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Failure of apoptosis-inducing factor to act as neuroglobin reductase

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ABSTRACT

Neuroglobin (Ngb) is a hexacoordinate globin expressed in the nervous system of vertebrates, where it protects neurons against hypoxia. Ferrous Ngb has been proposed to favor cell survival by scavenging NO and/or reducing cytochrome *c* released into the cytosol during hypoxic stress. Both catalytic functions require an as yet unidentified Ngb-reductase activity. Such an activity was detected both in tissue homogenates of human brain and liver and in *Escherichia coli* extracts. Since NADH:flavorubredoxin oxidoreductase from *E. coli*, that was shown to reduce ferric Ngb, shares sequence similarity with the human apoptosis-inducing factor (AIF), AIF has been proposed by us as a candidate Ngb reductase. In this study, we tested this hypothesis and show that the Ngb-reductase activity of recombinant human AIF is negligible and hence incompatible with such a physiological function.

Introduction

Neuroglobin (Ngb) is a member of the superfamily of vertebrate globins that is expressed at micromolar concentration in several areas of the CNS and even more abundantly in the retina [1,2]. Ngb has been suggested to participate in oxygen transport and/or sensing, as well as in the defence against radical species [3,4]. Ngb acts as a potent neuroprotectant, and its overexpression improves recovery after experimental stroke [5,6].

In vitro Ngb efficiently reduces cytochrome *c* (Cyt-c; [7]) and scavenges NO when Ngb is in its O_2-bound form [8]. Under hypoxic conditions, the increase in NO concentration has a number of detrimental consequences such as blockade of the respiratory chain, mitochondrial membrane depolarization and production of highly reactive species such as peroxynitrite, thereby eventually causing cell death [9]. Mitochondrial insults are associated with the release into the cytosol of Cyt-c, which, in its oxidized form, potently activates caspases and thus triggers apoptotic cell death [10]. Ngb could negatively regulate cell death by limiting the NO concentration and by reducing oxidized cytoplasmic Cyt-c. These catalytic activities may explain the neuroprotective action of Ngb under conditions of hypoxia and ischemia *in vivo* [4,11].

Both Cyt-c reduction and NO scavenging cause the oxidation of ferrous Ngb (Ngb(FeII)) to ferric Ngb (Ngb(FeIII)). Since only Ngb(FeII) is competent for O_2 binding and catalysis, Ngb can only act as a neuroprotectant if it is continuously reduced by a reductase that maintains redox cycling of the protein. Accordingly, an NAD(P)H-dependent Ngb-reductase activity has been detected in tissue homogenates of human brain and liver [12], but the enzyme(s) responsible for this activity has not been identified yet. *Escherichia coli* extracts also contain an Ngb-reducing system that has been identified as the NADH:flavorubredoxin oxidoreductase (FlRd-red) [13]. FlRd-red is the redox partner of flavorubredoxin, an enzyme implicated in the anaerobic detoxification of NO in *E. coli* [14,15]. Blast analysis of the sequence of *E. coli* FlRd-red against the human genome yielded, as the first hit, the FAD-binding domain of the mitochondrial apoptosis-inducing factor (AIF; 21% identical residues, 42% homologous residues and *E*-value = $2e^{-12}$; [13]), the main caspase-independent effector of cell death [16]. In healthy cells, AIF is

Abbreviations: Ngb, neuroglobin; Cyt-c, cytochrome *c*; Ngb(FeIII), ferric neuroglobin; Ngb(FeII), ferrous neuroglobin; FlRd-red, *Escherichia coli* NADH:flavorubredoxin oxidoreductase; AIF, apoptosis-inducing factor; NCtag-AIF, AIF carrying both N- and C-terminal histidine tags; Ctag-AIF, AIF carrying only a C-terminal histidine tag.

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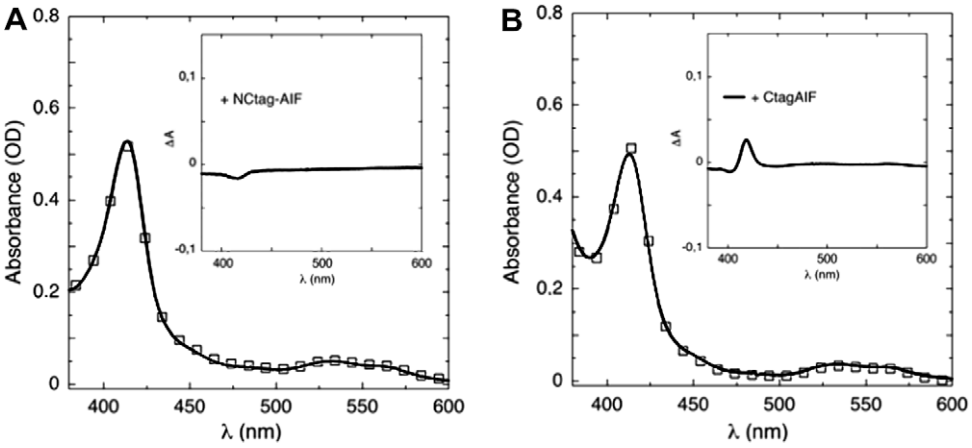

Fig. 1. Ngb reduction assay in the presence of catalytic amounts of AIF. In the presence of 100 μM NADH, no significant reduction of ferric Ngb (4.6 μM) was observed after incubation with 350 nM NCtag-AIF (A) or Ctag-AIF (B). Ngb spectra collected after 30 min of anaerobic incubation (squares) are compared with the spectrum of ferric Ngb (solid line). Insets: difference of the spectra depicted in the main panels.

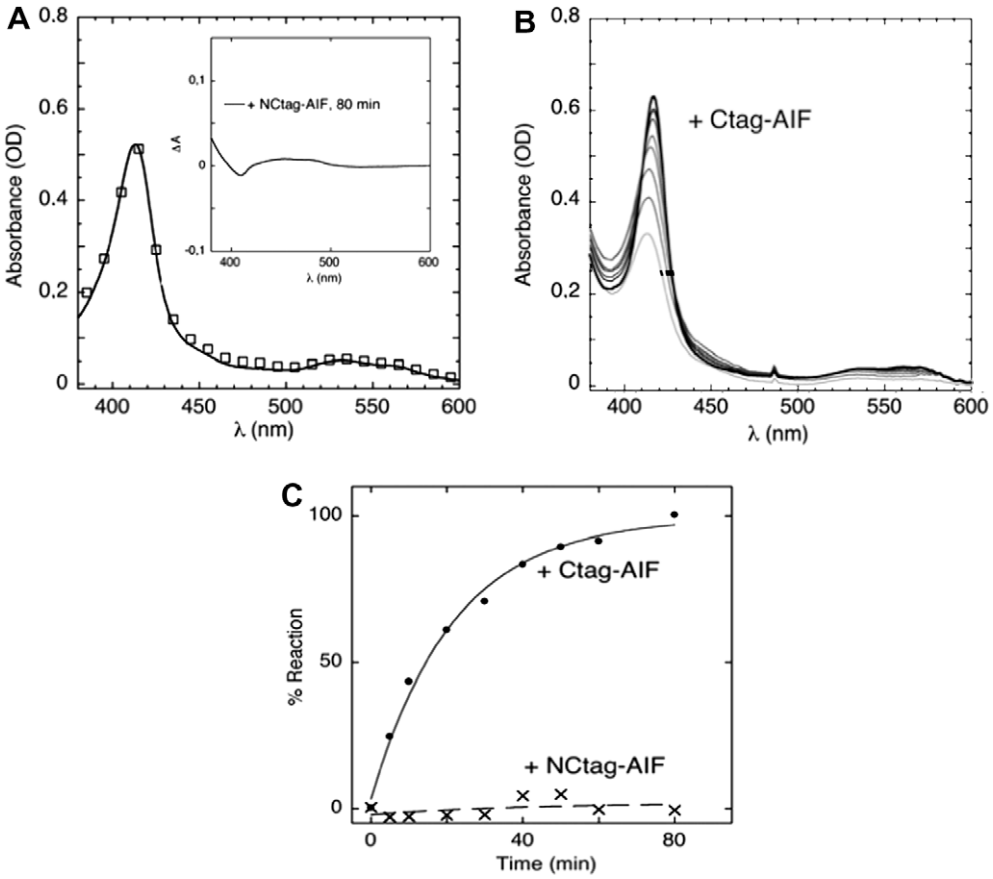

Fig. 2. Ngb reduction assay using reduced AIF in excess. Ferric Ngb, 5 μM (A) or 3 μM (B), was anaerobically incubated with 18 μM NCtag-AIF (A) or Ctag-AIF (B), pre-reduced by NADH, under CO atmosphere. After 80 min incubation, Ngb was not significantly reduced by NCtag-AIF, whereas a slow reduction occurred in the presence of Ctag-AIF. Inset: absorption changes after 80 min incubation. (C) Ngb reduction time courses, as monitored at 418 nm.

localized in the mitochondrial intermembrane space. Upon permeabilization of the outer mitochondrial membrane, AIF translocates first to the cytosol and then to the nucleus where it participates in chromatin condensation and DNA degradation [16]. However, AIF has also neuroprotective functions, and mice lacking AIF expression due to a hypomorphic mutation exhibit in the brain an increased level of cell death that causes neurodegeneration [17]. Based on its sequence similarity with FlRd-red, we hypothesized that cytoplasmic AIF might be able to reduce Ngb [13] and thereby maintain Ngb redox cycling, favoring neuron survival under hypoxia. Here, we show that *in vitro* AIF fails to efficiently reduce Ngb(FeIII) and therefore conclude that the hypothesis outlined above is untenable.

Materials and methods

Protein expression and purification. Mouse Ngb was purified as previously described [13]. Human Δ1–120 AIF was cloned into the expression vector pET28-b and expressed in *E. coli* strain Rosetta BL21(DE3) pLysS. The protein with histidine tags at both the N- and C-terminus (NCtag-AIF) was purified according to [18]. When specified, the N-terminal histidine tag was cleaved off by treatment with bovine thrombin. In this case, we will refer to the protein as Ctag-AIF (AIF carrying only a C-terminal histidine tag).

Neuroglobin reduction assay. Ngb reduction assays were carried out in 0.1 M K/phosphate buffer, pH 7.0, 20 μM EDTA, in the presence of 100–200 μM NADH; anaerobic conditions were obtained by extensively purging the buffer with N_2 gas. An aliquot of 1.5 ml reaction mixture, containing NADH, AIF and Ngb, was placed into a gas-tight cuvette and the process followed with an HP8453 UV–Vis spectrophotometer, collecting spectra in the UV–Vis range (200–650 nm) every 20 s for the first 3 min, then every 5 min.

NADH-oxidase activity of AIF. The NADH-oxidase activity of NCtag-AIF was measured spectrophotometrically. The assay was carried out at 25 °C in a total volume of 1.0 ml air-equilibrated buffer (50 mM Tris–HCl, pH 8) containing 200 μM NADH. The reaction was initiated by addition of AIF at a final concentration of 2 μM and, then, followed by monitoring the absorption decrease at 340 nm for 25 min.

Results and discussion

Upon induction of an apoptotic stimulus, AIF migrates from mitochondria to the nucleus and induces chromatin condensation [18]. We proposed that AIF present in the cytosol might intercept and reduce ferric Ngb [13]. Therefore, we tested if Ngb(FeIII) is reduced by recombinant AIF *in vitro*. Initially, we performed the Ngb(FeIII) reduction experiments following the same protocol successfully used with *E. coli* FlRd-red [13]. When catalytic amounts of recombinant AIF protein were added to a reaction mixture containing Ngb(FeIII) and 100 μM NADH under anaerobic conditions, no significant spectral changes occurred within 30 min (Fig. 1A). Since Churbanova and Sevrioukova [19] demonstrated that the redox properties of AIF are affected by the presence of N- or C-terminal His-tags, we compared two different preparations of recombinant AIF that were purified based on the presence of N-terminal and/or C-terminal His-tags. Both these AIF constructs were similarly deficient in their capacity to reduce Ngb(FeIII) *in vitro* (Fig. 1B).

Next, the putative existence of electron transfer was evaluated under anaerobic conditions, by mixing ferric Ngb with an excess of AIF that had been pre-reduced with NADH. Small amounts of contaminant O_2 might interfere with the apparent reduction time course, because O_2 binds to Ngb(FeII) and causes rapid protein autoxidation [20]. Therefore, in the experiment reported in Fig. 2, the sample was pre-equilibrated with 1 atm CO (≡1 mM), which binds with high affinity to Ngb(FeII) and thus competes with O_2 binding and re-oxidation of Ngb. When pre-reduced NCtag-AIF (which possesses two His-tags at both the C- and N-terminus) was employed, no detectable reduction of Ngb was observed (Fig. 2A); when an excess of pre-reduced Ctag-AIF (which lacks the N-terminal His-tag) was employed, a change in the Soret maximum from 413 nm (Ngb(FeIII)) to 417 nm (Ngb(FeII–CO)) was detected (Fig. 2B), although the process was too slow ($t_{½} \sim 15$ min, Fig. 2C) to be physiologically meaningful.

Since NCtag-AIF was totally ineffective in the aforementioned reaction, we verified its NADH-oxidase activity (see Materials and methods). The resulting k_{cat} for NADH was 0.025 min^{-1}, showing that NCtag-AIF was redox active towards NADH and O_2, though ~6–10 times less than the preparation employed by Miramar et al. [21]. It should be noticed that the experiment in Fig. 2 was carried out using pre-reduced AIF in excess over Ngb (4- to 6-fold), and therefore Ngb reduction could not be rate-limited by electron transfer from NADH to AIF.

From these results, we conclude that AIF can reduce Ngb(FeIII) in the presence of NADH but only at a very low rate, indicating that AIF is not the redox-active enzyme that recycles oxidized Ngb to Ngb(FeII) (Fig. 2). Thus, in spite of the intriguing sequence similarity between human AIF and *E. coli* FlRd-red, it appears that the Ngb-reductase activity detected in liver and brain homogenates cannot be attributed to AIF, at least based on the results obtained with the protein constructs herein tested. In conclusions, the hypothesis that we previously proposed [13] is falsified by the present data. Future studies will hopefully discover the molecular identity of the physiologically relevant Ngb(FeIII) reductase.

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