CLONING, EXPRESSION AND PURIFICATION OF ATLANTIC SALMON (*Salmo salar*, L.) NEUROGLOBIN

By

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Abbreviations: Ngb: neuroglobin, sNgb: salmon neuroglobin, hNgb: human neuroglobin, CO: carbon monoxide, ROS: reactive oxygen species, MBP: maltose binding protein.

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, hetereologously expressed in *E.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 psycrophilic proteins.

Introduction

Neuroglobin (Ngb) 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 hypothesised 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 seven (A to G) 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 harbour 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 share 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 optimised 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 immobilised metal ion affinity (IMAC) chromatography and gel filtration, the purified sNgb produced the typical spectra for oxidised 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 psycrophilic properties. This indicates that Ngb may possess differences in functions in fish and mammalia.

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]. Salmon Ngb were PCR cloned into a pETM41 vectors (a generous gift from G. Stier) by the forward primer; GCTTCCATGGGCGAGAAGCTGACAGAGAAAGAG harbouring a NcoI restriction site together with the reverse primer; GCTTGGTACCTTAGTCAGTCTTGTGTTCTCCGTTC harbouring 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 1mM 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 x g for 10 min.) and stored at -20 ^{o}C until use.

Protein purification

Typically, pellets from 100 or 200 ml cell culture were re-suspended in 4 ml 1x 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 x 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 x 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 x PBS pH 7.4 with 1mM DTT, and 0.2 M NaCl by passing through a PD10 column (GE Healthcare) The His-MBP were 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 the fractions containing sNgb were pooled, concentrated to about 5.8 mg/ml, aliquoted and stored frozen at – 80 ^{o}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 generous gift from G. Stier. The recombinant protease was purified after a standard procedure. In short, after transformation into BL-21 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 ^{o}C for 2 hrs. After harvesting and pelleting, the cells were re-suspended in 10 ml 1x PBS containing 10 mM imidazole and opened by sonication. After centrifugation for 20 min at 20 000 x 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 x PBS pH 7.4. The TEV protease was aliquoted into 25 µg portions and stored at -80 ^{o}C until use.

Specific antibody to sNgb

A rabbit polyclonal antibody was raised using a peptide containing the 15 last C-terminal 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

SDS-polyacrylamid gelelectrophoresis (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 hours 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 hours at 4^{o} 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^{TM} 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 a few solids 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 modelling of sNgb was carried out by the Molsoft software (Molsoft L.L.C. 11199 Sorrento Valley Road, S209 San Diego CA 92121, http://www.molsoft.com/). The 3D structure of mouse neuroglobin (3GLN) was used as template.

Results

Cloning, expression and purification

In order to optimise 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 ^{o}C is shown in Fig. 1, before and after induction with IPTG. Fig. 1 shows the amount of sNgb that was expressed over night at 15 °C, 3 hours at 30 °C and 1 hour at 37 ^{o}C. A crude measure of the quality of the expressed protein was obtained by comparing the amount of protein in the medium after expression to the protein content in the pellets from the cleared lysate preparations. As can be seen from the pellets (P) in the figure the fraction of non-soluble complexes of MBP-sNgb was highest in pellet from the 37 ^{o}C expression and lowest in the 15 ^{o}C expression. Inclusion of aminolevulinic acid in the bacterial growth medium increased the yield of high quality sNgb substantially (data not shown).

Ni-NTA chromatography of MBP-sNgb was monitored both as protein in general at A_{280} and heme containing protein specifically at A_{412}. Fig. 2 shows a comparison of Ni-NTA column elution profile of MBP-sNgb expressed at 15 and 37 °C. Soluble and predominantly monodisperse MBP-sNgb was identified as the protein that bound to the Ni-NTA column and eluted in the imidazole gradient whereas unbound “low quality” MBP-sNgb eluted in the flow through.

Thus, the area of the peaks monitored at A_{412}, from the HisTrap column profile at 15 and 37 ^{o}C were used to quantify the quality of MBP-sNgb produced at 15 compared to 37 °C. To do this we computed the areas of the 412 nm peaks in the gradient and the flow through. We regarded no-bonding MBP-sNgb in the flow through as low–quality protein and MBP-sNgb that eluted in the imidazole gradient as high-quality MBP-sNgb. Therefore we used the ratio as a rough quality measure. The data shown in Table 1 shows that the ratio between Ni-NTA bound and unbound MBP-sNgb expressed at 15 °C was considerable higher, 2.8, compared to sNgb expressed at 37 °C, 0.8. Thus, expression at 15 ^{o}C improves the quality of MBP-sNgb about four times compared to expression at 37 ^{o}C.

After buffer shift, concentration and TEV hydrolysis sNgb was separated from MBP and TEV by gel filtration. Figure 3 shows a typical gel filtration profile of TEV hydrolysed MBP-sNgb expressed at 15° C. As can be seen from the figure only minute amounts of aggregated MBP-sNgb and sNgb protein eluted in the void volume. The majority of protein elutes as MBP and sNgb with molecular sizes of about 44 (third peak) and 17 kDa (fourth peak), respectively. Gelfiltration of MBP-sNgb expressed at higher temperatures (37, 30 and 18 °C) showed higher contents of aggregated MBP-sNgb and unhydrolysed MBP-sNgb (data not shown).Thus, our finding was that the protein expressed at 15 ^{o}C resulted in almost completely monodisperse MBP-sNgb protein preparations.

Western analysis

The identity of sNgb was confirmed by a western analysis using antibodies raised against recombinant human Ngb (hNgb) and sNgb. The two blots in Fig. 4 showed

that the anti human Ngb antibody detects hNgb approximately 5 to 10 times better compared to salmon Ngb. Moreover, Fig. 4A shows the differences in molecular weight between the sNgB (17887 Da) and the hNgb (16933 Da). The blot in Fig.4B showed that the salmon specific antibody did not detect the human neuroglobin and recognized the salmon protein only.

Spectral analysis of sNgb

The absorption spectra of oxidised, reduced and CO-bound recombinant Ngb from Atlantic salmon is shown in Fig. 5. Shifts in the position for the Soret band from 411 nm (metNgb) via 415 nm (carboxyNgb) to 424 nm (deoxyNgb) for heme containing globins were evident. In addition to the strong absorption peak at 411 nm for metNgb a broad weak band at 510-555 nm is also shown. Reduced Ngb shows two weak peaks at 528 and 558 nm in addition to the strong absorption peak at 424 nm. These are typical absorption peaks shown in neuroglobin and also other globins [18-20].

Homology modelling of sNgb

Modelling of sNgb was carried out using the mouse neuroglobin (mNgb) 3D structure (3GLN) as template. Salmon and mouse Ngb structures differ in several aspects as shown in Fig. 6. Firstly, both the N- and C-terminals in the sNgb is longer compared to the mNgb. Secondly, most of the helixes are one to two turns shorter in sNgb compared to mNgb resulting in longer unstructured connections between the helixes. The long loop between helix B and C differs in the two neuroglobins. Thirdly, helix D in sNgb is disrupted. Interestingly, this helix harbours H68 (sNgb) (equal to H64 in mNgb) one of the two histidines that bind to the iron of the heme group. The sequence alignment and secondary structure prediction, shown in Fig. 6B, give an

overview of structural differences between the sNgb and mNgb. Many of these features are typical for psycrophilic proteins found in cold temperature adapted organisms [21, 22].

Discussion

As proteins from cold-water fishes often show psychrophilic properties [23], the effect of different temperatures were tested on the expression efficacy in terms of aggregation/mono dispersity. Psycrophilic proteins are thought to be more flexible compared to their meso- and thermophilic homologues and are thought to possess a more loosely built poly-peptide structure. These properties may intervene with proper folding of the protein when over expressed in a heterologous expression system. We also fused the sNgb to the MBP that has proved to have a chaperonic effect and has aided proper folding to a large number of hetereologously expressed proteins [24-29]. The data in Fig. 1 shows that 15 °C was the most optimal temperature for expressing MBP-sNgb. As can be seen from the figure a higher portion of soluble MBP-sNgb was in the cleared extract compared to aggregated MBP-sNgb in the pellet fraction expressed at higher temperatures. The elution profile of the Ni-NTA column also showed that the portion of MBP-sNgb that bound to the column resin was higher for the protein expressed at 15 °C compared to protein expressed at 37 °C. (Fig. 2). This probably also reflect more optimal expression conditions at 15 °C compared to 37 °C. In concert with this, the portion of unbound MBP-sNgb is larger for the high temperature expressed MBP-sNgb (red coloured A_{415} nm trace in Fig.2) compared to the imidazole gradient eluted MBP-sNgb. The reason for this is most probably that MBP-sNgb in the flow-through fraction consists of so-called soluble micelles where aggregated sNgb resides in the interior, whereas the soluble MBP is exposed to the solvent [29]. Such complexes are thought to be large and heterogeneous and may shield the His-tag for proper binding to the Ni-NTA resin.

The gel filtration analysis of the TEV hydrolysed MBP-sNgb show that even the 15 °C expressed protein contains minor amounts of large complexes of MBP-sNgb as well as sNgb that eluted in the void volume. For the same analysis of protein expressed at higher temperatures the proportion of larger MBP-sNgb and sNgb were considerably higher compared to the 15 °C expressed protein (data not shown). Thus expression at 15 °C improves the quality of sNgb considerably compared to expression at higher temperatures.

The western analysis showed that the polyclonal rabbit antibody raised against the hNgb only weakly recognised sNgb whereas the sNgb specific antibody did not recognise the hNgb at all. This is in accordance with that the salmon specific antibody was based on a C-terminal peptide that is absent in hNgb.

The spectra of sNgb in Fig. 5 showed the characteristic peaks for heme containing proteins. The rather broad absorption peak in the Soret region of met-sNgb indicates that the heme group is rather loosely connected to the protein. Binding of CO pulls the iron towards the protophorphyrin plane and the Soret peak of carboxy-sNgb becomes sharper indicating a closing of the grove.

The homology model of sNgb indicated interesting structural differences from the mNgb and hNgb that are typical for psycrophilic proteins. In order to function optimally at low temperatures, psycrophilic proteins are thought to possess a more flexible polypeptide chain that leads to a more loosely built 3D-structure compared to their meso- and thermophilic homologues [21]. In general the α-helixes in sNgb tended to be shorter compared to the human and mouse Ngb's. This leads to longer

unstructured connections between the helixes. In addition helix D (globin fold numbering) was different in the sNgb by being disrupted about two thirds to the C-end. Interestingly this helix contains the so-called distal histidine (His 65) that binds to the iron in the heme group. This could indicate a more flexible substrate-binding pocket that could encompass other types of ligands compared to the mammalian Ngb's. Moreover, the other differences, such as the prolonged N- and C-terminals, the shorter helixes and longer loops are hallmarks for psycrophilic behaviour in proteins [21, 22].

Conclusion

In this paper we have shown that a neuroglobin from Atlantic salmon has been cloned, hetereologously expressed and purified in milligram amounts. When expressed at low temperatures the quality sNgb was increased by being more monodisperse and less aggregated. Thus, sNgb could be concentrated to 6 mg/ml without any signs of precipitation. Also sNgb was specifically recognised by an antibody raised against a C-terminal peptide that is unique for the salmon neuroglobin. Homology modelling of sNgb suggests longer loops and local structural flexibility that is characteristic for psycrophilic proteins. The structural features for psycrophilic proteins correlates with improved expression conditions at low temperatures, were improved protein stabilising conditions would be expected.

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Table

Table 1. Areas under the A_{412} elution peaks for MBP-sNgb expressed at 15 °C and 37 °C eluted from the Ni-NTA columns. The peak areas are given in mA_{412}/min.

Expression temperature	Peak 1 (mA412/min)	Peak 2 (mA412/min)	Ratio peak 2/1
15 °C	1944	5383	2.8
37 °C	353	284	0.8

Legend to figures

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

Fig. 2. His-Trap column elution profile of protein and sNgb expressed at 15 ^{o}C (A) and 37 ^{o}C (B). MBP-sNgb that eluted in the flow-through and in the imidazole gradient was monitored by the absorbance at 412 nm (red trace). The insert in figure B is shown to clarify the ratio between soluble aggregates of MBP-sNgb compared to monodisperse MBP-sNgb that eluted in the imidazole gradient. The blue trace represents the protein profile monitored at 280 nm.

Fig. 3. Gel filtration profile of MBP-Ngb expressed at 15 °C and SDS-PAGE of eluted protein fractions.

Fig. 4. Western blot using polyclonal hNgb (A) and polyclonal sNgb (B). In 4A lane 1 represents recombinant sNgb (0.25 µg), and lane 2 and 3 represent recombinant hNgb, 0.05 µg and 0.15 µg respectively. In figure B, lane 1 contains hNgb (0.15 µg) and lane 2, 3 and 4 represent recombinant sNgb 0.25, 0.15 and 0.05 µg respectively,

Fig. 5. Spectra of recombinant sNgb in its oxidized state (red color), reduced state (blue color) and carboxyNgb (green color). Addition of a few grains of sodium dithionite resulted in reduced sNgb, and carboxyNgb was obtained by addition of CO. Absorbtion spectra were recorded from 350 to 600 nm.

Fig..6. (A) Homology model of sNgb, white, superimposed on the mNgb template (3GLN), red colored. The parts of largest structural differences, the N and C terminals together with helixes E and F are marked (B). A sequence alignment of sNgb and mNgb with outlined secondary structure elements (mouse) and postulated secondary structures (salmon). Alpha helixes are represented by cylinders and β-strands by arrows.

Figures

Fig. 1

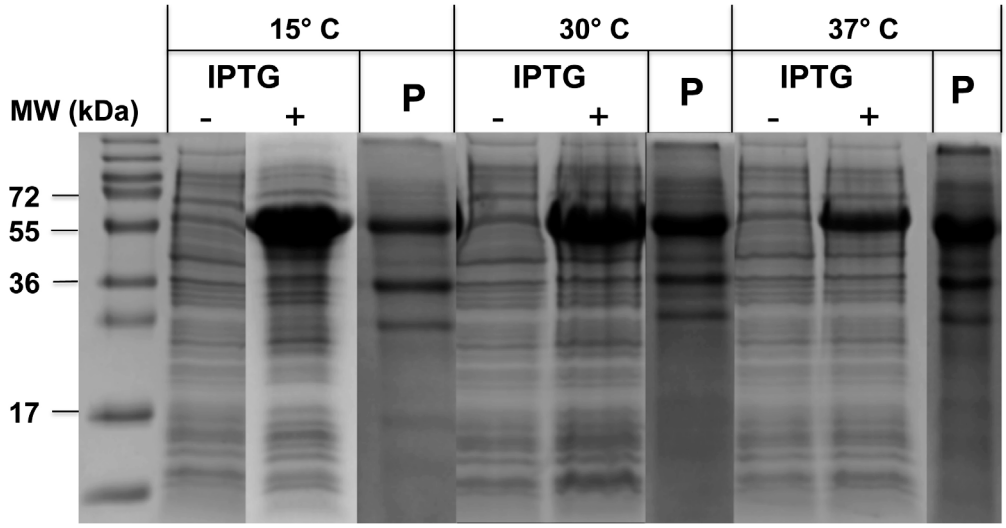

Fig. 2

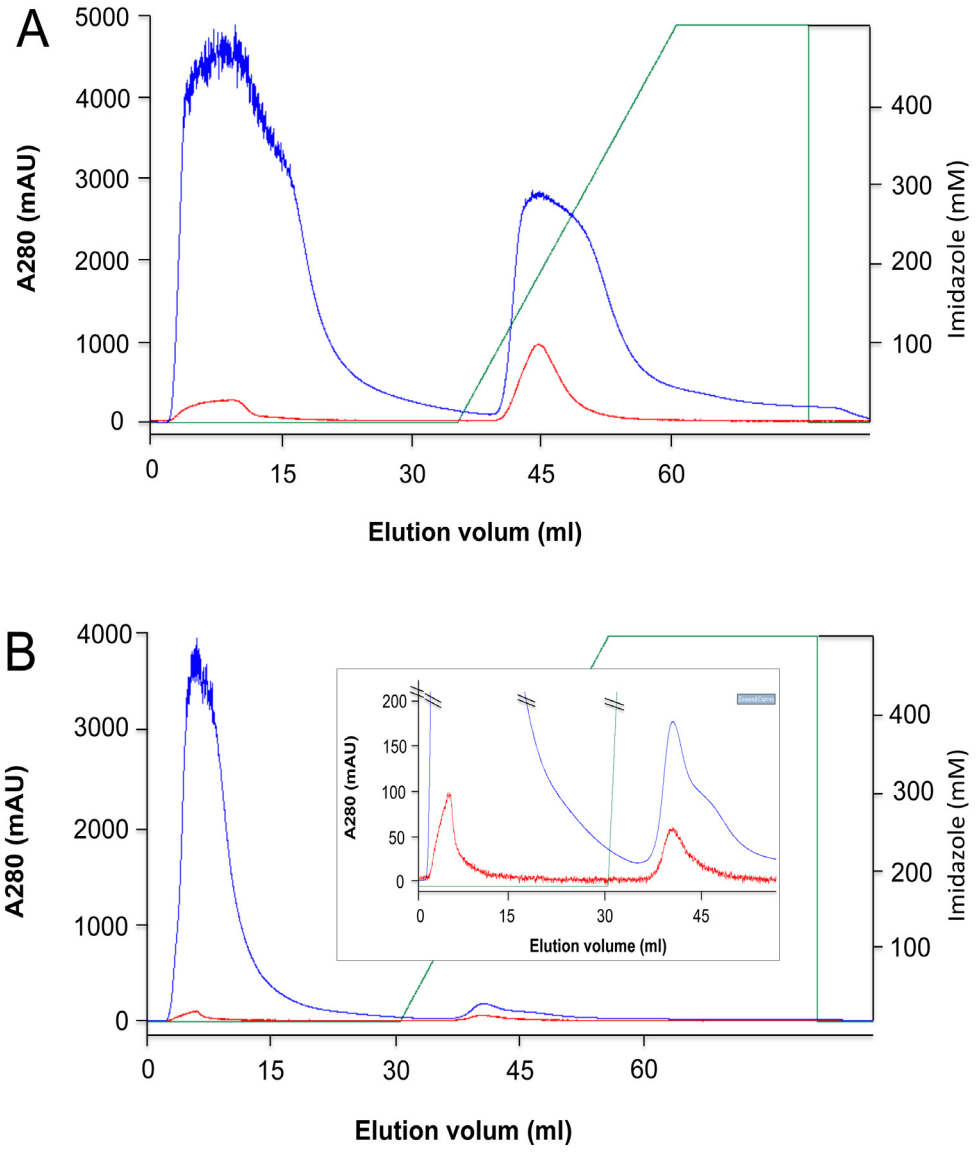
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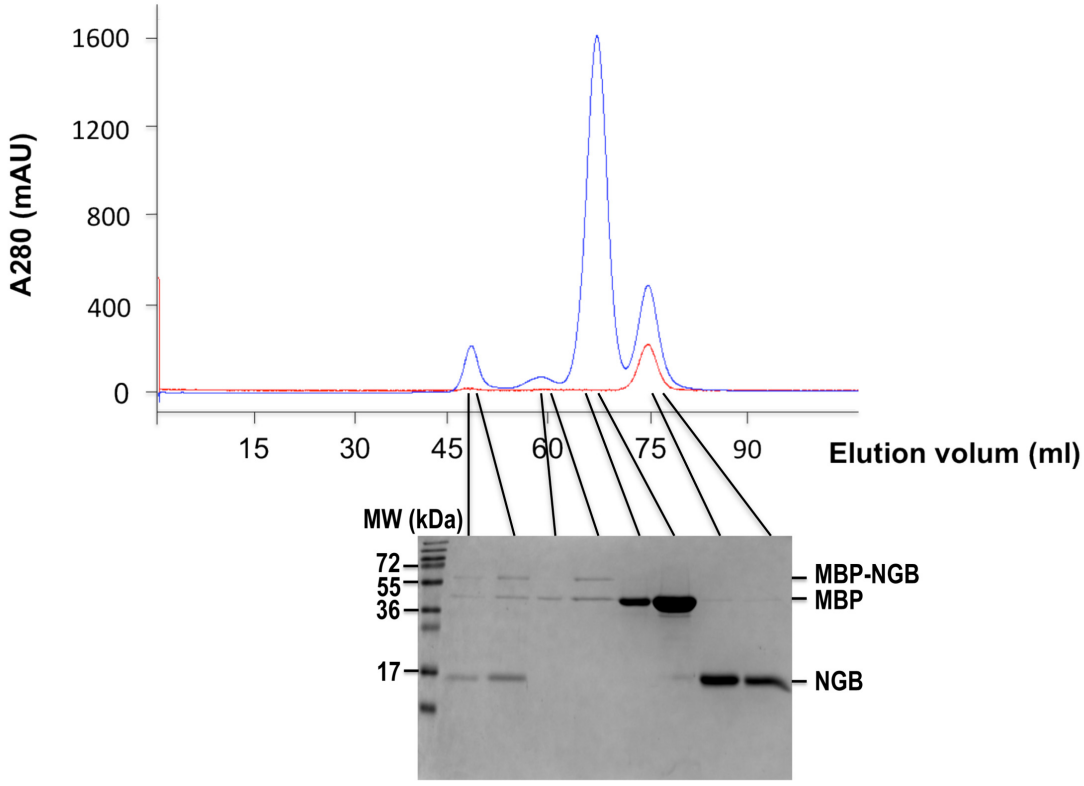
Fig. 3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75
90
Elution volum (ml)
MW (kDa)
72
55
36
17
MBP-NGB
MBP
NGB

Fig. 4

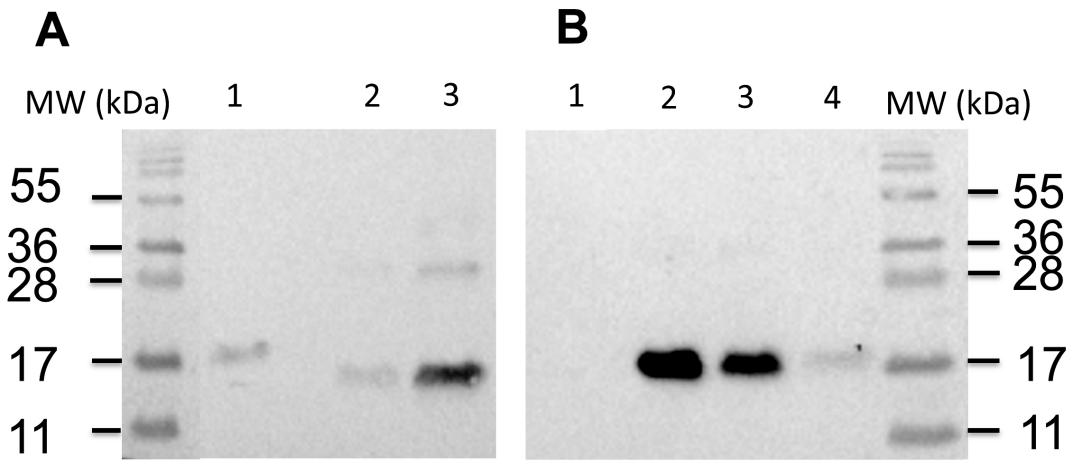


Fig. 5

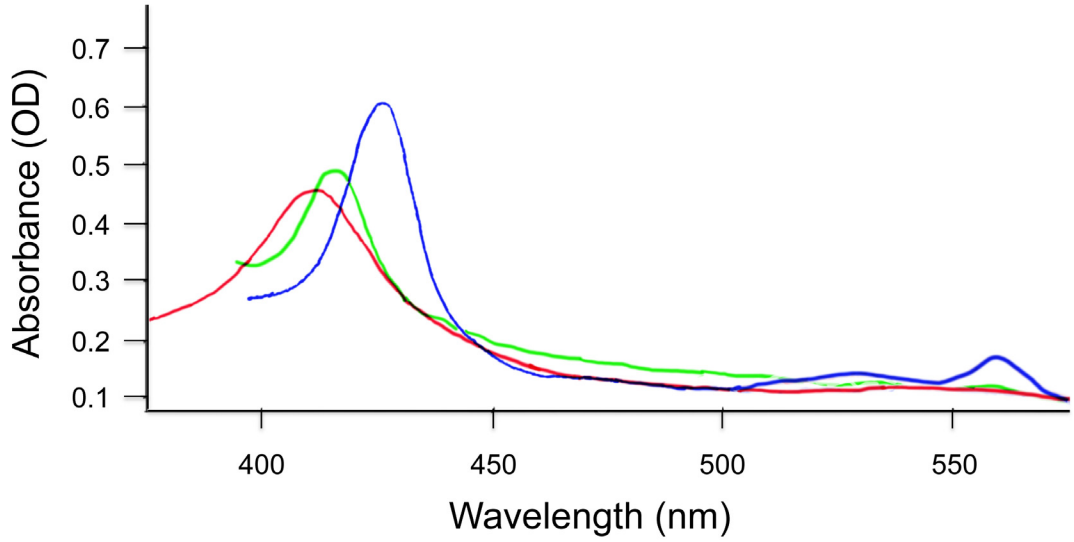
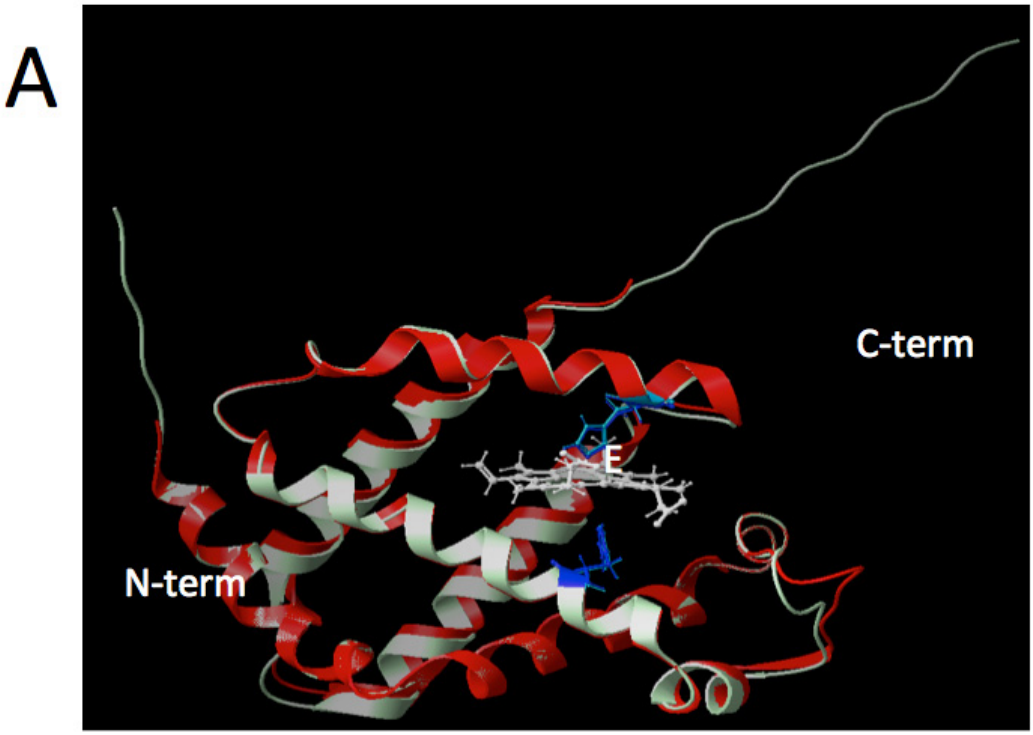

Absorbance (OD)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
400
450
500
550
Wavelength (nm)

Fig. 6


A
C-term
E
N-term
B
0 10 20 30 40 50 60 70 80
salmon .....E.ELIR.SW..#.+..#.HG.##F.RLF.LEP.LL.LF.YN...#....D.LSSPEFLDH#.KVMLVIDAAV..#-
mouse MEKLTEKEKELIRVSWESLGKNKVPHGVIMFSRLFELEPALLNLFHYN-TNCGTTQDCLSSPEFLDHVTKVMLVIDAAVSHLD
salmon -----RPESELIRQSWRVVSRSPLEHGTVLFARLFALEPSLLPLFQYNGRQFSSPEDSLSSPEFLDHIRKVMLVIDAAVTNVE
mouse
90 100 110 120 130 140 150 160
salmon .L..LE-#L..LG+KH.AVGV...SF..VGESLLYML..SLG..#T##.R.AW..#Y.#VV.AMSRGW.....
mouse NLHTLEDFLLNLGKKHQAVGVNTQSFAVVGESLLYMLQCSLGQGYTAPLRQAWLNMYTIVVAAMSRGWAKNGEHKTDp
salmon DLSSLEEYLTSLGRKHRAVGVRLSSFSTVGESLLYMLEKSLGPDFTPATRTAWSRLYGAVVQAMSRGW----------
mouse