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The reaction of neuroglobin with potential redox protein partners cytochrome b_5 and cytochrome c

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Abstract Previously identified, potentially neuroprotective reactions of neuroglobin require the existence of yet unknown redox partners. We show here that the reduction of ferric neuroglobin by cytochrome b_5 is relatively slow ($k = 6 \times 10^2\ M^{-1}\ s^{-1}$ at pH 7.0) and thus is unlikely to be of physiological significance. In contrast, the reaction between ferrous neuroglobin and ferric cytochrome c is very rapid ($k = 2 \times 10^7\ M^{-1}\ s^{-1}$) with an apparent overall equilibrium constant of 1 μM. Based on this data we propose that ferrous neuroglobin may well play a role in preventing apoptosis.

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Keywords: Neuroglobin; Cytochrome *c*; Cytochrome *b*5; Apoptosis

1. Introduction

Neuroglobin (Ngb) is a member of the globin protein family pre-dating the origins of myoglobin and hemoglobin [1] and belongs to the class of hexa-coordinate globins. The protein is found in all orders of vertebrates and in mammals is predominantly found in the neural tissues of the brain [2], in neurons with a high metabolic activity [3,4] and in particular in the retina [5].

The finding that mammalian Ngb binds O_2 led to speculation that the physiological role of the protein might be to act as a neuronal O_2 reserve analogous to myoglobin [2]. However, this function would be possible in vivo only if a reductase activity exists that is able to keep the heme in the ferrous form [6] as Ngb rapidly autoxidizes [7,9]. The relatively low intracellular concentration [2,5,8] and low in vitro oxygen affinity of Ngb [9] would also appear to rule out a major function in O_2 binding and delivery [8].

Ngb provides protection to neuronal cells during hypoxia and ischaemia [10,11]. In vitro kinetic studies have shown that Ngb in the oxygenated or nitrosylated ferrous form undergoes rapid redox reactions to scavenge either nitrogen monoxide (NO) or peroxynitrite, respectively [12–14] potentially fulfilling a neuroprotective role. In either reaction the end product is ferric Ngb. However, for Ngb to play a significant role in cell protection via NO or peroxynitrite scavenging, it must undergo a number of redox cycles, as it is present in cells at a relatively low concentration. This redox cycling would require a yet unidentified redox partner to reduce the ferric Ngb product back to the reactive ferrous form. A likely candidate for this reductant is cytochrome b_5 (Cyt b_5), which has numerous roles [15], including the reduction of the ferric forms of hemoglobin in red blood cells [16,17].

It is also possible that Ngb is able to enhance neuronal survival in ischaemic episodes [11] by an entirely different mechanism, such as suppression of apoptosis. Apoptosis is initiated by several factors and one major pathway of apoptosis involves the release of cytochrome *c* (Cyt *c*) from the mitochondrion. Cyt *c* binds to a partner protein, Apaf-1 in the presence of dATP and activates procaspase 9 in an assembled apoptosome [18,19]. This interaction specifically requires ferric Cyt *c* [20,21]. Immunohistochemical studies on retinal sections have shown that Ngb is present at high levels in the vicinity of mitochondria [5,22]. It is thus conceivable that Ngb may interfere with the apoptotic interaction of ferric Cyt *c* and Apaf-1 by rapidly converting Cyt *c* to the ferrous state.

In light of their possible physiological significance we report here a study of the redox reaction between ferrous Cyt b_5 and ferric Ngb and of the redox reaction of ferrous Ngb with ferric Cyt *c*.

2. Materials and methods

Recombinant murine Ngb was expressed and purified as previously described [7]. Cyt b_5 was prepared from fresh bovine liver as described earlier [17]. Bovine Cyt *c* was obtained from Sigma and purified further to remove aggregates. Steady state absorbance spectra and slow reaction time courses were measured using a Shimadzu UV PC 2500 spectrophotometer. The molar extinction coefficients ($mM^{-1}cm^{-1}$) used were $\varepsilon = 26.6$ at 555.6 nm for ferrous Cyt b_5 [23]; $\varepsilon = 27.6$ at 550 nm for ferrous Cyt *c* [24]; $\varepsilon = 10.7$ at 532 nm and $\varepsilon = 11.2$ at 561 nm for the ferric and CO form of Ngb, respectively [9].

Redox reactions were studied at room temperature under second-order conditions by mixing together appropriate aliquots of the ferrous Cyt b_5 and ferric Ngb (2.5-6.6 μM), in the presence of 1 mM CO, in a sealed spectrophotometer cell previously thoroughly purged with pure nitrogen gas. Absorbance was monitored over time at 416 nm. The equilibrium reaction between Ngb and Cyt *c* was investigated at room temperature by monitoring changes in absorbance in the wavelength range 500–600 nm during anaerobic titrations of ferric Cyt *c* with ferrous Ngb. The apparent equilibrium constant for the reaction was obtained by fitting a single hyperbolic function to the absorbance

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Abbreviations: Ngb, neuroglobin; Cyt b_5, cytochrome b_5; Cyt *c*, cytochrome *c*; NO, nitrogen monoxide

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data at 550 nm plotted against total Ngb concentration. Rapid kinetics were measured at 416.6, 545 and 557.5 nm under anaerobic conditions at 25 °C using an Applied Photophysics SX stopped flow apparatus coupled to an Applied Photophysics Π*. Control experiments made under pseudo-first order conditions using a Cyt *c* concentration of 20 μM after mixing. Reaction time courses were analysed by non-linear least squares fitting of either second-order or pseudo-first-order kinetic functions as appropriate, employing Table Curve 2D (Jandel Scientific, CA, USA). Kinetic traces obtained under second-order conditions were best fitted according to the equations $y = a + b(1 - 1/(1 + bcx))$ and $y = a + b/(1 + bcx)$ for a formation or decay, respectively. Rate constants ($M^{-1}\,s^{-1}$) were obtained from $k = bc/[A_0]$ where $[A_0]$ is the initial concentration of each protein, given that the half time is $t_{1/2} = 1/bc$ and $t_{1/2} = 1/k[A_0]$. Traces obtained under pseudo-first-order conditions were best fitted according to a monoexponential function $y = a + b(1 - e^{-cx})$.

3. Results and discussion

3.1. Ferrous Cyt b_5–ferric Ngb

Ngb and Cyt b_5 are both bis-His heme complexes and so any redox exchange is essentially "iso-spectral" (Fig. 1). These difficulties can be circumvented by taking advantage of the fact that ferrous Ngb can relatively rapidly dissociate the distal histidine [7,25] and bind CO at high rate ($k = 72 \times 10^6\ M^{-1}\,s^{-1}$) and with high affinity ($K = 0.18 \times 10^{-9}$ M) [7], yielding a CO complex with a distinct spectral signature (Fig. 1). In contrast Cyt b_5 does not bind CO. Thus when the reaction between ferrous Cyt b_5 and ferric Ngb is carried out in the presence of a large excess of CO the ferrous Ngb product is rapidly and quantitatively trapped as the CO complex. We have studied the redox reaction between Cyt b_5 and Ngb within the context of the reaction scheme (1):

$$\mathrm{Cyt}\,b_5^{2+} + {}_6\mathrm{Ngb}^{3+} \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} \mathrm{Cyt}\,b_5^{3+} + {}_6\mathrm{Ngb}^{2+} \underset{k_{-2}}{\overset{k_2}{\leftrightarrows}} {}_5\mathrm{Ngb}^{2+} + \mathrm{CO} \underset{k_{-3}}{\overset{k_3}{\leftrightarrows}} \mathrm{Ngb}^{2+}\text{–CO} \quad (1)$$

where $_6$Ngb and $_5$Ngb refer to hexa- and penta-coordinate Ngb, respectively. The CO binding rate (k_3) and dissociation rate (k_{-3}) and the rate of association (k_{-2}) and dissociation (k_2) of the distal histidine residue in Ngb have been previously reported [7,25].

The reaction of ferrous Cyt b_5 with ferric Ngb was followed at 416 nm, a wavelength at which the redox reaction is isosbestic but at which the CO complex of ferrous Ngb has a considerable absorption (Fig. 1). All reactions between ferric Ngb and ferrous Cyt b_5 demonstrated second-order behavior with observed rates far slower than the rate of CO binding to Ngb (Fig. 2). This indicates that the observed time courses reflect CO binding, which is rate limited by ferrous Ngb production as described in reaction scheme (1).

The reaction showed a noticeable dependence of rate on pH changing from 1200 $M^{-1}\,s^{-1}$ at pH 6.0 to ~400 $M^{-1}\,s^{-1}$ at pH 7.5 (data not shown).

Previous studies on the reactivity of Ngb have been focused almost exclusively on the reactions with gaseous ligands. Each of these reactions, whether the scavenging of NO by oxygenated Ngb [13] or scavenging of peroxynitrite by nitrosylated Ngb [14], or even the supply of O_2 to mitochondria [5], requires an electron donor such as Cyt b_5 to reduce the end-product ferric Ngb back to the active ferrous form. The reported redox potential of Cyt b_5 as compared to Ngb is adequate for the reduction process but would not provide a large driving force (88 mV) [7,26].

Our kinetic results show that the reaction of ferrous Cyt b_5 with Ngb is relatively slow compared to the rate constant for

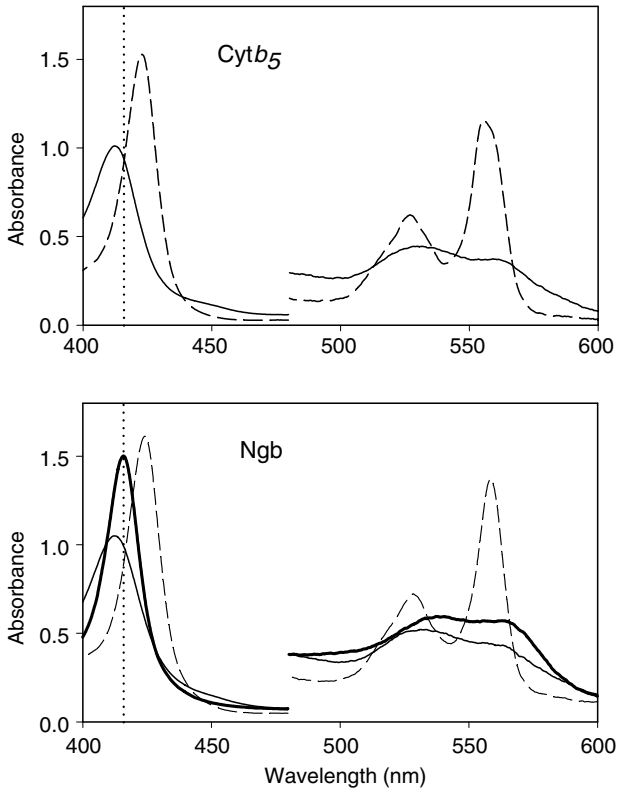

Fig. 1. Absorbance spectra of Cyt b_5 (upper panel) and Ngb (lower panel) in the ferric (continuous lines) and ferrous (discontinuous lines) forms. The Fe(II)CO spectrum of Ngb is indicated (thick line). The dotted line at 416 nm (isosbestic for ferrous and ferric Cyt b_5) shows the wavelength used for kinetic measurements. The 480–600 nm region is enlarged 5-fold.

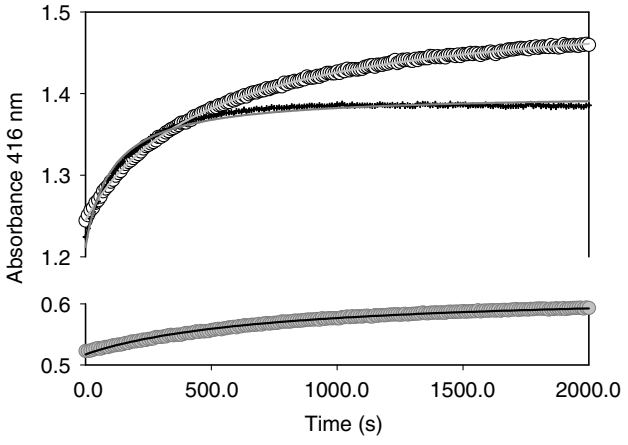

Fig. 2. Representative kinetic traces at 416 nm for the reaction between ferric Ngb and ferrous Cyt b_5 in the presence of 1 mM CO at a 1:1 Ngb:Cyt b_5 ratio at the following μM concentrations for each protein and pH values: Open circles, 5.5 μM heme, pH 7.5; crosshairs, 5.4 μM heme, pH 6.0; grey circles, 2.4 μM heme, pH 7.5. Lines indicate the fitting of the data according to second-order kinetic functions as described in the text. The buffer was 0.04 M bis Tris-propane.

its known physiologically relevant reaction with hemoglobin, which has a rate of 5×10^4 M^{-1} s^{-1} [27]. Although we cannot rule out a possible role for Cyt b_5 in the reduction of Ngb in vivo, its overall low rate suggests that this reaction is likely to be of little significance in the protection of cells against hypoxic ischaemic injury.

3.2. Ferric Cyt c–ferrous Ngb

The spectra of ferrous Ngb and ferric Cyt *c* are sufficiently different that their redox exchange reaction yields useful optical density changes across most of the visible and Soret spectral regions (Fig. 3). When ferric Cyt *c* was titrated, under anaerobic conditions, with aliquots of ferrous Ngb a rapid reaction occurred within the few seconds required for injection of Ngb and mixing of the contents of the cuvette. Successive spectra show the conversion of ferric to ferrous Cyt *c* after addition of each aliquot of ferrous Ngb (Fig. 4A). The titration followed at 550 nm showed that the reaction was associated with an apparent equilibrium constant of approximately 1 μM (Fig. 4B). Assuming a redox potential for Cyt *c* of 255 mV (NHE) [30,31], the equilibrium constant expected from the Nernst equation for a simple electron exchange between these partners with a redox potential difference of approximately 160 mV would be $\sim 10^{-3}$ M, suggesting that a reasonable degree of interaction exists between these proteins.

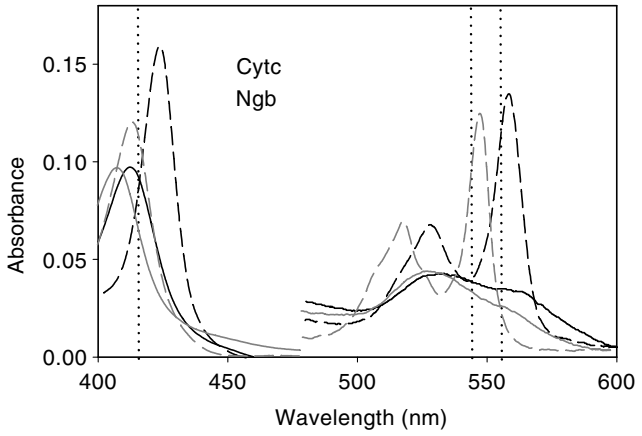

Fig. 3. Absorbance spectra of the ferric (continuous lines) and ferrous (discontinuous lines) forms of Cyt *c* (grey) and Ngb (black) measured at 1 μM protein concentration. Dotted lines show the isosbestic points for ferrous and ferric Ngb (416.6 and 545 nm) and ferrous and ferric Cyt *c* (557.5 nm) used in stopped-flow kinetic experiments. The 480–600 nm region is enlarged 5-fold.

Time courses for the reaction monitored at 416.6 nm, 545 nm (isosbestic points for the Ngb redox reaction, see Fig. 3) and 557.5 nm (isosbestic point for the Cyt *c* redox reaction, see Fig. 3) indicated rapid electron transfer from ferrous Ngb to ferric Cyt *c* (Fig. 5). The concentration dependence of the reaction obtained under both second-order and pseudo-first order conditions yielded a second-order rate constant for the reaction of 2.0×10^7 M^{-1} s^{-1} close to the fastest known inter-protein redox reaction rate constants, for example that between Cyt *c* and cytochrome *c* oxidase [31].

During ischaemic episodes cells can enter into an apoptotic pathway via the partial release into the cytoplasm of mitochondrial Cyt *c*, which is a required component of the caspase-cascade activating apoptosome [18,28,29]. It has previously been noted that Ngb exists in relatively high concentrations in cells which have an unusually high oxidative metabolic flux [3–5,22] and thus may be in increased danger of Cyt *c* leakage from mitochondria. Some years ago Zhivotovsky et al. showed that intracellular injection of Cyt *c* can initiate apoptosis [32] and the effect of the redox status of the intracellular Cyt *c* on the activation of apoptosis was later studied by Hampton et al. [33] who suggested that both ferrous and ferric Cyt *c* might be active. More recent studies have indicated that the redox status of Cyt *c* released from mitochondria [31] acts as a fail-safe mechanism in the regulation of programmed cell death [34] and suggest that ferrous Cyt *c* is inactive in the initiation of apoptosis [20,21], although apo-Cyt *c* also interferes with apoptosis in vitro [35]. In the highly metabolically active neuronal cells of the retina, where Ngb has been found at local concentrations as high as 100 μM [5] the very fast redox reaction between Ngb and Cyt *c* could well be of physiological significance.

Our findings thus suggest a mechanism whereby, in highly metabolically active cells, Ngb could reduce the small amounts of Cyt *c* leaking from damaged mitochondria and thus prevent unwanted initiation of the apoptotic process. Obviously, in situations in which apoptosis was the appropriate response to some change in the cellular situation then the larger amount

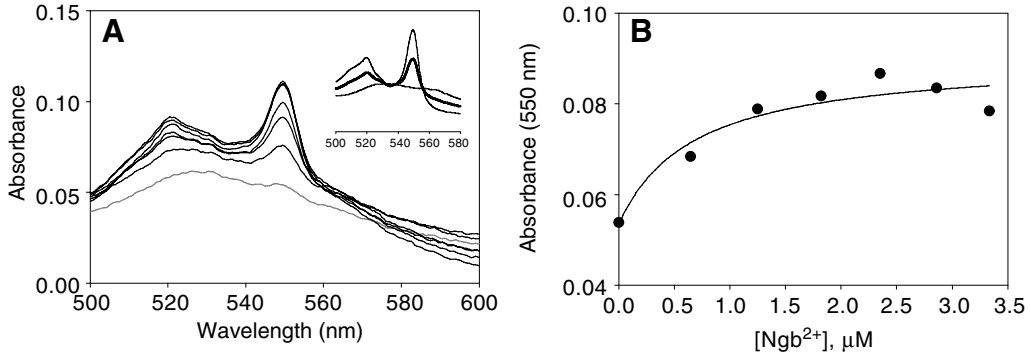

Fig. 4. (A) Changes in the absorbance spectrum of ferric Cyt *c* (1.5 ml, 4.6 μM heme, grey) upon serial additions of ferrous Ngb (20 μM heme) under anaerobic conditions. For reference the inset shows the absorbance spectra of ferrous Cyt *c*, ferric Ngb and of an equimolar mixture of these proteins (thick line). (B) Absorbance at 550 nm as a function of the concentration of added ferrous Ngb, as shown in (A). Data are corrected for the spectral contributions of ferric Ngb after each addition. The solid line shows the hyperbolic fit to the data used to derive the apparent equilibrium constant for the reaction. Experiments were performed in 0.05 M bis Tris-propane buffer at pH 7.4.

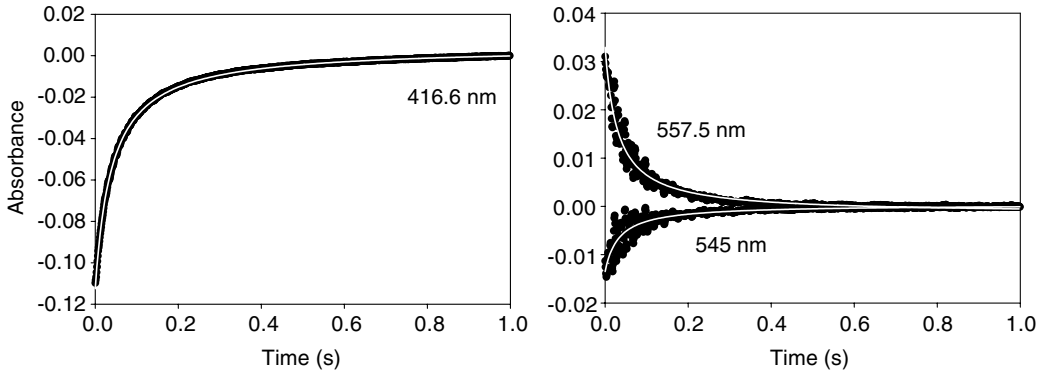

Fig. 5. Representative stopped flow reaction time courses for the anaerobic reduction of ferric Cyt *c* by ferrous Ngb measured at the indicated wavelengths as described in the text. Reactions were performed at pH 7.4 in 0.05 M bis Tris-propane. Concentration of each protein was 4 μM after mixing. The white lines indicate the fitting of the data (closed circles) according to second-order kinetic function as described in the text.

of released Cyt *c* would overwhelm the redox capability of Ngb and apoptosis would proceed. It should be noted that such a mechanism does not imply the existence of redox cycles and of a reductase activity for Ngb, but only requires that Ngb is at least in part in the ferrous (unliganded) state under normal in vivo conditions. Recent investigations of the oxygen affinity of Ngb, coupled with a likely cellular concentration of oxygen of ~1 Torr, suggest that in vivo more than 88% of Ngb is likely to be present in the cell in the ferrous deoxy form [8,9]. A plausible but previously unconsidered biological role for Ngb, at least in neurons and retinal cells might thus be to intercede in and control any inappropriate activation of apoptosis by rapidly reducing the released Cyt *c*. Further studies on the possible role of this reaction in the interception of the initial event in apoptosis are underway to test the hypothesis presented here.

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