

Strong modulation of nitrite reductase activity of cytoglobin by disulfide bond oxidation:
implications for nitric oxide homeostasis.

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ABSTRACT

Globin-mediated nitric oxide (NO) dioxygenase and nitrite reductase activities have been proposed to serve protective functions within the cell by scavenging or generating NO respectively. Cytochrome b5 has rapid NO dioxygenase activity, similar to other globins, however, the apparent rates of nitrite reductase activity have been reported as slow or negligible. Here we report that the activity of cytochrome b5 nitrite reductase activity is strongly dependent on the oxidation state of the two surface-exposed cysteine residues. The formation of an intramolecular disulfide bond between cysteines C38 and C83 enhances the nitrite reductase activity by 50-fold over that of the monomer with free sulfhydryl or 140-fold over that of the dimer with intermolecular disulfide bonds. The NO dioxygenase reactivity of cytochrome b5 is very rapid with or without disulfide bond, however, binding of the distal histidine following dissociation of the nitrate are affected by the presence or absence of the disulfide bond. The nitrite reductase activity reported here for the monomer with intramolecular disulfide is much higher than of those previously reported for other mammalian globins, suggesting a plausible role for this biochemistry in controlling NO homeostasis the cell under oxidative and ischemic conditions.

Keywords: Cytochrome b5, nitrite, reductase, nitric oxide, dioxygenase, disulfide

1. Introduction.

Cytoglobin (Cygb) is a hexacoordinate globin ubiquitously found in the cells of vertebrates [1]. There have been numerous suggested physiological functions of Cygb including oxygen sensing [2], peroxidase activity [3; 4; 5], lipid peroxidase activity [6; 7; 8], scavenging of reactive nitrogen species such as nitric oxide (NO) [9; 10] and scavenging of reactive oxygen species [11; 12; 13]. Nevertheless, the true cellular function (or functions) of Cygb remains unclear. Neuroglobin (Ngb) is a globin similar to Cygb found in high concentrations in neurons [14] and retinal tissue [15]. Both Ngb and Cygb have been proposed to function in NO regulation through NO dioxygenase (NOD) activity [9; 16; 17; 18; 19]. In addition to being scavenged by Ngb and Cygb, NO can be generated under conditions of hypoxia, a property particularly noted for Ngb [20; 21; 22; 23; 24; 25]. Hence it has been proposed that hexacoordinate globins such as Ngb and Cygb under normoxic conditions functions as a NO dioxygenase (NOD) to scavenging NO (Equation 1) but under hypoxic conditions the globin may switch its function to that of a nitrite reductase (NiR), generating NO (Equation 2). Therefore, the ferrous protein may serve to protect the functions of NO sensitive redox enzymes such as the terminal oxidase in the electron transport chain by consuming NO [17; 26; 27] and generate NO to facilitate vasodilation and increased blood flow to the hypoxic tissue [24; 25].



The high autoxidation rate of Ngb casts doubt on the role for Ngb homeostasis *in vivo* [28]. In contrast, the autoxidation rate of Cygb is low and, in combination with the rapid reduction of Cygb by ascorbate, cytochrome b5 and NADH, promoting the view that the oxidation state of the heme iron *in vivo* is normally ferrous [9; 29; 30]. The NOD activity of Cygb has previously been shown to be high in rat aorta cells with overexpressed protein and proposed to be a key component in determining the magnitude of the NO response in the vasculature [10]. Additionally, the co-localisation of Cygb with neuronal NO synthase in the brain strongly

supports a link between Cygb and NO metabolism [31], as does the protection of Cygb against hepatocyte damage during inflammation [32] and the angiotensin-mediated hypertension in aortic smooth muscle cells [33]. Knockout of Cygb in mice prolongs NO decay in aortic cells and that NO metabolism in vascular smooth muscle regulates NO flux [33]. Taken together, these studies strongly suggest that Cygb is a major pathway of NO metabolism.

The maximal observed rate of human Cygb NOD activity was measured at 430 s^{-1} with no discernible effect of NO concentration between $10 \text{ }\mu\text{M}$ and $30 \text{ }\mu\text{M}$ [34]. The NiR activity of human Cygb under anaerobic conditions have also been reported. One study has reported no NiR activity for Cygb [23], whilst others have reported some NiR activity of Cygb with rates of reaction that are comparable with other globins such as Ngb and the T (tense) state of human hemoglobin ($\sim 0.12\text{-}0.14 \text{ M}^{-1}\text{s}^{-1}$) [21; 35]. The reported rate of Cygb NiR activity is an order of magnitude slower than other globins such as myoglobin and the R (relaxed) state of hemoglobin ($2\text{-}6 \text{ M}^{-1}\text{s}^{-1}$) [35]. Consequently, the proposal that Cygb may function as a NiR has not gained support as a potential function of the protein *in vivo*.

The surface exposed cysteines of human Ngb and Cygb can form intramolecular disulfide bonds and are important for the ligand binding and enzymatic properties of both proteins [3; 8; 36; 37; 38]. The effect of cysteine oxidation on the NiR activity of Ngb is limited, exerting a twofold decrease in NiR activity with reduced disulfide compared to the protein with an intramolecular disulfide [25]. The intramolecular disulfide bond of Ngb is positioned on the CD loop of the protein structure, but the intramolecular disulfide bond of Cygb spans two adjacent α helices; Cys38 on helix B and Cys83 on helix E. Cys83 is close to the heme iron distal histidine (His81), potentially affecting the distal heme pocket structure depending on the oxidation state of the cysteines. Following generation of the protein by recombinant methods, three forms of Cygb can exist depending on the oxidation state of the cysteines: (i) dimer_{S-S}, where the cysteines form an intermolecular disulfide bond, producing a homodimer (ii) monomer_{S-H}, where the cysteines are reduced, forming a monomer with free sulfhydryls and (iii)

monomer_{S-S}, where the cysteines form an intramolecular disulfide on the same protein chain. The monomer_{S-S} form of the protein behaves significantly different to the dimer_{S-S} and monomer_{S-H} forms of the protein. This includes changes in ligand binding properties [3], switching of the ligand migration pathway [36] and lipid-induced heme iron coordination changes [6; 8; 37]. We have reported that this effect on the redox chemistry of the globin is related to an alteration of the distal histidine off-rate (His_{off}) by over 600-fold in the monomer_{S-S} form of the protein compared to other forms of Cygb [3]. However, the NO consumption as a function of oxygen concentration is only marginally affected by the oxidation state of the cysteines [39].

Here we have re-examined the capacity of recombinant human Cygb to act as NiR and NOD enzymes as a function of the oxidation state of the surface exposed cysteine residues. The oxidation state of the cysteines showed no discernible effects on the NOD activity of Cygb, the reaction being complete within the dead-time of the stopped-flow instrument. However, the oxidation state of the surface cysteines affected the kinetics of the relaxation of the protein from pentacoordinate to hexacoordinate (bis-his) states, exhibiting moderately higher rates of relaxation with the free sulfhydryl over that of the monomer_{S-S} and dimer_{S-S} forms. The effect of the oxidation state of the cysteines does, however, exert a considerable effect on the NiR activity of Cygb. The second-order rate constant of the NiR activity of Cygb in the dimer_{S-S} or monomer_{S-H} states are similar to the low NiR activities previously reported for Cygb and Ngb, but the monomer_{S-S} form of the protein is over 140 times that of the dimer_{S-S} form and 50 times that of the monomer_{S-H} form. These rates are greater than those previously reported for vertebrate globins such as myoglobin and the R state of hemoglobin.

2. Materials and Methods.

2.1 Materials

Aminoleuvelinic acid and Isopropyl β -D-1-thiogalactopyranoside (IPTG) were purchased from Molekula Ltd, UK. ProlinONONOate was purchased from Cayman chemicals, UK. Luria Bertani media was purchased from Melford Laboratories Ltd, UK. Carbon monoxide was purchased from BOC gasses, UK. Tris(2-carboxyethyl)phosphine (TCEP), sodium tetraborate, sodium phosphate (disodium hydrogen orthophosphate and sodium dihydrogen orthophosphate), kanamycin sulfate, sodium imidazole, sodium nitrite and sodium chloride were purchased from Sigma-Aldrich, UK.

2.2 Recombinant human cytoglobin expression and purification

Cygb plasmid was constructed from a pET28a inducible vector with the Cygb gene added as a cassette (Origene) with an N terminal cleavable his-tag as previously described [7]. Cygb was expressed in BL21 DE3 *E.coli* cells cultured in Luria Bertani media containing 50 $\mu\text{g ml}^{-1}$ kanamycin sulfate at 37 °C, 120 rpm following transformation using heat shock method (42 °C, 90 s). Cygb production was induced when optical density of cells was 0.8-1.0 at 600 nm by addition of 500 μM of IPTG. Aminoleuvelinic acid (250 μM) and ferric citrate (50 μM) were added to facilitate heme production. The flasks were bubbled with CO for ~30 s and flasks sealed and incubated overnight at 37 °C, 100 rpm. Cells were harvested by centrifugation (4000 g, 4 °C, 25 min) and cells frozen at -20 °C for storage. Cells were defrosted and lysed by pressure homogenization using an Avestin C3 emulsiflex (15000-20000 psi, two cycles). Cell debris was removed by centrifugation (22000 g, 4 °C, 20 min) and supernatant adjusted to 20 mM sodium phosphate buffer containing 150 mM NaCl and 20 mM imidazole pH 7.4. His-tagged protein was bound to an immobilised metal affinity column (nickel IMAC, 5 ml, GE healthcare), washed with 20 mM sodium phosphate buffer containing 150 mM NaCl and 20 mM imidazole and eluted using 20 mM sodium phosphate buffer containing 500 mM imidazole. Imidazole was removed by dialysis against sodium tetraborate buffer (1 mM) with at least three

buffer exchanges. His tag was cleaved by incubation with the protease thrombin (Sigma-Aldrich, UK) overnight at room temperature with gentle mixing (5 units per mg Cygb). His-tag free Cygb was purified as before using IMAC column using 20 mM imidazole to elute the tag-free protein. Imidazole was removed by dialysis, and protein concentrated using a Whatman centrifuge concentration (3 kDa cut-off) and stored at -80 °C.

Monomeric and dimeric Cygb were separated by Sephadex G75 column (600 mm x 16 mm) using a GE Healthcare Akta Prime as previously described [3; 37; 40]. The oligomeric state of the proteins was checked by native PAGE [37]. Monomeric protein with free sulfhydryl was generated by adding TCEP (5mM) at room temperature for 1 h before removal using a Sephadex G25 column (5x1 cm, GE healthcare). A double cysteine to arginine mutation of Cygb (Cys38Arg Cys83Arg) was generated by sequential site directed mutagenesis using primers synthesised by Eurofins Scientific, UK. Mutagenesis was performed using 1.5 U of *pfu* Turbo DNA polymerase (Agilent Technologies) using the Agilent Quikchange II protocol. Sequences were confirmed by Sanger sequencing (GATC Biotech, Germany) following transformation of PCR product into XL1Blue cells and plasmid purification (Qiagen, UK).

2.3 Measurement of nitrite reductase and nitric oxide dioxygenase activity

Cygb (5 μ M) was reacted with sodium nitrite (0-20 mM) in 0.1 M sodium phosphate buffer pH 7.4 in the presence of ~5 mM sodium dithionite. Dithionite was added immediately prior to nitrite addition to minimise the slow reduction disulfide [37]. Optical spectra were recorded using an Agilent 8453 diode array spectrophotometer fitted with a multi-cell carriage and temperature control. Rate constants were determined from the time courses Cygb (416 nm-430 nm) by fitting to exponential functions using the least squares method using Microsoft Excel or Synergy KaleidaGraph.

Cygb (10 μ M) in oxygenated 0.1 M sodium phosphate buffer pH 7.4 was reduced to oxyferrous Cygb by addition of 5 mM sodium ascorbate and left for at least 15 minutes to

reduce. Complete reduction was checked spectrophotometrically using an Agilent Cary 50 spectrophotometer. Sodium phosphate buffer (pH 7.4, 0.1 M) was degassed using a glass tonometer connected to a supply of argon gas and a vacuum pump via a custom build glass tap system to allow repeated degassing cycles. The degassed buffer was transferred anaerobically to a 10 ml glass syringe. Proli-NONOate was prepared in 25 mM sodium hydroxide to a concentration of 40mM and then purged with argon gas. Proli-NONOate was transferred using a glass Hamilton syringe to the degassed buffer (10-100 μ M), under these conditions the Proli-NONOate degrades to generate two molecules of NO. The Oxyferrous Cygb was mixed rapidly with degassed buffer containing in a 1:1 ratio using an Applied photophysics SX20 stopped-flow spectrophotometer pre-cooled to 11 °C.

3. Results

3.2 Effect of disulfide oxidation state on nitrite reductase activity

The reaction of deoxyferrous Cygb with nitrite in the presence of dithionite results in the generation of NO bound ferrous Cygb (Figure 1A and 1B). Optical changes show a hypsochromic shift of the Soret peak from 428 nm to 422 nm, accompanied with decrease in the α and β peaks and an appearance of a shoulder at ~580nm. The initial and end spectra for the reaction are shown in Figure S1. All ferric and dithionite-reduced deoxyferrous spectra are essentially identical, Cygb following nitrite reductase activity are predominantly ferrous-NO. Interestingly the oxyferrous spectra have differences between the monomer_{S-S} form and other forms of the protein. This is not due to a mixture of ferric and ferrous protein as full reduction by dithionite and rapid re-oxygenation by size exclusion chromatography yields identical oxyferrous spectra. The recombinant Cygb showed complex kinetics of NiR activity with fast and slow phases. The same reaction between equine myoglobin (Mb) and nitrite showed simple mono exponential kinetics, changing from deoxyferrous Mb to ferrous nitrosyl Mb. The observed rate constant for this reaction with Mb as a function of nitrite concentration showed linear

dependence and is presented in [Figure 2](#). The second order rate constant was measured as $2.70 \pm 0.19 \text{ M}^{-1}\text{s}^{-1}$, essentially identical to the previously reported value of $2.9 \text{ M}^{-1}\text{s}^{-1}$ under similar experimental conditions [21].

Following further purification of Cygb to isolate the monomeric and dimeric states of the protein the reactions with nitrite were repeated. Dimeric Cygb has intermolecular disulfide bonds between Cys38 of one chain and Cys83 of another ($\text{dimer}_{\text{S-S}}$). $\text{Dimer}_{\text{S-S}}$ Cygb exhibited monophasic reaction kinetics ([Figure 1C](#)) that was fitted to a single exponential function to obtain an observed rate constant (k_{obs}). This rate constant changed linearly as a function of nitrite concentration to yield a second order rate constant of $0.26 \text{ M}^{-1}\text{s}^{-1} \pm 0.02 \text{ M}^{-1}\text{s}^{-1}$ ([Figure 2](#)). The monomeric protein showed biphasic reaction kinetics with fast and slow phases. Assuming identical optical changes of both forms of the monomeric protein the protein exhibiting fast reaction kinetics consisted of 86 % of the protein under study, but did show some variation depending on the batch of protein expressed. We interpret this to imply that our monomeric protein was a mixture with an internal disulfide bond ($\text{monomer}_{\text{S-S}}$) and free sulfhydryls ($\text{monomer}_{\text{S-H}}$). Incubation with dithionite slowly reduces the disulfide as shown in [Figure S2](#), where the amplitude of the fast rate of $\text{monomer}_{\text{S-S}}$ decreases as a function of time as the $\text{monomer}_{\text{S-S}}$ is converted to slower kinetics of the $\text{monomer}_{\text{S-H}}$. Under the conditions of the experiments reported in [Figures 1 and 2](#), this reduction of disulfide bond resulting from the presence of dithionite was minimal.

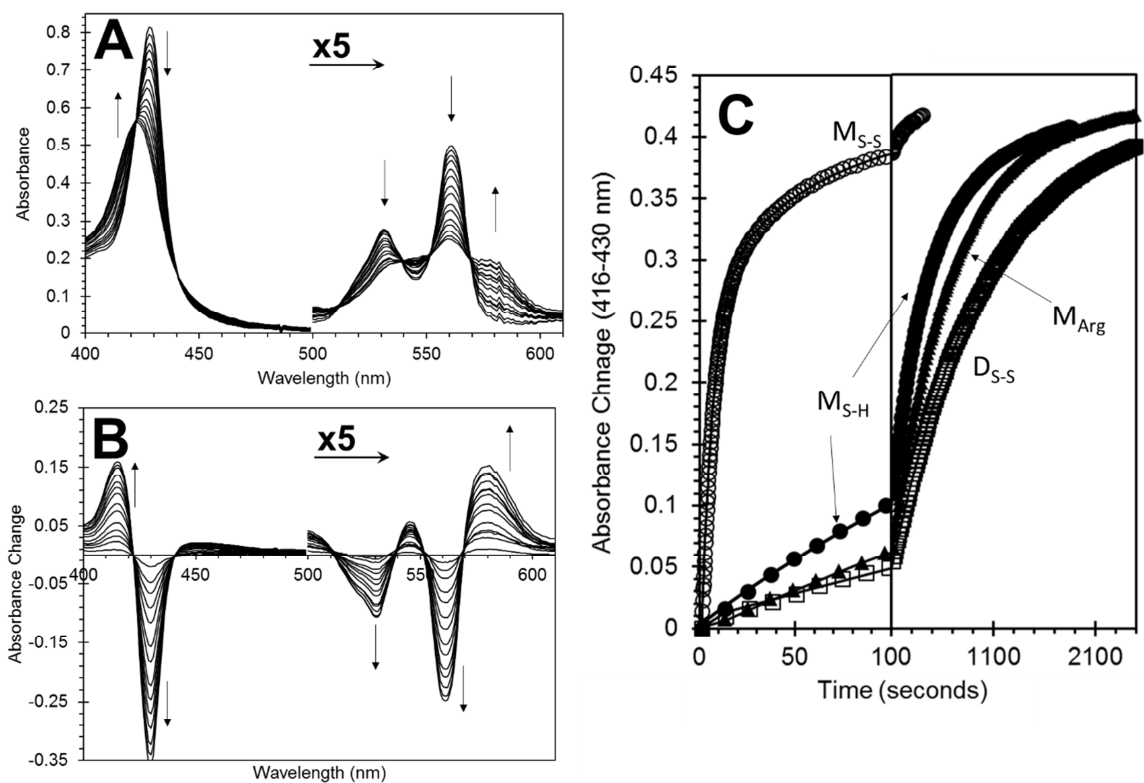


Figure 1. Nitrite-induced optical changes to deoxyferrous-Cygb. All experiments were performed in 100 mM sodium phosphate pH 7.4, all protein was made deoxyferrous by addition of ~5 mM sodium dithionite. (A) Sodium nitrite solution (5 mM) was added to deoxyferrous monomeric Cygb (5 μ M) and spectra recorded at 2 s intervals. (B) Difference spectra where the spectrum of deoxyferrous protein was removed from all subsequent spectra. (C) Example kinetic traces of ferrous-NO formation from reaction of Cygb and nitrite. Monomeric Cygb primarily consisting of intramolecular disulfide (M_{S-S} , \circ), monomeric Cygb double mutant C38R/C83R (M_{Arg} , \blacktriangle), TCEP reduced monomeric Cygb with free sulfhydryl (M_{S-H} , \bullet) and dimeric Cygb with intermolecular disulfide (\square). Solid lines represent fits to single exponential (monomer C38R/C83R and dimer) or double exponential (monomeric Cygb with intramolecular disulfide) graph is a split time base with linear time courses for 0 to 100 s and 100 to 2500 s.

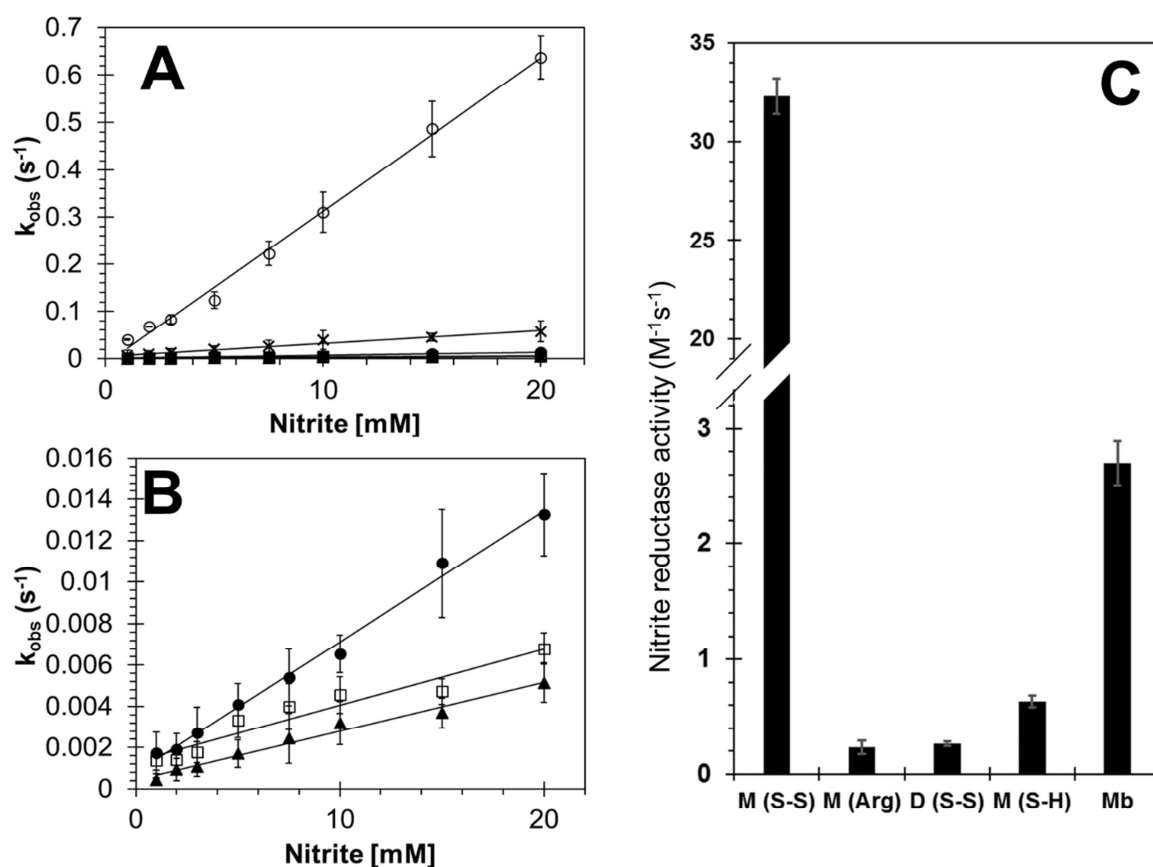


Figure 2. Nitrite reductase activities of monomeric, dimeric and C38R/C83R cytoglobin under anaerobic conditions (using ~5mM sodium dithionite). (A) Observed rate constants for the formation of ferrous-NO from the reaction of different forms of Cygb (deoxy) and nitrite. Monomeric Cygb with intramolecular disulfide (○), TCEP reduced monomeric Cygb (●) monomeric Cygb double mutant C38R/C83R (▲) and dimeric Cygb with intermolecular disulfide (□). Equine Mb is included for comparison (X) (B) Expansion of first panel to show data for double mutant and dimer. (C) Second order rate constant for the nitrite reductase activity of various forms of Cygb: M(S-S); monomer with intramolecular disulfide bond, M (Arg); monomer with no disulfide bond, D(S-S); dimer with intermolecular disulfide bond, M(S-H); TCEP reduced monomer with free sulfhydryl and Mb; myoglobin. $n=3$ for Mb, monomer with free sulfhydryl, dimer and C38R/C83R monomer, $n=4$ for Cygb with intramolecular disulfide.

The fast kinetics were interpreted to result from the monomer_{S-S} form of the protein and the slow phase from monomer_{S-H} as the slower kinetics were essentially identical to that of the TCEP reduced form of the protein and similar to the C38R/C83R mutant (Figure 1C and 2). The

kinetics of the dominant fast phase of the reaction, assigned to the monomer_{S-S} Cygb, was nitrite concentration dependent, giving a second order rate constant of $32.3 \text{ M}^{-1}\text{s}^{-1} \pm 0.90 \text{ M}^{-1}\text{s}^{-1}$. The monomer from the double cysteine mutant showed second order reaction kinetics of $0.23 \text{ M}^{-1}\text{s}^{-1} \pm 0.06 \text{ M}^{-1}\text{s}^{-1}$, essentially identical to that of the dimeric protein, in line with previously reported NiR rates for Cygb [21]. The TCEP reduced monomer_{S-H} Cygb was slightly faster than the dimer and mutant Cygb, giving a second order rate of $0.63 \text{ M}^{-1}\text{s}^{-1} \pm 0.05 \text{ M}^{-1}\text{s}^{-1}$.

3.2 Effect of disulfide on nitric oxide dioxygenase activity and heme iron coordination

The reaction of NO with oxyferrous Cygb in the monomer_{S-S} form of the protein is shown in [Figure 3 A and B](#). The reaction of the other forms of the protein exhibited identical optical changes. The initial spectrum, taken <2ms post-mixing, is typical of a mixed spin ferric Cygb with the heme iron partially hexacoordinate but primarily pentacoordinate. There was no evidence of the original pre-reaction oxyferrous Cygb, meaning that the reaction is essentially complete in the ~1.5 ms dead time of the stopped-flow, even at low NO concentration (10 μM). Therefore, the half-time of the reaction of NO with oxyferrous Cygb must be considerably below 1 ms, making the second order reaction greater than $10^8 \text{ M}^{-1}\text{s}^{-1}$, but likely less than $10^9 \text{ M}^{-1}\text{s}^{-1}$ due to diffusion rate limits. The end spectrum is that of the ferric protein with hexacoordinate heme iron. Therefore, the kinetics observed (200-400 s^{-1}) is likely that of distal histidine binding, i.e. the relaxation of the protein from pentacoordinate to hexacoordinate state following dissociation of the nitrate product. [Figure 3 C](#) shows the observed rate constants for the reaction of NO with oxyferrous Cygb at 80 μM NO. With all forms of the protein (monomer_{S-S}, monomer_{S-H} and dimer_{S-S} and C38R/C83R mutant) the rate constants of reaction are quite close, ranging from 200 to 400 s^{-1} . The observed rate constants are largely independent of NO concentration ([Figure 3 D](#)), with dimer_{S-S} slowest at $218 \pm 18 \text{ s}^{-1}$, followed by the monomer_{S-S} $241 \pm 35 \text{ s}^{-1}$, monomer_{S-H}, $320 \pm 26 \text{ s}^{-1}$ and C38R/C83R $393 \pm 47 \text{ s}^{-1}$.

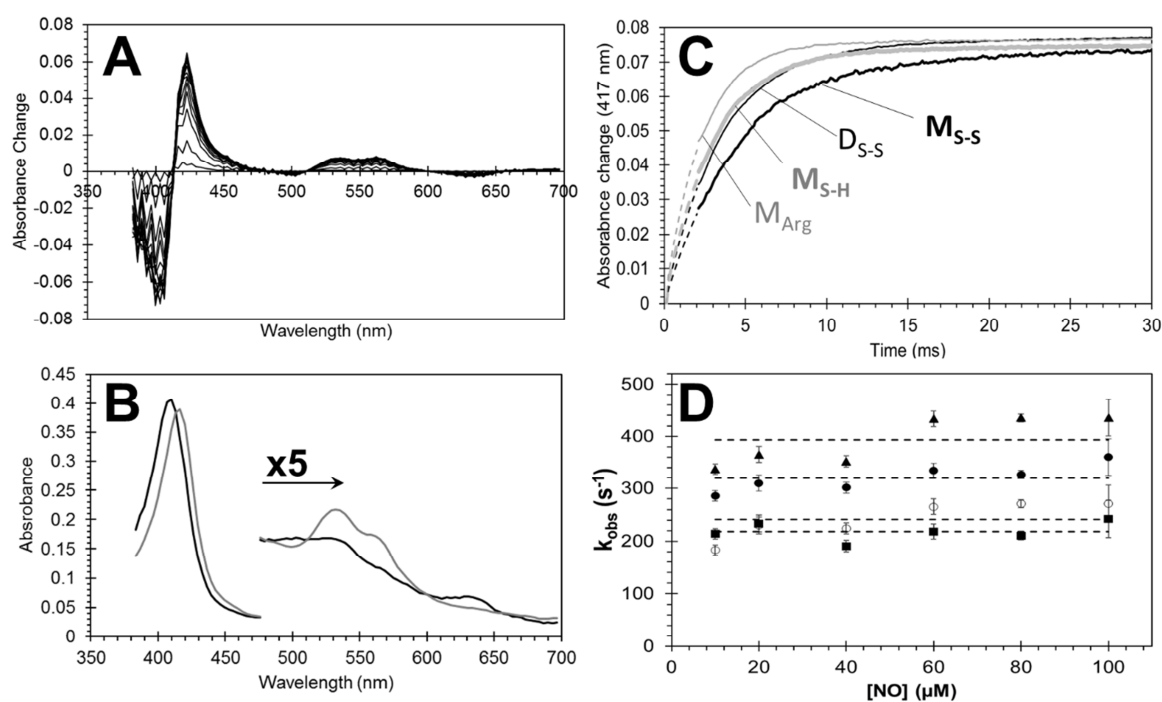


Figure 3. Optical changes following nitric oxide addition to oxyferrous monomeric, dimeric, free sulfhydryl and C38R/C83R cytoglobin. Protein concentration was 5 μM in 50 mM sodium phosphate buffer pH 7.4. (A) Optical changes of oxyferrous monomeric Cygb with intramolecular disulfide following mixing with NO (80 μM), each time point is 1.2 ms. (B) Initial and final spectra of Cygb following mixing with NO as fitted globally for a single pseudo-first order transition, initial spectrum (~ 1.5 ms following mixing, black line) is primarily that of pentacoordinate ferric Cygb. Final spectrum (50 ms following mixing, grey line) is that of hexacoordinate ferric Cygb. (C) Time course at 417 nm of monomeric Cygb with intramolecular disulfide (thick grey line), monomeric Cygb double mutant C38R/C83R (thin grey line) and dimeric Cygb with intermolecular disulfide (thin black line) and TCEP reduced Cygb (thick black line). Time courses within the deadtime of instrument (< 2 ms, dashed lines) were constructed using single exponential fits to the data. (D) Observed rates of optical changes following mixing are essentially NO concentration independent (monomer_{S-S}, \circ , dimer_{S-S} \square , TCEP reduced monomer_{S-H} \bullet and C38R/C83R mutant \blacktriangle , $n=3$).

4. Discussion

Previous reports on the NiR activity of Cygb have not taken into account different forms of the protein due to the oxidation state of the surface exposed cysteines. The study

presented here shows that the monomer_{S-S} form of the protein exhibits NiR activity far higher than that of the other forms of the protein, dimer_{S-S} and monomer_{S-H}. The published Cygb second order rate constant of NiR activity ($0.14 \text{ M}^{-1}\text{s}^{-1}$) most closely correlates with our measurement of dimer_{S-S} or monomer_{S-H} forms of the protein (0.26 and $0.63 \text{ M}^{-1}\text{s}^{-1}$ respectively); thus, it was likely a protein in the dimer_{S-S} or/and monomer_{S-H} form of the protein that was used in previous studies on NiR activity. Mutation of the cysteines slows the NiR activity further, probably due to an influence on the arginine residue on the nearby distal histidine. It is unclear whether the dimeric form of the protein has physiological relevance *in vivo*. The micromolar concentration of the protein in cells [21; 41] may yield monomeric protein rather than the dimer, but this has not been confirmed. Nonetheless, the monomeric forms of the protein, either with free sulfhydryl or intramolecular disulfide bond, are the likely form of the protein in the cytosol of cells and hence it is important to characterize the activities of both forms of the protein.

The NiR activity of the monomer_{S-S} form of Cygb is higher than that of other similar globins (Table 1). The allosteric state of human hemoglobin $\alpha_2\beta_2$ tetramer have a significant effect on the NiR activity of erythrocyte Hb, the T state showing a 50-fold lower NiR activity ($0.12 \text{ M}^{-1}\text{s}^{-1}$) than the R state Hb ($6 \text{ M}^{-1}\text{s}^{-1}$) (Table 1) [35]. This closely resembles the effect of the oxidation state of the cysteines in the monomeric protein, it too showing a 50-fold change in NiR activity. Cygb also has reported allosteric properties of O₂ binding [28], but this relates only to the dimeric protein and cannot account for the observations of the high NiR activity exhibited by the monomer_{S-S} protein. The likely explanation the intramolecular disulfide effect on NiR activity lies partially in the heme iron coordination. We have previously shown that the intramolecular disulfide enhances the distal histidine dissociation rate by over 1000-fold [3]. Furthermore, the NiR activity of Ngb is enhanced 2000-fold with distal histidine mutation to generate a 5-coordinate geometry [25]. Therefore, the more pentacoordinate-like geometry of the Cygb with intramolecular disulfide is likely to enhance NiR activity.

To our knowledge, the NiR activity of human Cygb with intramolecular disulfide is higher than that reported of any member of the hemoglobin superfamily in vertebrates to date. A recent report shows a high NiR activity of globin X from zebrafish (*Danio rerio*) at $26.7 \pm 2.0 \text{ M}^{-1}\text{s}^{-1}$ [42]. Globin X is a hexacoordinate globin that can be found in the blood of fish, amphibians and reptiles [43]. Concentrations of globin X *in vivo* are typically in the micromolar range, similar to cellular concentrations of Cygb [44], thus the oxygen carrying capacity of globin X in the blood is limited, suggesting a different role for this protein. The high NiR activity of globin X comes close to our reported value, although performed at higher temperatures, illustrating the biological relevance of ancient globins as important NiR enzymes [42]. A recent finding with zebrafish Cygb observed low rates of NiR activity with Cygb2 ($0.31 \text{ M}^{-1}\text{s}^{-1}$, 25 °C), but much high rates with Cygb1 ($14.6 \text{ M}^{-1}\text{s}^{-1}$, 25 °C) [45]. Interestingly, neither isoform of Cygb in zebrafish have the two cysteines to form intramolecular disulphides observed in human Cygb (Cygb2 has C38 only), although other cysteine residues are present but are unlikely to be in positions to form intramolecular disulfide bonds. However, the different sequence identity of Cygb1 results in a pentacoordinate protein, whereas Cygb2 is hexacoordinate [45]. This is consistent with the general observations that pentacoordinate and pentacoordinate-like globins have enhanced NiR activity in comparison to their hexacoordinate counterparts and supports our proposal that the higher NiR activity of monomer_{S-S} Cygb is, at least in part, due to the more pentacoordinate-like nature of this form of the protein.

Protein	$k_{\text{NR}} (\text{M}^{-1}\text{s}^{-1})$	Temperature (°C)	Reference
Cytoglobin _{S-S} (monomer)	32.3	25	This work
Zebrafish Cytoglobin 1	14.6 28.6	25 37	Corti et al. Nitric Oxide. 2016; 53: 22-34. [45]
Zebrafish Globin X	26.7	37	Corti et al. Proc. Natl. Acad. Sci. 2015; 113:8538-8543. [42]
Hemoglobin R state	6.0	25	Huang et al. J. Clin. Invest. 2005;115:2099–2107. [35]

Equine Myoglobin	2.9	25	Li et al. J. Biol. Chem. 2012;287:36623–36633. [21]
	2.7	25	This work
Cytoglobin _{S-H}	0.63	25	This work
Zebrafish Cytoglobin 2	0.31	25	Corti et al. Nitric Oxide. 2016; 53: 22-34. [45]
Cytoglobin _{S-S} (Dimer)	0.26	25	This work
Cytoglobin (unknown cysteine oxidation)	0.40	25	Corti et al. Nitric Oxide. 2016; 53: 22-34. [45]
	0.14		Li et al. J. Biol. Chem. 2012;287:36623–36633. [21]
	0		Peterson et al. J. Inorg. Biochem. 2008;102,1777-1782. [23]
Hemoglobin T state	0.12	25	Huang et al. J. Clin. Invest. 2005;115:2099–2107. [35]
Neuroglobin _{S-S}	0.12	25	Tiso et al. J. Biol. Chem. 2011;286:18277–18289. [25]
Neuroglobin _{S-H}	0.062	25	Tiso et al. J. Biol. Chem. 2011;286:18277–18289. [25]

Table 1. Nitrite reductase activity of various members of the hemoglobin family. All are human globins unless otherwise stated.

The observed kinetics following the NOD activity of Cygb are largely independent of NO concentration (Figure 3). This is due to the NO-independent relaxation of the pentacoordinate ferric Cygb to hexacoordinate, resulting from binding of the distal histidine (H81) to the space vacated by the nitrate product. The rate of distal histidine binding appears to be largely independent of the oxidation state of the cysteines. As one of these cysteines (C83) is close to the distal histidine one might expect some influence on histidine binding, but the influence of the cysteine appears to be minimal with only a factor of two separating the slowest and fastest rates. This is keeping with previous observations that the intramolecular disulfide only affects the histidine on-rate (k_H) by <2 fold following CO photo-dissociation [3]. The true reaction of NO with oxyferrous Cygb is faster than that observable by stopped-flow and is thus close to the

diffusion rate limit. Any effects of the oxidation state of the cysteines on this reaction must be lie between these upper and lower rate limits and is therefore limited.

Previous observations of Ngb also report an NO concentration-independent maximal rate (Table 2) with the reaction between NO and oxyferrous Ngb too quick to be observed [46]. This was interpreted as the initial species observed being ferrous-peroxynitrite, with the Soret maximum at 407-408 nm. This intermediate then decayed in a first order process to ferric Ngb consistent with a dissociation of the peroxynitrite leaving as nitrate. However, the spectra observed for the Cygb intermediate in Figure 3 is more consistent with a predominantly high spin ferric Cygb than peroxynitrite or nitrate bound ferric heme iron, with the observed rate of 200-400 s⁻¹ that of distal histidine binding. Either way, the true NOD activity of Cygb cannot be ascertained by the methods employed here, but the data does suggest that the NOD activity of Cygb is inherently faster than that measured by NO depletion kinetics that requires Cygb redox cycling [9; 39].

Protein	k _{NOD} (M ⁻¹ s ⁻¹)	Temperature	Reference
Hemoglobin N (<i>M. tuberculosis</i>)	7.5x10 ⁸	23 °C	Ouellet et al. 2002 Proc. Natl. Acad. Sci. U. S. A. 99, 5902-5907 [47]
Neuroglobin	>7x10 ⁷ 90 s ⁻¹ (maximal k) 300 s ⁻¹ (maximal k)	5 °C 5 °C 20 °C	Brunori et al. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 8483-8488 [46]
Myoglobin (Equine)	3.4x10 ⁷	20 °C	Eich et al. 1996 Biochemistry. 35, 6976-6983 [48]
Cytoglobin	3x10 ⁷ (by NO consumption) 200-400 s ⁻¹ (maximal k)*	20 °C 20 °C	Gardner et al. 2010 J. Biol. Chem. 285, 23850-23857 [9] This work.

Table 2. Nitric oxide dioxygenase activity of various members of the hemoglobin superfamily. All are human globins unless otherwise stated. * The k_{NOD} for cytoglobin measured here was distal histidine binding and did not represent true NOD activity, here we estimated at >1x10⁸ M⁻¹s⁻¹.

From the data presented here, the NiR activity of Cygb may have a more important biological role than previously understood. Although more studies are required to explore the role of NiR activity in cells, we can speculate that the NO generating activity of Cygb can be controlled through the oxidation of the surface exposed cysteines, enhancing NO production under conditions such as oxidative stress. Recently Cygb has been found to be co-localised with nNOS in various areas of the mouse brain, expressed in the cytoplasm of neurons [31] and downregulation of Cygb prevents angiotensin-mediated hypertension [33]. This illustrates the proposition that Cygb, like Ngb, is involved in NO homeostasis. Cygb has been noted to protect hypoxic tumors against oxidative stress [49; 50], particularly against the effects of cancer therapies such as chemotherapy and radiotherapy [50; 51]. It has also been previously suggested that Cygb regulates the levels of NO under hypoxic conditions has been suggested to indirectly regulate vascular tone in tissues under hypoxia by inducing vasodilation through cessation of NO scavenging, thereby alleviating the oxygen deficiency stress [49]. Our data supports these propositions, suggesting a role that can potentially unify the observed effects of the protein *in vitro* with the suggested roles of the protein *in vivo*.

Generation or exposure of cysteines to NO can promote S-nitrosylation. The S-nitrosylation of vertebrate Hb via beta-Cys93 has been reported contribute to blood pressure regulation and hence oxygen delivery [52; 53; 54; 55]. S-nitrosothiol formation in Ngb was observed following the reaction with nitrite, with considerably smaller fractions of S-nitrosylation observed with Cygb [23]. However, as no Cygb-dependent NiR activity was observed in the study, in contrast to ours and other studies, the level of S-nitrosylation as a function of cysteine oxidation, and its potential consequences on Cygb catalytic activities, may need reappraisal.

The role of Cygb in NO homeostasis is illustrated in [Figure 4](#). Cygb can, under conditions of normoxia, regulate NO through dioxygenase activity as previously suggested [49]. Under conditions of hypoxia, NO can be generated by NiR activity. However, under oxidative conditions the NiR activity of Cygb is significantly enhanced, upregulating NO generation to a point that it may be physiologically significant. Our data, together with that of others, suggests

that cysteine oxidation is crucial in modulating the redox chemistry of the protein, potentially acting as a redox switch. Under conditions of hypoxia-induced oxidative stress conditions may favour depletion of antioxidants such as glutathione and hence promotion of cysteine oxidation leading to an enhanced Cygb generation of NO in the presence of nitrite. The consequences of this for cell biochemistry is a complex issue due to the diverse NO cell signaling pathways. However, it is of note that the expression mechanism of induction of Cygb is HIF-1alpha dependent, with high specificity and sensitivity of its induction by hypoxia [56]. Ngb on the other hand, is less influenced by hypoxia.

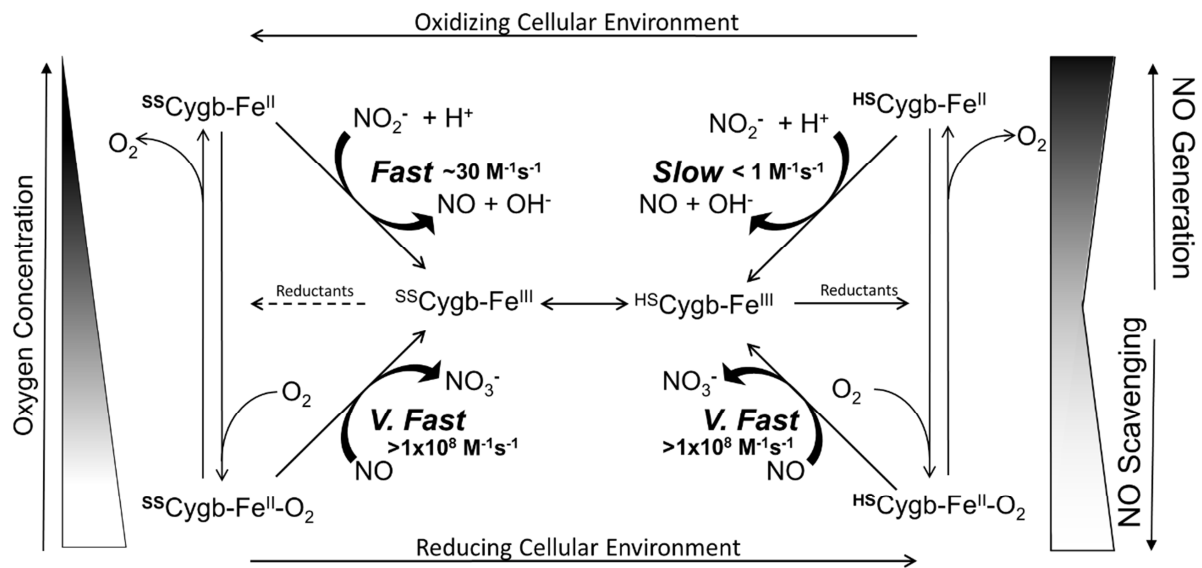


Figure 4. Cytoglobin-mediated control of NO homeostasis is controlled by the redox environment of the cell in addition to oxygen concentration. Under a normoxic cellular environment the cytoglobin sulfhydryl oxidation state does not influence the NO scavenging capability of cytoglobin. However, under hypoxic conditions the NO generating capacity of cytoglobin from nitrite is highly influenced by the oxidation state of its surface-exposed cysteine, which in turn is linked to the redox environment of the cell.

Abbreviations

The abbreviations used are: Cygb; cytoglobin, Ngb; neuroglobin, NiR nitrite reductase; nNOS; neuronal nitric oxide synthase; NOD; Nitric Oxide Dioxygenase.

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Highlights:

- Nitric oxide generation by cytoglobin nitrite reductase activity is modulated by the oxidation state of surface-exposed cysteines C38 and C83.
- With an intramolecular disulfide, cytoglobin generates nitric oxide at rates significantly greater than previously reported for other mammalian globins.
- Cysteine oxidation has no discernible effect on the rate of nitric oxide dioxygenase activity but does influence the rate of penta- to hexa- heme iron coordination following nitrate dissociation.

Supplemental section

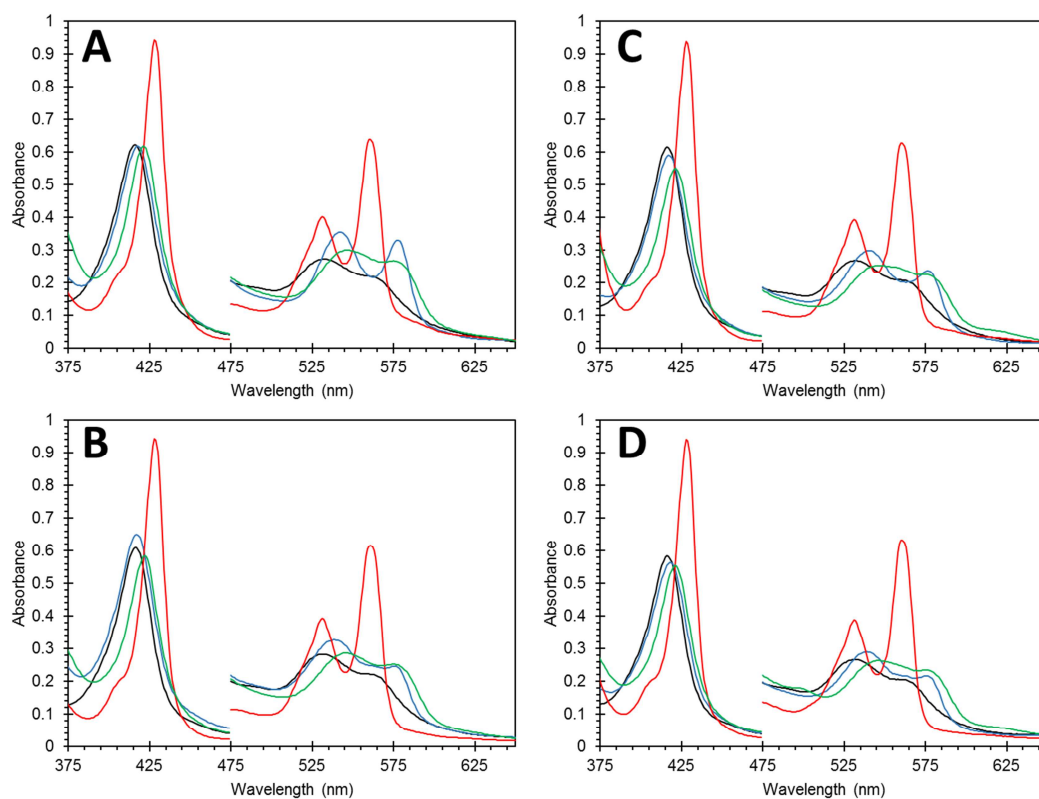


Figure S1. Optical spectra of cytoglobin monomers and dimer. Spectra of cytoglobin monomer with intramolecular disulphide (A), dimer with intermolecular disulphide (B), TCEP reduced monomer with free sulfhydryl (C) and monomer Cys38/83Arg mutant. Ferric (black line), deoxyferrous (red line), ascorbate-reduced oxyferrous (blue line) and protein following reaction with nitrite in presence of dithionite (green line).

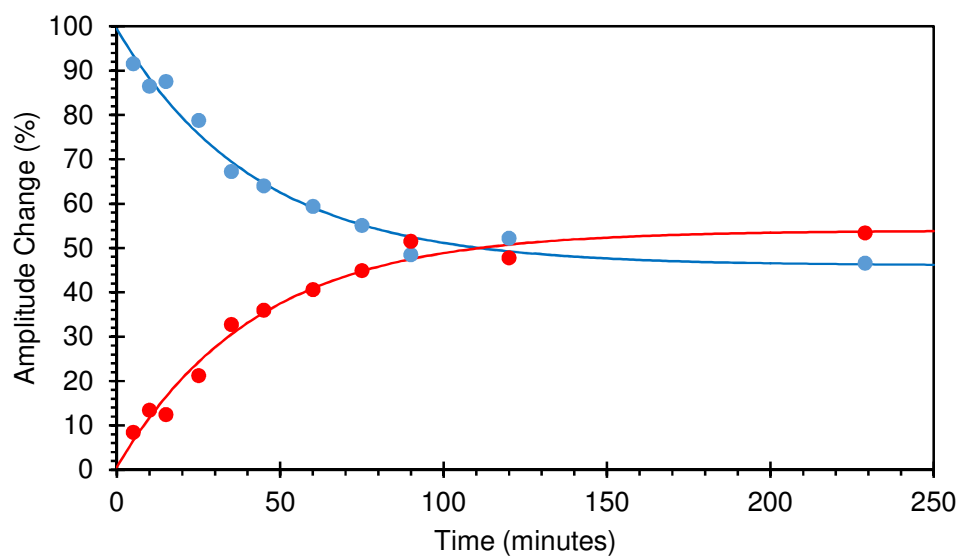


Figure S2. Effect of dithionite on Monomeric Cygb with intramolecular disulphide bond. Cytochrome (5 μM) was incubated with dithionite (5 mM) for various times and subsequently mixed with nitrite (10 mM). The changes in amplitude of the fast phase (blue line,) and slow phase (red line,) subpopulations are shown as a function of dithionite incubation time, showing the slow reduction of the disulfide bond. The amplitudes changes were each fitted with a single exponential, $k = 3.92 \times 10^{-4} \pm 5.8 \times 10^{-5} \text{ s}^{-1}$.