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A Globin Domain in a Neuronal Transmembrane Receptor of *Caenorhabditis elegans* and *Ascaris suum*:
Molecular Modeling and Functional Properties.

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*Running Title: *Characterization of the globin domain in a neuronal receptor*

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Background: GLB-33 is a putative neuropeptide receptor in *C. elegans* with a globin domain.

Results: Recombinant globin domain displays a ferric hydroxide-ligated form. When reduced, it can bind CO or O₂ and reduce nitrite to NO.

Conclusion: The globin domain may serve as an oxygen sensor or nitrite reductase.

Significance: Oxygen sensing mechanisms are relevant for neuropeptide receptor binding.

ABSTRACT

We report the structural and biochemical characterization of GLB-33, a putative neuropeptide receptor that is exclusively expressed in the nervous system of the nematode *Caenorhabditis elegans*. This unique chimeric protein is composed of a 7 transmembrane domain, GLB-33 7TM, typical of a G-protein-coupled receptor, and of a globin domain, GLB-33 GD. Comprehensive sequence similarity searches in the genome of the parasitic nematode, *Ascaris suum*, revealed a chimeric protein that is similar to a Phe-Met-Arg-Phe-amide neuropeptide receptor. The three-dimensional structures of the separate domains of both species, and of the full-length

proteins were modeled. The 7TM domains of both proteins appeared very similar, but the globin domain of the *A. suum* receptor surprisingly seemed to lack several helices, suggesting a novel truncated globin fold. The globin domain of *C. elegans* GLB-33, however, was very similar to a genuine 'myoglobin'-type molecule. Spectroscopic analysis of the recombinant GLB-33 globin domain showed that the heme is pentacoordinate when ferrous and in the hydroxide-ligated form when ferric, even at neutral pH. Flash-photolysis experiments showed overall fast biphasic CO rebinding kinetics. In its ferrous deoxy form, GLB-33 GD is capable of reversibly binding O₂ with a very high affinity and of reducing nitrite to nitric oxide faster than other globins. Collectively, these properties suggest that the globin domain of GLB-33 may serve as a highly sensitive oxygen-sensor and/or as a nitrite reductase. Both properties are potentially able to modulate the neuropeptide sensitivity of the neuronal transmembrane receptor.

Nematodes often inhabit low-oxygen environments where parameters such as food

supply, temperature and oxygen pressure vary. These fluctuations force the nematode to adapt its behavior in order to survive (1). Globins can play a role in this adaptation. The globin family is a heterogeneous group of proteins sharing the globin fold and burying a heme cofactor in a hydrophobic pocket, where diatomic gaseous ligands like O₂ can bind to the heme iron atom. *In silico* analysis of the genome of the free-living nematode *Caenorhabditis elegans* (*C. elegans*) resulted in the identification of a family of 33 globin genes, labelled *glb-1* to *glb-33*, that are all transcribed. The encoded globins, GLB-1 to GLB-33, are localized in a specific subset of cells (2-5). Characterization of some of these globins indicated that they are involved in a wide variety of cellular processes. For instance, GLB-1 may function in regulating facilitated O₂ diffusion in a small subset of cells (6). GLB-6 serves a role as redox sensor responding to oxidative stress, either directly or indirectly by the change in O₂ level (7). GLB-5 is an O₂ sensor that interacts with the NPR-1 receptor as part of a foraging strategy (8,9). GLB-26 is a myristoylated redox protein, involved in the defecation cycle under stress conditions (6,10). The role of all other globins in *C. elegans* remains unknown.

One of the *C. elegans* globin genes, *glb-33*, is expressed in neuronal cells and has been predicted to code for a 542 amino-acid-long chimeric protein, GLB-33.

The N-terminal domain of GLB-33 (residue nr 1-372) is composed of 7 hydrophobic α -helices and represents a transmembrane domain (GLB-33 7TM) typical of a G-protein-coupled receptor (GPCR) domain. In these receptors, ligand binding causes a conformational change, allowing a guanine nucleotide exchange in a G-protein. Their activating ligands vary widely in structure and characteristics (11-13) and include neuropeptides (14-16). In *C. elegans*, more than 100 neuropeptides have been identified by bioinformatic and mass spectrometric methods (17-19). They can be divided into 3 families: the FMRFamide (Phe-Met-Arg-Phe-NH₂)-related peptides or FLPs, the insulin-like peptides, also called INSs, and the remaining neuropeptide-like, non-insulin, non-FLP peptides, the NLPs (14,15,17,20-22). Although a specific functional role has been identified for only few of these neuropeptides, overall they have been shown to

play a crucial role in behavioral aspects as diverse as appetite (23,24), fat storage (25), locomotion (26-30), reproduction (31), and dauer formation (32). Peptides exert their biological functions via specific signal-transducing receptors by direct or indirect modulation of the synaptic activity or by acting as primary neurotransmitters.

The C-terminal domain of GLB-33 (residue nr 373-534), displays the major determinants of the globin fold, including 8 α -helices (A-H), the proximal HisF8 and the characteristic pattern of hydrophobic/hydrophilic residues (33,34) and is therefore named as GLB-33 globin domain (GLB-33 GD). Despite the occurrence of specific deviations from standard templates like ValCD1 and IleE7, this domain can be considered as a genuine globin (3).

In order to understand the functional role of the GLB-33 neuronal receptor, we have expressed and purified the recombinant GLB-33 GD in *Escherichia coli* (*E. coli*) and investigated its spectroscopic characteristics, kinetics and equilibrium of the binding of the gaseous ligands O₂ and CO, and the reactivity with nitrite. We also performed *in vitro* and *in vivo* localization studies. Furthermore, models of the 3D structures of the globin domain, as well as of the G-protein-coupled receptor domain were built and used as templates to search the databases for proteins with similar structures. We have identified a chimeric protein in the nematode *Ascaris suum* (*A. suum*) with a similar transmembrane domain, but with a structurally different globin domain. From this investigation we have identified several structural and functional characteristics of the globin domain of GLB-33 that are likely to be involved in the fine-tuning of the neuronal receptor response in *C. elegans*.

EXPERIMENTAL PROCEDURES

Localization of GLB-33

In vitro localization- The cDNA coding for GLB-33 (*glb-33*) is 1629 bp long and was cloned in the pEGFP-N1-vector with C-terminal GFP-Tag (Clontech) using BglIII and BamHI restriction enzymes (Biolabs, Westburg) and T4 DNA ligase (Novagen). 1 μ g of the expression construct was transfected into human neuroblastoma SH-SY5Y cells (ATCC® CRL-2266) with 3 μ l Lipofectamine 2000 (Invitrogen). To obtain detailed images of the subcellular localization of

GLB-33, an UltraVIEW Vox ERS microscope (PerkinElmer) was used, and images were created with the Velocity 6.0.1 software. GFP was excited at 488 nm with an argon laser and a bandpass filter was used to allow emission light between 500 nm and 550 nm.

In vivo localization - The translational reporter for *glb-33* was constructed using fusion PCR, as described by Hobert (35). The reporter contained 2.95 kb upstream and 0.653 kb downstream of the *glb-33* gene to include the endogenous promoter region and 3'UTR gene regulating elements, respectively. The *gfp*-gene was amplified from the pPD95.75 vector, and fused at the 3' side of the *glb-33* gene, thereby preceding the 3'UTR region. A coding region for a GA-linker between GLB-33 and GFP was also included. The sequences of the primers used were 5'-TAGACAGTTTGGCCACATCTT-3' (forward primer of the promoter of *glb-33*), 5'-GGGTACACTCTTCAAAAACA-3' (nested forward primer of the promoter of *glb-33*), 5'-TCCAGCGCTGCACCAGCTCCTGGCATTGACCAATTGTG-3' (reverse primer of the *glb-33* gene), 5'-GCATGGATGAACTATACAAATGACTTTTTCTAGATCTCGC-3' (forward primer of the 3'UTR region of *glb-33*), 5'-TGTTCTTCTCCCGATATGGTAND-3' (reverse primer of the 3'UTR region of *glb-33*), 5'-GACTCCGTGACAATTTCTC-3' (nested reverse primer of the 3'UTR region of *glb-33*), and 5'-GGAGCTGGTGCAGGCGCTGGAGCCGGTGCCTTGAGTAAAGGAGAAGAAC-3' (forward primer of *gfp*), and 5'-TTTGTATAGTTCATCCATGCC-3' (reverse primer of *gfp*).

In addition, the *unc-119* gene, including a 2.189 kb upstream region and a 1.228 kb downstream region of the *unc-119a* isoform, was amplified with forward primer 5'-TCAGTAAAAGAAGTAGAAT-3', and reverse primer 5'-GAATTTTAAACAATACTTC-3'. The PCR product was used as a co-injection marker, and rescues the locomotion defect of the *unc-119(ed3)* strain, the strain used for micro-injection. The final PCR products were injected into the gonads of young adult hermaphrodites using an AxioVert 135 (Zeiss) microscope and FemtoJet microinjection system (Eppendorf), at a

concentration of 50 ng/μl for the *glb-33* reporter and 20 ng/μl for the *unc-119* gene. Transformed lines were analyzed using a Nikon Eclipse TE2000-5 confocal microscope.

In silico identification of orthologs - The protein sequence of GLB-33 (accession nr. AAK68603) was used to search the non-redundant database of NCBI for orthologs using the protein-protein Basic Local Alignment Tool (BLAST) algorithm (BLASTP) (36,37). Additional similarity searches were performed on the *A. suum* CDS gene set (38) using the NemaBLAST algorithm available at Nematode.net v3.0 (39). GLB-33 orthologs were also identified using the BLASTP algorithm implemented in WormBase (40). All significant hits were manually aligned with GLB-33 using Genedoc (41), employing the procedure used earlier (42,43) based on the myoglobin fold (33) and the pattern of predominantly hydrophobic residues at 37 conserved solvent-inaccessible positions.

Cloning, expression and purification of recombinant GLB-33 GD and A. suum FMRF GD - *C. elegans* worms were grown as described previously (2). Young adult worms were collected and total RNA and cDNA were prepared as described (6). To confirm the presence of a globin domain in the *A. suum* FMRFamide receptor (accession nr. ADY45777.1), the cDNA coding for this globin domain was amplified and sequenced. *A. suum* cDNA (1365 bp) was kindly provided by Peter Geldhof and Edwin Claerebout (University of Ghent, Belgium) and was prepared as follows. Total RNA from L3-larvae was extracted with TRIzol reagent. After treatment with DNase (Invitrogen™), cDNA was generated from 1 μg of total RNA using the cDNA synthesis kit (iScript™, BIO-RAD). PCR was then performed with the gene-specific forward and reverse primers 5'-ATGTGGCGTCAAAAACGGCCAC-3' and 5'-TGGTTTCTTAAGGAACTCTGTCC-3', respectively. The PCR was performed in a final volume of 25 μl under the following conditions: 1× buffer, 1.5 mM MgCl₂ (Invitrogen™), 0.2 mM of each dNTP, 1 μM of each primer, and 1 U of Platinum® TaqPCRx DNA Polymerase (Invitrogen™). The PCR was started with 4 min at 95 °C, which was followed by 35 cycles consisting of 30 s at 95 °C, 30 s at 55 °C, and 60 s at 72 °C. A

final step of 10 min at 72 °C was added to the last cycle. The PCR product was analyzed on a 1.5% agarose gel.

The cDNAs coding for the GLB-33 GD (*glb-33 gd*, bp 1120 to 1629) and for the *A. suum*FMRF GD (*A. suum fmrfd* bp 991 to 1365) were cloned in the pET23a-vector with C-terminal His-Tag (Novagen) using NdeI and XhoI restriction enzymes (Biolabs, Westburg). The sequences of the forward and the reverse primers that were used to amplify the cDNAs were 5'-CATATGCTTCTCGGAGACAGGTTGTCC-3' and 5'-CCGCTCGAGTGGCATTGACCAATTGTGTC-3' for *glb-33 gd* and 5'-GGAATTCCATATGTGGCGTCAAAAACGGC CAC-3' and 5'-CCGCTCGAGTGGTTTCTTAAGGAAACTC-3' for *A. suum fmrfd*, respectively. Ligation of the cDNAs in the pET23a-vector was performed using T4 DNA ligase (Novagen). The ligated product was sequenced on both strands using BigDye terminator chemistry and an ABI 377 sequencer (Applied Biosystems). *In vitro* expression in BL21(DE₃)pLysS *E. coli* cells was performed as described previously (44). Alternatively, cells were resuspended in 50 mM Tris pH 7.5 and 300 mM NaCl. GLB-33 GD and *A. suum*FMRF GD were purified by nickel affinity chromatography (Clontech), eluted with 250 μM imidazole in resuspension buffer and dialyzed against 20 mM Tris pH 8.5 and 50 mM NaCl.

Optical, Resonance Raman, and Electron Paramagnetic Resonance spectroscopy - UV-Vis spectra were measured in a 250-700 nm range on a Cary-5 UV-Vis-NIR spectrophotometer (Varian). Expressed GLB-33 GD was dialyzed against different buffers with pHs ranging from 4 to 8.5 (100 mM sodium acetate pH 4, 100 mM sodium acetate pH 5, 100 mM bis-Tris pH 6, 100 mM HEPES pH 7, 100 mM Tris pH 8.5). Ferrous CO-bound and reduced deoxy protein samples were prepared by flushing 1 ml of 100 mM potassium phosphate buffer pH 7.0 for 15 minutes with CO- and N₂-gas respectively in a sealed cuvette. After addition of 10 μl of a saturated solution of sodium dithionite, highly concentrated, recombinant purified protein was added to obtain a final concentration of 50 μM. The ferrous oxy derivative of GLB-33 GD was prepared by

reducing the protein with 10 μl of a saturated sodium dithionite solution, prior to loading the sample on a PD10 gel filtration column (Amersham Biosciences) to remove the dithionite and allow O₂ to bind.

Resonance Raman (RR) measurements were carried out on an 80-cm Dilor XY-800 Raman scattering spectrometer consisting of a triple spectrograph operating in normal mode and a liquid nitrogen-cooled CCD detector. The excitation source was a Kr-ion laser (Spectra Physics 2020) at 413.1 nm. The protein solution was stirred at 6,000 rpm to avoid local heating. Five spectra (120-s recording time) were acquired and averaged after the removal of cosmic ray spikes by a program developed in-house. Laser powers of 1 and 50 mW were used.

X-band Electron Paramagnetic Resonance (EPR) measurements were performed on a Bruker Elexsys instrument equipped with Helium cryostat (Oxford Inc.). All measurements were done at 10 K. The continuous-wave (CW) EPR spectra were obtained with a modulation frequency of 100 kHz and a modulation amplitude of 0.5 mT. The electron spin echo (ESE) -detected EPR experiments were performed using the $\pi/2$ - τ - π - τ -echo sequence with $t_{\pi/2} = 16$ ns, $t_{\pi} = 32$ ns; τ was varied in steps of 8 ns from 88 to 3280 ns. The spectra recorded at the different τ values were subsequently summed.

CO association kinetics by flash photolysis - CO-bound GLB-33 GD samples were prepared in a sealed 4 × 10 mm quartz cuvette containing 1 ml of 100 mM potassium phosphate buffer, and 1 mM EDTA at pH 7.0. The buffer was equilibrated with different mixtures of CO and N₂-gas, using a High-Tech system (Bronkhorst, the Netherlands). To obtain the association rate constant for CO binding ($k_{on,CO}$), CO-ligated ferrous GLB-33 GD was prepared with CO concentrations ranging between 200 μM and 800 μM. 10 μl of a saturated solution of sodium dithionite was then added to the buffer to scavenge any residual O₂. A minimum amount of concentrated protein was injected to obtain a final concentration of ~ 5 μM. The heme iron atom was reduced by the added dithionite and conversion to the CO-ligated form was verified by UV-Vis spectroscopy.

Flash-photolysis experiments were carried out on a laser photolysis system (Edinburg

Instruments LP920) at 20 °C, using a frequency-doubled Q-switched Nd:YAG laser (Spectra Physics Quanta-ray) at 532 nm. CO-association was measured by photo-dissociating ligated GLB-33 GD by a short laser pulse (5-8 ns) and then following the recombination of the photo-dissociated CO-ligand at different time scales, ranging from 2000 ns for the geminate recombination of CO to 400 ms for the bimolecular rebinding of the ligand at 421 nm.

Exponential decays from the consecutive timescales were joined together to give the complete ligand-rebinding curve. All data were analyzed using the Matlab program (The Math Works Inc.). The rate of geminate rebinding (k_{gem}) was obtained by fitting a single exponential curve through the data points collected in the first 2000 ns after photodissociation :

$$\Delta OD_t = \Delta OD_{gem} \cdot \exp(-k_{gem} t) + \Delta OD_{obs} \quad (\text{Eq.1})$$

After logarithmic resampling of the data points, pseudo-first order fast observed rebinding rates ($k_{obs,f}$) and slow observed rebinding rates ($k_{obs,s}$) were determined by least-square fitting of the decays with bi-exponential equations :

$$\Delta OD_{obs} = \Delta OD_f \cdot \exp(-k_{obs,f} \cdot t) + \Delta OD_s \cdot \exp(-k_{obs,s} \cdot t) \quad (\text{Eq.2})$$

Fast and slow CO-rebinding association rates ($k_{on,f}$ and $k_{on,s}$ respectively) were calculated from the dependence of $k_{obs,f}$ and $k_{obs,s}$ on the CO-concentration according to the following equations (45) :

$$k_{obs,f} = k_{on,f} \times [\text{CO}] + k_{off,f} \quad (\text{Eq. 3})$$

$$k_{obs,s} = k_{on,s} \times [\text{CO}] + k_{off,s} \quad (\text{Eq. 4})$$

O₂ equilibria - O₂ equilibrium curves of GLB-33 GD were determined at 25 °C using a modified thin-layer diffusion-chamber technique described elsewhere (46-48). Prior to measurements, the sample was reduced with dithionite and desalted, as described above. A 4 µL sample (~100 µM GLB-33 GD in 100 mM sodium phosphate, 0.5 mM EDTA pH 7.4) was equilibrated with water-saturated gas-mixtures of varying O₂-tension (PO₂) created by Wösthoff gas mixing pumps. Reversible absorbance changes following changes in PO₂ were

recorded at 436 nm. Values for P₅₀ (PO₂ at half-saturation) and n₅₀ (cooperativity coefficient) were calculated from the zero intercept and slope of Hill-plots, log(Y)/(1-Y) vs. logPO₂, respectively, where Y is the fractional saturation.

Nitrite reductase activity - To study the reaction between deoxygenated GLB-33 GD and nitrite, a stock solution of nitrite (~20 mM in 100 mM sodium phosphate, 0.5 mM EDTA pH 7.4) and a sample containing GLB-33 GD (~10 µM heme in 100 mM sodium phosphate, 0.5 mM EDTA pH 7.4) were deoxygenated by equilibrating with N₂ gas for ~30 min. Nitrite solutions were freshly made every day, and nitrite concentrations were checked by the Griess method (49). A ~2-3 fold molar excess of a previously degassed dithionite solution was added (~20-30 µM final concentration) to the N₂-equilibrated GLB-33 GD sample and absorbance spectra were taken to verify full heme deoxygenation. The reaction between deoxy GLB-33 GD and nitrite (0.1-0.3 mM) was measured at 25 °C under pseudo-first order conditions in the presence of dithionite, as previously described (50,51). Observed rates were obtained by fitting absorbance traces at 579 nm to a single exponential function and apparent second order rate constant was obtained from the slope of a linear plot of observed rates as a function of nitrite concentration.

Autoxidation rate - The autoxidation rate of GLB-33 GD (~10 µM heme) was measured in air in 100 mM sodium acetate buffer pH 4 and in 100 mM sodium phosphate, 0.5 mM EDTA, pH 7.4 at 25 °C and 37 °C after previous reduction of the ferric protein with dithionite and desalting.

The decrease in absorbance at 577 nm was monitored spectrophotometrically every minute for the first 90 minutes and every 5 minutes for the next 60 minutes using a Cary-5 UV-Vis-NIR spectrophotometer (Varian) in experiments performed at pH 4.0, and every 50 minutes using a HP 8543 UV-visible diode array spectrophotometer in experiments performed at pH 7.4. Absorbance traces were fitted according to a single exponential function.

3D modeling of GLB-33 and A. suum FMRamide receptors - The three-dimensional structures of GLB-33 GD (residue 331-455), GLB-33 TM

(residue 1-373), *A. suum*FMRF GD (residue 331 to 455), and *A. suum*FMRF 7TM (residue 1 to 330) were predicted using the I-TASSER server (52,53) without structural restraints. The I-TASSER server builds 3D models using multiple-threading alignments by LOMETS and iterative TASSER assembly simulations. The Phyre2 program was used to identify proteins with a similar fold, as it uses the alignment of hidden Markov models via HHsearch to significantly improve the accuracy and the detection rate (54). The quality of the models was evaluated with Procheck (55) and Verify3D (56). ProSA-web (57) was utilized to examine the energy of residue-residue interaction using a distance-based pair potential approach in which the energy is transformed to a Z-score where residues with a negative score indicate reasonable side-chain interactions. The overlays between the predicted models and the figures were made using the PyMOL Molecular Graphics System (58). The full-length predictions were generated with a combination of manual approach and docking techniques. At first, the modeled N-terminal segment of the globin domains were approached to the C-terminal segment of the respective modeled 7TM domains. Secondly, the RosettaDock program (59) was launched to optimize the interface contacts of the two domains. A dimer of two GLB-33 GD models was also predicted using the known dimeric GLB-1 crystal structure (PDB: 2WTH) as a template. The interface of the dimer was optimized with the RosettaDock program.

RESULTS

In silico search for orthologs of GLB-33 - GLB-33 orthologs were found in the cluster of the Rhabditoidea (a subgroup of the Rhabditina, Nematoda) (60), more specifically in the genomes of *C. briggsae*, *C. remanei*, *C. brenneri*, *C. japonica*, *C. angaria*, *C. sp5* and *C. sp11*, which are all closely related species belonging to the *Caenorhabditis* genus as well as in *Heterorhabditis bacteriophora*. Furthermore, by computational analysis we identified a partially annotated orthologous sequence of GLB-33 in the recently sequenced genome of *A. suum* (geneset CDS database, GS_17715). Despite conservation of two out of three intron positions (EF10.0 and FG7.0), the predicted gene only encoded a

truncated form of the C-terminal globin domain and no N-terminal transmembrane domain. Most intriguingly, our comprehensive *in silico* searches also revealed similarities to a predicted FMRFamide receptor in the *A. suum* genome (*A. suum*FMRF, GS_06644), with an E-value of $2e^{-15}$ and a sequence identity of 24% compared to GLB-33. To exclude whether no orthologs were missed, we screened the sequenced genomes of all nematodes including *Ancylostoma* with GLB-33 GD, GLB-33 7TM, GLB-33, *A. suum*FMRF GD, *A. suum*FMRF 7TM, and *A. suum*FMRF as a template. No other receptors with a C-terminal extension similar to GLB-33 and *A. suum*FMRF were found.

Sequence analysis of A. suumFMRF GD - Alignment of the protein sequences of GLB-33 and *A. suum*FMRF (24 % identical and 44 % similar) showed that the physico-chemical properties of the majority of the amino acids are conserved (Figure 1). In the alignment, the positions of the transmembrane helices and α -helices are indicated as predicted by the 3D model of these proteins. It is clear that the amino acids building up the transmembrane helices are very similar, differing only by a few amino acids, thus resulting in transmembrane regions of comparable length (Figure 1A). However, in the globin-like domain of *A. suum*FMRF, the lack of the A- and D-helices and the extended C-helix is striking when aligned to GLB-33 GD (Figure 1A and 1B), resulting in a possible modified truncated globin domain. This globin domain is preceded by a helical linker region that connects it to the 7TM domain (Figure 1A). Therefore, we considered the possibility that the cDNA sequence of the globin domain of the *A. suum*FMRF reported in the database was incorrectly predicted, requiring a correction of the open reading frame. We therefore amplified the cDNA of *A. suum*FMRF GD and determined its sequence. Remarkably, the amplified cDNA was identical to the predicted one, supporting a correct protein sequence with an unusual tertiary structure.

In vivo and in vitro localization of GLB-33 - For the localization of GLB-33 *in vivo*, a translational reporter for the *glb-33* gene was constructed including the endogenous promoter region, and 3'UTR elements. The expression pattern of this

reporter was in line with the results of a previously used transcriptional reporter (4), and indicated that GLB-33 was exclusively expressed in the nervous system (Figure 2A). The GLB-33 protein was present in a large number of neurons in the head and tail region, the nerve ring, the ventral and dorsal nerve cord, and several lateral nerve cords (Figure 2B and 2D). However, despite its wide expression in the nervous system, GLB-33 did not seem to be expressed in any of the amphid, cephalic, labial, or phasmid sensory neurons; the typical expression pattern for these neurons was not observed, nor was any overlap seen between GFP-expression and DiI-staining, a compound that selectively stained several amphid and phasmid neurons (data not shown). This indicates that GLB-33 is expressed in motor- or interneurons, which are involved in locomotory behaviour or information processing, and not in neurons that sense environmental cues. Finally, the expression pattern of the reporter was in line with the membrane bound localization of the receptor (Figure 2C). The membrane localization of GLB-33 was confirmed by the transfection of *glb-33-gfp* in human neuroblastoma SH-SY5Y cells (Figure 2E).

Spectral characterization of GLB-33 GD and A. suumFMRF GD -

UV-Vis spectra - The UV-Vis absorption spectra of GLB-33 GD in the as-expressed, the dithionite-reduced (ferrous deoxy), the ferrous CO-bound, and the ferrous oxy-form, prepared at pH 8.5 and the aquo-met-form, prepared at pH 4, are shown in Figure 3. The ferrous deoxy form has the Soret band at 433 nm and a Q-band maximum at 558 nm. These maxima are typical for pentacoordinate high-spin ferrous deoxy heme proteins (45). The absorption spectrum of the CO-form of GLB-33 GD is similar to those of sperm whale myoglobin (*swMb*), with peaks at 420, 539 and 568 nm (45). After reduction with dithionite and desalting, the ferrous oxy form has peaks at 413, 543 and 581 nm (Figure 3A). Although the absorption spectrum of as-expressed GLB-33 GD (pH 6 - 8.5) is apparently very similar to that of a ferrous oxy-globin, with the Soret band located at 413 nm and the Q-bands at 544 and 577 nm, these maxima are also similar to those of the ferric hydroxide-ligated form of *swMb* (61), and to those

of low-spin heme oxygenase at alkaline pH (62). EPR (see below) confirms that the ligated state of the as-expressed GLB-33 GD can be assigned to the hydroxide-ligated ferric form. Note that in the as-purified samples at pH 8.5, there was still a small fraction of ferrous oxy-form present capable of reversible O₂ binding.

The UV-Vis spectra of the protein remained practically unchanged during the purification process, after several freeze-thaw cycles, and after prolonged exposures to air, indicating that the hydroxide-ligated ferric derivative is very stable. Dialysis of GLB-33 GD against 10 mM sodium acetate pH 4 resulted in the aquo-met form (Figure 3B), with absorption maxima comparable to hexacoordinate high-spin heme oxygenase (62). This acidic transition was completely reversible, since redialysis to buffers with pH 6 - 8.5 reconverted GLB-33 GD to the ferric hydroxide-ligated form, thus confirming that the as-expressed protein was not in the ferrous oxy state (data not shown).

In Figure 3C, the UV-Vis absorption spectra of the as-expressed and deoxy reduced *A. suumFMRF GD* are depicted as prepared in 50 mM Tris-HCl pH 7.5. The as-expressed protein has the Soret-band at 412 nm and Q-bands at 534 nm indicating that the globin is in a hexacoordinate low-spin ferric state. Upon reduction, the Soret band shifts to 424 nm with Q-band maxima at 534 nm and 558 nm, indicative of ferrous hexacoordinate globins, in contrast with the ferrous pentacoordinate heme of the deoxy GLB-33 GD. The absorption maximum at 661 nm, typical for free biliverdin (63), in both spectra indicates the presence of denatured protein as the intensity of the peaks remained unchanged after reduction of the heme iron atom (Figure 3B). Indeed, *A. suumFMRF GD* was very unstable, and extensive precipitation occurred during the whole purification process, as well as during attempts to bind other ligands to the heme iron atom, as could be deduced from the disappearance of the Q-band maxima (data not shown). Stable liganded species could not be produced for this reason.

Resonance Raman spectra - Figure 4A shows the high-frequency region of the RR spectra of GLB-33 GD in the as-expressed, the ferrous and the CO-bound ferrous forms in 50 mM Tris-HCl pH 8.5. The high-frequency region of the RR

spectra of heme proteins contains a number of so-called “marker bands”, that depend on the oxidation state, the coordination state and the spin state of the heme iron atom.

RR spectra confirmed that GLB-33 GD is expressed in the hydroxide-ligated ferric form under the conditions used, (Figure 4A (a)), with a first set of marker bands at $\nu_4=1376\text{ cm}^{-1}$, $\nu_3 = 1502\text{ cm}^{-1}$, $\nu_2 = 1581\text{ cm}^{-1}$. The ν_4 line at 1376 cm^{-1} and a ν_3 line at 1503 cm^{-1} are typical of hexacoordinate, low-spin ferric heme complexes (64). The frequencies of ν_3 and ν_2 are very close to the hydroxide-ligated form of met-globins, that are characterized with ν_3 at $\sim 1505\text{--}1507\text{ cm}^{-1}$ and ν_2 at $\sim 1585\text{ cm}^{-1}$ (65-67). A second set of marker bands is located at $\nu_3 = 1476\text{ cm}^{-1}$ and $\nu_2 = 1558\text{ cm}^{-1}$, that likely stems from a small fraction of a high-spin ferric form (68,69).

The high-frequency region of dithionite-reduced, deoxy GLB-33 GD (Figure 4A (b)) has a single set of marker bands at $\nu_4 = 1356\text{ cm}^{-1}$, $\nu_3 = 1470\text{ cm}^{-1}$, $\nu_2 = 1560\text{ cm}^{-1}$, typical for a pentacoordinate, high-spin ferrous heme. Two ν_4 bands at 1371 cm^{-1} and 1354 cm^{-1} are found in the RR spectra of the CO-bound ferrous protein recorded at low laser power (0.5 mW) (Figure 4A (c)). These stem from the CO-bound ferrous and photolysed form of the globin, respectively. The latter is confirmed by the fact that the intensity of the ν_4 band at 1354 cm^{-1} increases when the laser power is increased to 100 mW (Figure 4A (d)). This illustrates that, the CO-bound ferrous form is photo labile. The ν_4 frequency of the CO-bound form is lower than that of the as-expressed ferric form, suggesting that the GLB-33 GD distinguishes -OH from CO as a ligand, as also found for a bacterial heme-based aerotactic transducer (HemAT-*Bs*(O₂), Ref. (70)).

Figure 4B shows the low-frequency region of the RR spectra of the different GLB-33 GD ligated forms. The low-frequency region of the RR spectra of heme proteins contains a number of bending modes of the heme peripheral vinyl and propionate substituents. The frequency of these bending modes depends on the strength of the interactions between these side chains of the heme and amino acid residues in the heme pocket of the protein. The assignment of the RR bands is based on the work of Spiro and coworkers on Mb (68). The propionate bending mode, $\delta(\text{C}_\beta\text{C}_\alpha\text{C}_\delta)$, of as-expressed GLB-33 GD (Figure 4B (a)) is located at 376 cm^{-1} (broad signal) indicating a strong H-bond

interaction between the heme propionates and nearby amino-acid residues. A vinyl bending mode is found at 416 cm^{-1} . The broad profile of this band indicates the presence of a second vinyl mode at a higher frequency of $\sim 430\text{ cm}^{-1}$. A broader signal is found in the $490\text{--}515\text{ cm}^{-1}$ region where the $\nu_{\text{Fe-OH}}$ signal is expected (71). For dithionite reduced deoxy GLB-33 GD (Figure 4B (b)), the frequency of the propionate bending mode is located at a higher frequency of 382 cm^{-1} , indicating an even stronger interaction between the heme propionates and nearby amino acids. Furthermore, a larger separation between the two vinyl modes is observed. One is located at 414 cm^{-1} , while the second mode is located at 432 cm^{-1} . These data show significant interactions between the protein moiety and the heme that are dependent on the heme-ligation state.

The Fe-CO stretching mode can be found in the low-frequency region of the RR spectra of CO-bound ferrous heme proteins (72). In the current case, a clear determination of the Fe-CO-modes is hampered by the high photolability of the CO-ligated GLB-33 GD form (Figure 4B (c,d)), although a broader signal is seen in the $489\text{--}504\text{ cm}^{-1}$ region typical of the Fe-CO modes.

EPR - CW-EPR (not shown) and ESE-detected EPR (Figure 5a) both confirm the presence of two low-spin ferric forms in the as purified GLB-33 GD sample at pH 8.5. The two low-spin components have the following principal g values: $(g_1, g_2, g_3) = (2.62, 2.20, 1.815)$ (Figure 5b, red) and $(g_1, g_2, g_3) = (2.845, 2.12, 1.69)$ (Figure 5b, green). The former g -values are similar to those of a low-spin Antarctic fish hemoglobin (73), and point to the formation of a hydroxide-ligated ferric heme form (74). The g -values are also similar to those observed for alkaline cytochrome *c* peroxidases (75,76). The second set of g -values is very similar to that of horse-radish peroxidases $((g_1, g_2, g_3) \sim (2.9, 2.1, 1.6))$ showing low-spin hydroxide- ligation of the ferric heme in alkaline conditions (77,78). A recent study on *Thermobifida fusca* hemoglobin revealed the presence of two ferric forms with similar EPR parameters as those found here. The component with a g_1 value around 2.8 was assigned to the hydroxide-ligated ferric complex where the OH group is strongly hydrogen bonded, while the form characterized by g_1 at ~ 2.6 is ascribed to a

coordination mode in which the OH group is not so strongly H-bonded (79). Interestingly, also in *T. fusca* hemoglobin, the hydroxide-ligated complexes were still observable at pH 6.0. No significant contribution of a high-spin ferric form (i.e. aquo-met) is found in the CW EPR or ESE-detected EPR of GLB-33 GD at pH 8.5 (data not shown). The EPR spectra thus show that low-spin ferric heme complexes are already dominantly formed at lower pH values than in most other globins. The UV-visible absorption and RR spectra are in agreement with this finding. The difference between the two low-spin ferric forms most probably stems from a change in the hydroxide stabilization by the surrounding protein matrix.

Ligand binding kinetics of GLB-33 GD -

CO rebinding kinetics - Flash photolysis was performed at pH 7 as described in Materials and Methods. CO rebinding from within the protein matrix to the heme iron atom is referred to as the [CO]-independent, geminate rebinding phase (occurs in the ns-timescale after flash photolysis). For GLB-33 GD, this geminate rebinding process occurred with a rate constant of $9.87 (\pm 0.07) \times 10^7 \text{ s}^{-1}$ (Table 1), about 18 times as fast as the geminate rebinding of CO to *swMb* ($k_{\text{gem}} = 0.55 (\pm 0.08) \times 10^7 \text{ s}^{-1}$, Table 1). About half of the CO molecules rebind immediately, resulting in a high fraction of geminate rebinding of 0.50 ± 0.05 (Table 1), an indication that the CO molecules cannot easily escape from the protein matrix in comparison with *swMb* ($F_{\text{gem}} = 0.043 \pm 0.006$, Table 1). On longer timescales, a fraction of the gaseous ligands can escape to the bulk solvent and rebind from outside the protein matrix in a [CO]-dependent way. This bimolecular rebinding phase in GLB-33 GD is a biphasic process, characterized by a fast and a slow rebinding rate constant: $k_{\text{on},f} = 0.90 (\pm 0.10) \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and $k_{\text{on},s} = 0.35 (\pm 0.01) \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, respectively (Table 1, Figure 6). The latter is comparable to the CO rebinding rate of *swMb* (Table 1).

O₂ equilibria - The apparent P_{50} measured with the diffusion-chamber method was 0.07 ± 0.01 torr at 25 °C, pH 7.4 (data not shown). However, we cannot exclude that at these very low levels of PO_2 the protein within the diffusion-chamber was partially oxygenated even when equilibrated with pure nitrogen (i.e. to obtain zero

saturation). Therefore it is possible that the true P_{50} is lower than 0.07 torr. The equilibrium association constant ($K_{\text{O}_2}, \text{M}^{-1}$) for O_2 derived from the P_{50} value is $\geq 8.5. \mu\text{M}^{-1}$ when assuming an O_2 solubility in water of $1.67 \mu\text{M torr}^{-1}$ at 25 °C (80). A Hill coefficient close to 1 (~ 0.9) indicated absence of cooperativity (not shown).

Autoxidation rate of GLB-33 GD - Autoxidation of oxy GLB-33 GD at pH 4 (25 and 37 °C) and at pH 7.4 (25°C) showed a single exponential process with a rate of 1.81 h^{-1} (25 °C) and 15 h^{-1} (37 °C) at pH 4, ~ 278 times faster than native *swMb* (0.054 h^{-1}) at pH 7 and 37 °C (81), and ~ 62 times faster than the *swMb* His(E7)68Glu mutant (0.24 h^{-1}) at pH 7 and 37 °C (82). At pH 7.4 the rate constant is $0.09 \pm 0.02 \text{ h}^{-1}$. As the absorbance peaks of the oxy and hydroxide-ligated ferric forms are almost identical (Figure 3A), the spectral change over time was small. However, the fact that isosbestic points (e.g. 531, 555, 568 and 585 nm at pH 7.4) were maintained over time (not shown) is consistent with a single oxy to ferric transition at both pH 4.0 and 7.4. For pig myoglobin, a larger increase of the oxidation rate constant (several thousands) (83) was found when going from pH 7 to pH 4 in comparison to GLB33GD. However this smaller increase can be due to the fact that there is a conversion from the hydroxide-ligated form to the aquo-met form for the latter.

Nitrite-reductase activity of GLB-33 GD The nitrite reductase activity was measured in the presence of a slight excess of dithionite. The data show that deoxygenated GLB-33 GD was able to convert nitrite to NO and the nitrite-induced conversion of deoxy heme to NO-bound heme was confirmed spectrophotometrically (Figure 7A). The visible absorption spectrum changed over time from that of deoxy GLB-33 GD (Fe^{2+}) with a single peak at 558 nm to that of a NO-bound globin ($\text{Fe}^{2+}\text{-NO}$) with peaks at 545 and 570 nm, indicating that the reaction followed the general scheme for nitrite reduction by deoxy globins in the presence of dithionite (51,84). When plotting the fitted observed rates (k_{obs}) as a function of nitrite concentration, the second order rate constant was $36.3 \text{ M}^{-1} \text{ s}^{-1}$ (Figure 7B), which is ~ 10 -fold higher than that for Mb ($2.9 \text{ M}^{-1}\text{s}^{-1}$, (85)).

3D modeling of GLB-33 GD and GLB-33 7TM domain - The structural model of the N-terminal GLB-33 7TM domain was predicted using the I-TASSER software (52,53). The best predicted model shows a C-score of -0.30 and an expected TM-score of 0.67 ± 0.12 . Both these values indicate a satisfactory model with reliable topology. The stereo-chemical quality of GLB-33 7TM model was analyzed with Procheck. The resulting Ramachandran plot displayed 91.9% of all amino-acid side-chain angles in allowed regions and 8.1% in disallowed regions. Verify3D analysis showed about 50% of the residues with a score over 0.15, and ProSA-web evaluation confirmed this result with an overall calculated Z-score of -2.52. Phyre² identified the human β_2 -adrenergic GPCR (β_2 -AR, PDB: 2RH1; (86)) as the most similar protein of known structure, with 41% similarity with the query sequence, and which was chosen as reference structure for the comparison. An overlay of the model with the β_2 -AR structure was generated using PyMOL (Figure 8A). A root mean square deviation (r.m.s.d.) value of 1.80 Å was calculated over 243 C $_{\alpha}$ atom pairs, suggesting the presence of several differences. The position of every helix of the modeled GLB-33 7TM is very well superimposed on the β_2 -AR structure throughout the whole length, with exceptions in correspondence of numerous loops : N-terminal and C-terminal extremities, as well as the loop connecting helix 5 to 6 are not present in the PDB file of β_2 -AR (evidence of strongly flexible regions), the GLB-33 7TM model shows a 17 residues longer loop connecting helix 3 to 4 compared to the reference structure, and the amino-acid stretch that connects helix 4 to 5 presents a short ordered helix in the β_2 -AR structure, whereas it was predicted as disordered in our model. The most significant distances in the C $_{\alpha}$ positions of the α -helices are comprised between a minimum of 1.8 Å (N-terminal of helix 5) and a maximum of 6.5 Å (N-terminal of helix 1).

Similarly, a prediction model was generated for GLB-33 GD. The C-score (-0.62) and the expected TM-score (0.63 ± 0.13) indicate the good quality of the best model predicted. Procheck evaluation indicated that the geometry of the 97.6% of the residues is in allowed regions and as little as 2.4% is in disallowed regions. Verify3D showed that all the residues have a score higher than 0.2, and ProSA-web results confirm this

finding through a negative Z-score for every residue, and a calculated Z-score of -7.44 for the global molecule. All these data indicate a reliable model. For comparison, an overlay of the predicted model on the known high resolution 3D structure of GLB-1 (PDB: 2WTG; (6)) was made (Figure 8B). The r.m.s.d. of the overlay resulted in 2.30 Å calculated on 134 C $_{\alpha}$ atom pairs. The 3-over-3 globin fold is conserved in the GLB-33 GD model, nevertheless the different orientation of the modeled helices compared to the reference structure causes a global non-correspondence of the C $_{\alpha}$ backbone. A closer look at the amino-acid residues in the distal site of the GLB-33 GD model highlights its exceptional hydrophobic composition: Leu(41)CD3, Ile(69)E7, Arg(72)E10, and Ile(73)E11 (Figure 8C). This highly apolar environment leaves Arg(72)E10 as the only residue available for ligand stabilization. Likewise, *C. elegans* GLB-1 displays a majority of hydrophobic residues in its distal pocket (Phe34, Phe38, Phe49, Phe66, Ile73, and Phe114), but O₂ is directly stabilized by an unusual combination of the distal residues Tyr(35)B10 and Gln(69)E7 (6).

Until now, no 3D structure of a globin associated with a transmembrane domain has been solved. As a consequence, homology modeling of the full-length protein was impossible. To visualize the full structure of GLB-33, a manual approach of the assembly of the GLB-33 GD model to the GLB-33 7TM model was attempted. The N-terminal of the globin domain was located manually in the proximity of the C-terminal of the 7TM domain. Subsequently, the docking program RosettaDock was used to optimize the interface interactions between the two models. The result is shown in Figure 8D. Despite the limitation of this approach, the docking shows a wide interaction surface between the two domains and a possible connection between the two terminal loops of the predicted models. Indeed, the interaction surface involves the N-terminal of the A-helix, the E-F-corner (i.e. C-terminal E-helix and N-terminal F-helix), 70% in length of the H-helix, and the C-terminal loop of the GD domain. Thus, these data suggest that any conformational change that occurs in the GD can be readily transmitted to the 7TM domain.

3D modeling of *A. suum*FMRF 7TM and *A. suum*FMRF GD - Similarly to GLB-33, the full-length FMRFamide receptor of *A. suum* was modeled separating first the N-terminal *A. suum*FMRF 7TM from the C-terminal *A. suum*FMRF GD and secondly rejoining manually the two predicted domains. The *A. suum*FMRF 7TM model predicted by the I-TASSER software (52,53) shows a C-score and an expected TM-score (0.41 and 0.77 ± 0.10 respectively) that indicates a good quality model. The geometry analysis performed with Procheck resulted in 94.5% of the residues in allowed regions and in 5.5% in disallowed regions. The Verify3D program highlighted that about 38% of the residues shows a score over 0.15 and the Z-score calculated with ProSA-web of -0.49 confirmed this result. As for GLB-33-7TM, Phyre² identified the human β_2 -adrenergic GPCR (β_2 -AR, PDB: 2RH1; (86)) as the most similar protein of known structure with 35% similarity. An overlay of the *A. suum*FMRF 7TM model with the GLB-33 7TM model was generated using PyMOL (Figure 9A). Over 282 C _{α} atoms, a r.m.s.d. of 1.18 Å was calculated, suggesting a low level of variety. Indeed, the two models superimpose very well, except for longer N- and C-terminal loops in the GLB-33 7TM model, and for the longer interhelical loop between helices 3 and 4 already noticed in its comparison with the β_2 -AR structure, that is absent in the prediction of *A. suum*FMRF 7TM.

Strikingly, the three-dimensional model for the putative C-terminal *A. suum*FMRF GD indicated a globin fold non referable to any known globin family. It can be recognized as a classical 3-over-3 α -helical globin fold, but lacking the A- and D helices, and exhibiting a longer C-helix (Figure 9B). For this model, we obtained a C-score of -0.90 and an expected TM-score of 0.56 ± 0.14 with the I-TASSER software. The geometry quality assessment performed with Procheck indicated that 98.4% of the residues lay in allowed regions and 1.6% in disallowed regions. Verify3D and ProSA-web display positive concordant results (about 93% the residues have a score higher than 0.2, and a negative Z-score for every residue and a calculated Z-score of -5.2 for the global molecule, respectively). All these data confirm the good geometry of this model. The Phyre² similarity search identified the globin domain of GLB-6 from

C. elegans (PDB: 3MVC; (7)) as the most similar structure with 42% of similarity. Since *A. suum*FMRF GD lacks several helices, we modeled its structure with a variety of 2/2 truncated hemoglobin (trHb) structures as a template to exclude bias in the modeling program. These tests did not result in a good model however, and suggested that the α -helical fold of *A. suum*FMRF GD is more related to the 3/3 classical globin fold than to the 2/2 trHbs, even though some deviations can be detected (data not shown). Superimposing the models of *A. suum*FMRF GD and of GLB-33 GD (Figure 9B), we obtained a r.m.s.d. value of 2.57 Å over 88 C _{α} atom pairs. Obviously, this comparison emphasizes the lack of the A-, B-, and D-helices in *A. suum*FMRF GD. The shorter F-helix of GLB-33 GD is also noticeable, as well as the similar open conformation of the E-helix in both predicted models. A detailed look at the amino-acid composition of the heme pocket reveals that it is more polar than the heme pocket of GLB-33 GD, with Phe(10)B10, Glu(38)E7, Cys(41)E10 and Ile(42)E11 (Figure 9C).

Finally, the full-length appearance of *A. suum*FMRF was investigated using the model (Figure 9D). The two docked domains display a discrete interaction area and the possibility of a covalent connection between the two termini of the predicted models. The heme pocket of the docked *A. suum*FMRF GD is even more exposed to the solvent than in GLB-33 GD (Figure 9D), as shown in Figure 9D. Obviously this is also due to the peculiar truncated globin fold of *A. suum*FMRF GD.

DISCUSSION

The similarity in primary and tertiary structure between the *C. elegans* GLB-33 and the *A. suum* FMRFamide receptor, as indicated by modelling analysis (Figure 9), suggests that they could serve a similar role *in vivo*. The modeled N-terminal extracellular loops create a structural pocket where FLPs could bind thus leading to a conformational change, resembling that of other FMRFamide receptors (Figure 8A). Nothing is known, however, about the process regulated by these chimeric proteins, but some indications can be found by studying the *in vivo* transcription pattern. Analysis of transcriptome data of *A. suum* during development indicates that the FMRFamide

receptor is predominantly expressed in the L3 larval stage, and peaks when the larvae have just hatched from the egg (Peter Geldhof, University of Ghent; and Robin Gasser, University of Melbourne, personal communication), but it is unclear so far what role the FMRFamide receptor could play in this developmental stage. In contrast, the *C. elegans* GLB-33 is expressed throughout all life stages of the nematode (3). Following the *in vivo* expression pattern of GLB-33, we detected this protein predominantly in inter- and motorneurons, and it might therefore be involved in movement following processing sensory cues (Figure 2).

To explore the function of the globin domains of GLB-33 and of the *A. suum* FMRFamide receptor, recombinant GLB-33 GD and *A. suum*FMRF GD were prepared and their biochemical properties investigated. It has to be noted that in *A. suum* the recombinant globin domain was very unstable, probably due to the lack of several helices as compared to genuine globins and the lack of the FMRFamide receptor domain that might compensate this truncation. In the absence of various gaseous ligands, the globin domain of GLB33 show an optical spectrum that is characteristic of a pentacoordinate ferrous globin, whereas the globin domain of *A. suum* is hexacoordinate. Raman and EPR spectra of *C. elegans* GLB-33 GD show tight interactions between the heme group or its ligand and the surrounding protein moiety (Figures 4B, 5). Since changes in the ligation state of the heme iron atom are accompanied by conformational changes in the globin core, this suggests that the heme ligation and/or redox state of the globin domain could influence the conformational state of the coupled receptor domain. In this way the globin domain could modulate the sensitivity of the effector domain for the binding of neuropeptides. Likewise, the binding of a neuropeptide to the receptor domain could introduce a conformational change in the globin domain, thus altering the ligand-binding properties of the latter. More arguments for the modulation of ligand affinity can be found in our modelling data. Docking the model of GLB-33 GD to that of GLB-33 7TM showed that the opening to the heme pocket is directed to the outer environment (Figure 8D), suggesting that in the chimeric protein ligand binding and dissociation is still possible.

The reactivity of a globin domain towards ligands depends mostly on the amino-acid residues in the heme pocket that can stabilize the bound ligand. The distal B10, E7, E10 and E11 residues are known to be involved in this stabilization in several globins (87-89), and are occupied by a different set of residues in the two globin domains investigated here (Figure 1B): Ala31, Ile69, Arg71, and Ile73 in GLB-33 GD, and Phe10, Glu38, Cys41 and Ile42 in *A. suum*FMRF GD, respectively. Interestingly, neither globin domain contains the distal HisE7. Few examples of globins that contain Ile as the E7 distal residue, as GLB-33 GD, are known, including the truncated Hb of the cold adapted bacterium *Pseudoalteromonas haloplanktis* TAC125 (*Ph-2/2HbO*), where the B10, E7, E10 and E11 positions are occupied by Tyr, Ile, Val and Phe, respectively (90). The electronic absorption spectra of *Ph-2/2HbO* at physiological pH and low temperature (12K) are very similar to those of GLB-33 GD at the same pH at room temperature, with absorption maxima in the Q-band region at 535 nm and 570 nm, indicating a hexacoordinate low-spin ferric species with a hydroxide group as the 6th ligand. In the case of *Ph-2/2HbO*, this ligand is donated to the heme iron by the TyrB10 residue (90). At room temperature, the absorption spectrum of *Ph-2/2HbO* indicates the additional presence of a hexacoordinate high-spin His-Fe-H₂O form (90) and is similar to the spectrum of GLB-33 GD at pH 4 (Figure 3B). Also *T. fusca* hemoglobin, with Tyr, Ala, Arg and Leu on positions B10, E7, E10 and E11, exhibits hydroxide-ligated forms, which are in equilibrium with an aquo-met form at pH 6 (79). In the latter protein, EPR contributions of hydroxide-ligated heme forms with similar principal *g* values as for the GLB-33 GD case were observed, with the exception that the *g*₁~2.6 contribution was only a minority species. In the case of GLB-33 GD, both components contribute approximately equal to the overall EPR spectrum. This is in line with the amino-acid residues in the distal pocket of the two proteins. In *T. fusca* hemoglobin the hydroxide group bound to the ferric heme is stabilized by TyrB10, TyrCD1 and TrpG8 in agreement with the dominance of the *g*₁~2.8 contribution in the EPR spectrum indicative of strong H-bonding to OH. In contrast, in GLB-33 GD, the B10 position is occupied by an Ala residue unable to stabilize the hydroxide ligand.

The only polar side chain in the largely apolar heme pocket is ArgE10. This explains the larger fraction in which the heme-bound hydroxide is more weakly H-bonded to the protein than in the *T. fusca* hemoglobin, as shown by the larger EPR $g_1 \sim 2.6$ contribution (Figure 5). Since no amino-acid residues are present in the distal pocket of GLB-33 GD that can donate a hydroxide group to the ferric heme-iron (Figure 8C), it is most likely that the hydroxide ligand originates from a water molecule as is also the case for *T. fusca* hemoglobin. This hypothesis is supported by the observation that the conversion between the high-spin aquo-met species and the low-spin hydroxide-ligated form is completely reversible upon pH change through dialysis (data not shown).

Interestingly, a similar heme environment is found in the distal pocket of the PAS-domain of the O₂ sensor Fix-L of *Bradyrhizobium japonicum* (*Bj* Fix-L). Although the structures of the globin domain and of the PAS-domain differ significantly, they both bind a heme cofactor, and some comparisons can be made. In *Bj* Fix-L, ligand interaction is possible through the guanidinium group of G_β2Arg (91). This residue has been shown to assist in the sensing of O₂. This is not accomplished by stabilizing the oxy-form though, but instead the side chain is involved in triggering a conformational switch upon binding of regulatory ligands (92). In GLB-33 GD, the ArgE10 residue could be involved in a similar mechanism. It appears plausible that when O₂ binds with high affinity to the ferrous heme, it will induce a conformational switch of the Arg(72)E10 residue upon formation of the oxy derivative, which may then act as a trigger to transduce the signal to the transmembrane receptor domain. The observation of two ferric hydroxide-ligated forms observed in EPR spectra (Figure 5) suggests the existence of two different protein conformations with potentially different roles in signal activation or deactivation. .

Another functionally important property of ferrous GLB-33 GD is its higher reactivity with nitrite, compared to other globins. This may be due to the positively charged Arg(72)E10 residue, which may help in facilitating electron transfer from the heme to the bound nitrite. The relatively fast conversion of nitrite to NO mediated by the ferrous protein could also be involved in efficient

neuronal signaling and affect local activities of nearby neurons. Moreover, it has been shown that NO mediates the inhibitory effects of an FMRFamide-like neuropeptide in *A. suum*, linking NO with neuropeptide signaling (93). Clearly, the effects of such reaction on the effector domain of the full-length protein remain to be investigated, particularly because *C. elegans* does not express nitric-oxide synthase enzymes and could then in principle be more reliant on nitrite-reductase activities for NO signaling.

The covalent structural coupling of a globin domain and a 7TM domain is unique, and has never been observed before. However, a functional non-covalent interaction has been shown for the O₂ sensor GLB-5, one of the globins encoded in the genome of *C. elegans*, and NPR-1, an FMRF-amide receptor activated by the binding of the neuropeptides FLP-18 and FLP-21 (8,9,94). GLB-5 modulates the same O₂ sensing neurons as NPR-1 (AQR, PQR and URX), which also express GCY-35 and GCY-36, subunits of soluble guanylate cyclases (sGCs) that also act as O₂ sensors (9,95-100). It was proposed that GLB-5 acts as a signaling molecule, inhibiting neuronal activation when the O₂ concentration drops below 21% (9). Interestingly, sGCs can also be activated by NO, emphasizing a possible similar mechanism between GLB-33 and GLB-5 (103). Because of its very high affinity for O₂, t GLB-33 may function as a highly sensitive O₂ sensor that would modulate neuronal sensitivity to neuropeptides and allow *C. elegans* to move towards nutrient rich substrates in the hypoxic soil. Furthermore, the ability of GLB-33 GD to generate NO from nitrite (largely available in the soil) under hypoxic conditions would then add another level of complexity to this response.

CONCLUSION

Overall, we can conclude that GLB-33 is composed of a 7TM domain, similar to FMRFamide receptor domains, covalently coupled to a globin domain that is able to bind external ligands at the heme group. Our modeling data show that the conformational switch of GLB-33 7TM upon ligand binding, likely a neuropeptide ligand, can result in a conformational change of the globin domain, and conversely that reactions

in the heme pocket may modulate the affinity of neuropeptides for the 7TM domain.

Characterization of GLB-33 GD showed that it possesses characteristics typical of an O₂ sensor when present in the ferrous and ferric forms. Furthermore, the ferric heme occurs as a combination of two hydroxide-ligated ferric forms under physiological conditions. In addition, since nitrite-reductase activity is fast compared to that of other globins, GLB-33 GD could also be

involved in local NO production from available nitrite when in the ferrous unliganded state, provided that close targets of NO bioactivity and heme reducing pathways are present. We identify Arg(72)E10 as the most likely trigger for a conformational switch that could be transmitted to the 7TM domain and as such activate yet unknown neuronal signalling pathways.

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FOOTNOTES

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1 Abbreviations: Transmembrain domain of GLB-33 (GLB-33 7TM); G-protein-coupled receptor (GPCR); GLB-33 globin domain (GLB-33 GD); resonance raman (RR); Electron Paramagnetic Resonance (EPR).

FIGURE LEGENDS

Figure 1 : Alignments based on the 3D structures and 3D models of the 7M and globin domains. A) GLB-33 and *A. suum*FMRF. Transmembrane helices and α -helices are framed. Gray-scale shading indicates iso-functional or identical amino acid residues. The linker region is depicted in bold. B) GLB-33 with the relevant reference proteins *swMb* (Accession number BAF03579), GLB-1 (Accession number CAA77458), GLB-6 (Accession number CCD65109) and orthologs in *C. briggsae* (Cbri, WormBase ID CBG06424), *C. remanei* (Crem, WormBase ID CRE05562), *C. brenneri* (Cbri WormBase ID CN07673), *C. japonica* (Cjap, WormBase ID JA64867), *C. angaria* (Cang, WormBase ID Cang_2012_03_13_00952.g15095.t1), *C. sp5* (WormBase ID Csp5_scaffold_00786.g15463.t1), *C. sp11* (WormBase ID Csp11.Scaffold506.g2439.t1) *Heterorhabditis bacteriophora* (Hrab, WormBase ID Hba_01784) and *Ascaris* (WormBase ID GS_17715, GS_06644). Helices are the annotated helices from the crystal structure of *SwMb*. Conserved residues at buried sites in the 'Helices' are indicated with a capital letter according to (33). Gray-scale shading indicates iso-functional or identical amino acid residues.

Figure 2 : *In vivo* and *in vitro* expression pattern. A. Overview of the *in vivo* expression pattern of the *glb-33* translational reporter. B. head region. C. subcellular expression of the reporter, and D. tail region of the worm. GLB-33 is membrane bound and is expressed in a high number of neurons in the head and tail region of the worm, the nerve ring, the ventral (VNC) and dorsal nerve cord (DNC) and several lateral nerve cords. E. *In vitro* membrane localization of GLB-33 in human neuroblastoma SH-SY5Y cells.

Figure 3 : UV-Vis absorption spectra of A) GLB-33 GD in the as-expressed form pH 8.5 (black) (Soret band: 413 nm; Q-bands: 577 and 544), the ferrous deoxy form (blue) (Soret band: 433 nm; Q-band: 558 nm), the CO-bound ferrous form (red) (Soret band: 420 nm; Q-bands: 568 and 539 nm), and the ferrous oxy-form (green) (Soret band: 413 nm; Q-bands: 581 and 543 nm) at pH 8.5. B) GLB-33 in the as expressed form at pH8.5 (black) and pH4 (red). The latter gives a spectrum for the aquo-met form (Soret band: 408 nm; Q-bands: 503 and 633 nm), C) *A. suum*FMRF GD in the as-expressed form (black) (Soret band: 412 nm; Q-bands: 565 and 534 nm) and the deoxy ferrous form (red) (Soret band: 424 nm; Q-bands: 558 and 534 nm).

Figure 4 : A. High-frequency region and B. low-frequency region of the resonance Raman spectra of GLB-33 GD in a. the as-expressed hydroxide-ligated ferric form, b. the ferrous deoxy form at 50 mW laser power and the CO-bound ferrous form recorded at c. less than 0.5 mW, and d. 100 mW laser power.

Figure 5 : (a) ESE-detected EPR spectrum of as-purified GLB-33 GD (sum of 400 τ values between 88 ns and 3280 ns) and (b) first derivative of spectrum (a) in black with simulation of ferric hydroxide GLB-33 GD (red) and ferric azide GLB-33 GD (green). The measurements were performed at 9K.

Figure 6 : CO rebinding to GLB-33 GD. CO rebinding curves after flash photolysis recorded at 421 nm at different CO concentrations. Continuous lines in red are non-linear least-squares fitting of data obtained according to Eq. 2 and Eq. 3.

Figure 7 : Nitrite-reductase activity of GLB-33 GD. A. Representative spectral changes accompanying the transition of deoxy-GLB-33 GD to NO-bound GLB-33 GD. Arrows indicate the direction of change. An example of mono-exponential fitting of the absorbance trace at 579 nm over time (s) for the reaction between GLB-33 GD and 0.2 mM nitrite is shown in the inset. B. Plot of k_{obs} as a function of nitrite concentration. The second-order rate constant is given by the slope of the linear regression (pH 7.4, 25 °C).

Figure 8: 3D models of GLB-33. A. 3D model of GLB-33 7TM and overlay with the human β 2-G protein coupled receptor. B. 3D model of GLB-33 GD and overlay with GLB-6. C. Detailed view of the heme pocket of GLB-33 GD. D. Manual approach of the GLB-33-GD model to the GLB-33-7TM model using RosettaDock.

Figure 9 : 3D models of *A. suum* FMRFamide receptor. A. 3D model of *A. suum* FMRF 7TM and overlay with GLB-33 7TM. B. 3D model of *A. suum* FMRF GD and overlay with GLB-33 GD. C. Detailed view of the heme pocket of *A. suum* FMRF GD. D. Manual approach of the *A. suum* FMRF 7TM model and of the *A. suum* FMRF GD model using RosettaDocking.

TABLES

Table 1: Rate constants of ligand binding of *C. elegans* globins compared to rate constants reported for other globins. N.d. = not detectable

	GLB-33 GD	GLB-1	GLB-26	swMb	<i>A. suum</i> Hb
CO					
$k_{\text{on,f}}$ ($\mu\text{M}^{-1} \text{s}^{-1}$)	0.90 ± 0.10	-	23	-	-
$k_{\text{on,s}}$ ($\mu\text{M}^{-1} \text{s}^{-1}$)	0.35 ± 0.01	-	-	0.51 ± 0.06	0.21 ± 0.02
k_{gem} ($\times 10^7$) s^{-1}	9.87 ± 0.01	-	-	0.55 ± 0.08	3.0 ± 0.2
F_{gem}	0.50 ± 0.05	-	-	0.043 ± 0.006	0.45 ± 0.05
O ₂					
k_{on} ($\mu\text{M}^{-1} \text{s}^{-1}$)		23	n.d	14	3
k_{off} (s^{-1})		0.5	n.d	15	0.013
K_{O_2} (μM^{-1})	$\geq 8.55^1$	46^2	n.d	1.1	215
P_{50} (torr)	$\leq 0.07^1$	0.003^2	n.d	1	0.0072
k_{oxid} (h^{-1}) pH 4	1.81 (25 °C) 15 (37°C)				
k_{oxid} (h^{-1}) pH 7.4	0.09 ± 0.02 (25°C)			0.054 (37°C) 0.005 (25°C)*	

Values for *GLB-26* and *GLB-1* are taken from (6), for swMb from (101) and for *A. suum* Hb from (102).

¹from equilibrium experiments; ²from kinetic experiments; *pH 7.2. From (103).

Figure 1

A

GLB33 : APLTTTISPLYEEHDDPYINYSLCYTPLEHDEYKTFVSVLLNGYITTVGLVLTGNTGNLVKSVHVTSLDKNRGVAVSMALALAFWDTVLLWSAFFYVYGAKKLVNVLNNL : 109
 AsumFMRF : -----NEILRYECITDNIIDWTWTII-TGEVTTIIAVLGVGNVLSLKIIFR--SLISIRPSVRLYLVSVALANSFVS--FCSIWYVSLAEMLKPLLE : 85

GLB33 : YLDRIINTIPYFHALSHWANTASIVCVVAITIQRFMATRDPFFRTSRTTVIVQSFRTERRISFTYCATYRRLFKMPLYVSLCALLFNLPAFFEIRSKSCFNRTDNETLVT : 218
 AsumFMRF : TIRFFQYVTVIMHPVFSLSITAAVWMLLTVTADRYFALARPLRHR-----AHDLSLRARCVVVS-----ILFAAVLYSLPTLFEMRVSDCARYTNDTRLI : 176

GLB33 : LSPTRLRITNP-YFTVYRCSRMLMVSVGNILIVCSALTLWFLRGSNRRSRQLFQMTDNLLERYASRESMNTMISVMLVVKFILFRSLTFFLDIMEVTV---VMDNY : 323
 AsumFMRF : VVPTDLRLDDEYISYSVTLNMIFFTVGPFCLL-TILSIRMIFFVAKARSLRIGMDARATLSGRSTSQENT-TVMLLAVAVKFLLCNYMLIAVNIWEHLAAGSCIAVGTLL : 283

GLB33 : Y--IIDISNLLILVNSATNCLTYLKAETWLNSEFVERKTIKRRKTIKCS--GQLGDRLSILKSSWEKANEMTNGEIGVRVA-WNMVRKHP---NLCKNDEPEKVSLL : 423
 AsumFMRF : YKLCVELSNLLVVVNSASDSIVYFR---NRQKRPVAVCSNALKTLTTPDE-----ANLLKNEFM---CHQSSKRMIGSQTLTDLMLV : 358

GLB33 : NGSKRSIDHA-KFQEIIGGRITSFISELLELMQNNQEPES-YIVMRIRRVGAVHYDKGIVF-TS-SVWKEFKHTIQTIISEVQFSQPQEREALDANNIFISFIIREMKM : 528
 AsumFMRF : DN-----EELARDERICIQTLLLEEMSSIGDP-TAEQNIANKLNFVGSQHAQ---ISIPASTWNKFKTTLIEEMS--TNSR-----HLWSRLACMFIEKFRCC : 445

GLB33 : GIWAIIGDTIIGMP : 541
 AsumFMRF : QSFLKKEP----- : 453

B

Helices : -----aaaaaaaaAaaaAaa-----bbbbbbBbbBbbBbbCccccc-----D-----ddddddeeeEeeEeeEeeEeeEe : 67
 SwMb : -----VISEGEWQLVHVWAKVEA-----DVAGHQDILIRLFKSHPETLEK-----FDRFKHLKTEAEMKASEDLKKHGVTVLTALGAILK : 77
 GLB1 : -----SMNRQEISDLCKVKSLEGRMVGTEAQNIENGNAFYRYFTNFP-----DLRVY-----FKGA--EKYTADDVKKSERFDKQQRILLACHLLANVYTN : 86
 GLB6 : -----HLTQPQILFVRKTNWHARN--QGALEPAISIFRNSFFKNP-----EIRQMIM--FGT-----KNEGHERLKKHAQLFTVLMDDLIALN : 74
 GLB33GD : -----GQLLGDRLSILKSSWEKANE--MTNGEIGVRVA-WNMVRKHP---NLCKNDEPEKVS---LLNGSKRSIDHA-KFQEIIGGRITSFISELLELM--QNNQP : 89
 Cbri_GLB33 : -----GQLLGDRLSILKSSWEKANE--MTNGEIGVRVA-WNMVRKHP---TMCKKTEPEKVS---LLNGSKRSIDHA-KFQEIIGGRISGFIAELLELM--RTNQA : 89
 Crem_GLB33 : -----GQLLGDRLSILKSSWEKANE--MTNEEIGVRVA-WNMVRKHP---TMCKKTEPEKVS---LLNGSKRSIDHA-KFQEIIGGRISGFITELLELM--QSNQA : 89
 Cbre_GLB33 : -----GQLLGDRLSILKSSWEKANE--MTNGEIGVRVA-WNMVRKHP---TMCKADEPEKVS---LLNGSKRSIDHA-KFQEIIGGRISAFITELLELM--QNNQP : 89
 Cjap_GLB33 : -----GQLLGDRLSILKSSWEKANE--MTNGEIGVRVA-WNMVRKHP---TMY--KEPEKVS---LLNGSKRSIDHA-KFQEIIGGRISAFITELLELM--QNNQA : 87
 Sp5_GLB33 : -----GQLLGDRLSILKSSWEKANE--MTNGEIGVRVA-WNMVRKHP---TMCKNDEPEKVS---LLNGSKRSIDHA-KFQEIIGGRITSFITELLELM--QNNQA : 89
 Sp11_GLB33 : -----GQLLGDRLSILKSSWEKANE--MTNGEIGVRVA-WNMVRKHP---TMCKNDEPEKVS---LLNGSKRSIDHA-KFQEIIGGRISAFITELLELM--QNNQP : 89
 Cang_GLB33 : -----GQLLGDRLTILCTSDKVMK--NTGGELGTRIA-WNMLRKH---TMLNDEPEKVS---LLNGSKRSIDHV-KLKEIGNRITAFIYELLELM--KNSQT : 89
 Hrab_GLB33 : -----GQLGLRVLYMLRKHPSLFG--VFCVNPMMYT--NCDRVAEP---ELVSLTNGNRRSFDLL-----TNTKQVEADRTISFISELLELM--RTGK : 83
 AsumGS_17 : -----MTRQFGLRIVYAMLRKDPSS-----LFDSIAPS--ORNSEEEP---LSNSTRTGIERRSFDLL-----TCPYVEVGERIENFIFIGELIRMM--QEQG : 80
 AsumFMRFGD : -----DE-----ANLLKNEFM---CHQSSKRMIGSQTLTDLM--VLDN-----EELARDERICIQTLLLEEMSSIGDP-I : 57

Helices : -----FffFffFff-----ggggGggGggGGGgGggg-----hhhhhHhhHhhHhhHhhHhhhhhh : 122
 SwMb : -----KKGHHEAEKPLAQSHATKHK-I-----PIKYLEFISEAIIHVLSRHP--GDFGADRQGMNKALELFRKDIIAKYKELGY : 151
 GLB1 : -----EVFKGYVRETIHRHRIYKM-----PALMMAFFTVFTGYLESVGC-----LNDQKAAWALGKEFNAESQTHLKNSNLPHV : 157
 GLB6 : -----GLREAGEKHVWPTNRQYGCFFHAHLDDQFAIAMIERTLEWGE---KKDRTEITQRGWTIKVLFVTEQLKEGFQDEQKRA : 150
 GLB33GD : ES-YIVMRIRRVGAVHYD---KGIVF-TS-SVWKEFKHTIQTIISE--VFSSPQEREALDANNIFISFIIREMKMGIWAIGDTIGQMP : 171
 Cbri_GLB33 : ES-YIIMRIRRVGAVHYD---KGIVF-SS-SVWKEFKYTIQSIISE--VFSSPQEREALDANNIFISFIIREMKMGIWAIGDTIGQMP : 171
 Crem_GLB33 : ES-YIIMRIRRVGAVHYD---KGIVF-SS-SAWKEFKNTIQTIISE--VFSTSPQEREALDANNIFISFIIREMKMGIWAIDDAIGQMP : 171
 Cbre_GLB33 : ES-YIVMRVRRVAVHYD---KGIVF-TS-SVWKEFKHTIQTIISE--VFSSPQEREALDANNIFISFIIREMKMGIWAIGDTIGQMP : 171
 Cjap_GLB33 : ES-YIVMRVRRVAVHYD---KGIVF-TS-SVWKEFKYTIQTIISE--VFSSPQEREALDANNIFISFIIREMKMGIWAIGDTIGQMP : 169
 Sp5_GLB33 : ES-YIIMRIRRVGAVHYD---KGIVF-TS-SVWKEFKHTIQTIISE--VFSSPQEREALDANNIFISFIIREMKMGIWAIGDTIGQMP : 171
 Sp11_GLB33 : ES-YIVMRVRRVAVHYD---KGIVF-TS-SVWKEFKHTIQTIISE--VFSSPQEREALDANNIFISFIIREMKMGIWAIGDTIGQMP : 171
 Cang_GLB33 : ED-VITCRIRRVGAVHYD---RKINF-KT-SLFKEFKNTILCAVAD--CEYDTPERDLAIESWNIETSFIIKEMKMTWVMDNVHQVE : 171
 Hrab_GLB33 : PEEYIIMRIRRVGAVHYD---KGIQF-PS-SVWKEFKASTIISIIE--CEFKTQDEREALDANNIFISFIIREMKMGIWAIGDTIGAI : 166
 AsumGS_17 : SEQAIIERIRRVGAVHYE---HKVLFSS-SVWKEFKASTLAIIE--CPFESETIRMETINAWSSVILVIREMKMGWMSRHLP : 159
 AsumFMRFGD : AEQNIANKLNFVGSQHAQ-----ISIPASTWNKFKTTLIEEMS--TNSR-----HLWSRLACMFIEKFRCCQSFLKKEP : 124

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Figure 2

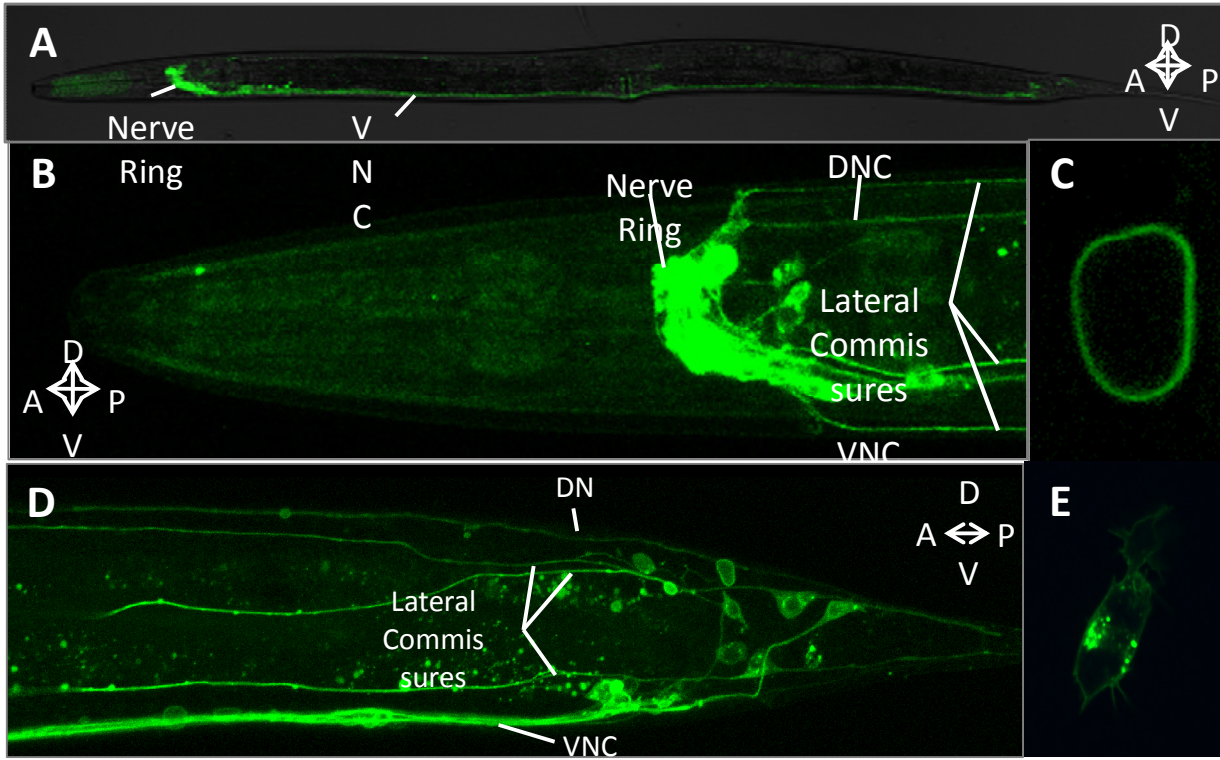
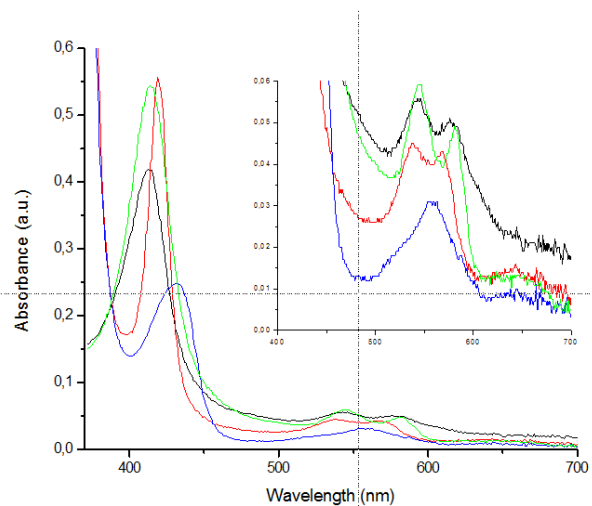
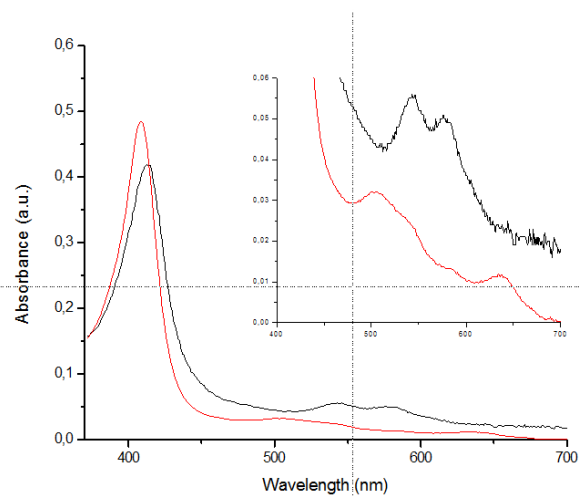


Figure 3

A



B



C

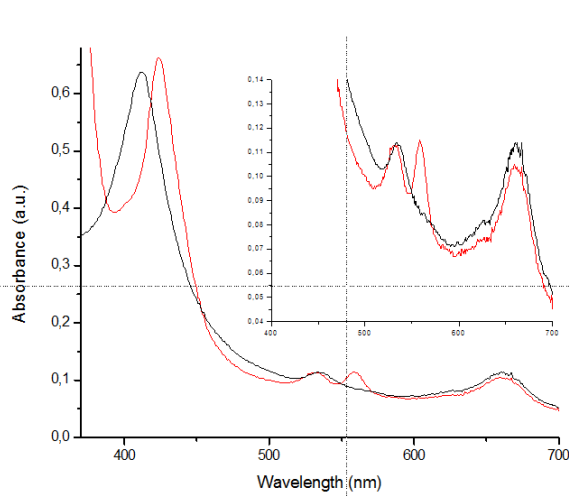


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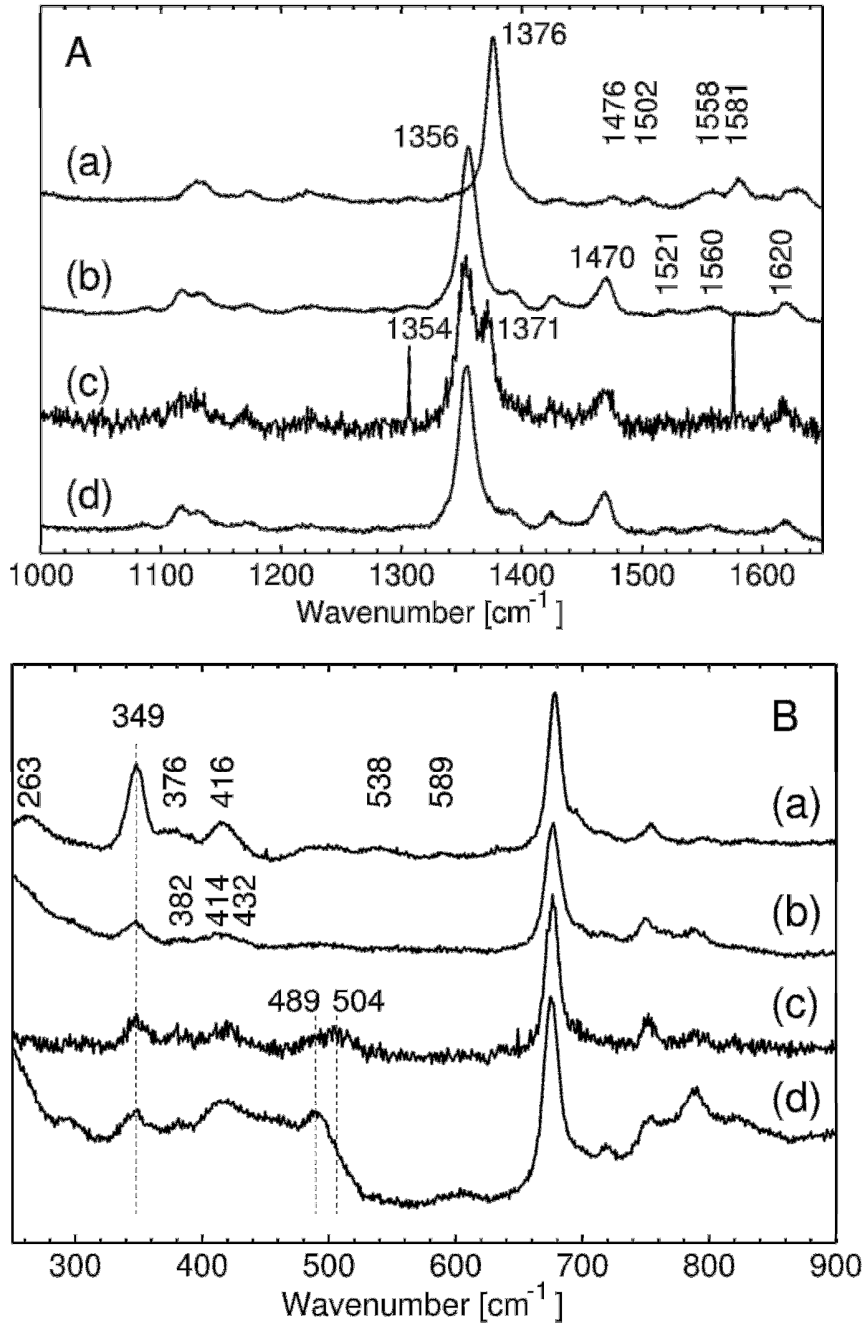


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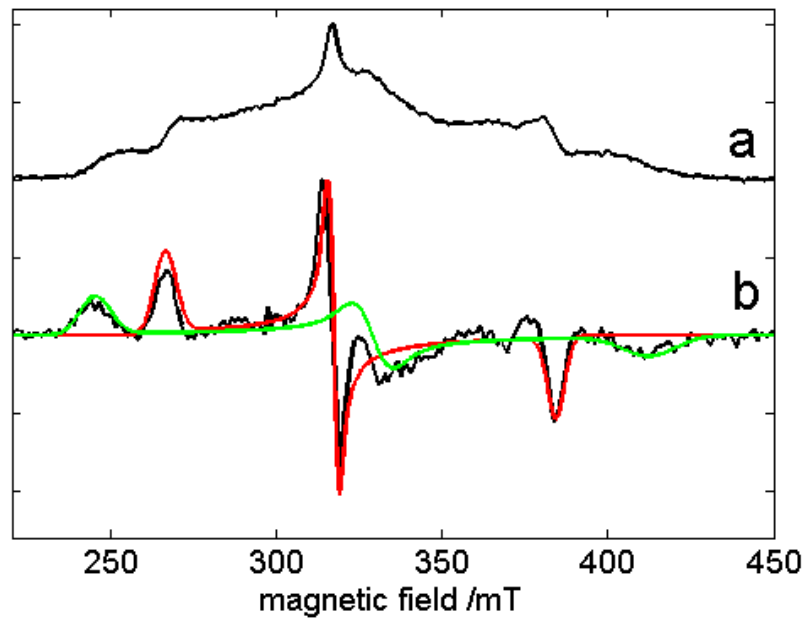


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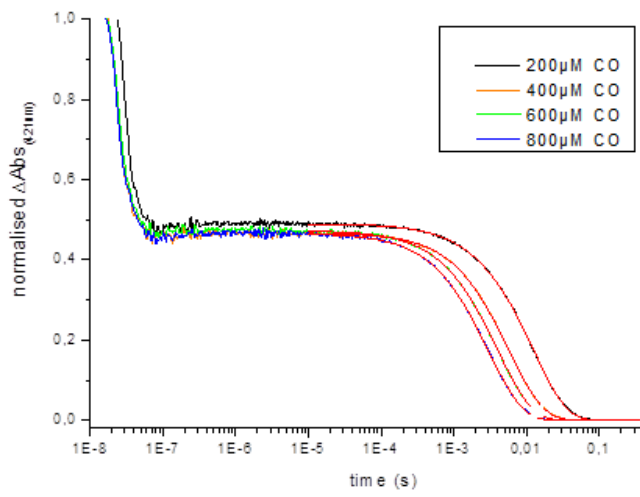


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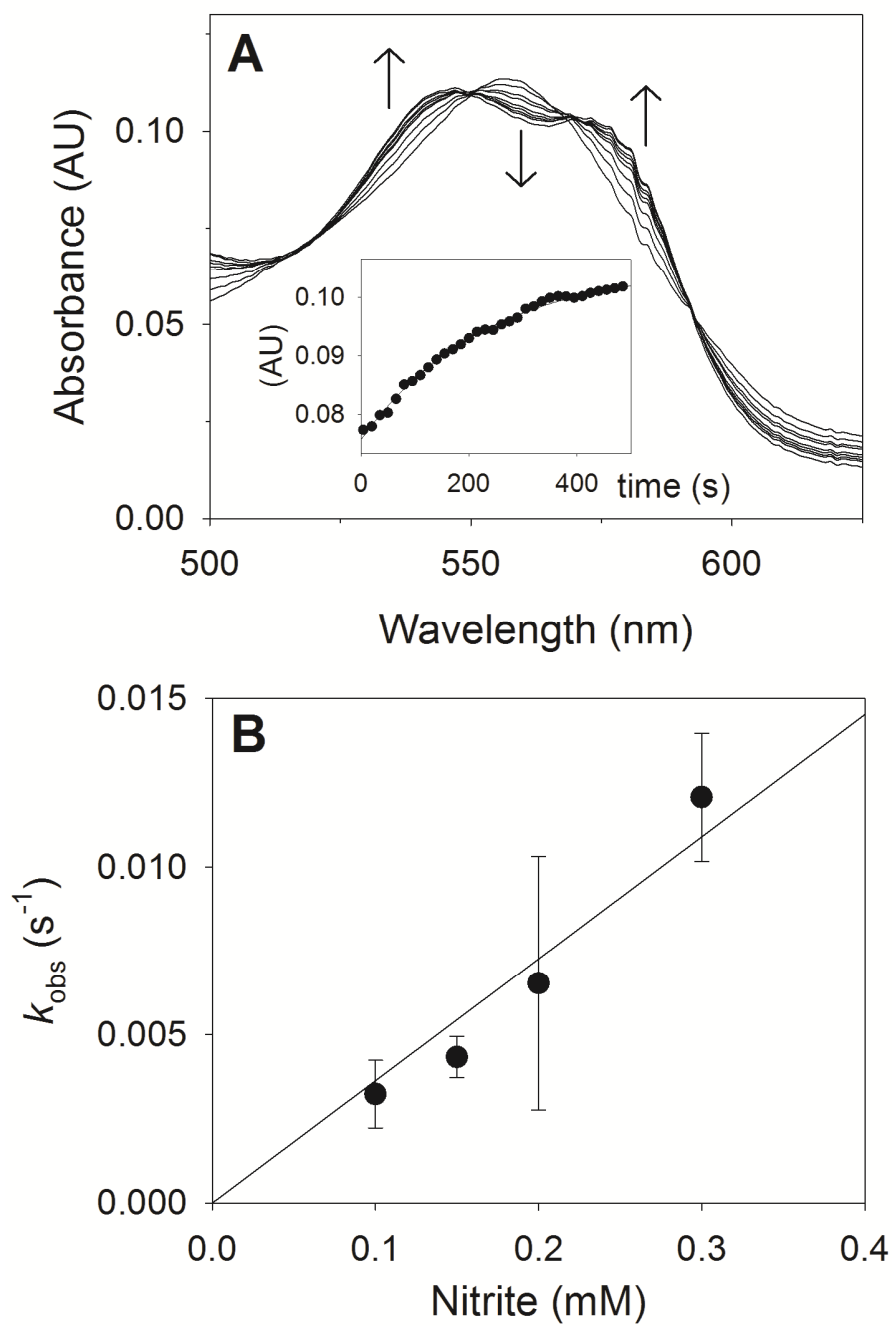
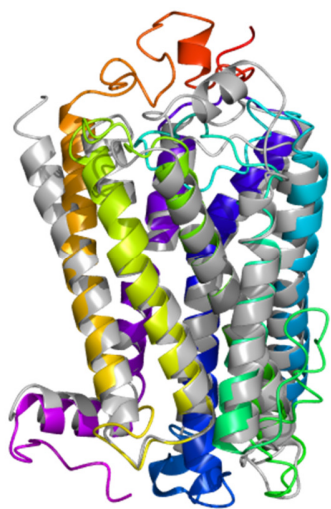
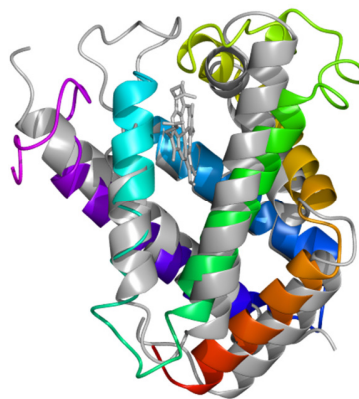


Figure 8

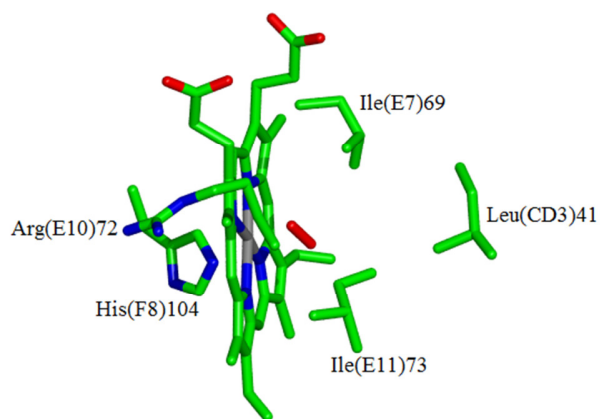
A



B



C



D

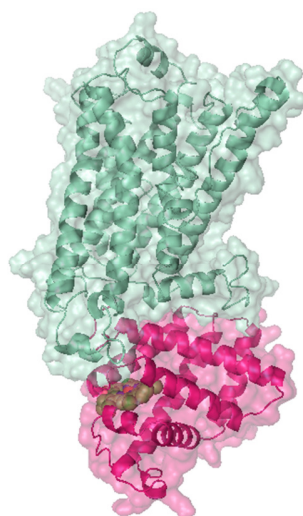
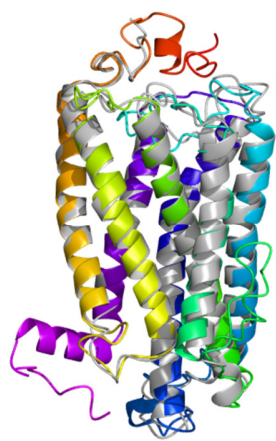
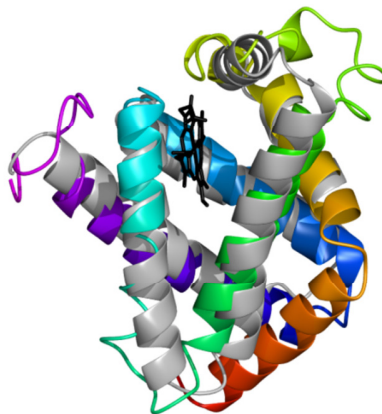


Figure 9

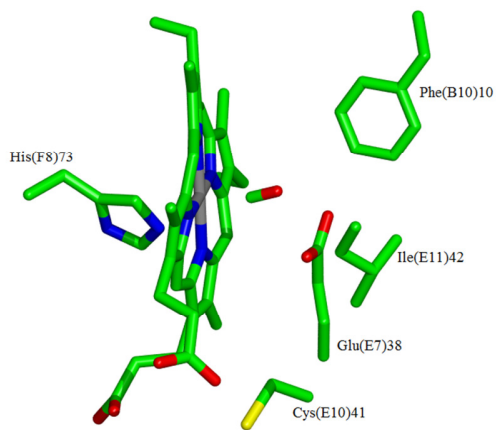
A



B



C



D

