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Effect of temperature, pH and heme ligands on the reduction of Cygb(Fe³⁺) by ascorbate

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Abstract

Cytoglobin (Cygb) plays a role in regulating vasodilation in response to changes in local oxygen concentration by altering the rate of nitric oxide (NO) metabolism. Because the reduction of Cygb(Fe³⁺) by a reductant is the control step for Cygb-mediated NO metabolism, we examined the effects of temperature, pH, and heme ligands on the Cygb(Fe³⁺) reduction by ascorbate (Asc) under anaerobic conditions. The standard enthalpy of Cygb(Fe³⁺) reduction by Asc was determined to be 42.4 ± 3.1 kJ/mol. The rate of Cygb(Fe³⁺) reduction increased ~6% per °C when temperature varied from 35 °C to 40 °C. The yield and the rate of Cygb(Fe³⁺) reduction significantly increases with pH (2-3 times per pH unit), paralleling the formation of the Asc ion (A²⁻) and the increased stability of reduced state of heme iron at high pH values. Heme ligand cyanide (CN⁻) decreased the yield and the rate of Cygb(Fe³⁺) reduction, but ligands CO and NO allowed the process of Cygb(Fe³⁺) reduction to continue to completion. Critical information is provided for modeling and prediction of the process of Cygb-mediated NO metabolism in vessels in a range of temperature and pH values.

Keywords

Cytoglobin; Ascorbate; temperature; pH; heme ligand

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Introduction

Globins are small, respiratory proteins existing in a wide range of organisms from archaea to animals [1, 2]. They have a compact helical conformation and an ability to bind heme that allows reversible binding of gaseous diatomic molecules, including oxygen (O_2), nitric oxide (NO), and carbon monoxide (CO) [3, 4]. It is well known that hemoglobin (Hb) in blood serves as a vehicle to load the respired O_2 in the lungs and deliver it to all locations within the body [5]. Myoglobin (Mb) in skeletal and cardiac muscle plays a role in storing O_2 and facilitating O_2 diffusion [6]. Neuroglobin (Ngb) is expressed in brain neurons, the retina, and endocrine tissue [7]. Cygb is predominantly expressed in fibroblasts and related cell types in different organs and tissues [8-10].

Recent evidence has shown that Cygb is expressed not only in adventitial fibroblasts but also in vascular smooth muscle cells, where it plays a role in vascular NO metabolism [11] and functions as a nitrite reductase in the vascular wall [12, 13]. NO is metabolized in tissues in an O_2 -dependent manner. When the O_2 concentration is decreased, the extent of vascular NO metabolism is reduced [11]. The rate of O_2 -dependent NO consumption was found to be high enough to regulate the NO concentration and the diffusion distance in both the intervascular tissues and the vascular wall [14, 15]. Because NO is a potent endogenous vasodilator that dilates blood vessels in a dose-dependent manner, an increase in NO diffusion distance or an increase in the level of NO in hypoxia will induce blood vessel dilation, allowing more blood into the hypoxic tissue [16-18].

The Cygb concentration in tissue is in the micromolar range [19-23]. It has been shown that ferric Cygb (Cygb(Fe^{3+})) can be reduced by cellular reductants such as ascorbate (Asc) [23-25]. Asc concentrations are in the range of tens of micromolar in human serum [26-28], ~0.1 mM in vascular smooth muscle cells [29] in the presence of 50 μ M extracellular Asc, ~300 μ M in rat liver and brain cells [30], and 0.5-1 mM in human fibroblasts and endothelium [31, 32]. In the presence of O_2 , reduced Cygb binds O_2 to form oxyCygb that rapidly reacts with NO generating ferric Cygb. Then Cygb(Fe^{3+}) is reduced back to Cygb(Fe^{2+}) by a cellular reductant. This recycling process for Cygb can allow Cygb to efficiently metabolize NO at low micromolar Cygb concentrations. The rate of consumption of NO by Cygb is O_2 -dependent [21, 24] and limited by reducing Cygb(Fe^{3+}) to Cygb(Fe^{2+}) [21].

We have recently proposed a reaction mechanism to describe how Cygb regulates the rate of NO consumption in an O_2 -dependent manner and suggested that Cygb may act as an oxygen sensor in the vascular wall to regulate the NO decay rate and diffusion distance in response to changes in O_2 concentration [24]. We can see from this reaction mechanism that the rate of NO metabolism by Cygb is largely affected by the rate of Cygb reduction by Asc, the key control step in the process of Cygb-mediated NO metabolism. In this study, we further examine how the reduction of Cygb(Fe^{3+}) by Asc is affected by pH, temperature, and ligands of heme Fe.

Experimental Methods

Reduction of Cygb(Fe^{3+}) by ascorbate under different conditions

The expression and purification process of Cygb was described in our prior reports [24, 25]. The reduction of Cygb(Fe^{3+}) by Asc was performed under anaerobic conditions and monitored using a Cary 50 UV/Vis spectrophotometer by scanning from 350 nm to 650 nm or by fixing the wavelength at the absorbance peak of the corresponding absorption bands to measure the changes in absorbance with time. Cellular Asc concentrations can reach the low-millimolar range [31, 32]. The range of Asc used in our in vitro experiments this study was from 0.1 mM up to 44.4 mM. Using the high Asc concentrations (10 mM - 44.4 mM) in the experiments was necessary for accurately measuring the reaction equilibrium constants of Cygb reduction by Asc [25]. The buffer solution (pH 4, 7, 8, 9, or 10) in the cuvette was deaerated by bubbling argon gas into the solution for at least 15 minutes through a tube. Before adding 3 μM deaerated Cygb(Fe^{3+}) into the cuvette, the tube was moved to the gas phase above the solution surface and the argon gas flow was maintained in the cuvette during the experiments. Five to ten minutes after the injection of Cygb(Fe^{3+}) into the solution, varying concentrations of deaerated Asc were added into the chamber to start the reduction. The temperature of the cuvette was controlled at 25°C, 30°C, 35°C, 37°C, or 40°C with a temperature controller attached to the spectrophotometer. In some experiments, a certain concentration of NO or NaCN was injected into the cuvette during Cygb reduction by Asc to examine the effect of NO or NaCN on the concentration of the reaction product Cygb(Fe^{2+}) at pH 7.0 and temperature of 37°C. NO solution was prepared as described [14, 33].

Results

Reduction of Cygb(Fe^{3+}) and formation of nitrosyl-Cygb (Cygb(Fe^{2+} -NO))

Figure 1 demonstrates some typical UV-Vis spectra of the Cygb species examined in this study. The Soret band of Cygb(Fe^{3+}) (416 nm) shifts to that of the Cygb(Fe^{2+}) (428nm) after adding dithionite or Asc into the solution, indicating a reduction of Cygb(Fe^{3+}) to Cygb(Fe^{2+}). Cygb(Fe^{3+}) was completely reduced by 100 μM dithionite, but only partially reduced by Asc even with Asc concentrations as high as 40 mM [25]. From Fig. 1A we can see that 10 mM Asc only partially reduced Cygb(Fe^{3+}). However, it is interesting to note that in the presence of CO, the final product Cygb(Fe^{2+} -CO) (421 nm) is almost the same (spectral curves overlap) regardless of the complete reduction of Cygb(Fe^{3+}) by the excess of dithionite or partial reduction of Cygb(Fe^{3+}) by Asc (Fig. 1B). The same result was observed when adding 15 μM NO rather than CO to both dithionite- and ascorbate-reduced Cygb(Fe^{3+}) (Fig. 1C). The Soret band of Cygb(Fe^{2+} -NO) has a peak at 410 nm with a relatively lower absorbance than the Soret band of Cygb(Fe^{2+} -CO) at 421 nm. The decrease of Cygb(Fe^{3+}) concentration or the increase of Cygb(Fe^{2+}) concentration with each Asc addition shows a stair-step pattern and both reach an equilibrium concentration after each addition of Asc [25].

Effects of temperature and pH on the equilibrium constant of Cygb(Fe³⁺) reduction by Asc

Spectroscopic measurements were performed to examine the effect of temperature on the equilibrium constant of Cygb(Fe³⁺) reduction by Asc. It was observed that at a constant [Asc] and pH 7.4, the ratio [Cygb(Fe²⁺)]/[Cygb(Fe³⁺)]₀ is nearly linear with temperature in the temperature range examined (25 °C – 40 °C) (Fig. 2A). We have previously demonstrated that the reduction of Cygb(Fe³⁺) to Cygb(Fe²⁺) by Asc consists of two reaction steps:

and the ratio of [Cygb(Fe²⁺)]/[Cygb(Fe³⁺)]₀ (or y/c_0) can be expressed as the following equation [25]:

$$\frac{y}{c_0} = \frac{K_1 K_2 [\text{Asc}]}{1 + K_1 (1 + K_2) [\text{Asc}]} \quad (2)$$

where K_1 and K_2 are equilibrium constants of the reaction steps one and two, respectively; and [A] is Asc concentration. The measured value of $K_1(1+K_2)$ is 0.075 mM⁻¹, so Eq. (2) can be simplified as below when [Asc] ≥ 2 mM:

$$\frac{y}{c_0} = K_1 K_2 [\text{Asc}] \text{ or } K_1 K_2 = \frac{y}{c_0 [\text{Asc}]} \quad (3)$$

Where $K_1 K_2$ is the overall equilibrium constant of the reduction of Cygb(Fe³⁺) to Cygb(Fe²⁺). By plotting ln(K) vs. 1/T at [Asc]=1.4×10⁻³ M, we got a straight line (Fig. 2B). From the slope of the straight line, we determined the value of the standard state enthalpy change, H° which is 42.4±3.1 kJ/mol, for the reduction of Cygb(Fe³⁺) to Cygb(Fe²⁺) by Asc. In Fig. 2C, we demonstrate two typical experimental curves of Cygb(Fe³⁺) reduction by 1 mM Asc at 35 °C and 40 °C. The initial slope of the two kinetic curves after 1 mM Asc is injected into the solution (designated by the arrow) at 40 °C (k_{40C}) is slightly greater than at 35 °C (k_{35C}) with a ratio of initial slopes k_{40C}/k_{35C} of 1.34 ± 0.11 (n=3), indicating that the rate of Cygb(Fe³⁺) reduction by Asc increased 34% when temperature varied from 35 °C to 40 °C or changed ~6%/°C in the temperature range between 35 °C and 40 °C. The plateau of the kinetic curve measured at 40 °C ($P_{40C} = 0.096 \pm 0.012$, n=3) is slightly higher than that at 35 °C ($P_{35C} = 0.083 \pm 0.032$, n=3).

The ratio of [Cygb(Fe²⁺)]/[Cygb(Fe³⁺)]₀ increased with pH at a given Asc concentration and temperature (37 °C) (Fig. 3A). The kinetic curves of Cygb(Fe²⁺) formation (Fig. 3B) show that the plateau of these curves increases by a factor of ~2.3 when pH increases by 1 unit ($P_{pH6} = 0.027 \pm 0.001$, $P_{pH7} = 0.068 \pm 0.004$, $P_{pH8} = 0.134 \pm 0.022$, n=3). Similarly, the rising slope of the curves after 1 mM Asc was injected (designated by the arrow) into the reaction solution increases by a factor of ~2.8 when pH increases by 1 unit.

Effect of NaCN and NO on the Reduction of Cygb(Fe³⁺) by Asc

Since the cyanide ion (CN⁻) can bind to Fe³⁺ as a ligand, we further examined if NaCN could affect the reduction of Cygb³⁺ by Asc. We measured the formation of Cygb(Fe²⁺)

from Cygb(Fe³⁺) reduction by different Asc concentrations in the presence and absence of 5 mM NaCN by recording absorbance change vs. time at the wavelength 428 nm (Fig. 4A). The kinetic curves of Cygb(Fe²⁺) formation from the reduction of Cygb(Fe³⁺) by 1 mM Asc in the presence of 5 mM NaCN or in the absence of NaCN are demonstrated in Fig. 4B. The results show that the plateau of the curves ($[\text{Cygb(Fe}^{2+})]/[\text{Cygb(Fe}^{3+})]_0$) measured in the absence of NaCN ($P_{\text{CN}} = 0.065 \pm 0.003$) increases by a factor of ~2.2 compared to those in the presence of 5 mM NaCN ($P_{+\text{CN}} = 0.029 \pm 0.002$). Similarly, the rising slope of the curves after 1 mM Asc was injected into the reaction solution measured in the absence of NaCN increases by a factor of ~2.7 compared to those in the presence of 5 mM NaCN. Thus, NaCN not only decreases overall Cygb(Fe²⁺) formation, but also decreases the rate of Cygb(Fe²⁺) formation. Because the yield of the product of Cygb(Fe³⁺) reduction by Asc, Cygb(Fe²⁺), increases with Asc concentration, the Cygb(Fe³⁺) concentration must decrease as Asc concentration increases (Fig. 5, measured at 416 nm). To examine the effect of NO on the reduction of Cygb(Fe³⁺) by Asc, we measured the kinetic curves of Cygb(Fe²⁺-NO) formation by adding 10 μM NO into the solution after the reduction of Cygb(Fe³⁺) by Asc reached equilibrium. It was observed that the absorbance at 416 nm, which is proportional to $[\text{Cygb(Fe}^{3+})]/[\text{Cygb(Fe}^{3+})]_0$, decreased to different levels after 0.5 mM and 5 mM Asc are added, but the absorbance increased to the same level (the formation of Cygb(Fe²⁺-NO)) after 10 μM NO was injected into the solution.

Discussion

Spectroscopy of the pure protein species Cygb(Fe³⁺), Cygb(Fe²⁺), Cygb(Fe²⁺-CO), Cygb(Fe²⁺-NO), and the mixture of Cygb(Fe²⁺) and Cygb(Fe³⁺) in the presence of Asc under anaerobic conditions enables determination of the concentrations that the different Cygb species form in solution. From Eq. (3) and the relationship between the equilibrium constant and temperature, we determined the standard enthalpy, ΔH° , for the overall reduction of Cygb(Fe³⁺) to Cygb(Fe²⁺) by Asc. The positive ΔH° indicates that this reaction is an endothermic reaction. If the equilibrium constant at a given temperature is known, one can easily calculate the equilibrium constant at other temperatures using the determined ΔH° . It is notable that both the product of Cygb(Fe²⁺) formation and the rate of Cygb(Fe³⁺) reduction only slightly increase when temperature increases from 35 °C to 40 °C (Fig. 2C). It has been suggested that Asc binds to Cygb at a site near a heme propionate prior to Cygb(Fe³⁺) reduction [23, 25]. It is likely that increasing temperature may increase the rate constant of Cygb(Fe³⁺) reduction but decrease the binding affinity of Asc (see Eq. (1)), resulting in a small increase in the rate of Cygb(Fe²⁺) reduction when temperature changes from 35 °C to 40 °C.

The reduction product Cygb(Fe²⁺) and the rate of Cygb(Fe²⁺) formation also increases with pH (Fig. 3). It was shown that Mb(Fe³⁺) can be reduced by anions of ascorbate in the following manner [34]:

Both HA^- and A^{2-} can reduce $Mb(Fe^{3+})$ to $Mb(Fe^{2+})$, but the rate constant of $Mb(Fe^{3+})$ reduction by A^{2-} is nearly 4 orders of magnitude greater than that by HA^- . Our results suggest that the reduction of $Cygb(Fe^{3+})$ by Asc follows a similar reaction mechanism as shown in Eqs. (4)-(7). The values of pK_1 and pK_2 are 4.03 and 11.35 at 25 °C, respectively. At pH 4, the main species of Asc are H_2A and HA^- , and their concentrations are close to each other (nearly a 1:1 ratio). At pH 6.0, the main species of Asc in the solution is HA^- and the total concentration of H_2A and A^{2-} is only 1%. With increasing pH, the A^{2-} concentration gradually increases. The experimental curves in Fig. 3B suggest that the increased reduction product $Cygb(Fe^{2+})$ is likely caused by the increased A^{2-} concentration. It has demonstrated that increasing pH will stabilize the reduced state of the heme iron as shown in the case of alkaline cystathionine beta-synthase [35] and pseudoperoxidase from *Leishmania major* (LmPP) [36]. This stabilization may also contribute to the increase in the rate constant of $Cygb(Fe^{3+})$ reduction by Asc with increasing pH. Our results show that increasing pH by 1 pH unit between pH 6 and pH 8 can change the rate of $Cygb(Fe^{3+})$ reduction by a factor of ~2.8. Because $Cygb(Fe^{3+})$ reduction is the control step in the NO dioxygenation by Cygb, it implies that pH can significantly affect the rate of NO metabolism by Cygb in biological systems.

CO, NO and CN^- can bind to heme Fe as a ligand, but CN^- mainly binds on heme Fe^{3+} to form $Fe^{3+}-CN^-$, while CO and NO bind to heme Fe^{2+} to form $Fe^{2+}-CO$ and $Fe^{2+}-NO$ [13, 37, 38]. The formation of $Fe^{3+}-CN^-$ inhibits $Cygb(Fe^{3+})$ reduction (Fig. 4). In contrast, the formation of $Fe^{2+}-CO$ and $Fe^{2+}-NO$ greatly reduces the free $Cygb(Fe^{2+})$ concentration. As a result, the rate of the reverse reaction from Fe^{2+} to Fe^{3+} greatly decreases so that almost all $Cygb(Fe^{3+})$ can be reduced to $Cygb(Fe^{2+})$ and stored in the form of $Cygb(Fe^{2+}-NO)$. As shown in Fig. 5, the yield of the reduction of $Cygb(Fe^{3+})$ by 5 mM Asc is significantly greater than that by 0.5 mM Asc. However, after excess NO is added into the reaction system, the final product from the reduction of $Cygb(Fe^{3+})$ by Asc in the presence of NO is the same for both Asc concentrations. This result, consistent with those shown in Figures 1B and 1C, indicates that the formation of $Cygb(Fe^{2+}-NO)$ pushes the reaction continuously toward the right side in the reaction equations (1). Thus, Cygb not only metabolizes NO in the presence of O_2 and reductants [11, 21, 23-25], but it also functions as a repository for the storage of excess NO in the absence or lack of O_2 during hypoxia in tissues. In the vascular wall, the formation of $Cygb(Fe^{2+}-NO)$ largely reduces the rate of NO metabolism by Cygb, leading to an increase in the vascular NO concentration to dilate blood vessels and allowing more oxygenated blood to flow into the hypoxic tissue.

In conclusion, the reduction reaction of Cygb(Fe^{3+}) by Asc is reversible and the reduction is an endothermic reaction. Temperature changes near body temperature between 35 °C and 40 °C have a relatively small effect on the rate of Cygb(Fe^{3+}) reduction and the yield of the Cygb(Fe^{2+}) product. However, changes in pH values from 6 to 8 can significantly increase the rate of Cygb(Fe^{3+}) reduction by a factor of ~2.8 per pH unit. The ligand of heme Fe^{3+} , CN^- , reduces both the rate and the yield of Cygb(Fe^{3+}) reduction. However, in the presence of NO, Cygb(Fe^{3+}) can be completely reduced to Cygb(Fe^{2+}) due to the formation of Cygb(Fe^{2+} -NO). The formation of Cygb(Fe^{2+} -NO) may protect NO activity both in hypoxia and in the re-oxygenation process of tissues by slowing down the rate of NO metabolism. These results provide helpful information for quantitative or qualitative understanding of how the rate of NO metabolism can be regulated by temperature, pH and ligation of the heme iron.

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Highlights

- The reduction of Cygb by ascorbate is examined under different conditions.
- Temperature slightly increases the rate of Cygb reduction between 35 °C and 40 °C.
- pH significantly increases the rate of Cygb reduction between pH6 and pH8.
- Heme Fe³⁺ and Fe²⁺ ligands have opposite effect on the Cygb reduction.

Fig. 1.

Spectral curves of Cygb species. All measurements were performed under anaerobic conditions at 37 °C. (A) Cygb(Fe^{3+}), Cygb(Fe^{3+}) + 10 mM Asc, and Cygb(Fe^{3+}) + excess of dithionite. (B) Cygb(Fe^{3+}), Cygb(Fe^{3+}) + 10 mM Asc + 15 μM CO, and Cygb(Fe^{3+}) + excess of dithionite + 15 μM CO. (C) Cygb(Fe^{3+}), Cygb(Fe^{3+}) + 10 mM Asc + 15 μM NO, and Cygb(Fe^{3+}) + excess of dithionite + 15 μM NO.

Fig. 2.

Effect of temperature on the equilibrium constant and the rate of $\text{Cygb(Fe}^{3+})$ reduction by Asc at pH 7.4. A: The product ($\text{Cygb(Fe}^{2+})$) increases as temperature increases at different Asc concentrations ([Asc] increasing from bottom to top are: 0.1, 0.4, 1.4, 4.4, 14.4 and 44.4 mM). B: The plot of the logarithm of the equilibrium constant of the overall reduction of $\text{Cygb(Fe}^{3+})$ by Asc ([Asc]=1.4 mM) vs. $1/T$. The value of ΔH° determined from the slope of the fitted line is 42.4 ± 3.1 kJ/mol. C: Typical experimental curves of $\text{Cygb(Fe}^{3+})$ reduction by 1 mM Asc at two different temperatures (35 °C and 40 °C).

Fig. 3.

Effect of pH on the yield and the rate of $\text{Cygb(Fe}^{3+}\text{)}$ reduction by Asc. A: $[\text{Cygb(Fe}^{2+}\text{)}]/[\text{Cygb(Fe}^{3+}\text{)}]_0$ increases as pH increases at different Asc concentrations (for the curves from the bottom to top of the graph: [Asc] is 0.1, 0.4, 1.4, 4.4, 14.4 and 44.4 mM). B: The kinetic curves of $\text{Cygb(Fe}^{2+}\text{)}$ formation at pH 6, pH 7 and pH 8. Asc (1 mM) was added at the time designated by the arrow.

Fig. 4.

Effect of NaCN on reduction of Cygb(Fe³⁺) by Asc. A: The ratio $[\text{Cygb}(\text{Fe}^{2+})]/[\text{Cygb}(\text{Fe}^{3+})]_0$ in the presence of 5 mM CN⁻ is less than in the absence of CN⁻ at different Asc concentrations. Asc was added into the solution at the time designated by the vertical lines. B: Kinetic curves of Cygb(Fe²⁺) formation from the reduction of Cygb(Fe³⁺) by 1 mM Asc in the presence or absence of 5 mM NaCN.

Fig. 5.

Effect of NO on the Cygb(Fe^{3+}) reduction by 0.5 mM and 5 mM Asc. Measurements were performed at 37 °C and pH 7. Asc and NO were injected into the solution at the time designated by the arrows.