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Neuroglobin: Enzymatic reduction and oxygen affinity

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Abstract

Neuroglobin (Ngb) is a hexacoordinate globin expressed in the nervous system of vertebrates, involved in neuroprotection. O_2 equilibrium measurements on mouse Ngb yielded significantly different P_{50} values, ranging from ~2 torr to ~10 torr. By a kinetic approach minimizing the effects of protein autoxidation, we measured $P_{50} = 2.2$ torr at 20 °C. As predicted from the structure, O_2 binds to the Y44D Ngb mutant more quickly ($k = 2.2\ s^{-1}$ vs $0.15\ s^{-1}$) and with slightly higher affinity ($P_{50} = 1.3$ torr) than wild-type. In addition, we introduced a novel reduction protocol for metNgb based on NADH:flavorubredoxin oxidoreductase (FlRd-red) from *Escherichia coli*, a candidate for the Ngb reducing activity recently identified in *E. coli* extracts. Interestingly, *E. coli* FlRd-red shares sequence similarity with the FAD-binding domain of the human apoptosis-inducing factor, a finding which may have unexpected significance with reference to the mechanism of neuroprotection by Ngb.

Keywords: Hexacoordinate globin; Enzymatic reduction; Rapid kinetics; Oxygen affinity; Mutagenesis; Neuroprotection

Neuroglobin (Ngb) is a heme protein discovered by Burmester et al. [1] which belongs to the extended globin family and in vertebrates is expressed in the brain at a fairly low concentration (~μM) compared for instance to myoglobin in red muscles (~0.2 mM). The interest in Ngb raised considerably after the discovery that (i) its expression in neurons is up-regulated under hypoxia and (ii) its overexpression reduces the extent of ischemic damage after experimental stroke in rats [2–4]. The mechanism underlying this Ngb-mediated neuroprotection is still unclear [5–13]. It seems to us unlikely that Ngb is primarily an O_2 reservoir (or transporter), because of its fairly low average tissue concentration. Alternative functions that have been discussed group into the following categories: (i) signaling of hypoxia; (ii) detoxification of O_2- or NO-derived reactive species; (iii) NADH oxidase to sustain anaerobic glycolysis. Wakasugi and coworkers [14–16] proposed Ngb to be a signal transducer, based on the evidence that metNgb (but not NgbCO) is a guanine-nucleotide dissociation inhibitor (GDI), which binds to the GDP-bound state of the α-subunit of the heterotrimeric $G_{\alpha\beta\gamma}$-protein.

The 3D structure of metNgb (from man and mice) has the typical globin fold [17,18], but it is peculiar because: (i) the ferric and ferrous heme iron are both hexacoordinate, with the distal His(E7)64 and the proximal His(F8)96 directly bound to the metal ion, and (ii) binding of CO to ferrous Ngb is associated to a large conformational change involving a sliding motion of the heme and a moderate shift in the position of helix F and loops CD and EF [19,20]. Endogenous hexacoordination implies that binding of O_2 or other external ligands (such as CO or NO) to the heme iron can only occur upon rupture of the 6th coordination bond with His(E7)64, as shown by extensive kinetic data obtained by stopped flow and laser photolysis [21–27].

The O_2 affinity of mouse Ngb was initially reported by Burmester and coworkers [1] to be fairly high ($P_{50} \approx 2$ torr). However Fago et al. [28] in a study involving also the characterization of the effects of pH and temperature, found a $P_{50} > 10$ torr for the murine protein (at pH ≈ 7.1, $T = 25$ °C). A source of complication is that $NgbO_2$ is quite unstable, yielding fairly rapidly metNgb [21,28]. Up to date the reason for the discrepancy in the

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reported values of the O_2 affinity of Ngb and the mechanism underlying the rapid autoxidation are unknown. In an attempt to resolve this discrepancy, we resorted to a kinetic approach to estimate the O_2 affinity using time resolved optical spectroscopy. This kinetic assay was extended to the mutant Y44D because examination of the structure indicates that, in addition to His(E7)64, stabilization of the hexacoordinate state may depend also on electrostatic interactions involving Lys(E10)67, Tyr(CD3)44 and one of the heme propionates (Fig. 1). We therefore assumed that this network may oppose the ligand-linked sliding of the heme [19,20] and thus formation of the pentacoordinate state competent for O_2 binding; this hypothesis was also based on the observation that the heme sliding seen in NgbCO is associated to a shift in the CD loop position, increasing the distances between Lys(E10)67 and Tyr(CD3)44 and the heme propionate.

A quantitative kinetic assay for O_2-binding demands full reduction of Ngb under controlled experimental conditions without using dithionite. Therefore, we set up a novel protocol for the reduction of metNgb using NADH:flavorubredoxin oxidoreductase (FlRd-red) from *Escherichia coli* [29], in the presence of NADH as the electron donor. It so happened that a very recent paper [30] reported the presence in *E. coli* extracts of a NADH-dependent Ngb reductase system, not yet identified; based on preliminary experiments [30], a similar enzyme may be present also in mammalian brain and liver. Data reported below suggest that a good candidate for the Ngb reductase activity present in the *E. coli* extracts may be FlRd-red, which we discovered to be quite efficient. This is of some pathophysiological significance since it was proposed [27] that $NgbO_2$ acts as a scavenger of NO, yielding rapidly metNgb and nitrate; this reaction would have the double effect of (i) quenching overproduction of endogenous NO by NOS (which is overexpressed under hypoxic stress), and (ii) yielding metNgb which is the only state capable of acting as a GDI (see above). Clearly, for this mechanism to be effective in the brain, metNgb must be efficiently re-reduced to sustain a quasi-enzymatic redox activity. We therefore propose that the relevant system is a flavin containing enzyme.

Materials and methods

Cloning, expression, and purification of recombinant Ngb. The synthetic gene for mouse Ngb (mutant C55S/C120S) was cloned in pET14b vector and expressed as N-terminal His_6-tagged protein in *E. coli* BL21(DE3)-pLysS cells. Cells were grown at 37 °C in TB medium and induced with 1 mM IPTG, in the presence of 1 mM 5-deoxyaminolevulinic acid and CO. After 23 h at 25 °C cells were harvested, resuspended in 100 mM Hepes/Na buffer, pH 7.4, 300 mM NaCl, and 30 mM imidazole and lysed by sonication. After centrifugation, the lysate was applied to a Ni–NTA column and the protein eluted with an imidazole gradient up to 300 mM. The His-tag was removed by treatment with 5 U/mg bovine thrombin for 16 h at RT. Ngb was finally purified by anionic exchange chromatography, concentrated and stored at −80 °C. Concentration of Ngb in the deoxy state was assessed spectrophotometrically using $\varepsilon_{528nm} = 16.2\ mM^{-1}\ cm^{-1}$ and $\varepsilon_{558nm} = 28.9\ mM^{-1}\ cm^{-1}$ [28]. *E. coli* FlRd-red was purified according to [29]. Experiments were carried out in 0.1 M K/phosphate buffer, pH 7.0, 20 μM EDTA.

Optical measurements. Absorption spectra were collected using an HP8453 spectrophotometer with a 1 cm light path. Stopped-flow experi-

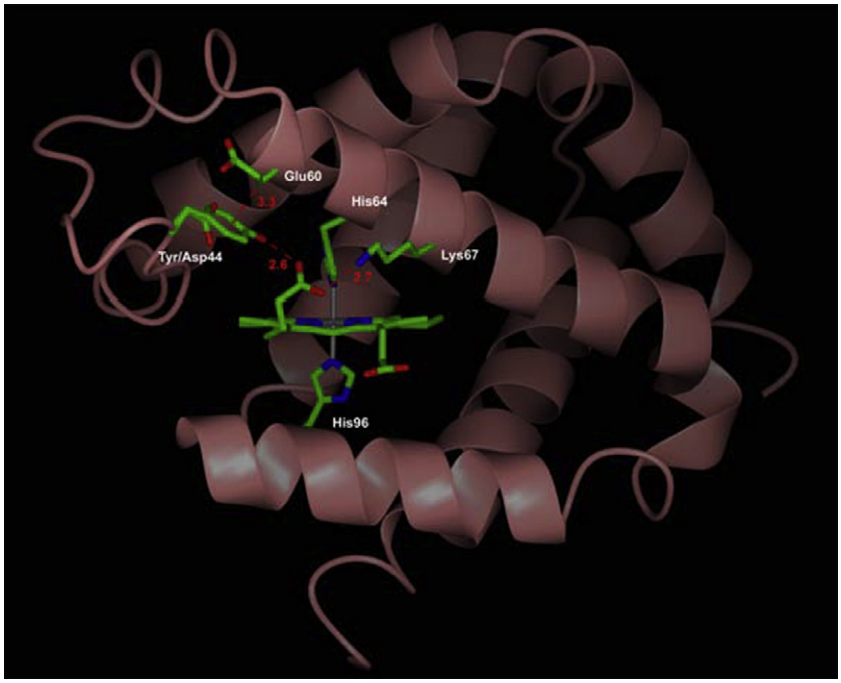

Fig. 1. The structure of metNgb. Cartoon representation of the mouse metNgb structure showing the interactions established by Tyr(CD3)44 with the heme proprionate on one hand, and with Glu(E3)60 at the N-term of helix E on the other. These contacts were presumed to stabilize the heme hexacoordinate state and to fix the CD loop position, thereby opposing the sliding motion of the heme observed upon binding of CO to the ferrous state [19]. The distance between Lys(E10)67 and the heme propionate is also highlighted.