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Efficient reduction of vertebrate Cytoglobins by the Cytochrome b_5/Cytochrome b_5 reductase/NADH system

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Abstract

Cytoglobin is a heme-containing protein ubiquitous in mammalian tissues. Unlike the evolutionarily related proteins hemoglobin and myoglobin, cytoglobin shows a six-coordinated heme binding, with the heme iron coordinated by two histidine side chains. Cytoglobin is involved in cytoprotection pathways through yet undefined mechanisms and it has recently been demonstrated that cytoglobin has redox signaling properties via nitric oxide (NO) and nitrite metabolism. The reduced, ferrous cytoglobin can bind oxygen and will react with NO in a dioxygenation reaction to form nitrate, which dampens NO signaling. When deoxygenated, cytoglobin can bind nitrite and reduce it to NO. This oxidoreductase activity could be catalytic if an effective reduction system exists to regenerate the reduced heme species. The nature of the physiological cytoglobin reducing system is unknown, although it has been proposed that ascorbate and cytochrome b_5 could fulfill this role. Here we describe that physiological concentrations of cytochrome b_5 and cytochrome b_5 reductase can reduce human and fish cytoglobins at rates up to 250-fold higher than those reported for their known physiological substrates, hemoglobin and myoglobin; and up to 100-fold faster than 5 mM ascorbate. These data suggest that the cytochrome b_5/cytochrome b_5 reductase system is a viable reductant for cytoglobin *in vivo*, allowing for catalytic oxidoreductase activity.

Graphical abstract

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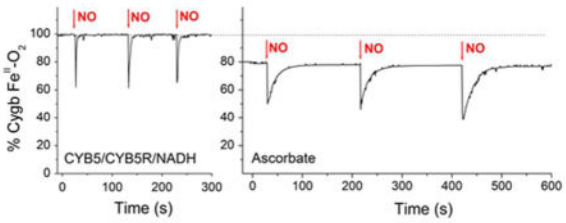

A number of six-coordinate globins, such as cytoglobin (Cygb) and neuroglobin (Ngb) have been recently discovered in mammals and other organisms [1–4]. In these six-coordinate globins, the distal histidine residue coordinates directly with the heme iron in both the ferrous (Fe^{II}) and ferric (Fe^{III}) states. This is in contrast to the evolutionarily related five-coordinate globins myoglobin (Mb) and hemoglobin (Hb), where the distal histidine residue participates in stabilizing bound ligands, but does not interact with the heme iron directly.

Cygb was initially discovered in the stellate cells of the liver, and soon thereafter found in virtually all human tissues with highly variable expression [1, 2, 4]. Subsequent studies suggested that Cygb, while present in virtually all organs, seemed to be found mostly in fibroblasts and other related cell types (osteoblasts, chondroblasts, and hepatic stellate cells), as well as specific neuronal populations within the brain [5, 6]. Other tissue types have since been shown to express Cygb, such as muscle (including vascular smooth muscle), endothelium, hepatocytes, and macrophages [7, 8]. Subcellularly, Cygb is thought to be a cytoplasmic protein in most cell types, although there is evidence of both cytoplasmic and nuclear localization in some cell types, specifically neurons [7]. In contrast with the presence of a single gene product in mammals, fish show a gene duplication with two products – Cygb 1 and Cygb 2 [9]. These two proteins have evolved significant functional differences, with Cygb 1 showing properties more akin to oxygen transport proteins and Cygb 2 being more similar to the mammalian Cygb protein [10]. Very recently, experiments in zebrafish detected mRNA for Cygb1 in the blood of the zebrafish, although it is still unknown which cell types within the blood are the source of this mRNA [10].

While structural and functional properties of Cygb have been well-characterized, their functions *in vivo* remain unknown. Many of these possible functions rely on electron and/or oxygen transfer from the globin to various targets, which may impact different pathways. For example, Cygb has a lipid peroxidase activity that may generate signaling lipids under oxidative stress conditions [11, 12]. In the ferrous state, Cygb can bind oxygen with high affinity [4, 13]. The ferrous oxy species is not as stable as for Hb/Mb, and undergoes autoxidation (Equation 1) with a half-life of ≈ 2.6 min at 37 °C for the human protein and 1.3 min for Cygb 2 at 25 °C. In contrast the oxygen-bound Cygb 1 shows higher stability with a half-life of 42 min at 25 °C [10]. In any case, the observed oxygen release rates outcompete the autoxidation rates [4, 10], and thus an involvement of Cygb in oxygen transport processes is possible. In addition, Cygb and Ngb can both modulate nitric oxide (NO) levels by carrying out reactions that scavenge or produce NO [14,15]. Ngb and Cygb can function as NO scavengers through their NO dioxygenase activity [16–18], where the oxygen-bound form of the protein consumes NO and oxygen to produce nitrate (Equation 2). Interestingly, both molecules also produce NO from nitrite through nitrite reduction reactions [8, 10, 19–22], where

the unbound, reduced form of the protein transfers an electron to nitrite, producing NO (Equation 3). As the relative concentrations of the deoxy and oxygen-bound forms depend on the concentration of oxygen, these reactions provide a pathway to regulate NO concentrations in response to the oxygen tension.

$$Fe^{II}-O_2+NO \rightarrow Fe^{III}+O_2^{-\bullet} \quad \text{(Equation 1)}$$

$$Fe^{II}-O_2+NO \rightarrow Fe^{III}+NO_3^{-} \quad \text{(Equation 2)}$$

$$Fe^{II}+NO_2^{-}+H^{+} \rightarrow Fe^{III}+NO+OH^{-} \quad \text{(Equation 3)}$$

It should be noted that the three reactions result in the generation of the oxidized form of the protein. Thus, a sustained, catalytic function of Cygb in these reactions necessitates the presence of a reducing system that is able to maintain the globin heme in the reduced (ferrous or ferrous-oxy) states [23]. Previous work has documented the reduction of Cygb by ascorbate (Asc), a result that suggests Asc may mediate Cygb reduction *in vivo* [17, 18, 24, 25]. Previous reports have indicated that cytochrome b_5 (CYB5) can reduce Cygb and support NO dioxygenation by Cygb. However, the efficiency of ferric Cygb reduction by CYB5 in physiological conditions, as compared to other reducing compounds such as Asc, is not well established. The reduction of ferric Cygb by CYB5 appears to be slower than the reported rates of NO dioxygenation, raising questions about the mechanism of this reaction [17]. Moreover, previous reports have only studied the reaction of reduced CYB5 with Cygb, but the ability of CYB5 to support Cygb-mediated catalysis in combination with the complete CYB5 reducing system; which includes cytochrome b_5 reductase (CYB5R) and NADH, has not been tested. Here we study the reduction of human and fish Cygbs by CYB5 in the presence and absence of cytochrome b_5 reductase (CYB5R) and NADH. We conclude that the CYB5/CYB5R/NADH system provides a feasible reduction system for Cygb that has been conserved through evolution and can provide a preferred route for physiological Cygb reduction, complemented by the possible tissue-specific role of other reductants.

MATERIALS AND METHODS

Reagents

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

Expression and Purification of Recombinant Globins

Human cytoglobin (hCygb) and the zebrafish globins, cytoglobin 1 (Cygb1) and cytoglobin 2 (Cygb2), were expressed recombinantly in *E. coli* as described with minor modifications [10]. In order to decrease the amount of protoporphyrin-IX or other heme

precursors incorporated in both hCygb and Cygb2, the final growth step for these proteins was performed under slightly different conditions than previously reported. Specifically, after induction of protein expression, the cultures were transferred into 1 l flasks and continued growing at 30°C, 100 rpm until they were harvested 20–24 h later.

Cell lysis was accomplished by sonication (30 pulses of 30s at 5% amplitude) using a Misonix S-4000 sonicator (Qsonica, Newtown, CT). The crude lysate was clarified by centrifugation at 18000×*g* for 60 min. All three Cygb proteins incorporate N-terminal His tags, and were purified by Ni-nitrilotriacetic acid (Ni-NTA) agarose chromatography using an Akta Purifier 10 FPLC system (GE Healthcare) with UNICORN software.

Zebrafish globin X and human Ngb were expressed in the same fashion as Cygb1, as described previously [20, 26]. GbX incorporated an N-terminal His tag, and was purified by Ni-NTA agarose chromatography as described above for the Cygb proteins. The Ngb plasmid did not include an N-terminal His tag, and was purified using anion exchange and size exclusion chromatography as described previously [20]. Protein purity was assessed by SDS-PAGE and UV-visible spectroscopy; spectral data was used to compare the protein properties to previously reported standards [21, 26]. All proteins were buffer exchanged into 100 mM sodium phosphate, pH 7.4 (Fisher Scientific, Pear Lawn, NJ) and stored at −80° C.

Expression and Purification of Human Cytochrome b_5 and Human Cytochrome b_5 Reductase

Recombinant human mitochondrial CYB5 (Cytochrome b_5 type b, accession number NP_085056.2) was overexpressed in *E. coli* and subsequently purified as previously described with minor modifications [27, 28]. Eluted fractions were concentrated using a 10 kDa cutoff Amicon Ultra centrifugal filter (Millipore). This concentrated protein solution (1–2 ml) was then further purified via gel chromatography by passing through a Sephacryl S200HR column (GE Healthcare) equilibrated in 50 mM Tris, 1 mM EDTA, pH 8.0. The eluted fractions from this step were again concentrated using 10-kDa molecular mass cutoff filters, buffer exchanged into 100 mM sodium phosphate, pH 7.4, and stored at −80° C. The expression plasmid for CYB5b (pET11a:CYB5) [28], encoding the soluble portion of the human mitochondrial CYB5, was a kind gift from Dr. Mario Rivera (University of Kansas). Human CYB5R (Cytochrome b_5 reductase type 3, isoform 2; accession number NP_015565.1) was expressed and purified as described previously [27]. The identity/similarity percentages for the human and zebrafish proteins (with similarities based on BLOSUM 62 matrix scores [29]) are 56.1%/70.5% for CYB5 and 75.5%/87.8% for CYB5R. Thus we expected that the human proteins would be suitable to replace the zebrafish counterparts although at a somewhat decreased efficiency.

Steady-state reduction of globins by NADH + CYB5 + CYB5R or Asc

Steady-state reactions were conducted anaerobically in 1 ml optical cuvettes (Starna Cells, Atascadero, CA) closed by a screw cap. Samples of heme globins were oxidized to the ferric form with excess potassium ferricyanide at room temperature and then passed through a Sephadex G25 column (PD10, GE Healthcare) equilibrated with 100 mM sodium phosphate buffer, pH 7.4, to remove ferricyanide. The resulting oxidized globin was diluted in 1 mL of

buffer to approximately 20 μM. For CYB5-mediated reduction, a mixture of NADH (Calbiochem, San Diego, CA), CYB5R, and CYB5 was added to yield the desired final concentrations of 100 μM NADH, 0.2 μM CYB5R, and 2 μM CYB5. This reaction was also conducted without CYB5 to determine if direct electron transfer from CYB5R to the globins was possible. In order to estimate the apparent K_M for Cygb or Mb, the initial rates for Cygb/Mb reduction were determined at a range of Cygb (5–100 μM) or Mb (10–500 μM) concentrations while keeping the NADH, CYB5R and CYB5 concentrations unchanged (100 μM NADH, 0.2 μM CYB5R, and 2 μM CYB5). The reactions for Mb were followed in a Cary 50 spectrophotometer (Agilent); however the reactions for the Cygbs proceeded at a much faster rate (timescale 1–5 s) and were studied on an SX20 stopped-flow spectrophotometer equipped with either a single wavelength or a diode-array absorbance detector (Applied Photophysics). For Asc-mediated reduction, 500 mM Asc (JT Baker, Phillipsburg, NJ) was added to yield final concentrations between 0.1 and 50 mM. UV-visible spectra and kinetic data were recorded on an HP8453 UV-vis spectrophotometer (Agilent Technologies, Palo Alto, CA). In all experiments, reduction was followed at the absorbance peak for the ferrous form of the globins around 560 nm. Reactions were followed at 37 °C in 100 mM sodium phosphate, pH 7.4.

Fast-kinetics reduction of ferric globins by ferrous CYB5

Reduction of CYB5 and electron transfer from ferrous CYB5 to the ferric globins was studied under anaerobic conditions. UV-visible spectra and kinetic data were recorded on an SX20 stopped-flow spectrophotometer equipped with a diode-array detector (Applied Photophysics). CYB5 was reduced to the ferrous form with excess sodium dithionite, which was removed using a Sephadex G25 column (PD10, GE Healthcare) equilibrated with 100 mM sodium phosphate buffer. Globins were oxidized with excess potassium ferricyanide (ferricyanide solutions ~1 M were added in a 1:10 ferricyanide:protein ratio (v/v) and incubated for 5–60 seconds at room temperature) and then excess oxidant was removed as described above. The absorbance at 568 nm was used to follow globin reduction, as this wavelength was found to be isosbestic for the transition of CYB5 from the ferrous to the ferric state, but not for that of any of globins tested. The final concentration of all globins was maintained between 5 and 10 μM, while the CYB5 concentration was varied from as high as 60 μM to less than 10 μM. Reactions were followed at 25 °C in 100 mM sodium phosphate, pH 7.4.

Kinetic data analysis

The reaction of the globins with CYB5 is considered to proceed according to an elementary bimolecular process as described in Equation 4:

$$\mathrm{Globin{-}Fe^{III}{+}CYB5_{red}} \iff \mathrm{Globin{-}Fe^{II}{+}CYB5_{ox}} \quad \text{(Equation 4)}$$

Where the reaction can proceed in either direction, with k_{FWD} and k_{REV} being the bimolecular rate constants for the forward (globin reduction) and reverse (globin oxidation) reactions. Forward rate constants (k_{FWD}) for the reduction of each globin were calculated based on the initial rates observed in the stopped-flow experiments. The absorbance changes

were converted into protein concentration changes by dividing the absorbance by the difference between the extinction coefficients of each globin's reduced and oxidized states, and the initial velocity, in units of mol per second (M s^{-1}), was then divided by the concentration of the globin to calculate the initial rate (s^{-1}). The initial rates were plotted versus the concentration of CYB5 and the data were fitted to a linear expression (Equation 5) to yield an estimate of the forward rate constant for the electron transfer from CYB5 to the globin.

$$k_0 = k_{FWD}[CYB5] \quad \text{(Equation 5)}$$

Additional analysis of the stopped-flow data was conducted according to the method described by Malatesta for non-pseudo-first order conditions [30]. Absorbance traces were fit to Equation 6,

$$\Delta A_\lambda = \Delta A_{EQ}\frac{1-e^{-\eta t}}{1+\omega e^{-\eta t}} + C \quad \text{(Equation 6)}$$

Where ΔA_λ represents the change in absorbance at a specific wavelength (568 nm in this case), ΔA_{eq} represents the equilibrium change in absorbance at that same wavelength, η is the observed rate constant, ω is a constant that varies between −1 and 1 and accounts for deviation from first-order behavior, and C is a constant.

In order to calculate the bimolecular rate constants for the forward and reverse reactions, the observed rate constants were plotted against the concentration of CYB5 and the data were fitted to Equation 7:

$$\eta = \sqrt{k_{FWD}^2([globin]-[CYB5])^2 + 4k_{FWD}k_{REV}[globin][CYB5]} \quad \text{(Equation 7)}$$

Where again η is the observed rate constant, k_{FWD} and k_{REV} are the bimolecular rate constants for the forward and reverse reactions, and [globin] and [CYB5] are the total concentrations of the globin and CYB5, respectively. We observed large standard deviations in the parameters k_{FWD} and k_{REV} calculated via equations 6 and 7. Thus, in order to estimate values for k_{REV} more accurately, the k_{FWD} value obtained by the analysis of initial rates (Equation 5) was substituted in Equation 7. Data were analyzed using Origin 8.0 (OriginLab Corp.).

Kinetic simulations

In order to calculate the time course of the different Cygb species during Cygb reduction and NO dioxygenation reactions, in the presence of either the CYB5/CYB5R/NADH system or Asc, the reaction was modeled using the COPASI software, v. 4.19 [31]. The reactions used to model the process are as follows:

$$\mathrm{Cygb\,Fe^{III}} + \mathrm{CYB5_{red}} \iff \mathrm{Cygb\,Fe^{II}_{6c}} + \mathrm{CYB5_{ox}} \quad (1)$$

$$\mathrm{Cygb\,Fe^{III}} + \mathrm{Asc} \rightarrow \mathrm{Cygb\,Fe^{II}_{6c}} + \mathrm{Asc_{ox}} \quad (2)$$

$$\mathrm{Cygb\,Fe^{II}_{5c}} + \mathrm{O_2} \iff \mathrm{Cygb\,Fe^{II}O_2} \quad (3)$$

$$\mathrm{Cygb\,Fe^{II}O_2} + \mathrm{NO} \rightarrow \mathrm{Cygb\,Fe^{III}} + \mathrm{NO_3^-} \quad (4)$$

$$\mathrm{Cygb\,Fe^{II}O_2} \rightarrow \mathrm{Cygb\,Fe^{III}} + \mathrm{O_2^{-\bullet}} \quad (5)$$

$$\mathrm{Cygb\,Fe^{II}_{5c}} + \mathrm{NO} \iff \mathrm{Cygb\,Fe^{II}NO} \quad (6)$$

$$\mathrm{CYB5R} + \mathrm{CYB5_{ox}} \iff \mathrm{CYB5R{:}CYB5_{ox}} \quad (7)$$

$$\mathrm{CYB5R{:}CYB5_{ox}} \rightarrow \mathrm{CYB5R} + \mathrm{CYB5_{red}} \quad (8)$$

$$\mathrm{Cygb\,Fe^{II}_{5c}} \iff \mathrm{Cygb\,Fe^{II}_{6c}} \quad (9)$$

Where Cygb-Fe^{II}_{6c} denotes the six-coordinate ferrous Cygb, Cygb-Fe^{II}_{5c} indicates the five-coordinated ferrous Cygb, Cygb-Fe^{III} is the ferric (met) Cygb, and CYB5R:$CYB5_{ox}$ is the Michaelis complex between CYB5R and $CYB5_{ox}$. In reaction 2, Asc_{ox} indicates the oxidation product of Asc, which could be Asc radical or dehydroascorbate. For the sake of simplicity, and as NADH is present in saturating concentrations during our assays with CYB5R, we consider CYB5R to be in a fully reduced form through the reaction and hence use the approximation $[CYB5R_{red}] \approx [CYB5R]_T$.

Cytoglobin NO dioxygenase activity

To study the ability of CYB5 + CYB5R + NADH and Asc to support a catalytic, Cygb-dependent NO dioxygenase activity, we studied the reaction as follows: in a 1 ml cuvette,

closed with a screw cap with a rubber septum, 20 μM ferric Cygb (metCygb) was mixed with either 2 μM CYB5 + 0.2 μM CYB5R + 200 μM NADH or 5 mM Asc, in buffer saturated with 100% oxygen. The reaction was monitored by UV-Vis spectroscopy until the spectra stabilized showing characteristic peaks for oxyCygb. Then NO-saturated buffer was added to the cuvette to achieve a final NO concentration of 5 μM. The spectral changes were monitored until the spectra reached again a stable, oxyCygb species and then NO was added again, the process was repeated for 3–5 cycles. The reaction was studied on an HP8453 UV-vis spectrophotometer (Agilent Technologies, Palo Alto, CA). Spectral deconvolution, based on Cygb and CYB5 standard spectra was used to calculate the amount of each Cygb species (oxyCygb, deoxy Cygb, metCygb, Cygb-NO) during the reaction. The calculated concentrations of deoxyCygb were negligible. Reactions were followed at 37 °C in 100 mM sodium phosphate, pH 7.4.

RESULTS

Reduction of globins by Asc

We investigated the ability of Asc, a reducing compound ubiquitously found in cells, to act as reductant for ferric Cygb and other globins. We monitored the reaction of three Cygbs and Mb (20 μM) with increasing concentrations of Asc under anaerobic conditions. Our results are shown in Figure 1. We observe that the reaction of the globins with Asc proceeds with a hyperbolic behavior as reported for hCygb [18, 24, 25]. It is notable that higher concentrations of Asc do not increase the rates of the reaction with hCygb in a linear fashion, in agreement with previous observations [18]. We observe that even at high, supraphysiological concentrations of Asc (50 mM, with physiological concentrations reported in the 0.05–12.5 mM range [32]) the Cygbs are not completely reduced after 15 minutes. The results are consistent with a reversible reaction, probably involving an intermediate where Asc is bound to the ferric form of Cygb [24]. Mb, while reduced more slowly initially, is completely reduced by 10 mM Asc within 15 minutes (Figure 1).

To determine the bimolecular reaction rate constants for the reaction of the oxidized globins with Asc, we calculated the initial rates of the reaction of hCygb, Cygb1, Cygb2 and Mb with Asc. The observed initial rates are shown in Figure 2. As expected from the data in Figure 1, the reaction rate for the Cygbs is not proportional to the Asc concentration and varies in a hyperbolic fashion consistent with Michaelis-Menten kinetics and the presence of a Cygb-Asc intermediate.

In the case of Mb the rates show a linear relationship, with a calculated rate constant of 0.11 $M^{-1}s^{-1}$ (Table 1). The plots also indicate that in the physiological range of Asc concentrations (0–10 mM) the rates for the three Cygbs change in a near-linear fashion, and an approximate bimolecular rate can be estimated. Fitting the observed initial rates in this range (0–10 mM Asc) to a linear fit, we calculated bimolecular rate constants of 1.05 $M^{-1}s^{-1}$ (hCygb), 0.77 $M^{-1}s^{-1}$ (Cygb1) and 1.26 $M^{-1}s^{-1}$ (Cygb2) (Table 1). These rates are in good agreement with other values reported for hCygb [17, 33] and Mb [17, 24]; the rate for hCygb is, however, one order of magnitude slower that the value reported by Liu et al [18].

Reduction of globins by CYB5/CYB5R/NADH

We then tested the ability of the CYB5/CYB5R/NADH system to reduce Cygbs and other globins in similar conditions to those used for Asc in the preceding experiments. The ferric globins (20 μM) were incubated with 0.2 μM CYB5R and 2 μM CYB5 (consistent with the physiological range and stoichiometry for CYB5R/CYB5 [34, 35]) and the reaction was initiated by the addition of NADH (final concentration 100 μM) to the reaction mixture. The concentration of NADH used amply exceeds the reported K_M value (0.6 μM) whereas CYB5 is present in a physiological, but non-saturating concentration (K_M = 20 μM) [36]. The reduction of the globins was monitored by UV-Visible spectroscopy. The reduction rates for the different globins studied varied widely (Figure 3A). We observe that the CYB5/CYB5R/NADH system is very efficient at reducing the Cygbs, whereas the known physiological substrates, Hb and Mb, are reduced in a ≈10-fold longer timescale. The three Cygbs were entirely reduced within 2–3 minutes, whereas Hb and Mb were fully reduced in 10–15 minutes and GbX and Ngb were only partially reduced after 15 minutes. The slow reaction of Ngb with CYB5 is consistent with other reports [37]. GbX and Ngb have a more negative redox potential than the other globins [20, 26, 38, 39], and thus their reduction will be less favorable thermodynamically (Table 2). However it is apparent that other factors are at play as Hb and Mb have more positive potentials [40] than Cygbs [10, 41] and yet they are reduced more slowly than the Cygbs (Table 2). Compared to the reduction of the same globins with 5 mM Asc, we observe that the CYB5/CYB5R/NADH system achieves a faster and more complete reduction of the Cygbs, and the rate of reduction for Mb is similar for the two different reducing systems. (Figure 3B). In order to establish if the reduction is mediated by CYB5, or if CYB5R is in fact able to directly reduce the Cygbs in the presence of NADH, we conducted additional experiments where CYB5 was omitted from the reaction mixture (Figure 3C). We observe that CYB5R can reduce the Cygbs in the absence of CYB5 but at a very slow rate. Among the proteins tested, the reaction is fastest for Cygb1, but even in this case only half of the protein is reduced after 15 minutes, whereas the addition of CYB5 results in complete reduction within 2 minutes. Overall, we observe that CYB5R can reduce the Cygbs as expected from its very negative redox potential, but clearly CYB5 is necessary to efficiently mediate the electron transfer and achieve fast globin reduction (Figure 3A).

To further characterize the steady-state behavior of the CYB5/CYB5R/NADH system with the three Cygbs we studied the apparent K_M for Cygb by varying the concentrations of Cygb in the presence of the previously studied concentrations of CYB5, CYB5R and NADH (2 μM, 0.2 μM, and 100 μM respectively). Although the values are very dependent on the existing concentrations of CYB5, CYB5R and NADH they can provide an indication of the physiological relevance of the system and its apparent catalytic and binding specificity. For example, studies with Hb indicate an apparent K_M towards Hb of 0.11–2.2 mM [46], a low apparent affinity but consistent with the high physiological concentrations of Hb in erythrocytes. Our results are shown in Figure 4, and indicate that the Cygbs uniformly have low apparent K_M values, with calculated values of 3.6 ± 2.5 μM, 14 ± 2 μM, and 1.6 ± 0.8 μM for hCygb, Cygb1, and Cygb2, respectively. The maximum reaction rate (V_{max}) for each of these proteins was calculated to be 5.0 ± 3.0 μM/s, 3.7 ± 1.0 μM/s, and 5.0 ± 2.5 μM/s for hCygb, Cygb1, and Cygb2, respectively. These values indicate a surprisingly high affinity for the Cygbs in physiologically relevant concentration of CYB5, CYB5R and NADH. We

note that the high concentrations of CYB5 (2 μM) relative to the observed apparent K_M towards Cygb can influence the accuracy of the observed K_M values, however it is significant that these values are one or two orders of magnitude lower than those reported for other globins. It is also remarkable that the apparent K_M values are in the μM concentration range observed for hCygb *in vivo* [47]. For Mb, in contrast, the relationship of initial velocity to globin concentration seems to remain mostly linear in the range studied. While this prevents an accurate calculation of Michaelis-Menten parameters, we estimated values of K_M = 790 ± 250 μM, in good agreement with the value for Hb and the physiological concentrations of Mb.

Kinetics of ferric globin reduction by ferrous CYB5

As we have shown that the CYB5/CYB5R/NADH system is a fast and efficient reductant for Cygbs, it is important to determine the rate of the electron transfer between ferrous CYB5 and ferric Cygbs in order to know whether the concentration of reduced CYB5 or its rate of reduction by CYB5R are the limiting steps for the reduction of Cygb by the CYB5/CYB5R/NADH system *in vivo*. The study of the reaction between heme proteins with similar spectral properties makes the use of pseudo-first order conditions challenging. A classical strategy for the study of the electron transfer from cytochromes to heme globins involves the addition of excess carbon monoxide (CO) to the reaction mixture. As the six-coordinated cytochromes do not bind CO whereas most heme globins bind CO at almost diffusion-controlled rates, the formation of the ferrous heme globin can be monitored by the absorbance of the ferrous-CO species. This provides a better spectroscopic signal and also prevents any backwards reaction where the ferrous globin reduces the ferric cytochrome. However, in the case of six-coordinate globins, the binding of gaseous ligands is limited by the dissociation of the distal histidine ligand. This reaction can occur in similar timescale to that of the observed globin reduction rates (4.5 s^{-1} for hNgb [38]; 2–5 s^{-1} for hCygb [4, 48]) and thus the use of CO can yield inaccurate reduction rates.

In order to avoid this problem we studied the reaction in non-pseudo first order conditions, restricting our analysis to the initial rates of the reaction. Our data (Figure 3) indicates a fast reaction, and thus we used stopped-flow techniques to monitor the reaction by UV-Visible spectroscopy. The analysis of the spectra of CYB5 in its oxidized and reduced states shows several isosbestic points [37]. Given the changes in absorbance of the different globins used, the isosbestic point of 568 nm was preferred to monitor the reduction of the globins.

Sample traces for the reactions between the globins and CYB5 are shown in Figure 5. Following similar trends to those observed for the complete reducing system (Figure 3) the reaction is rapid for all three Cygbs, although in this case is faster for hCygb and Cygb2, followed by Cygb1, whereas Cygb2 was reduced more slowly than hCygb and Cygb1 in the presence of the complete reducing system. In comparison, the reaction of Mb in the same conditions occurs more slowly (40 to 200-fold timescale).

The observed initial rates as a function of the concentration of CYB5 are shown in Figure 6. The data was fitted to Equation 5, thus calculating the bimolecular rate constants for the forward reactions (Table 3). Our results indicate that ferrous CYB5 reacts faster with hCygb and Cygb2, with very similar rate constants. Some deviation from the linearity is observed

for both hCygb and Cygb2 at high CYB5 concentrations, which may be related to a transient complex formation as hinted by the low apparent K_M values (Figure 4). The reaction with Cygb1 shows a 5-fold lower rate constant, whereas the rate constant for Mb is around 250-fold lower than for Cygb/Cygb2 and 50-fold lower than Cygb1. Our calculated value is for Mb is comparable to other reports (8.2×10^2 $M^{-1}s^{-1}$) [49].

Computer simulations and experimental assessment of Cygb reduction and catalysis

With our calculated reaction rates and available data from the literature we estimated the parameters for the kinetic model describing the reactions of the globins with CYB5 or Asc (Table 4). We used this model to simulate the ability of different concentrations of CYB5 or Asc to maintain Cygb in the ferrous state in the presence of oxygen. The results of our computer simulations are shown in Figure 7. The calculated traces indicate that low concentrations of CYB5 can overcome the autoxidation of the Cygb Fe^{II}-O_2 complex and maintain a high proportion of Cygb in the ferrous-oxy state (>95% for 1 μM CYB5 or higher). However, at the same oxygen concentration, very high Asc concentrations are needed to maintain Cygb in the ferrous state. The simulations indicate that even at 10 mM Asc, less than 75% of the Cygb is in the ferrous-oxy state, and at 1 mM Asc the expected concentration of ferrous-oxy Cygb is less than 20% (Figure 7).

These results suggest that Asc concentrations in most tissues will have a very limited capacity to support Cygb-mediated reactions. To test this hypothesis, we studied the dioxygenation reaction of oxygenated hCygb and (NO) in the presence of the CYB5/CYB5R reduction system or Asc (Figure 7C, D). In this experiment, we studied the regeneration the oxyCygb species from ferric, metCygb after additions of NO. Figure 7C, D shows the percentage of oxyCygb *versus* time. NO additions cause a sudden decrease of oxyCygb, with concomitant formation of metCygb, as expected from the fast reaction in Equation 2. Consistent with the simulated data, CYB5 supports higher levels of reduced protein (very near 100%), and recovers these levels within 5–10 seconds after NO dioxygenation (Figure 7C). These results suggest that the rates of Cygb reduction by CYB5 that we determined can describe well the reduction process by the complete CYB5/CYB5R/NADH system, and extra steps including other species that may be reduced at a different rate by CYB5 (e.g. a Fe^{III}-$OONO^-$ intermediate) do not need to be invoked to describe the process. Also in agreement with the simulation, Asc is not able to keep Cygb in a fully reduced state, although it performs slightly better than the simulations (around 75% oxyCygb versus 55% expected). Asc reduces hCygb at a lower rate than CYB5/CYB5R, and takes roughly 60–90 seconds to recover nearly the initial level of reduction after NO dioxygenation (Figure 7D). It should be noted that still the recovery time for Asc is around 5-fold shorter than expected from the simulation. This appears to indicate that the reduction rates used for Asc in the dioxygenation reaction are underestimated. Increasing the reduction rate constant in the computer simulations from 1.05 $M^{-1}s^{-1}$ to 6 $M^{-1}s^{-1}$ provides a better fit to the experimental data (data not shown). A possible explanation for this fact is the faster reaction of Asc with a reaction intermediate, as proposed by Gardner et al. [17]. Overall, these results suggest that NADH/CYB5/CYB5R is a more efficient system than Asc for the support of Cygb-dependent NO dioxygenation.

DISCUSSION

Most globin functions involve an active, reduced ferrous heme. Particularly in the case of six-coordinate globins, which show autoxidation rates notably faster than the canonical oxygen carriers Hb or Mb, an active heme reduction system is required [23]. The nature of this system *in vivo* remains unclear and may well be different according to the cell type. The ability of Asc to reduce six coordinate globins, in particular Cygb, has been widely explored [17, 18, 24, 25].

Our results indicate that the CYB5/CYB5R/NADH system has favorable properties to function as an *in vivo* reductant of Cygb, as even in very high concentrations of Asc, it outperforms Asc by keeping most of the Cygb reduced and can regenerate the oxyCygb species at least 5-fold faster. This does not completely preclude a role for Asc in Cygb reduction, as several tissues expressing Cygb can achieve high (around 10 mM) Asc levels, for instance neurons [50]. However, most tissues have lower Asc levels, often well below 1 mM [32]. In particular, vascular smooth muscle cells (0.1–0.2 mM) [51, 52] and RBCs (0.04–0.08 μM) [32, 53], have relatively low Asc content and Asc seems unlikely to be an effective reductant in these conditions. For example, recent studies indicate that some of the six-coordinate globins may have significant roles in the red blood cells (RBCs) of zebrafish and other organisms. GbX [26] and Ngb [54] have been detected in fish RBCs, and Cygb1 [10] is also present in zebrafish blood, likely in RBCs as well. As CYB5 and CYB5R are present in RBCs in concentrations around 1 μM [55–57], where they provide the canonical route for Hb reduction, we hypothesize that this system is the main physiological reductant of Cygb, and probably GbX, in the RBCs.

It is remarkable that CYB5 reduces Cygbs with faster rate constants than for Hb and Mb, even though the redox driving force favors Hb/Mb reduction by CYB5 (Table 2). This indicates that the interaction of CYB5 and Cygbs is favored, probably through optimal electrostatic interactions. The electrostatic surfaces of Cygb and Mb show a number of positively charged residues around the heme cavity, complementary to the negative charges in CYB5 surface (Figure 8). In the case of Ngb, a positive surface patch is also observed in the putative interaction area, but less pronounced than for Cygb/Mb, suggesting a weaker electrostatic interaction with CYB5. Given the large difference in redox potentials (≈ 90 mV) and the lack of complementary surfaces, is not surprising that CYB5 is a poor reductant for Ngb. However, the preference of CYB5 towards Cygb and not Mb/Hb is not apparent and deserves a further analysis of the docking interactions of the putative complexes.

The higher efficiency of the reduction of Cygb by CYB5, as compared to Hb/Mb, could derive form the longer coevolution of Cygb with CYB5 and/or the higher electron demand posed by the fast autoxidation rates of Cygb as compared to Mb/Hb. As Cygbs predate the origin of Mb/Hb [59] it begs the question if CYB5 has been optimized by evolution to reduce Cygbs and not its canonical substrates Hb and Mb. In this regard, it is noteworthy that we observe similar high efficiency of a human CYB5 for the reduction of human or zebrafish Cygbs. As many other species, including zebrafish, are known to express CYB5, it seems likely that CYB5 and CYB5R may serve as the physiological reductants of Cygbs not only in humans, but in many other species as well. Certainly CYB5 and CYB5R are important to

mammalian Hb physiology as defects in both proteins are linked to methemoglobinemia [60–63]. Nevertheless, it is also clear that the evolutionary pressure for Hb and Mb reduction is lower than for other putative globin substrates as these proteins have slow autoxidation rates (Table 2). Some mammals do have very inefficient reducing systems for Hb, for instance swine are particularly susceptible to methemoglobinemia due to their low concentrations of CYB5R in RBCs [64].

We also observe that the CYB5 interaction with Cygb is highly efficient at reducing Cygb following NO deoxygenation, reducing the protein more rapidly and more thoroughly than even 5 mM Asc (Figure 7), near the highest level documented in any human cells [32]. NO deoxygenation has been suggested as an important physiological function of Cygb, specifically in the vascular wall. This reaction may be critical to limit the relaxing effects of NO in the vasculature and help regulate blood pressure levels [16, 18, 24, 65], probably complementing other heme-based systems as the Hb α-chain in myoendothelial junctions [66, 67]. Considering the low levels of Asc in many cells in the vascular wall, including smooth muscle cells, it seems likely that CYB5 plays an important role in supporting Cygb's role in NO deoxygenation.

Altogether, our results suggest that Cygb reduction will be preferably mediated by CYB5 over Asc. The role of Asc as Cygb reductant could be limited to particular cell types with high Asc levels, such as hepatocytes, fibroblasts and neurons. CYB5, in conjunction with CYB5R and NADH may mediate the reduction of Cygb in most physiological conditions. The three proteins can constitute a metabolon involved in the regulation of the NO signaling.

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ABBREVIATIONS

Asc	ascorbate
CYB5	cytochrome b_5
CYB5R	cytochrome b_5 reductase
Cygb	cytoglobin
hCygb	human cytoglobin
Cygb1	zebrafish cytoglobin 1
Cygb2	zebrafish cytoglobin 2
GbX	zebrafish globin X

Hb	Human hemoglobin
Mb	Human myoglobin
Ngb	human neuroglobin

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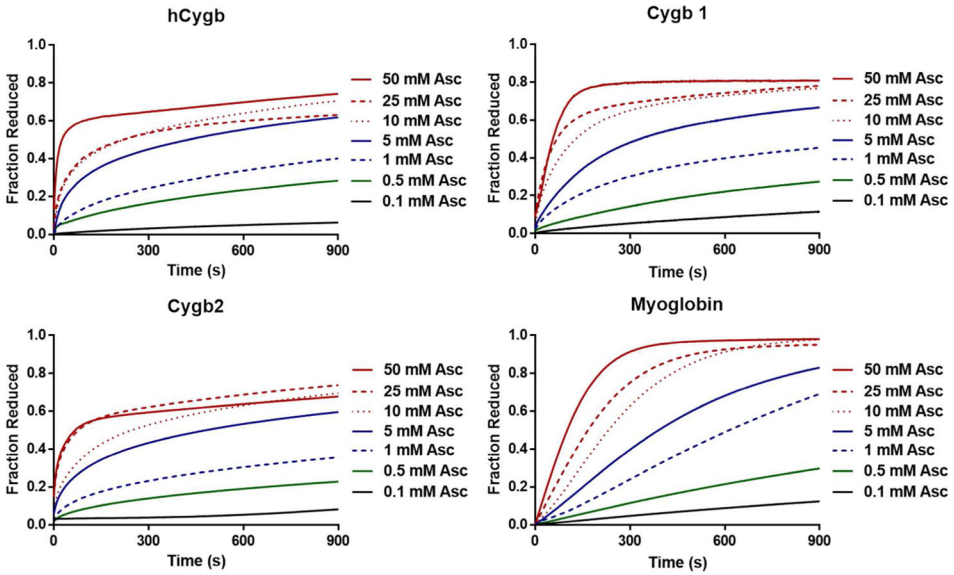

Figure 1. Reduction of globins by Asc

The reduction of the three Cygbs and Mb (20 μM) by increasing concentrations of Asc was monitored by UV-Visible spectroscopy. For the three Cygbs, the reduction shows a hyperbolic behavior with a faster initial rate that decays into a second, more linear phase. All experiments were conducted at 37° C in 100 mM sodium phosphate, pH 7.4. Each trace is the average of 3 individual traces.

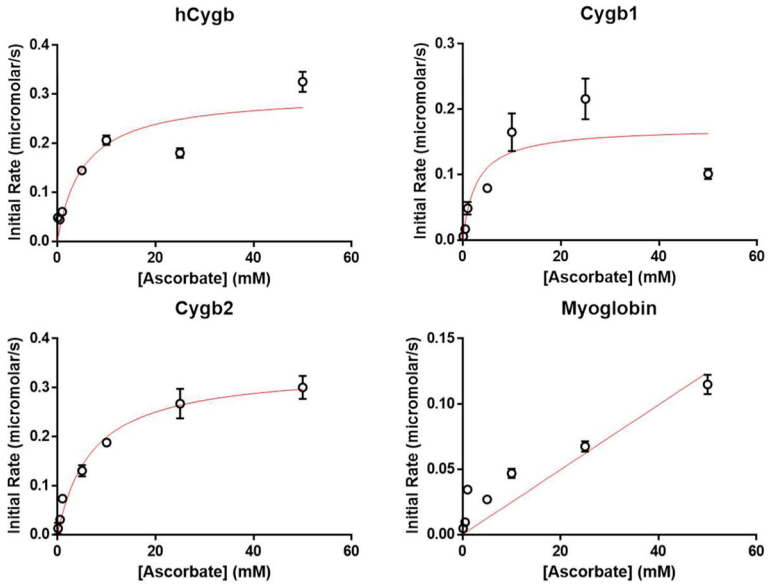

Figure 2. Rate constants for Asc-mediated reduction of Cygbs and Mb

Plots show the initial rate of globin reduction for a mixture of 20 μM ferric Cygb or Mb and varying concentrations of Asc. While the relationship becomes non-linear for Cygbs at high (>10 mM) Asc concentrations, a linear fit to all points at ≤10 mM Asc allows estimation of the second-order rate constants for the reduction of each protein by Asc. Red lines indicate the fits to the Michaelis-Menten equation (Cygbs) or a linear fit (Mb).

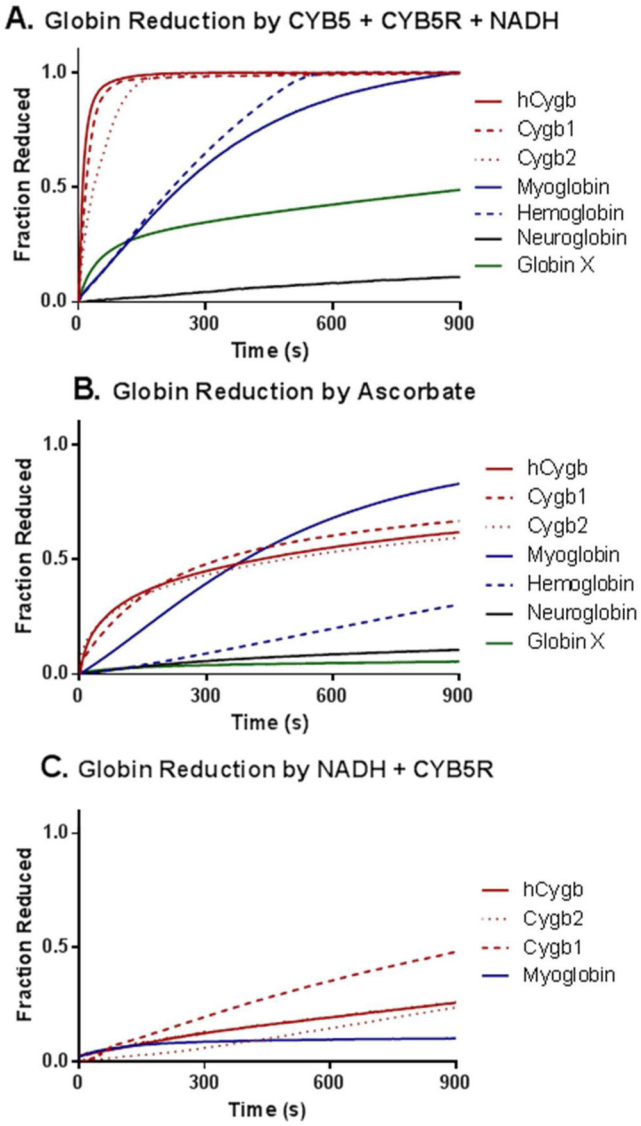

Figure 3. Reduction of globins by the CYB5/CYB5R/NADH system as compared to Asc

Panel A; Globins (20 μM) were incubated with 2 μM CYB5, 0.2 μM CYB5R. The reaction was initiated by the addition of 100 μM NADH. Panel B; Globins (20 μM) were mixed with 5 mM Asc. Panel C; Globins (20 μM) were incubated with 0.2 μM CYB5R. The reaction was initiated by the addition of 100 μM NADH. The reduction of the globins was monitored by UV-Visible spectroscopy. All experiments were conducted at 37° C in 100 mM sodium phosphate, pH 7.4. Each plotted trace is the average of 2 or more individual traces.

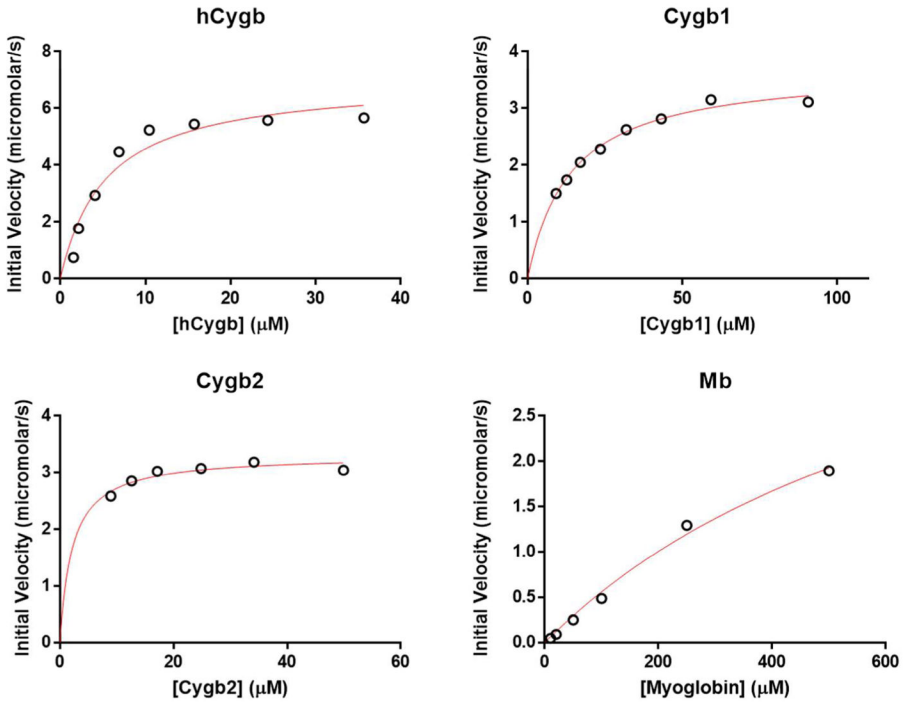

Figure 4. Determination of apparent K_M values of the CYB5/CYB5R/NADH reducing system for Cygb and Mb

2 μM CYB5, 0.2 μM CYB5R and 100 μM NADH were premixed and the reactions was initiated by the addition of the indicated concentration of Cygb/Mb. The plots indicate the observed initial rates at each globin concentration. Red lines denote the fit of the data to the Michaelis-Menten equation. Reactions were carried out in 100 mM sodium phosphate, pH 7.4 at 37 °C.

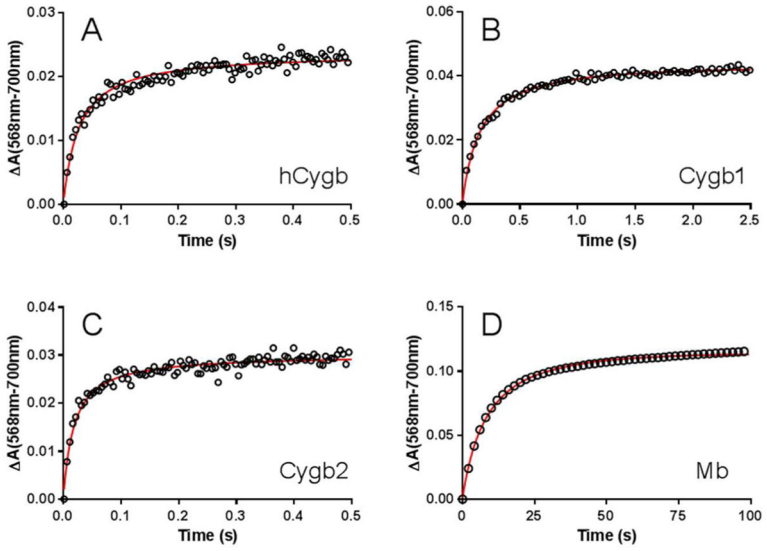

Figure 5. Stopped-flow kinetics for the reaction of ferric globins with ferrous CYB5

Ferric globins (8–10 μM) were mixed with ferrous CYB5 (5–60 μM) in the stopped-flow instrument. The plots show the changes in absorbance at 568 nm (open circles) after subtracting the absorbance value at 700 nm at each time point. The traces are fitted according to Equation 6 (red lines). Reactions were carried out in 100 mM sodium phosphate, pH 7.4 at 25 °C.

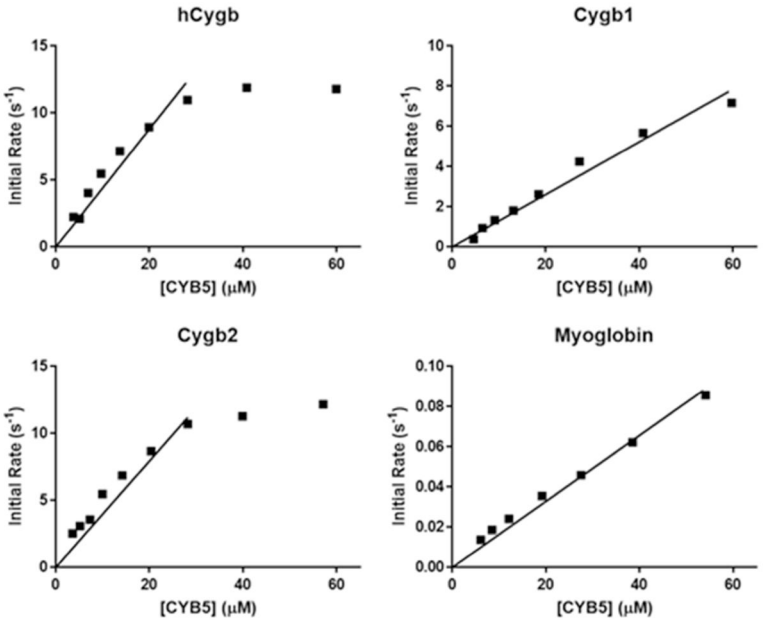

Figure 6. Determination of reaction rate constants for the reaction of CYB5 with Cygb and Mb

The plots indicate the observed initial rates *versus* the CYB5 concentration for each of the three Cygbs and Mb. Lines denote the fit to Equation 5. All data was gathered at 25° C. At least three experimental sets generated for each protein, the points indicate a representative experiment for each protein.

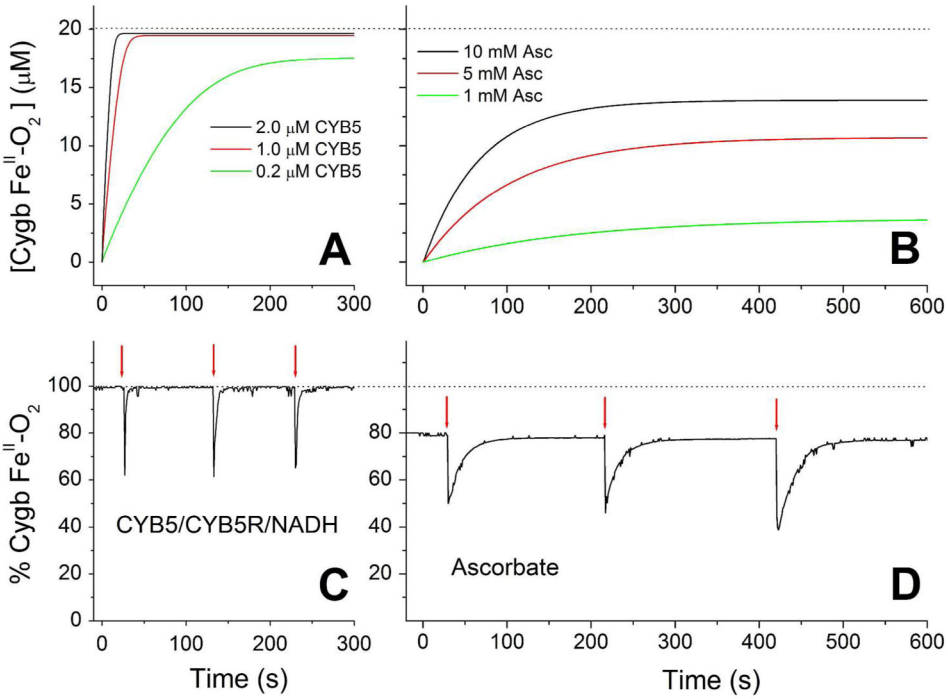

Figure 7. Computer simulations and experimental assessment of the NO dioxygenase activity of Cygb

Panels A and B, simulated traces for the reduction of Cygb (20 μM) by different concentrations of CYB5 (Panel A) or Asc (Panel B). For the CYB5 calculations, the concentration of reduced CYB5R was kept constant at 0.2 μM. Oxygen concentration was kept constant at 130 μM (10% O_2). Only the concentrations of the ferrous-oxy species (Fe^{II}-O_2) are shown; the rest of the Cygb is mostly in the ferric (Fe^{III}) form. See methods for details. Panels C and D, measured NO dioxygenase activity of Cygb in the presence of 2 μM CYB5 (Panel C) or 5 mM Asc (Panel D) in O_2 saturated buffer. The red arrows indicate the additions of NO-saturated buffer (5 μM NO final concentration). The reactions with Asc are shown in wider panels to maintain same timescales. Dotted lines indicate 100% ferrous-oxy Cygb levels.

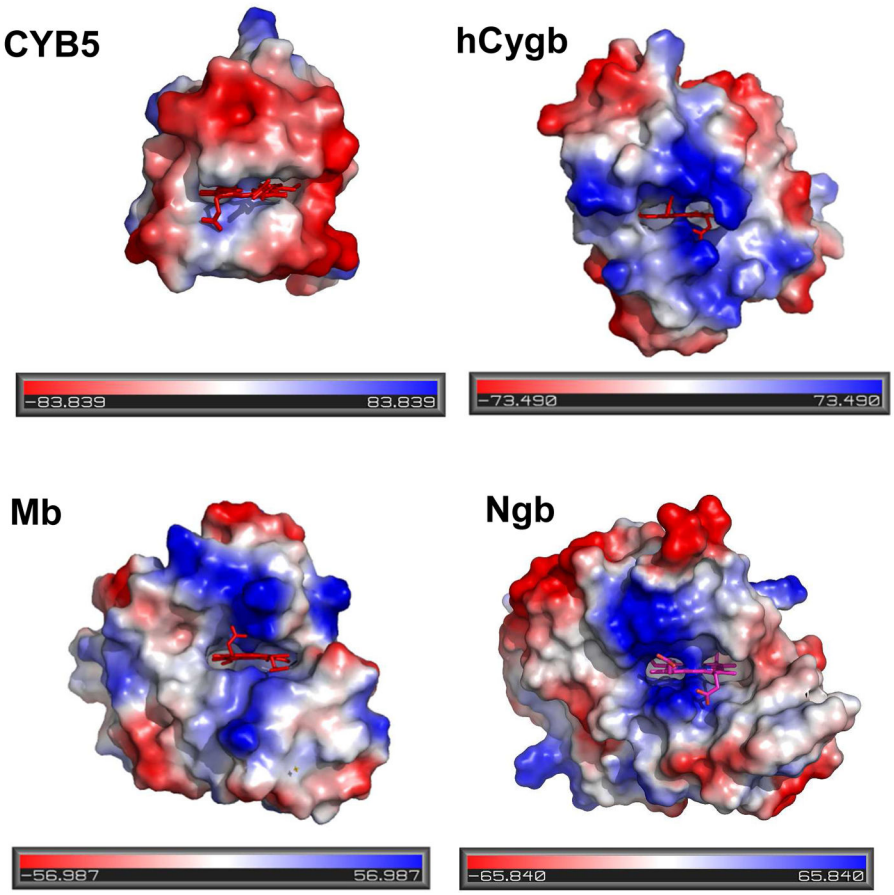

Figure 8. Electrostatic surfaces of CYB5 and selected heme globins

The surface potential for CYB5, hCygb, Mb and Ngb is shown. Positive potential is represented in blue, negative surface potential in red. The heme group is represented as red sticks. The scale under each structure indicates the relative magnitude of the color scale. The PDB structures used are 3NER (CYB5), 1UMO (hCygb), 5MBN (Mb), and 1OJ6 (Ngb). Protein structures and electrostatic surface potentials were generated with PyMOL [58].

Table 1

Bimolecular reaction rate constants for the reaction of ferric globins with Asc.

Protein	Bimolecular reaction rate constant ($M^{-1}s^{-1}$)
hCygb	1.14 ± 0.17 [a]
hCygb (m)	2.68 ± 0.13 [b]
hCygb (d)	1.58 ± 0.10 [b]
hCygb	1.3 ± 0.2 [c]
hCygb	36.1 ± 4.4 [d]
Cygb1	0.83 ± 0.07 [a]
Cygb2	1.04 ± 0.15 [a]
Mb	0.124 ± 0.016 [a]
Mb	0.13 [c]
Mb	0.087 ± 0.003 [e]

[a] This work.

[b] Value from ref 33.

[c] Value from ref 17.

[d] Value from ref 18.

[e] Value from ref 24

Table 2

Autoxidation rate constants and redox potentials (*vs* Standard Hydrogen Electrode) for selected heme proteins.

Protein	Autoxidation rate (min^{-1})	Redox potential (mV)
hCygb	0.270 ± 0.020 [a]	−37 ± 3 [a]
Cygb1	0.0239 ± 0.0024 [a]	−58 ± 26 [a]
Cygb2	1.44 ± 0.15 [a]	−26 ± 11 [a]
Ngb	0.230 ± 0.030 [b]	−118 ± 4 [b]
GbX	0.32 ± 0.05 [c]	−119 ± 3 [c]
swMb	0.00085 ± 0.00008 [d]	59 [e]
Hb	0.0013 ± 0.0001 (α chains) [f] 0.00018 ± 0.00003 (β chains) [f]	85 [g]
CYB5	NA	−40 [h]

[a] Value from ref [10].

[b] Value from ref [20].

[c] Value from ref [26].

[d] Value from ref [42].

[e] Value from ref [43].

[f] Value from ref [44].

[g] Value from ref [45].

[h] Value from ref [28].

NA, not applicable.

Table 3

Calculated rate constants for the reaction of ferric Cygbs with ferrous CYB5.

Protein	Forward Rate Constant ($M^{-1}s^{-1}$)
Cygb	$2.87 \pm 1.39 (\times 10^5)$
Cygb1	$1.11 \pm 0.18 (\times 10^5)$
Cygb2	$2.96 \pm 1.50 (\times 10^5)$
Mb	$1.61 \pm 0.38 (\times 10^3)$

Table 4

Rate constants used for the simulation of the reactions of Cygb with CYB5/CYB5R/NADH and Asc.

	Reaction	Forward rate		Reverse rate		Reference
1)	$Fe^{III} + CYB5_{rd} \Leftrightarrow Fe^{II}_{6c} + CYB5_{ox}$	2.87×10^5	$M^{-1}s^{-1}$	6.03×10^5	$M^{-1}s^{-1}$	This work
2)	$Fe^{III} + Asc \rightarrow Fe^{II}_{6c} + Asc_{ox}$	1.05	$M^{-1}s^{-1}$	*NA*		This work
3)	$Fe^{II}_{5c} + O_2 \Leftrightarrow Fe^{II}O_2$	3×10^7	$M^{-1}s^{-1}$	3.5×10^{-1}	s^{-1}	4
4)	$Fe^{II}O_2 + NO \rightarrow Fe^{III} + NO_3^-$	3×10^7	$M^{-1}s^{-1}$	*NA*		18
5)	$Fe^{II}O_2 \rightarrow Fe^{III} + O_2^{-\bullet}$	4.5×10^{-3}	s^{-1}	*NA*		10
6)	$Fe^{II}_{5c} + NO \Leftrightarrow Fe^{II}NO$	1×10^8	$M^{-1}s^{-1}$	8×10^{-4}	s^{-1}	18
7)	$CYB5R + CYB5_{ox} \Leftrightarrow CYB5R{:}CYB5_{ox}$	6×10^6	$M^{-1}s^{-1}$	33	s^{-1}	46
8)	$CYB5R{:}CYB5_{ox} \rightarrow CYB5R + CYB5_{rd}$	5.67×10^2	s^{-1}	*NA*		46
9)	$Fe^{II}_{5c} \Leftrightarrow Fe^{II}_{6c}$	4.3×10^2	s^{-1}	5	s^{-1}	4

Reaction rates were determined in the current work or taken from the literature as noted. Some values were estimated as follows: for reaction 4, forward and reverse rates were approximated from the estimated binding constant (8×10^{-12} M) [18]; for reaction 7, forward and reverse rates were estimated from the reported rate for reaction 8 (3.4×10^3 min^{-1} = 567 s^{-1}) [46] and the reported Michaelis-Menten constant (K_M) for the combined reactions 7 and 8 (100 μM) [46]. NA, not applicable.