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Cloning, expression and purification of Atlantic salmon (*Salmo salar*, L.) neuroglobin

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ABSTRACT

Neuroglobin (Ngb) exists only in small amounts in salmon brain. In order to study the protein in more detail salmon neuroglobin (sNgb) was cloned, heterologously expressed in *Escherichia coli* and purified. The protein had red color and showed the characteristic peaks at 411 nm (metNgb), 415 nm (carboxyNgb) and 424 nm (deoxyNgb). Western analysis showed that sNgb reacted weakly against a rabbit anti human neuroglobin (hNgb) and strongly to a sNgb specific antibody. Our 3D-homology model of the sNgb indicated modifications adjacent to and in the O_2/CO binding site. This may correlate to differences in substrate affinities for the sNgb compared to the hNgb. Also sNgb contained shorter helixes and longer interhelical loops typical for psychrophilic proteins.

Introduction

Neuroglobin (Ngb)[1] is a recently discovered globin [1–4] and it is thought to be of ancient evolutionary origin. Ngb is found in all vertebrates in small amounts, and is expressed mainly in nervous tissues [1]. The biological function of Ngb is still not clear, but it has been hypothesized that it is involved in transport and storage of O_2, in scavenging of ROS (reactive oxygen species) and NO (nitric oxide), and in G-protein signalling and binding of CO [5]. It has been shown that Ngb plays a neuronal protective role during hypoxia [6,7]. In zebra fish and hypoxia tolerant turtles the expression of Ngb were shown to be up regulated in response to hypoxic conditions [8,9].

Ngb, similar to myoglobin (Mb) and hemoglobin (Hb), possesses the typical globin fold of eight (A–H) α-helixes, and is a respiratory porphyrin-containing protein that binds oxygen reversibly. Ngb differs from Hb and Mb by displaying a hexacoordinated heme structure, seen in both the ferrous deoxygenated (Fe^{2+}) and ferric (Fe^{3+}) forms. The proximal HisF7 and the distal HisE7 is directly coordinated to the heme-Fe atom, the fifth and sixth coordination positions, respectively [10]. Moreover, spectroscopic studies as well as simulations indicate [11,12] that the distal part of the binding pocket in Ngb is different compared to other globins and can harbor multiple bound conformations and docking sites for ligands. Conformational disorder in helix C to helix D and the PheB10 residue may explain the heterogeneity of the distal part of the binding pocket [13]. Sequence alignments show that salmon Ngb shares little amino-acid sequence similarity with mammalian such as mouse and human Ngb's (Kvamme et al., unpublished).

A practical interest in Ngb originates from the ban on using CO_2 sedation of salmon prior to slaughter that will be effectuated soon in Norway. A possible alternative procedure is the use of carbon monoxide (CO) [14,15]. The CO_2 stunning is currently replaced by electrical or percussion stunning, however, both these methods have drawbacks and both would benefit for a calm fish entering the slaughter machines. Due to its high affinity for CO and location in neural tissues neuroglobin may be a possible target for regulating the efficiency of sedation by CO.

Thus, in order to study Atlantic salmon neuroglobin (sNgb) in more detail, we cloned sNgb into an appropriate expression vector and optimized the expression conditions. The sNgb was fused to MBP (maltose binding protein) and expressed at low temperatures in order to improve the solubility and the monodispersity of the protein. After immobilized metal ion affinity (IMAC) chromatography and gel filtration, the purified sNgb produced the typical spectra for oxidized and reduced forms and reacted strongly to a sNgb specific antibody raised against a sNgb specific peptide. Moreover, homology modelling indicates that the active site of sNgb may be more flexible and showed typical psychrophilic properties. This indicates that Ngb may possess differences in functions in fish and mammalia.

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[1] *Abbreviations used:* Ngb, neuroglobin; sNgb, salmon neuroglobin; hNgb, human neuroglobin; CO, carbon monoxide; ROS, reactive oxygen species; MBP, maltose binding protein.

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Materials and methods

Cloning and expression

A plasmid containing the mRNA sequence for a salmon neuroglobin (Acc. no: BT059199) was generously provided by Dr. B.F. Koop [16]. The salmon Ngb coding region were PCR cloned into a pETM41 vectors (a generos gift from G. Stier) by the forward primer; GCTTCCATGGGCGAGAAGCTGACAGAGAAAGAG harboring a NcoI restriction site together with the reverse primer; GCTTGGTACCTTAGTCAGTCTTGTGTTCTCCGTTC harboring an Acc65I restriction site. This vector construct fused the sNgb to the MBP separated by a short amino acid sequence containing a TEV-protease cleavage site. After transformation, the bacteria were grown in LB-medium containing 25 μg/ml Kanamycin and 1 mM aminolevulinic acid (Sigma–Aldrich/FLUCA). When the cell medium reached A_{600} of 0.6, the medium was equilibrated to the appropriate temperature, and expression of MBP–sNgb was initiated by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After this, sNgb was expressed at the specific temperatures for different times. After harvesting the cells were pelleted by centrifugation (2500*g* for 10 min.) and stored at −20 °C until use.

Protein purification

Typically, pellets from 100 or 200 ml cell culture (0.3 or 0.6 g (w/w cells)) were re-suspended in 4 or 8 ml 1× PBS (phosphate buffered saline) pH 7.4 containing 0.2 M NaCl and 50 μg/ml RNase A EDTA-free protease-inhibitor, opened by sonication and centrifuged at 20,000*g* for 15 min. The cleared extract was applied on a Ni–NTA column and the histidine-tagged MBP–sNgb fusion protein was eluted in a gradient from 20 to 500 mM imidazole in 1× PBS pH 7.4 with 1 mM DTT and 0.2 M NaCl. The pooled protein fractions were concentrated to 2.5 ml and the buffer was changed to 1× PBS pH 7.4 with 1 mM DTT, and 0.2 M NaCl by passing through a PD10 column. (GE Healthcare) His-MBP was cut from the sNgb by incubating with TEV protease (25 μg) overnight at room temperature and concentrated by a Vivaspin concentrator to 2 ml. After gel-filtration through a Superdex G75 (16/60) column (GE healthcare), in 1× PBS pH 7.4, 1 mM DTT and 0.2 M NaCl, the fractions containing sNgb were pooled, concentrated to about 5.8 mg/ml, aliquoted and stored frozen at −80 °C. Elution of protein and heme containing proteins were monitored at A_{280} and A_{412}, respectively.

Purification of TEV protease

The plasmid containing the TEV protease was a modified His pET24d, a generos gift from G. Stier. The recombinant protease was purified after a standard procedure. In short, after transformation into BL-21 (DE3) cells, the protease expression was induced with 1 mM IPTG at a cell density of 0.6 A_{600}, and expressed in 100 ml LB-medium with 25 μg Kanamycin at 30 °C for 2 h. After harvesting and pelleting, the cells were re-suspended in 10 ml 1× PBS containing 10 mM imidazole and lysed by sonication. After centrifugation for 20 min at 20,000*g* the cleared lysate was applied onto a Ni–NTA column and the histidine-tagged TEV protease was eluted in a gradient from 10 to 400 mM imidazole. The pooled fractions were concentrated to 2 ml and the buffer was changed to 1× PBS pH 7.4. The TEV protease was aliquoted into 25 μg portions and stored at −80 °C until use.

Specific antibody to sNgb

A rabbit polyclonal antibody was raised using a peptide containing the 15 last C-terminial amino acids of sNgb. The antibody was produced by GenScript (GenScript USA Inc., USA) using their custom affinity-purified peptide polyclonal antibody service (Cat. no SC1031) in rabbit.

Western analysis

Polyacrylamide gel electrophoresis (SDS–PAGE) was carried out essentially according to Laemmli [17]. Recombinant hNgb (50 ng/μl; PromoKine, C-60210) was used as a positive control. For Western analysis, proteins separated on SDS–PAGE were transferred to PROTRAN^{R} Nitrocellulose Transfer Membrane (Whatman GmbH, Germany). After transfer, nonspecific binding sites on the membrane were blocked by incubation for 1.5 h at room temperature with 5% non-fat dry milk and 5% BSA in PBS. The membranes were then incubated with a polyclonal rabbit anti-salmon Ngb or a polyclonal rabbit anti-human Ngb (Santa Cruz Biotechnology, sc-30144) for 24 h at 4 °C using a 1:50 diluted antibody in PBS containing 1% (w/v) non-fat dry milk. After washing, the membranes were incubated with horseradish peroxidase linked donkey anti-rabbit IgG antibody (GE Healthcare, UK). Ngb was visualized by chemo luminescence and recorded by using Molecular Imager Chemidoc™ XRS + Imagine systems (BIORAD).

Spectra of sNgb

Absorption spectra were measured on recombinant sNgb in oxidized and reduced states and with CO added. Recombinant sNgb was diluted in PBS pH 7.4 containing 1 mM DTT and 0.2 M NaCl. Reduced sNgb was obtained by adding 20 mg of sodium dithionite, and carboxy-sNgb was obtained by adding CO directly to the cuvette. Absorption spectra were recorded from 350 to 600 nm on a Agilent 8453 spectrophotometer (Agilent Technologies, Waldbronn, Germany).

Homology modelling of sNGB

Homology modeling of sNgb was carried out by the Molsoft software (Molsoft L.L.C.) http://www.molsoft.com/). The 3D structure of mouse neuroglobin (3GLN) was used as template.

Results

Cloning, expression and purification

In order to optimize proper folding the salmon neuroglobin was fused to MBP. The effect of expression temperature on the protein quality was also investigated by SDS–PAGE analysis of the cleared lysates and the resuspended pellets. The amount of sNgb expressed at 37, 30 and 15 °C is shown in Fig. 1, before and after induction

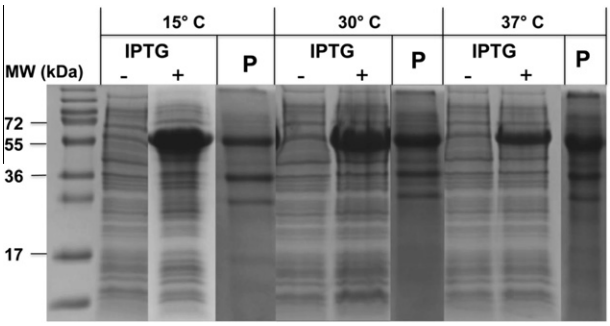

Fig. 1. SDS–PAGE analysis of the amount of soluble MBP–sNgb expressed at different temperatures, 15, 30 and 37 °C, before (−) and after (+) induction with 1 mM IPTG, compared with the amount of aggregated MBP–sNgb in the pellet (P) fractions.