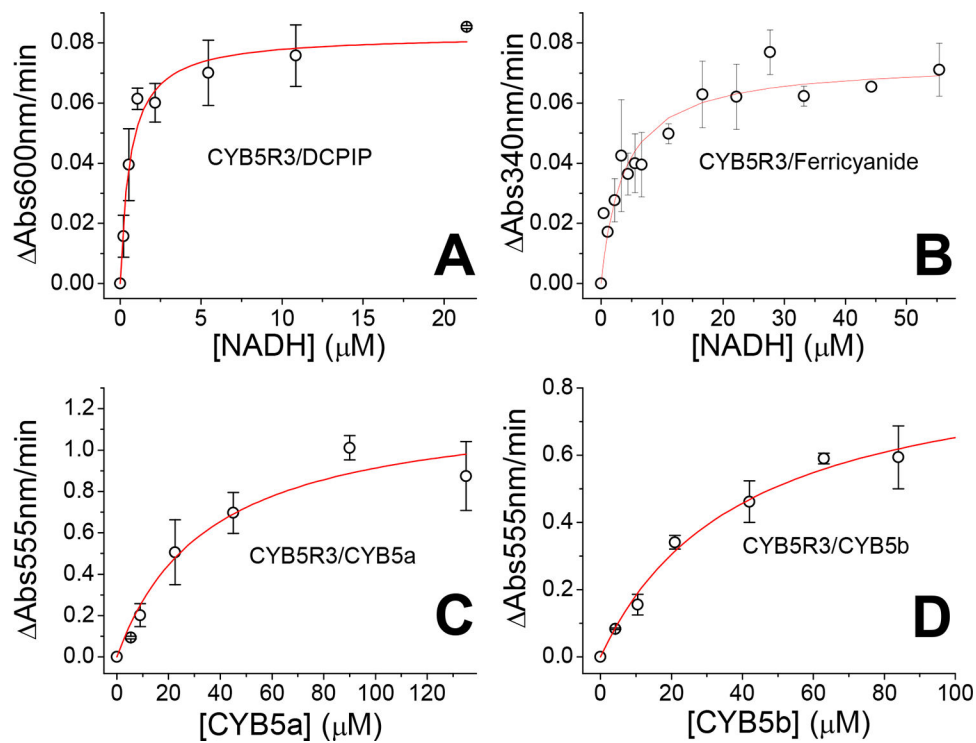


Figure 3. Steady state kinetics of zebrafish Cytochrome b_5 reductase.

Panels A and B, determination of the V_{\max} and K_M towards NADH for the NADH-diaphorase activity of CYB5R with DCPIP (Panel A) or ferricyanide (Panel B) as electron acceptor. Panels C and D, determination of the V_{\max} and K_M towards zebrafish CYB5a (Panel C) or CYB5b (Panel D) in the presence of saturating concentrations of NADH (60 μ M). Experiments conducted in 50 mM Bis-Tris propane, pH 7.4, 25 $^{\circ}$ C.

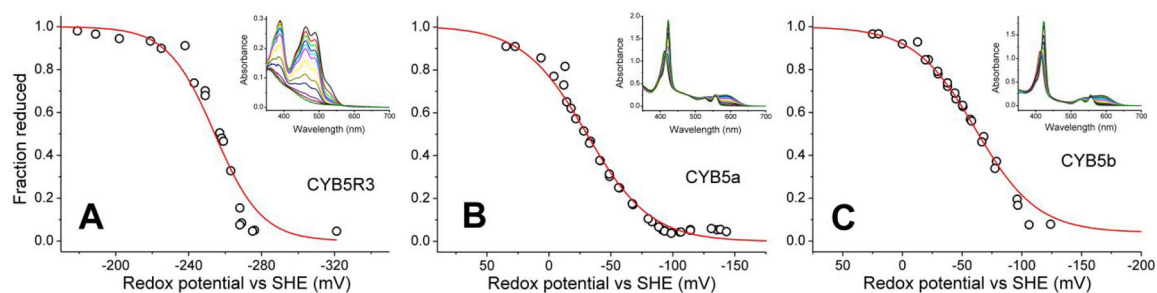


Figure 4. Redox potentials of zebrafish Cytochrome b_5 reductase and cytochromes b_{5a} and b_{5b} . Panel A, CYB5R; Panel B, CYB5a; Panel C, CYB5b. The plots show the fit of the fraction reduced (as determined from absorbance spectra, open circles) to the Nernst equation (solid lines). The insets show the absorbance changes during the reductive titrations. Peaks at 510nm (CYB5R) or 590nm (CYB5a/b) are due to the mediators.

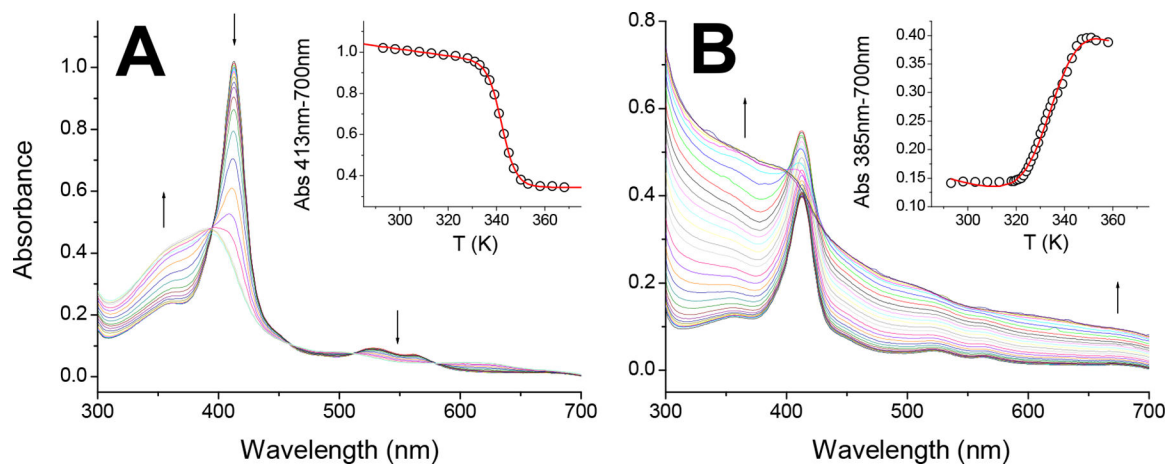


Figure 5. Thermal denaturation of zebrafish Cytochrome *b*_{5a} and cytochrome *b*_{5b}. Panel A, CYB5a; Panel B, CYB5b. The spectra obtained at increasing temperatures (20–95 °C) are shown, the direction of the absorbance changes is indicated by arrows. The insets show the changes in the Soret peak absorbance with the temperature and the fit of the absorbance changes to the Santoro-Bolen equation (red lines).

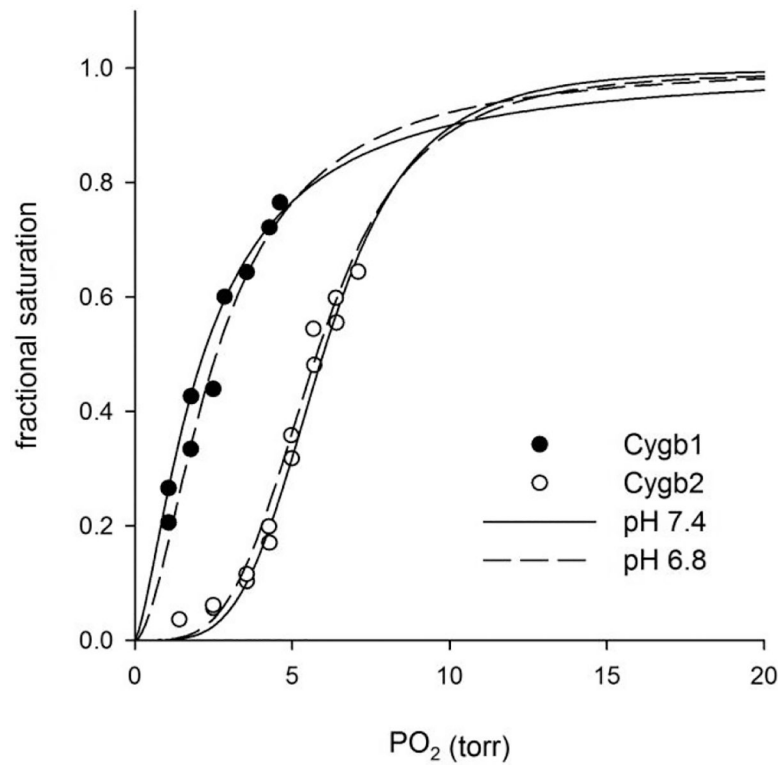


Figure 6. Oxygen equilibrium curves of zebrafish cytoglobins 1 and 2 at 37 °C. The points for Cygb1 (solid circles) and Cygb2 (open circles) are indicated. The lines show the fit to the Hill sigmoidal equation.

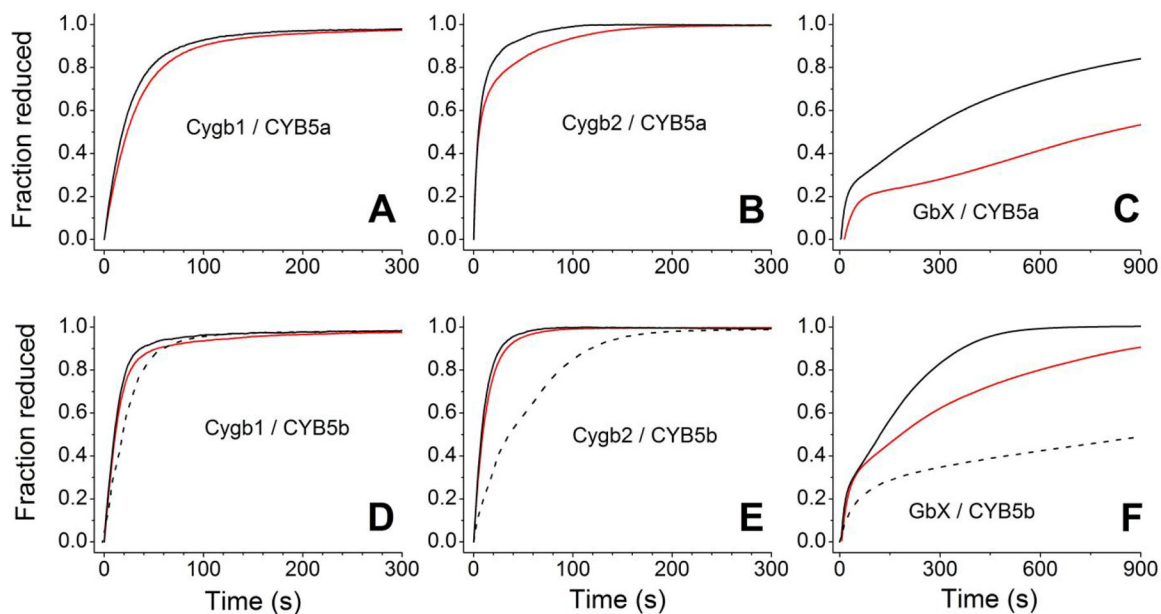


Figure 7. Reduction of zebrafish cytoglobins 1 and 2 and GlobinX by cytochrome b_5 reductase and cytochrome b_5 .

Panel A, Reduction of Cygb1 by zebrafish CYB5a and CYB5R. Panel B, Reduction of Cygb2 by zebrafish CYB5a and CYB5R. Panel C, Reduction of GlobinX by zebrafish CYB5a and CYB5R, Panel D, Reduction of Cygb1 by zebrafish CYB5b and CYB5R (solid lines) or the human CYB5b/CYB5R system (dotted lines). Panel E, Reduction of Cygb2 by zebrafish CYB5b and CYB5R (solid lines) or the human CYB5b/CYB5R system (dotted lines). Panel F, Reduction of GlobinX by zebrafish CYB5b and CYB5R (solid lines) or the human CYB5b/CYB5R system (dotted lines). Red lines, reactions monitored at 25 °C, Black lines, reactions monitored at 37 °C.

Table 1.

Kinetic parameters for the reaction of zebrafish cytochrome *b*₅ reductase and related proteins with DCPIP, ferricyanide, cytochrome *b*₅a, and cytochrome *b*₅b

CYB5R	Diaphorase activity - DCPIP		Diaphorase activity - Ferricyanide		Cytochrome <i>b</i> ₅ reductase activity				Reference
	kcat (s ⁻¹)	k _M NADH (μM)	kcat (s ⁻¹)	k _M NADH (μM)	kcat (s ⁻¹)	K _m CYB5a (μM)	kcat (s ⁻¹)	K _m CYB5a (μM)	
Zebrafish	26 ± 2	0.65 ± 0.12	235 ± 13	3.7 ± 0.9	165 ± 20	36 ± 12	121 ± 11	40 ± 8	This work
Fly	50	<i>nd</i>	332	44	<i>nd</i>	13		<i>nd</i>	42
Rat		<i>nd</i>	800	6.0	400	13		<i>nd</i>	43
Pig		<i>nd</i>	630	2.5	510	8.8		<i>nd</i>	44
Human	21	0.48	827	0.6	<i>nd</i>	35		<i>nd</i>	4546

nd, not determined.

Table 2.

Redox potential of zebrafish Cytochrome *b*₅ reductase, cytochrome *b*_{5a}, cytochrome and *b*_{5b} and related mammalian proteins

Protein	Redox potential vs SHE (mV)	References
CYB5R		
zebrafish	-264 ± 3	This work
dog	-272	47
Pig	-258	48
rat	-268, -272	43, 49
CYB5a		
zebrafish	-28 ± 2	This work
bovine	-2, +5	50, 51
Pig	0, +13	48
rat	-7	52
CYB5b		
zebrafish	-62 ± 2	This work
human	-40	25
rat	-102	53, 54

Table 3.Melting temperatures of zebrafish cytochrome *b*₅a, cytochrome *b*₅b and related mammalian proteins

Protein	T _m (°C)	References
CYB5a		
zebrafish	68.0 ± 1.6	This work
bovine	67.6 ± 0.3	25, 26
	73.1 ± 0.4	26
CYB5b		
zebrafish	59.6 ± 4.5	This work
human	85.7 ± 0.1	25
rat	85.5 ± 0.1	26

Table 4.

Oxygen binding parameters for zebrafish Cytoglobins

Protein	P ₅₀ (torr)	Hill coefficient (<i>n</i>)
Cygb1		
25°C, pH 6.8	0.39 ± 0.12	1.05 ± 0.09
25°C, pH 7.4	0.53 ± 0.06	1.43 ± 0.20
37°C, pH 6.8	2.54 ± 0.11	1.77 ± 0.01
37°C, pH 7.4	2.20 ± 0.01	1.43 ± 0.01
Cygb2		
25°C, pH 6.8	3.61 ± 0.21	3.45 ± 0.08
25°C, pH 7.4	4.44 ± 0.49	2.99 ± 0.16
37°C, pH 6.8	5.72 ± 0.07	3.78 ± 0.13
37°C, pH 7.4	6.09 ± 0.15	4.28 ± 0.11