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The zinc-mediated sulfide-binding mechanism of hydrothermal vent tubeworm 400-kDa hemoglobin

Jason F. FLORES[1]* and Stéphane M. HOURDEZ[2]
(1) *Department of Biology, The Pennsylvania State University*
**Corresponding author: The University of North Carolina at Charlotte, Department of Biology, 9201 University City Boulevard, Charlotte, NC 28223, USA, FAX: 704-687-3128, E-mail: jflores1@uncc.edu*
(2) *Université Pierre & Marie Curie-Paris 6, CNRS-UMR 7144 AD2M, Equipe Ecophysiologie Adaptation et Evolution Moleculaires, Station Biologique de Roscoff, 29680 Roscoff, France*

Abstract: Hydrothermal vent and cold seep tubeworms possess two hemoglobin (Hb) types, a 3600-kDa hexagonal bilayer Hb as well as a 400-kDa spherical Hb. Both Hbs can reversibly and simultaneously bind and transport oxygen and hydrogen sulfide used by the worm's endosymbiotic bacteria to fix carbon. The vestimentiferan 400-kDa Hb has been shown to consist of 24 polypeptide chains and 12 zinc ions that are bound to specific amino acids within the six A2 globin chains of the molecule. Flores et al. (2005) determined that the ligated zinc ions were directly involved in the sulfide binding mechanism of this Hb. This discovery contradicted previous work suggesting that free-cysteine residues were the sole sulfide binding mechanism of the 400-kDa Hb. In the present study, we investigated the effects of acidic pH pretreatment and zinc chelator concentrations on the binding of sulfide by the Hb. We show that acidic pH pretreatment, as well as NEM capping of free-cysteines, does not affect sulfide binding by the purified Hb. In addition, complete inhibition of sulfide binding was reached when the concentration of the zinc chelator, TPEN, was increased compared to the initial study. These results demonstrate the essential role of zinc in sulfide binding by the 400-kDa Hb from *Riftia pachyptila*.

Keywords: Hydrothermal vent • *Riftia pachyptila* • *Oligobrachia mashikoi* • Hemoglobin • Sulfide binding • Zinc chelation

Introduction

Deep-sea hydrothermal vents and cold seeps offer a striking contrast to the barren appearance of the usual deep-sea at similar depths. These communities are based on local primary production by bacteria using reduced chemicals such as sulfide and methane. However, the presence of hydrogen sulfide in hydrothermal vent and cold seep fluids makes it necessary for metazoans inhabiting these environments to have mechanisms to cope with this toxic chemical. Mobile organisms such as crabs and fish can physically avoid or limit sulfide exposure. Most of the successful animals at hydrothermal vents and cold seeps, however, have developed symbioses with sulfide-oxidizing bacteria. These include many annelid and molluscan species (Fisher, 1990).

Vestimentiferan tubeworms (Polychaeta; Siboglinidae) may be the most emblematic symbiotic organisms in the deep sea. They are found around many hydrothermal vents and cold seeps all over the globe. They are devoid of mouth, gut, and anus and rely solely on their symbionts for nutrition. These latter are located deep inside the worm's

body and, for this reason, the host must transport all nutrients necessary for the bacteria (CO_2, H_2S, O_2). The same family of polychaetes also contains pogonophoran tubeworms, a group in which at least two species, *Siboglinum poseidoni* and *Oligobrachia mashikoi*, have been shown to possess methanotrophic symbiotic bacteria (Schmaljohann et al., 1990; Kimura et al., 2003).

In addition to the protective and nutritional benefits provided by their bacterial endosymbionts through sulfide oxidation, vestimentiferan tubeworms themselves express at least two giant extracellular hemoglobins (Hbs) that are capable of simultaneously binding and carrying large quantities of both oxygen and hydrogen sulfide (Terwilliger et al., 1980; Arp & Childress, 1981; Zal et al., 1996). One of these Hbs assembles into a giant 3600-kDa hexagonal bilayer structure commonly found in many annelid groups (For review see Weber & Vinogradov, 2001). The other giant Hb is a 400-kDa spherical assembly that is the only Hb present in the pogonophoran tubeworms (Terwilliger et al., 1980; Yuasa et al., 1996; Flores et al., 2005). Both Hbs are made of several copies of different globin chains while the 3600-kDa Hb also possesses structural chains called linkers that assist with maintaining the massive assembly (Royer et al., 2000; Suzuki & Vinogradov, 2003). The binding of both sulfide and oxygen by these Hbs is essential to prevent the spontaneous reaction of these chemicals before they reach the bacteria as well as to limit the activity of free sulfide, which is toxic for the host. Oxygen binds to the heme group but the site of sulfide binding is still debated (Zal et al., 1998; Flores et al., 2005). Three mechanisms have been proposed, one involving abundant disulfide-bonds found in the linker chains, a second involving zinc ions bound to the vestimentiferan A2 polypeptide chain, whereas the third proposed mechanism involves free-cysteine (free-Cys) residues (those not involved in disulfide bonds) found within the A2 and B2 polypeptide globin chains (Arp et al., 1987; Suzuki et al., 1990; Zal et al., 1998; Flores et al., 2005).

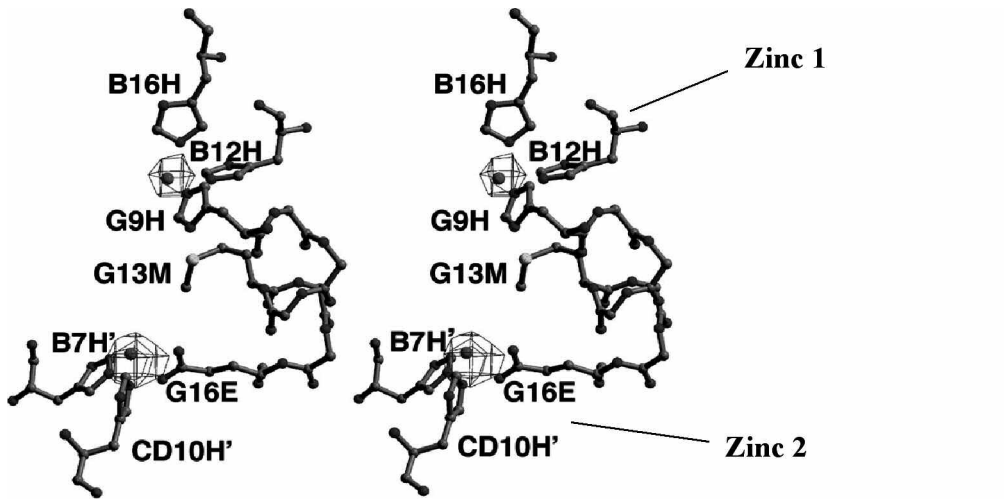
To test binding through cysteine side chains, Arp et al. (1987) showed that sulfide binding could be inhibited by treatment of the vestimentiferan Hbs with a sulfhydryl blocker (N-ethylmaleimide (NEM)) following reduction of disulfide bonds using dithiothreitol (DTT) indicating the linker disulfide bridges (only found in the 3600-kDa Hb) as sulfide binding sites. Later, based on structural investigations, Suzuki et al. (1990) suggested that sulfide might also bind to free-Cys residues in the globin chains. Following that suggestion, Zal et al. (1998) conducted experiments in which they tested both disulfide and free-Cys hypotheses. Using mass-spectrometric determination of the number of cysteines in the polypeptides (Zal et al., 1996), these authors concluded that the free-Cys mechanism was responsible for 75% of the sulfide bound to the 3600-kDa Hb and 100% of the sulfide bound to the 400-kDa Hb and that the disulfide-rich linker chains were indeed a plausible location for binding the remainder of sulfide in the 3600-kDa Hb. The final mechanism involving zinc was discovered only recently (Flores et al., 2005). Using data obtained from the crystal structure of the 400-kDa Hb from

Figure 1. A. Stereo view of the key amino acids in the zinc binding domains of the 400-kDa Hb from *Riftia pachyptila* (modified after Flores et al., 2005). The first letter of each label denotes the helix, the number the amino acid position in that helix and the last letter the amino acid identity. The prime sign (‘) indicates amino acids found on the other A2 chain. **B**. Sequence alignment of annelid A2 globin chains. Key amino acid positions for each zinc ion (Z1 and Z2) are indicated and conserved amino acids for these sites are shaded. Riftia: *Riftia pachyptila* (accession number P80592); Tevnia: *Tevnia jerichonana* (accession number AAP04530); Oasisia: *Oasisia alvinae* (accession number AAP04531); LamJap: *Lamellibrachia* from Japan (accession number P15469); LamMed: *Lamellibrachia* from the Mediterranean (accession number AAP04528); Slend: Slender vestimentiferan (accession number AAA03291); Lam sp1: *Lamellibrachia* sp. 1 (accession number AAB27511); Lam sp2: *Lamellibrachia* sp. 2 (accession number AAB27512); O.mash: *Oligobrachia mashikoi* (accession number BAD86542); A.mar: *Arenicola marina* (accession number CAI56308); L.terr: *Lumbricus terrestris* (accession number P02218); Tyl: *Tylorrhynchus heterochaetus* (accession number P09966); S.spall: *Sabella spallanzanii* (accession number CAC37412).

Figure 1. A. Vue stéréoscopique des acides aminés clé dans les domaines de liaison du zinc de l’Hb de 400-kDa de *Riftia pachyptila* (modifié d’après Flores et al., 2005). La première lettre de chaque nom fait référence à l’hélice, le nombre à la position de l’acide aminé dans cette hélice et la dernière lettre à l’identité de l’acide aminé. Le signe prime (‘) indique les acides aminés de l’autre chaîne A2. **B**. Alignement des séquences de chaînes de globine A2 d’annélides. Les positions des acides aminés clé pour chaque ion zinc (Z1 et Z2) sont indiquées et les acides aminés conservés pour ces sites sont grisés. Riftia: *Riftia pachyptila* (numéro d’accession P80592); Tevnia: *Tevnia jerichonana* (numéro d’accession AAP04530); Oasisia: *Oasisia alvinae* (numéro d’accession AAP04531); LamJap: *Lamellibrachia* from Japan (numéro d’accession P15469); LamMed: *Lamellibrachia* from the Mediterranean (numéro d’accession AAP04528); Slend: Slender vestimentiferan (numéro d’accession AAA03291); Lam sp1: *Lamellibrachia* sp. 1 (numéro d’accession AAB27511); Lam sp2: *Lamellibrachia* sp. 2 (numéro d’accession AAB27512); O.mash: *Oligobrachia mashikoi* (numéro d’accession BAD86542); A.mar: *Arenicola marina* (numéro d’accession CAI56308); L.terr: *Lumbricus terrestris* (numéro d’accession P02218); Tyl: *Tylorrhynchus heterochaetus* (numéro d’accession P09966); S.spall: *Sabella spallanzanii* (numéro d’accession CAC37412).

Riftia pachyptila, Flores et al. (2005) determined that the pockets surrounding the free-Cys are formed from hydrophobic residues that likely restrict access for hydrogen sulfide (HS^-). The crystal structure also revealed 12 zinc ions interacting with specific amino acids at the interface between pairs of A2 chains (Fig. 1). Sulfide binding experiments were subsequently conducted on both vestimentiferan Hb types following pre-treatment of the Hbs with either a sulfhydryl inhibitor (NEM) or zinc chelator (N,N,N',N'-Tetrakis-(2-pyridylmethyl)-Ethylenediamine (TPEN)) to assert a possible function of this metal in sulfide binding (Flores et al., 2005). When the zinc-chelation data were compared to control data, it was determined that zinc ions were responsible for 18% of bound sulfide in the vestimentiferan 3600-kDa Hb and 57% of the sulfide bound to the 400-kDa Hb. Thiol-inhibited Hbs did not show a decrease in bound sulfide compared to controls. In the case of the 3600-kDa Hb, the remaining sulfide could be bound via disulfide exchange to the cysteine-rich linker polypeptide chains of this protein (Zal

A



B

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                                                  Z2   Z1  Z1              Z2
                                                  B7   B12 B16             CD10
Riftia          ------------------------------WAEAYGSGNSREEFGHFIWSHVFQHSP-AARDMFKRVRGDNIHTPAFRAHATRVLGGL
Tevnia          ---------------------------------------SREDFGHFIWSHVFQHSP-GARDMFNRVRGDNIHTPAFRAHATRVLGGL
Oasisia         ----------------------------------------------HFMWAHVFQHSP-AARDMFKRVRFDNIHTPAFRAHATRVLGGL
LamJap          ---------------YECGPLQRLKVKRQWAEAYGSGNDREEFGHFIWTHVFKDAP-SARDLFKRVRGDNIHTPAFRAHATRVLGGL
LamMed          ------------------------------------------------WALVFKDAP-SARDLFKRVRGDNIHTPAFRAHATRVLGGL
Slend           -------------DTHVCGPLQRLKVKRQWAEAYGSGGRREDFGHYIWAHVF
Lam sp2         --------------DHVCGPLQRLKVKRQWAEAYGSGNRREDFGHYIWAHVF
Lam sp1         ---------------YECGPLQRLKVKRQWAEAYGSGNDREEFGHFIWTHVF
O.mash          -KSLIVFACLVAYAAADCTSLNRLLVKRQWAEAYGEGTNRELLGNRIWEDLFANMP-DARGLFSRVNGNDIDSSEFQAHSLRVLGGL
A.mar           MKSLVVLFALVAMVAAECGPMQRLLVKTQWNKVYGTSKVRDEAGHVLWKAIFAQDP-ETRALFKRVNGDDIYSPEFMAHSARVLGGL
L.terr          --------------KKQCGVLEGLKVKSEWGRAYGSGHDREAFSQAIWRATFAQVP-ESRSLFKRVHGDDTSHPAFIAHAERVLGGL
Tyl             -------------SSDHCGPLQRLKVKQQWAKAYGVGHERVELGIALWKSMFAQDN-DARDLFKRVHGEDVHSPAFEAHMARVFNGL
S.spall         -MYKWLLCLALIGCVSGCNILQRLKVKNQWQEAFGYADDRTSXGTALWRSIIMQKPESVDKFFKRVNGKDISSPAFQAHIQRVFGGF

                                                  Z1  Z1 Z2
                                                  G9  G13G16
Riftia          DMCIALLDDEPVLNTQLAHLAKQHETRGVEAAHYDTVNHAVMMGVENVIGSEVFDQDAW-----------------
Tevnia          DMCIALVDDEPVLNTRLAHLAKQPETRGVGAAPYDPVYPA-----------------------------------
Oasisia         DMCIALLDDQSVLDTQLTHLATQHVSRGVDAEHY-------------------------------------------
LamJap          DMCIALLDDEGVLNTQLAHLASQHSSRGVSAAQYDVVEHSVMMGVEHEIGQNVFDKDAWQACLDVITG-GIQGN-
LamMed          dmcialldegvlntqlghlasqhsprgvsp-------------------------------------------
Slend           ------------------------------------------------------------------------
Lam sp2         ------------------------------------------------------------------------
Lam sp1         ------------------------------------------------------------------------
O.mash          DMCVASLDDVPVLNALLARLNSQHDSRGIPAAGYPAFVASAISAVRATVGARSFDNDAWNSCMNQIVS-GISG--
A.mar           DIAISLLDNQADLDVALAHLHVQHVERHIPTRYFDLFKNALMEYAPSALGR-CFDKTAWSSCFDVIAN-GIKE--
L.terr          DIAISTLDQPATLKEELDHLQVQHEGRKIPDNYFDAFKTAILHVVAAQLGR-CYDREAWDACIDHIED-GIKGHH
Tyl             DRVISSLTDEPVLNAQLEHLRQQHIKLGITGHMFNLMRTGLAYVLPAQLGR-CFDKEAWAACWDEVIYPGIKHD-
S.spall         DMCISMLDDSDVLASQLAHLHAQHVERGISAEYFDVFAESLMLAVESTIES-CFDKDAWSQCTKVISS-GIGSGV
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et al., 1996; Flores et al., 2005) whereas the remaining sulfide bound to the 400-kDa Hb could not be easily explained based on current structural knowledge of the molecule.

In an attempt to reconcile results from Flores et al.'s (2005) and Zal et al.'s (1998) studies, we carried out additional experiments. Experimental procedures in the latter study included a step to remove all excess bound sulfide prior to beginning the experiment using pH alterations and flushing under a nitrogen flow. This was not performed in Flores et al.'s series of binding experiments because it had been determined that the purification process alone was enough to remove bound sulfide from these Hbs (Flores, pers. obs.). It is possible that in Zal et al.'s (1998) study, the pH alteration induced a conformational change in the purified Hb that allowed inhibition by NEM in that work.

The data presented in this paper provide further support for the presence of a zinc-based sulfide binding mechanism in vestimentiferan tubeworm Hbs and conclusively show that free-cysteine residues are not involved in any direct way in sulfide binding by the Hbs of this group of worms. We also address recent findings on the structure and sulfide-binding function of the extracellular Hb of *Oligobrachia mashikoi*, a related species of worm.

Materials and methods

Effect of pH pre-treatment on binding inhibition by NEM and TPEN

Previous investigations of sulfide binding by vestimentiferan Hbs by Childress et al. (1984), Fisher et al. (1988), as well as Zal et al. (1998) used acidic pH and nitrogen gas flushing to remove hydrogen sulfide bound to the blood prior to experimental treatments. In an effort to determine if alterations in pH could affect the opening of the free-Cys pockets, samples of purified 400-kDa Hb were pre-treated for 12 hrs in saline buffer (Fisher et al., 1988; Flores et al., 2005) at two different pH values (5.5 and 7.5) and kept under a nitrogen flow. The pH of the Hb solution was then brought back to 7.5 by overnight dialysis against 500 times the volume of the samples of buffer at pH 7.5. Each batch of Hb (pH 5.5- and pH 7.5-treated) was used to investigate the sulfide binding mechanism. Three treatments were tested on each batch: TPEN (chelation with 10:1 TPEN:zinc molar ratio), NEM inhibition, and TPEN+NEM treatment. The chelation experiment was carried out with a larger amount of TPEN than the one previously used (3:1 TPEN:zinc molar ratio; Flores et al., 2005). As a control, the same volume of solvent as the treated sample was added to the untreated samples.

Gas chromatography measurements showed that none of the samples (pH 5.5- and pH-7.5 treated) contained any sulfide before they were used for the equilibrium dialysis experiment against 1 mM sulfide for 18 hours. All bound sulfide was reported to the heme concentration (determined by the cyan-metHb method) for comparative purposes.

Results and Discussion

Effect of pH pre-treatment on sulfide binding and inhibition by NEM and TPEN

After equilibrium dialysis, there was no significant difference in bound sulfide between the untreated pH 5.5- and pH 7.5-pretreated samples (Fig. 2), indicating that the intrinsic ability of the Hb to bind sulfide was not affected by the pretreatment. NEM-treated samples did not show any significant difference in bound sulfide between the two pH pretreatments when compared to the untreated controls (Fig. 2). This shows that the pH 5.5 pretreatment did not induce a conformational change that could have explained the discrepancies between Flores et al.'s (2005) and Zal et al.'s (1998) studies.

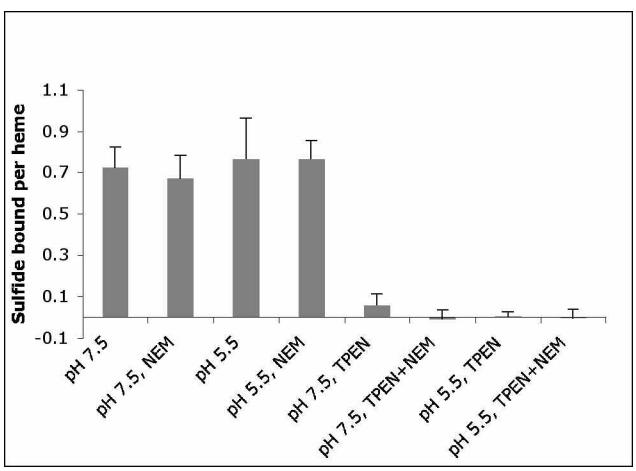

Figure 2. Effect of different treatments on sulfide binding in *R. pachyptila* 400-kDA HB. pH 7.5, pH 5.5, NEM and TPEN were used as indicated in the text.

Figure 2. Effet des différents traitements sur la liaison des sulfures par l'Hb de 400 kDa de *R. pachyptila*. pH 7.5, pH 5.5, NEM et TPEN ont été utilisés comme indiqué dans le texte.

After treatment by the zinc chelator TPEN, as well as combined NEM and TPEN, sulfide binding was completely cancelled for both pH pretreatments (Fig. 2). This confirms the essential role of zinc in the mechanism of sulfide binding (Flores et al., 2005).

Effect of chelator concentration on sulfide binding inhibition

Flores et al. (2005) reported a 57% of inhibition of the sulfide binding by the 400-kDa Hb. In their experiments, a chelator:zinc molar ration of 3:1 was used. Here, we used a ratio of 10:1 and obtained nearly 100% of inhibition. It is possible that complete chelation was not reached in Flores et al.'s study (2005). The average sulfide bound per heme was calculated to be 0.73 ± 0.04 (mean ± SD). With each molecule bearing 24 heme groups, the native molecule can bind 17.1 ± 1.1 sulfide molecules (mean ± SD), i.e. an average 1.5 sulfide per zinc ion. It is not clear how two zinc ions can bind three molecules of sulfide but inter-species comparisons could bring very useful indications (see below).

Zinc-sulfide binding domains in vestimentiferans and other annelids

Figure 1B shows an alignment of the published amino acid sequences of globins belonging to the A2 subfamily, from Siboglinidae and other annelids. Amino acids involved in the binding of zinc ions (Fig. 1A) in *R. pachyptila* A2 globin pairs are highlighted. The amino acids involved in the binding of zinc 2 (positions B7, CD10, and G16) are conserved between *Lamellibrachia* from Japan (LamJap) and *Riftia* A2 chains. Residues B7 and CD10 are also conserved for *Lamellibrachia* from the Meditteranean (LamMed), *Tevnia* and *Oasisia* but the sequences are incomplete and we do not know what amino acids are located at G16 for these three species. The amino acids involved in the binding of zinc 1 (positions B12, B16, G9, and G13) show more variations than the residues in the zinc 2 site. The B12 position is well conserved in the Japanese *Lamellibrachia*, *Tevnia*, *Oasisia* and *Riftia* A2 chains. In the Mediterranean *Lamellibrachia*, this residue is replaced by Leu. The G9 and G13 positions are also conserved in *Riftia* and the Japanese *Lamellibrachia*, G9 His is replaced by a Pro in *Tevnia*. Due to the incompleteness of sequences for *Tevnia* and *Oasisia*, it is difficult to draw conclusions for the other key amino acids. Short fragments of other vestimentiferan species (Lam sp1, Lam sp2, and Slend) also show the conserved B7 and B12 amino acids. Another vent vestimentiferan, *Ridgeia piscesae*, possesses A2 chain sequence that displays all amino acids found in *R. pachyptila* A2 (S.L. Carney, pers. com.).

Although the presence of these residues is not proof that sulfide binding occurs via a zinc sulfide binding mechanism, it is at least possible that this is a conserved functional mechanism in these vent and seep species. In *Lamellibrachia luymesi* from the Gulf of Mexico, the 400-kDa Hb binds only 0.5 sulfides per heme (Freytag, 2003), giving a total of 12 sulfide molecules for each native Hb in this species, 6 fewer sulfide molecules than *R. pachyptila*. Most of the amino acids corresponding to essential amino acids in *R. pachyptila* A2 chains are conserved in one other species of *Lamellibrachia* (LamJap), except the B16 His that is replaced by an Asp (fig. 1B). The same replacement is found in the Meditterranean *Lamellibrachia*. We could speculate that a similar change is found in *L. luymesi* and that this amino acid is involved for the binding of one of the three sulfide molecules. Incidentally, COI sequencing for the Mediterranean species of *Lamellibrachia* shows a very close relationship with *L. luymesi* (Hourdez, unpub. data).

The crystal structure of the 400-kDa hemoglobin from *Oligobrachia mashikoi* (Siboglinidae) shows a remarkably similar quaternary structure to the vestimentiferan *R. pachyptila* 400-kDa Hb (Flores et al., 2005; Numoto et al., 2005). The 24-subunit hollow sphere of each species is roughly the same size (120-130 Å outer diameter, 50 Å inner diameter). It has also been shown that the zinc binding domains found in other vestimentiferans are not present in the A2 chain of *O. mashikoi* (Numoto et al., 2005; fig. 1B). The major difference between the two Hbs is the absence of zinc ions in the *O. mashikoi* crystal structure. In addition, the authors reported that methyl-mercury used in the crystallization process was able to bind to a free cysteine similar to the mechanism Zal et al. (1998) hypothesized to bind sulfide in vestimentiferans, suggesting that the hydrophobic pocket did not prevent movement of large ions. Based on these results, the authors attempted an investigation of the sulfide binding mechanism in this Hb (Numoto et al., 2005). The authors showed a decrease of free sulfide in solution when Hb was added. However, they did not attempt to release the bound sulfide and the disappearance could very well correspond to oxidation by the oxygen originally bound to the heme groups of the Hb. In addition, binding of sulfide for transport may not be relevant as the species was shown to possess methanotrophic symbionts (Kimura et al., 2003).

The sequences of the A2 chains from other annelids that were analyzed (*Lumbricus*, *Sabella*, *Tylorrhynchus* and *Arenicola*) do not contain the classic zinc 1 and zinc 2 binding motifs. All these globin sequences come from 3600-kDa Hbs, the 400-kDa Hb being only found in Siboglinidae. The absence of these residues at these locations, however, is not necessarily evidence that zinc sulfide binding does not occur in these species as has been suggested (Nakagawa et al., 2005). In these other species, functional studies using chelators and inhibitors should be conducted and the findings coupled with structural investigations before conclusions are made. Caution should be exercised when attempting to assert function from sequence and/or structure information alone. Other amino acid residues both within the annelid A2 chain and in other chains from other species could form binding pockets for

zinc-sulfide complexes. The importance of sulfide as both an electron acceptor and inhibitor of electron transport makes it necessary for organisms encountering sulfide to cope with it and, more importantly, prevent disruption of respiration. Alternative mechanisms for binding sulfide are possible and have been shown experimentally (Kraus & Wittenberg, 1990; Childress et al., 1993; Zal et al., 1998). For example, in *R. pachyptila* 3600-kDa Hb, the presence of numerous cysteine residues involved in disulfide bonds in the non-globin linker chains of the assembled hexagonal bilayer Hb may account for part of the sulfide bound to that Hb (Arp et al., 1987; Zal et al., 1998). However, some key amino acids may also be required here as *Lumbricus* Hb does possess these cysteine-rich linkers but is not able to bind sulfide (Zal et al., 1998).

Conclusion and perspectives

The mechanism of sulfide binding by siboglinid Hb is still debated. As NEM may react with free sulfide as well as with the free-Cys residues, we preferred to treat the Hbs with NEM before dialysis instead of adding it to the dialysis buffer. In previous experiments conducted at pH 7.5 (where HS- makes up approximately 80% of total sulfide present) using NEM in the buffer, sulfide reaction was apparent and most -if not all-H_2S likely reacted with the NEM (either directly or indirectly) thereby decreasing greatly the amount of free sulfide in solution and preventing binding by the Hb (Flores et al., 2005). However, it is possible that, following our experimental protocol, NEM separates from the free-Cys during dialysis and therefore does not prevent sulfide binding. Nevertheless, the complete inhibition of sulfide binding by TPEN indicates that if zinc ions are not the sulfide-binding sites themselves, they are essential to the process.

In order to better understand the mechanism(s) of sulfide binding, it would be essential to crystallize other Siboglinid 400-kDa Hbs and associate these data to functional data for species showing variations in the amino acids of the zinc binding motifs found in *R. pachyptila* A2 chains. The crystallization of such Hbs saturated with sulfide would provide the definite demonstration of the binding site.

Zinc-based sulfide binding is present in at least one other thiophilic organism, the vesicomyiid clam, *Calyptogena* spp. This symbiont-containing bivalve lives in shallow as well as deep water reducing environments exposed to relatively high concentrations of hydrogen sulfide. It has an intracellular Hb that only binds oxygen and also expresses an extracellular, non-heme zinc-containing protein in its serum that is responsible for the uptake of sulfide (Childress et al., 1993; Zal et al., 2000). The presence of a zinc-based sulfide binding mechanism in two distantly-related symbiotic taxa suggests that this is a successful mode of sulfide transport.

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References

Arp A.J. & Childress J.J. 1981. Blood function in the hydrothermal vent vestimentiferan tube worm. *Science*, **213**: 342-344

Arp A.J., Childress J.J. & Vetter R.D. 1987. The sulphide-binding protein in the blood of the vestimentiferan tube worm, *Riftia pachyptila*, is the extracellular haemoglobin. *Journal of Experimental Biology*, **128**: 139-158.

Childress J.J., Fisher C.R., Favuzzi J.A., Arp A.J. & Oros D.R. 1993. The role of a zinc-based, serum-borne sulphide-binding component in the uptake and transport of dissolved sulphide by the chemoautotrophic symbiont-containing clam *Calyptogena elongata. Journal of Experimental Biology*, **179**: 131-158.

Childress J.J., Arp A.J. & Fisher C.R. 1984. Metabolic and respiratory characteristics of the hydrothermal vent tube worm *Riftia pachyptila*. *Marine Biology*, **83**: 109-124.

Fisher C.R. 1990. Chemoautotrophic and methanotrophic symbioses in marine invertebrates. *Reviews in Aquatic Sciences*, **2**: 399-436.

Fisher C.R., Childress J.J. & Sanders N.K. 1988. The role of vestimentiferan hemoglobin in providing an environment suitable for chemoautotrophic sulfide-oxidizing endosymbionts. *Symbiosis*, **5**: 229-246.

Flores J.F., Fisher C.R., Carney S.L., Green B.N., Freytag J.K., Schaeffer S.W. & Royer W.E. 2005. Sulfide binding is mediated by zinc ions discovered in the crystal structure of a hydrothermal vent tubeworm hemoglobin. *Proceedings of the National Academy of Science of the USA*, **102**: 2713-2718.

Freytag J.K. 2003. Ecological physiology and biochemistry of sulfide acquisition by two hydrocarbon seep vestimentiferans, *Lamellibrachia luymesi* and *Seepiophila jonesi*. The Pennsylvania State University, Ph.D. thesis. 129 pp.

Gorodezskey L.A. & Childress J.J. 1994. Effects of sulfide exposure history and hemolymph thiosulfate on oxygen consumption rates and regulation in the hydrothermal vent crab *Bythograea thermydron*. *Marine Biology*, **120**: 123-131.

Kimura H. M., Sato Y., Sasayama Y. & Naganuma T. 2003. Molecular characterization and in situ localization of endosymbiotic 16S ribosomal RNA and RuBisCO genes in the pogonophoran tissue. *Marine Biotechnology*, **5**: 261-269.

Kraus D.W. & Wittenberg J. 1990. Hemoglobins of the *Lucina pectinata*/bacteria symbiosis I. Molecular properties, kinetics and equilibria of reactions with ligands. *Journal of Biological Chemistry*, **265**: 16043–16053.

Nakagawa T., Onoda S., Kanemori M., Sasayama Y. & Fukumori Y. 2005. Purification, characterization and sequence analyses of the extracellular giant hemoglobin from *Oligobrachia mashikoi*. *Zoological Science*, **22**: 283-291.

Numoto N., Nakagawa T., Kita A., Sasayama Y., Fukumori Y. & Miki K. 2005. Structure of an extracellular giant hemoglobin of the gutless beard worm *Oligobrachia mashikoi*. *Proceedings of the National Academy of Science of the USA*, **102**: 14521-14526.

Royer W.E., Strand K., vanHeel M. & Hendrickson W.A. 2000. Structural hierarchy in erythrocruorin, the giant respiratory assemblage of annelids. *Proceedings of the National Academy of Science of the USA*, **97**: 7107-7111.

Schmaljohann R., Faber E., Whiticar M.J. & Dando P.R. 1990. Co-existence of methane- and sulphur-based endosymbioses between bacteria and invertebrates at a site in the Skagerrak. *Marine Ecology Progress Series,* **61:** 119-124.

Suzuki T., Takagi T. & Ohta S. 1990. Primary structure of a constituent polypeptide chain (AIII) of the giant haemoglobin from the deep-sea tube worm *Lamellibrachia*: a possible H_2S-binding site. *Biochemical Journal*, **266**: 221-225.

Suzuki T. & Vinogradov S.N. 2003. Globin and linker sequences of the giant extracellular hemoglobin from the leech *Macrobdella decora*. *Journal of Protein Chemistry*, **22**: 231-242.

Terwilliger R. C., Terwilliger N. B. & Schabtach E. 1980. The structure of hemoglobin from an unusual deep sea worm (Vestimentifera). *Comparative Biochemistry and Physiology*, **65B**: 531-535.

Weber R.E. & Vinogradov S.N. 2001. Nonvertebrate hemoglobins: functions and molecular adaptations. *Physiological Reviews*, **81**: 569-628.

Yuasa H.J., Green B.N., Takagi T., Suzuki N., Vinogradov S.N. & Suzuki T. 1996. Electrospray ionization mass spectrometric composition of the 400 kDa hemoglobin from the pogonophoran *Oligobrachia mashikoi* and the primary structures of three major globin chains. *Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology*, **1296**: 235-244.

Zal F., Lallier F.H., Green B.N., Vinogradov S.N. & A. Toulmond 1996. The multi-hemoglobin system of the hydrothermal vent tube worm *Riftia pachyptila*. 2. complete polypeptide chain composition investigated by maximum entropy analysis of mass spectra. *Journal of Biological Chemistry*, **271**: 8875-8881.

Zal F., Leize E., Lallier F.H., Toulmond A., VanDorsselaer A. & J.J. Childress 1998. S-sulfohemoglobin and disulfide exchange: The mechanisms of sulfide binding by *Riftia pachyptila* hemoglobins. *Proceedings of the National Academy of Science of the USA*, **95**: 8997-9002.

Zal F., Leize E., Oros D.R., Hourdez S., Van Dorsselaer A. & Childress J.J. 2000. Haemoglobin structure and biochemical characteristics of the sulphide-binding component from the deep-sea clam *Calyptogena magnifica*. *Cahiers de Biologie Marine*, **41**: 413-423.