Novel dissociation mechanism of a polychaetous annelid extracellular haemoglobin

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The giant extracellular hexagonal bilayer haemoglobins (HBL-Hbs), found in most terrestrial, aquatic, shallow-water and deep-sea annelids (including vestimentiferans) are complexes of globin and nonglobin linker chains, of \( \approx 3.6 \) MDa. They represent a summit of complexity for oxygen-binding haem proteins [1,2] and a remarkable hierarchical organization, as evidenced by the crystal structure of \textit{Lumbricus} Hb [3]. A model of the quaternary structure of \textit{Arenicola marina} HBL-Hb has been proposed by Zal and collaborators based on electrospray ionization (ESI)-MS analysis and multangle laser light scattering (MALLS) measurements [4]. The authors provided an inventory of the constituting polypeptide chains and identified the existence of 10 subunits (eight of which are globins), including two monomers \((a_1\) and \(a_2)\) of \(\approx 15\) kDa, and five disulfide-bonded trimers \(\approx 49\) kDa). The remaining two chains are linkers that are disulphide bonded to form homo- and heterodimers \(\approx 50\) kDa. These latter polypeptide chains are essential for maintaining the integrity of the HBL-Hb molecule [5,6]. Three and six copies of each of the two monomer subunits, and one copy of the trimer, form a dodecamer subunit \([(a_1)_3(a_2)_6T]\), of a mean mass close to 200 kDa. The molecular mass of the dodecamer subunit has been determined, by ESI-MS, to be \(204 \pm 0.08\) kDa [7], which is in good agreement with the model of the quaternary structure proposed by Zal and collaborators [4]. Twelve such complexes of globin chains are linked together by 42 linker chains to reach a total mass of \(3648 \pm 24\) kDa. Therefore, each of the 12 subunits of the whole molecule is then associated to an average of 3.5 linkers, leading to the overall formula \([(a_1)_3(a_2)_6T]L_{3.5}\).

\textbf{Keywords} 

dissociation; ESI-MS; hemoglobin; MALLS; polychaete

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The extracellular haemoglobin of the marine polychaete, \textit{Arenicola marina}, is a hexagonal bilayer haemoglobin of \(\approx 3600\) kDa, formed by the covalent and noncovalent association of many copies of both globin subunits (monomer and trimer) and nonglobin or ‘linker’ subunits. In order to analyse the interactions between globin and linker subunits, dissociation and reassociation experiments were carried out under whereby \textit{Arenicola} hexagonal bilayer haemoglobin was exposed to urea and alkaline pH and the effect was followed by gel filtration, SDS/PAGE, UV-visible spectrophotometry, electrospray-ionization MS, multangle laser light scattering and transmission electron microscopy. The analysis of \textit{Arenicola} haemoglobin dissociation indicates a novel and complex mechanism of dissociation compared with other annelid extracellular haemoglobins studied to date. Even though the chemically induced dissociation triggers partial degradation of some subunits, spontaneous reassociation was observed, to some extent. Parallel dissociation of \textit{Lumbricus} haemoglobin under similar conditions shows striking differences that allow us to propose a hypothesis on the nature of the intersubunit contacts that are essential to form and to hold such a complex quaternary structure.

\textbf{Abbreviations} 

ESI, electrospray ionization; Hb, haemoglobin; HBL, hexagonal bilayer; MALLS, multangle laser light scattering; RI, refractive index; RW, average gyration radius; TEM, transmission electron microscopy.
Polymerization is needed in extracellular respiratory proteins for retention in the vascular system and for adequate oxygen capacity at a manageable osmotic pressure, but this size requirement poses issues for spontaneous assembly. The in vivo association of such complex proteins remains unclear in polychaete annelids. The pathway of folding of HBL-Hbs has been reported to involve independent folding of individual domains, followed by domain interaction for the oligochaete, \textit{L. terrestris} Hb [5]. Moreover, it was found that oligomeric proteins might require the presence of molecular chaperones to promote the assembly of the functional units [8]. However, to date, such proteins have not been described for the in vivo assembly of HBL-Hb. Since 1996, significant efforts have been devoted, by several laboratories, to elucidate, in greater detail, the arrangements between the subunits from a structural point of view [3,9]. The stability of the quaternary structure of annelid HBL-Hb has been studied by changing the chemical composition of the medium, as follows (a) by varying pH, (b) incubation in the presence of chaotropic salts or (c) incubation in the presence of denaturating agents. The dissociation–reassociation process of \textit{Arenicola} Hb has never been investigated in detail and remains poorly understood despite several electrophoretic and gel-filtration studies [10,11]. There is an increasing interest in understanding the dissociation and association process of this Hb because it provides useful information about subunit interactions necessary to maintain the quaternary structure. Moreover, \textit{Arenicola} Hb has been proposed as a useful model system for developing therapeutic extracellular blood substitutes [12] and requires a detailed study of subunit interactions in order to identify the optimal composition of storage and transfusion buffer.

This article reports the results of an in-depth study of the dissociation of \textit{Arenicola} Hb followed by gel filtration, SDS/PAGE, spectrophotometry, light scattering and ESI-MS. Two different dissociation techniques were employed: alkaline pH and addition of urea at pH 7.0. In this investigation, our attention was focused on the mechanism of subunits dissociation and on the reassociation of the subunits after dissociation. This was accomplished, in part, by comparison with the well-studied extracellular Hb of the oligochaete, \textit{L. terrestris} [3,5,6,13–16].

\section*{Results}

\subsection*{Subunit composition of native \textit{Arenicola} Hb, and dissociation products}

\textbf{Native \textit{Arenicola} Hb}

The subunit composition of freshly prepared samples of native \textit{Arenicola} Hb was re-examined by SDS/PAGE and ESI-MS to permit comparison with previous data (Fig. 1) [4]. The deconvoluted ESI-MS spectra (Fig. 1A) and the SDS/PAGE pattern (Fig. 1B) of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1}
\caption{Subunit composition of native \textit{Arenicola} haemoglobin (Hb). (A) MaxEnt-processed electrospray ionization (ESI-MS) spectrum of denatured \textit{Arenicola} Hb. The insets show the details of monomeric chains (I), linker subunits unobserved previously (II), trimeric globin complex T and the homodimer \textit{D}_1 (III). (B) Left lane: SDS/PAGE of unreduced \textit{Arenicola} Hb which confirms the presence of the three groups of subunits: I, II and III. (B) Right lane: migration of low molecular weight standards (Amersham). Results of a single representative experiment are presented.}
\end{figure}

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\textit{Self-assembling properties of \textit{A. marina} haemoglobin}


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the unreduced Arenicola Hb revealed three groups of subunits: I, II and III. Group I consists of the two monomeric globin chains a1 and a2 (15 952 ± 1.0 and 15 975 ± 1.0 Da); a new linker subunit group (group II) was observed, which is composed of three constant monomeric chains (23 122 ± 1.0, 24 065 ± 1.0 and 24 219 ± 1.0 Da); and group III is composed of the five disulfide-bonded globin trimers (49 581 ± 4.0, 49 612 ± 4.0, 49 657 ± 4.0, 49 708 ± 4.0 and 49 750 ± 4.0 Da) and the linker homodimer, D1 (50 323 ± 4.0 Da).

Spectrophotometric titration of Arenicola Hb

In order to investigate the presence of any pH- or urea-dependent change surrounding the haem pocket, the optical spectra (300–700 nm) of Arenicola Hb were recorded between pH 2.0 and 12 and exposed to an increasing concentration of urea (1–8 M) for 48 h (Fig. 2). The absorption spectrum of oxyhaemoglobin over the range 300–700 nm is not significantly altered at pH 7.0 over 48 h (Fig. 2A). At acidic pH (Fig. 2B), the spectrum gradually changes from that of oxyhaemoglobin to that of methaemoglobin: the Soret band becomes broader and slightly less intense, with a shift to a lower wavelength, a decrease in the intensity of the α (574 nm) and β (540 nm) bands, and the formation of a distinct absorption at 630 nm and near 500 nm. Spectrophotometric data showed an important decrease in the intensity of the Soret band, characteristic of haem loss, for pH values of < 3.0 (data not shown), > 8.0 (Fig. 2C) and in the presence of an increasing concentration of urea (Fig. 2D).

Gel filtration and SDS/PAGE patterns of the dissociated subunits

Figure 3 shows typical gel filtration elution profiles of partially dissociated Arenicola Hb and Lumbricus Hb at alkaline pH 8 (Fig. 3A, B, respectively) and in the presence of 4 M urea at pH 7 (Fig. 3C, D, respectively). The elution profile of Lumbricus Hb (Fig. 3B, D) is in agreement with results published previously [14]. In addition to the undissociated Hb (Fig. 3, HBL), three peaks corresponding to the dodecamer subunit (D), the trimer + linker (T + L) subunits, and the monomer (M) subunit are observed. The Arenicola Hb profile is different (Fig. 3A, C) because only two peaks are

![Fig. 2. Spectrophotometric titration of Arenicola haemoglobin (Hb). Overlay of UV-visible spectra of Arenicola Hb, dissociated under various conditions for 48 h (A–C) at ambient temperature: (A) 0.1 M Tris/ HCl buffer at pH 7.0; (B) 0.1 M Tris/HCl buffer at pH 5.0; and (C) 0.1 M Tris/HCl buffer at pH 9.0. (D) Arenicola Hb immediately after exposure to increasing concentrations (1–8 M) of urea at pH 7.0. The arrows indicate the evolution of the absorbance with time (A–C) or with an increasing concentration of urea (D). AU, absorbance unit. Results are presented for a single representative experiment.](image-url)
observed. The nonreduced SDS/PAGE of collected fractions (Fig. 3, inset) showed that the initial subunit content of the first peak (Fig. 3, lanes 1 and 4) is similar to that of native Arenicola Hb, corresponding to undissociated Hb (I<sub>HBL</sub>) (the concentration of each sample loaded on the gel are slightly different). Peak I<sub>D</sub> which has the size expected for a putative one-twelth of the whole molecule of Arenicola Hb comprises the trimers and the monomers (Fig. 3, lanes 2 and 5), confirming that it corresponds to the dodecamer. Two additional, less intense, bands are also observed and they are present in all the other lanes in the middle of the gel [17]. These bands have previously been reported for Arenicola Hb as polymerization of the monomer or partial dissociation of the disulphide-bounded trimers, during the preparation of the samples before migration on the gel [18]. Moreover, no corresponding polypeptide chains were observed during MS analysis (see below, Fig. 4A). After the dissociation of Lumbricus Hb, all the subunits (trimer, linker and monomer) are present in the dissociated fractions (lane 7 and 8). The pattern corresponding to dissociated fractions of Arenicola Hb (Fig. 3, lane 3 and 6) exhibits alterations with the absence of the bands corresponding to the linker subunits. Control experiments were carried out in the presence of reducing agent or protease inhibitor and revealed similar gel filtration and SDS/PAGE patterns, indicating that the differences are not the result of degradation by a protease.

**Dissociated subunits observed by ESI-MS**

Figure 4 shows ESI-MS spectra for dissociated Arenicola Hb at alkaline pH. The spectra are similar for the dissociation in the presence of urea. The deconvoluted mass spectrum of undissociated Arenicola Hb (Fig. 4A) is similar to that for the native Arenicola Hb (Fig. 1A). The dodecamer subunit (Fig. 4A), was found to contain all the subunits <i>T</i> and <i>M</i>, and a small amount of the linker homodimer D<sub>1</sub>, which had not dissociated from the dodecamer. The deconvoluted spectrum of fully dissociated Arenicola Hb (Fig. 4A) reveals the absence of the linker subunits at 50 319 Da and at 23 122, 24 065 and 24 219 Da and the less intense...
Relative intensity of the trimers. These observations are in agreement with the observation of the disappearance of linker subunits on the SDS/PAGE patterns (Fig. 3, lanes 2, 3, 5 and 6). Moreover, the multicharged spectra for fully dissociated Arenicola Hb (Fig. 4B) revealed several new peaks for \textit{m/z} < 900, indicating possible degradation of the protein.

**Kinetic of dissociation of *Arenicola* Hb**

Dissociation of *Arenicola* Hb followed by gel filtration

The extent of dissociation of purified *Arenicola* Hb over the pH range 2–12 and at increasing concentrations of urea (from 1 M to 8 M in 0.1 Tris/HCl buffer, pH 7.0), at 4 °C for 25 h, was investigated by gel filtration (Fig. 5). The pH stability curves at three different incubation times is represented in Fig. 5A and is divided into four sections (a) \(h\), pH around the isoelectric point (4.0–5.0): spontaneous release of the haem from the protein pocket and simultaneous protein unfolding, (b) \(d\), pH 3.0–4.0 and pH 7.0–12.0: *Arenicola* Hb dissociation, (c) \(p\), pH around the isoelectric point (4.0–5.0): *Arenicola* Hb precipitate, and (d) \(s\), pH 5.5–7.0: the quaternary structure of *Arenicola* Hb is maintained. The dissociation of *Arenicola* HBL-Hb is a rapid time- and pH-dependent process at alkaline pH, as revealed by the slope of the percentage HBL curve (Fig. 5A). Between 1 and 4 M urea, the dissociation of HBL-Hb is faster within the first 2 h and slows down to reach an equilibrium at \(\approx 20\) h (Fig. 5B). The HBL-Hb is fully dissociated immediately after exposure to 6 M urea. Figure 6 represents the overlaid chromatograms of typical elution profiles of dissociated *Arenicola* Hb at alkaline pH (Fig. 6A,B) and in 4 M urea (Fig. 6C,D) at three incubation times. The profiles are similar and even if the formation of dodecamer is less rapid in
urea, its dissociation is faster. As soon as Arenicola HBL-Hb is fully dissociated, the dodecamer dissociates slowly (Figs 5 and 6) into smaller subunits containing haem (absorbance at 414 nm) but also nonhaem-containing fragments, with retention times corresponding to molecular masses of < 15 kDa (framed Fig. 6B,D). The ratio of the absorbance $A_{414}$ : $A_{280}$ of the dodecamer peak increases during the first hour of the dissociation from 2.75 to 2.85 ($A_{414}$ : $A_{280}$ native Arenicola Hb = 2.23). Then, it remains constant to decrease progressively with time. The same variation is observed for the two other peaks at alkaline pH and in the presence of urea.

**Effect of divalent cations at alkaline pH**

Figure 7 reveals the effect of divalent cations on the dissociation of Arenicola Hb at alkaline pH immediately after exposure to the buffer. No dissociation is observed when Arenicola Hb is diluted in seawater (pH 7.8), while it is almost completely dissociated upon dilution in 0.1 M Tris/HCl buffer at pH 7.8. The presence of Ca$^{2+}$ and Mg$^{2+}$ either prevents (Fig. 7) or decreases the extent of dissociation of these molecules at alkaline pH. While slightly further dissociation is observed in the presence of EDTA at alkaline pH (presumably by competitive complexing of the divalent cations), no significant dissociation occurs at neutral and acidic pH. A similar experiment was carried out for the dissociation of Arenicola Hb in 4 M urea in the

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**Fig. 6.** Formation of disrupted apoglobin induced by dissociation. Gel filtration elution profile on a Superose 6-C column of dissociated Arenicola haemoglobin (Hb) in 0.1 M Tris/HCl buffer showing the formation of disrupted apoglobins at 280 nm (framed). The haemoglobin is dissociated, as described in the Experimental procedures. Elution profile of Arenicola Hb at (A) 414 nm and (B) 280 nm, immediately after exposure at pH 8.0 (solid line), after 5 h (broken line) and 25 h (dotted line). Elution profile of Arenicola Hb at (C) 414 nm and (D) 280 nm, immediately after exposure in 4 M urea at time zero (solid line), after 4 h (broken line) and 24 h (dotted line). The undissociated peak is labeled HBL, and the major dissociated peak is the dodecamer D. Results are presented for a single representative result.

**Fig. 7.** Structure stabilization induced by divalent cations at alkaline pH. Dissociation of Arenicola haemoglobin (Hb) immediately after exposure to the buffer, under different conditions over the pH range 6.0–8.0, followed by gel filtration on a Superose 6-C column. The percentage of undissociated Hb was determined by integrating the chromatogram at 414 nm using the MILLIENNIUM software and is represented as a function of pH. The dissociation, expressed as percentage of undissociated Arenicola Hb, in different conditions, was shown as follows: diamonds, 0.1 M Tris/HCl buffer; crosses, 0.1 M Tris/HCl buffer and 5 mM EDTA; triangles, 0.1 M Tris/HCl buffer and 50 mM Mg$^{2+}$; squares, 0.1 M Tris/HCl buffer and 50 mM Ca$^{2+}$ and asterisks, sea water (pH 7.8). Results are the means ± SD for three individual experiments at each point.
presence and absence of Ca$^{2+}$, and no stabilizing effect was observed (data not shown).

Dissociation pattern followed by MALLS

MALLS analysis of partially dissociated *Arenicola* Hb at pH 7.8 yielded profiles shown in Fig. 8, with molecular mass (Fig. 8A) and gyration radius (RW) (Fig. 8B) estimated during the elution profile at three different incubation time-points. The estimated molecular mass (Fig. 8A) decreases during incubation, and the polydispersity (estimated by the molar mass slopes) assumes a downward curvature shape, particularly for peaks I$_{HBL}$, I$_1$ and I$_2$, characteristic of a less homogenous population. The polydispersity of the peak I$_{HBL}$ indicates that it includes intermediates of dissociation which are truncated HBL *Arenicola* Hb (Fig. 8). Truncated HBL-Hbs (partially dissociated HBL-Hb particles lacking one-sixth to one-half of the HBL structure) are also observed on the transmission electron microscopy (TEM) images of the I$_{HBL}$ fraction purified by gel filtration (Fig. 9A). Even if the estimated average RW values (Fig. 8B) are close to the angular variation detection limit of 10 nm, the RW decreases after 2 h with an important scattering and increase observed, after 24 h of incubation, for I$_1$ and I$_2$.

Reassembly of HBL structure

The extent of reassociation of *Arenicola* Hb was investigated by MALLS after dissociation at alkaline pH. As scattering intensity is strongly dependent on particle radius, a small amount of large particles in the sample would give a large response with the light scattering detector, although their amount, as measured by the refractive index (RI) response, is low. These interesting properties allowed us to observe a reassembly of *Arenicola* Hb, which was not so easily observed using gel filtration only. Figure 10 shows MALLS representative results obtained with the reassembly of HBL-Hb structures from dissociated *Arenicola* HBL-Hb, immediately after exposure to alkaline pH 8.0 and 9.0 (Fig. 10A,B respectively) and after 1 h at pH 8.0 (Fig. 10C). Similar results were observed in the presence of 4 M urea. While different ionic composition buffers at pH 7.0 were tested, the reassembly was only observed in a buffer containing an ionic composition similar to that of *A. marina* blood (see Experimental procedures), at pH 7.0, and after a very short dissociation incubation time (< 5 min). The reassociation is limited, as revealed by the RI profiles of the I$_{HBL}$ peak after reassociation and the proportion of reassociated HBL-Hb (Fig. 10A,B). The observation of the reassociation is characterized by the differences of the light scattering signals for the I$_{HBL}$ peak, before and after the reassociation (Fig. 10A,B). The reassociation is not observed after 1 h of dissociation at alkaline H (Fig. 10C) and is less important as pH increases (Fig. 10B) and coincides with the absence of truncated HBL-Hbs (retention time between 20 and 25 min), as revealed by the MALLS profile (Fig. 10C). Control experiments using a reducing agent or protease inhibitors during the dissociation process, did not improve the reassociation. The reassociation is confirmed by the TEM images of I$_{HBL}$ isolated by gel filtration after the reassociation.

![Fig. 8](image-url)
process (Fig. 9B). We can distinguish truncated HBL-Hbs in a more structured conformation than before reassociation (Fig. 9A) and structured HBL-Hb similar to native Arenicola HBL-Hb (Fig. 9C).

Discussion

The structural data (Fig. 1) confirmed published data on native Arenicola Hb to some extent [4], but also revealed some differences. One difference is the absence of the heterodimer D2 (51981 ± 4.0 Da) and the observation of smaller chains, of ≈24 kDa, which might correspond to the putative linker L2 or to linkers that were not previously observed [4]. The linkers are cysteine-rich proteins which, in A. marina [19] as in Riftia pachyptila [20], were found to bind H2S at slightly alkaline pH, resulting in the formation of persulfides for detoxification purpose in nonsymbiotic species. However, the role of cysteines in binding H2S appears to be controversial, as revealed by recent studies [21,22], and is still under active investigation. In an acidic environment, as used for ESI-MS analysis under denaturing conditions, H2S is released and some rearrangement could occur, resulting in a possible cleavage of the heterodimer, D2, into smaller subunits. Moreover, the animals used to collect blood were obviously different from those used in previous studies, and it is possible that different alleles exist in different populations of A. marina.

A complex mechanism of dissociation

Dissociation profile of Arenicola Hb

The dissociation of Arenicola Hb was investigated in detail at alkaline pH and in the presence of urea. Our results are in agreement with studies by Daniel and collaborators [23] who found that Arenicola Hb is less stable at alkaline pH than Lumbricus Hb. Extracellular annelid Hbs usually dissociate at pH ≥ 8.0 [13,24] according to an equilibrium process, as observed in L. terrestris and Tubifex tubifex Hbs [24]. The peculiarity of A. marina extracellular Hb is that the dissociation occurs even at pH values between 7.0 and 8.0, in a buffer that does not contain any other ions, such as alkaline earth cations (Figs 5A and 7). In addition, this is not an equilibrium process. Indeed, the dissociation is almost immediately complete at pH 8.0 and is time-dependent (Figs 5 and 8). The dissociation profiles of Arenicola Hb in urea are similar (Figs 6 and 8), suggesting that the mechanism of dissociation is common to both denaturing treatments, even if the kinetics are different. The formation of the dodecamer is faster at alkaline pH and its dissociation occurs more rapidly in the presence of urea (Fig. 6). This reveals the importance of hydrogen bonds in the structure of the dodecamer. Several simultaneous dissociations of an HBL-Hb structure can be envisioned, as proposed for Lumbricus Hb dissociation [14]. However, the dissociation process of Arenicola Hb is more complex to
The quaternary structure is rapidly affected at alkaline pH or in the presence of urea (Fig. 5). The dissociation leads to the rapid formation of the one-twelfth protomers (D + L) through truncated HBLs. Indeed, results from gel filtration, MALLS and TEM analyses reveal the presence of a small amount of truncated HBLs at the early stage of the dissociation process (Figs 8, 9A and 10) and the formation of one major peak, I\textsubscript{D} (Figs 3A, B and 6), interpreted as the dodecamer, according to structural analysis (Fig. 3, lanes 2 and 5, Fig. 4A). However, the higher molecular mass of peak I\textsubscript{D} (MALLS results, Fig. 8A), the presence of D\textsubscript{1} on the ESI-MS spectra of the dodecamers (Fig. 4A), and the \(\frac{A_{414}}{A_{280}}\) value, which increases during the first incubation hour for peak I\textsubscript{D} (Fig. 6), all indicate that the dodecamer is still associated with linkers at the start of the dissociation. Then, the linkers dissociate from the dodecamer, resulting in a decrease of molecular mass (peak I\textsubscript{D}, Fig. 8A). The dodecamer does not dissociate into stable trimers and monomers, as observed for \textit{Lumbricus} Hb [14], but into higher molecular mass units (peaks I\textsubscript{1} and I\textsubscript{2}, Fig. 8A), in low abundance and transitory. The denaturation of these subunits is evident from the variation of the RW value (Fig. 8B). The RW increases for I\textsubscript{1} and I\textsubscript{2}, while the molecular mass decreases after 24 h of dissociation. These variations of RW are characteristic of an extended unfolded conformation during the dissociation process. The decrease of RW after 2 h of dissociation is explained by the formation of smaller subunits with smaller radius. The important scatter is caused by the presence of a mix of small structured subunits and small destructed subunits, which have a higher RW value. After 24 h of dissociation, most of these dissociated subunits are denaturated, so the scatter is less important.

**Structural alterations of \textit{Arenicola} Hb**

UV-visible spectroscopy around the Soret band provided information about the haem environment. An observation by Ascoli and collaborators [25] suggested that oxidation of earthworm Hb affected its quaternary structure, leading to dissociation. In \textit{Arenicola} Hb, however, by comparing the dissociation profiles at alkaline and acidic pH (Fig. 5A) and the light absorption spectrum (especially between 500 and 700 nm) (Fig. 2B, C), it appears that the spectral changes are only partially related to the dissociation. Indeed, at pH 8.0 and above, the extensive dissociation of \textit{Arenicola} Hb was accompanied by a relatively small change in the visible absorption region of the spectra (Figs 2C and 5A) and the methaemoglobin formation (at pH 6.0) is not accompanied by an extensive dissociation (Figs 2B and 5A). The dissociation pattern of \textit{Arenicola} Hb is similar in the presence of a reducing agent, confirming that dissociation is not induced by oxidation of...
the haem. The important decrease of the Soret band observed at alkaline pH with time (Fig. 2C) and in the presence of an increasing concentration of urea (Fig. 2D), reveals a significant alteration in the haem pocket, leading to a dissociation of haem from the haemoglobin. These analyses revealed that the denaturation is accompanied by local changes in the haem cavity, potentially having profound effects on the protein structure, as it is known that haem clearly stabilizes intact myoglobins and haemoglobins with respect to the apoglobin [26–28]. The formation of apoglobin and its degradation are confirmed by the following observations, namely (a) the decrease of the A414 : A280 of each elution peak, which is characteristic of a loss of haem and (b) the increasing formation of nonhaem-containing subunits, observed by gel filtration for both dissociation processes (Fig. 6B,D). These nonhaem, smaller, products (Fig. 6) are interpreted as degradation products of the subunits, as they do not correspond to unfolded linkers (which should elute later or we should see them by SDS/PAGE (Fig. 3, lanes 3 and 6) and ESI-MS (fully dissociated haemoglobin, Fig. 4A). Finally, the degradation products on the ESI-MS multicharged spectra of fully dissociated Arenicola Hb associated with a less intense signal for the disulphide-bounded trimers (Fig. 4b). The removal of haem is followed by proteolytic degradation of the apoglobin, perhaps initiated by the presence of free hemin, which has been reported to enhance oxidant-mediated damage [29].

Disappearance of the linkers

The linkers are thought to be degraded during the dissociation process. Indeed, they are not observed by SDS/PAGE (Fig. 3, lanes 3 and 6) or on the ESI-MS spectra (Fig. 4A) of fully dissociated Arenicola Hb. Recently, Suzuki & Riggs [30] and Chabasse et al. [31] showed that Arenicola linker chains possess a conserved cysteine-rich domain [a low-density lipoprotein A (LDL-A) module] homologous to the cysteine-rich region of the ligand-binding domain of the low-density-lipoprotein receptor (LDLR) family [30,31]. Studies investigating free hemin-induced modifications in LDL revealed that hemin associates with LDL and undergoes oxidative breakdown, releasing free iron, which is well known to catalyze oxidant degradation [32]. The haem dissociates easily from Arenicola Hb after dissociation at alkaline pH or in the presence of urea (Fig. 2C,D). The product of hemin peroxidation was found to be either aggregation or fragmentation [33,34]. Aggregation of linkers has previously been observed for Lumbricus Hb [5], and could attenuate the volatilization into the gas phase necessary for observation by ESI-MS (B. N. Green, and S. N. Vinogradov, personal communication). However, we should then observe bands of higher molecular mass on the SDS/PAGE gel (Fig. 3, lanes 3 and 6). Further studies on the identification and characterization of Arenicola Hb subunits isolated by preparative gel electrophoresis using a proteomics approach (M. Rousselot et al., unpublished data) revealed that molecular mass bands (< 15 kDa) observed after dissociation of Arenicola Hb at alkaline pH or in the presence of urea, are composed of globins and also of linker fragments that are not observed for the native Arenicola Hb SDS/PAGE pattern. This confirmed that the dissociation of Arenicola Hb at alkaline pH or in the presence of urea, induces fragmentation of the linker chains, probably as a result of their oxidation in the presence of free hemin.

The effect of potential protease was considered and control experiments using protease inhibitor were performed; the linker still disappeared during dissociation, as evidenced by SDS/PAGE and ESI-MS experiments (data not shown). The same phenomenon was observed in the presence of a reducing agent. The disappearance or the severe reduction in the relative intensities of the linker chains from the ESI-MS spectra has previously been observed in Eudistylia chlororcuorin [35] and in other HBL-Hbs (B. N. Green, personal communications).

Stabilizing effect of divalent cations at alkaline pH

Arenicola Hb is stable at slightly alkaline pH (pH 7–8) when salts are present at concentrations similar to physiological concentrations. Among those salts that are important for structure, alkaline earth cations (Ca2+ and Mg2+) play a major role (Fig. 7). These cations also stabilize the HBL structure of other annelid Hbs [38] and Myxicola chlo-rocuorin [39], divalent cations are not necessary to maintain the HBL-Hb structure at neutral pH, even in the presence of EDTA. The divalent cations probably scavenge side-chain anionic groups ionized at alkaline pH. Moreover, LDL-A modules, found on linker chains, possess a cluster of four conserved acidic residues [31], which may be involved in calcium-dependent protein folding [40].

A limited association–dissociation equilibrium

At alkaline pH values, annelid extracellular Hbs dissociate irreversibly into one-twelfth of the whole molecule [41,42]. However, extracellular Hbs from the...
earthworm *L. terrestris* and *T. tubifex* dissociate further into several smaller subunits, which are in association–dissociation equilibrium with one another [6,13,14,24,43,44]. After dissociation of *Arenicola* Hb, some rearrangements are observed when the sample is returned to neutral pH with a salt composition similar to its physiological fluid. This rearrangement is only observed in the presence of partially dissociated HBL-Hbs and the one-twelfth protomers and led to a recovery of the structure of the one-twelfth protomers at different degrees of polymerization, and up to a completely reassociated HBL-Hb (Fig. 9B). In the absence of these structured subunits (truncated HBL-Hbs or one-twelfth protomers), no reassociation is observed: the smaller subunits lost the ability to reassemble to form whole Hb molecules, probably as a result of the fragmentation of linkers essential to maintain the quaternary structure [3,5], and the formation of apoglobin with important structural alteration. Control experiments using protease inhibitor and reducing agent during the dissociation process were performed in order to evaluate the potential effect on linker and globin degradation, but the reassociation was not improved.

**Intersubunit contacts**

At alkaline pH and even more with urea, *Lumbricus* Hb dissociates to form dodecamers which further dissociate into trimers, linkers and monomers [13,36]. This pattern contrasts with that observed for *Arenicola* Hb: the HBL-Hb is preferentially dissociated at alkaline pH, where the dodecamer is stabilized compared with urea-induced dissociation (Fig. 6). Even if the overall structure of the HBL-Hbs is similar, the dissociation analyses reveal that the nature of the major intersubunit contacts, which contribute to the quaternary structure, is different. Comparatively, *Lumbricus* Hb is less stable in the presence of urea, suggesting that the interactions involved in the quaternary structure here are rather hydrogen bonds. This view is supported by the ease of the dissociation of *Arenicola* Hb at slightly alkaline pH (pH 7–8, Fig. 5A) with the stabilizing effect observed in the presence of divalent cations over this pH range, while *Lumbricus* Hb remains structured. This suggests salt bridge interactions between ionic side-chains, but also that salts of the physiological fluid are mainly involved the quaternary structure of *Arenicola* Hb. Incidentally, the blood of *A. marina* has higher concentrations in salts, including divalent cations, than *L. terrestris* blood [45].

A comparison from the 3D volume reconstruction by cryoelectron microscopy and X-ray crystallography of *Arenicola* [46] and *Lumbricus* Hbs [3,46], shows that their bilayered architecture mainly differs by the offset of the hexagonal layers. Interlayer contacts are different in the two Hbs, leading to a 16° rotation of the two hexagonal layers in *Lumbricus* Hb, while they are eclipsed in *Arenicola* Hb [46]. The distance between the hexagonal layers is larger in *Arenicola* Hb than in *Lumbricus* Hb [46], thus the haemoglobin : haemoglobin contact between one-twelfth protomers of the opposite layers observed in *Lumbricus* Hbs are weaker in *Arenicola* Hb – if they exist – and the surface contact of the linkers to the solvent is larger in *Arenicola* Hb. In addition, the linker–linker interaction seems stronger in *Arenicola* Hb than in *Lumbricus* Hb [46]. All these data suggest that the linker–linker interlayer contacts, which play a central role in the assembly of the quaternary structure are mostly hydrogen bonds in *Lumbricus* Hb and salt bridges in *Arenicola* Hb.

The globins and linkers are glycosylated in *Lumbricus* Hb [47] but the polypeptide chains in *Arenicola* Hb are not glycosylated [4,48]. Work on *Lumbricus* Hb suggests that carbohydrate-gluing, mediated by lectin-like interactions, could help maintain the quaternary structure [48].

In conclusion, we have shown that *Arenicola* Hb is able to reassociate after dissociation at alkaline pH. However, its dissociation follows a novel and complex mechanism, different from that previously reported for other extracellular annelid Hbs; *Arenicola* Hb is less tolerant to pH and salt variations, which induced rapid degradation of the complex (fragmentation of the linker and apoglobin formation). A parallel study on the dissociation of *Lumbricus* Hb revealed that the nature of the intersubunit contacts, essential in the preservation of the quaternary structure, is different. The dissociation pattern suggests the importance of the salt bridge interactions in the stabilization of the quaternary structure and the importance of the salt composition of the buffer to maintain the integrity of *Arenicola* Hb at slightly alkaline pH. These conclusions are of prime importance for storage and transfusion conditions, considering the development of *Arenicola* Hb as blood substitute, for which the medium required is different from *A. marina* physiological conditions.

**Experimental procedures**

**Animal collection**

Individuals of *A. marina* were collected at low tide from a sandy shore near Roscoff (Penpoull beach), Nord Finistère, France, by the crews of the marine station facilities, and
kept in local running sea water for 24 h. Blood samples were withdrawn from the lugworm’s ventral vessel into a needle and centrifuged (15 min, 10 000 g, 4 °C) to remove insoluble material. The blood extraction was performed at 4 °C in an Arenicola saline buffer compatible for trans-fusion to vertebrates (4 mM KCl, 145 mM NaCl, 0.2 mM MgCl2 and 10 mM Hepes/0.1 mM NaOH, pH 7.0), in the presence of a commercial protease inhibitor cocktail (Complete; Roche, Basel, Switzerland). The resulting samples were purified immediately and kept frozen (−40 °C) until use. Purified Lumbricus Hb was kindly provided by S. Vinogradov (Wayne State University, MI, USA).

**Purification techniques**

Analytical gel filtration was performed on a 1 × 30-cm Superose 6-C (fractionation range: 5–5000 kDa) and Superose 12-C (fractionation range: 1–300 kDa) column (Amersham Biosciences Biotechnology, Uppsala, Sweden) using a high-pressure HPLC system (Waters, Milford, MA, USA). The column was equilibrated with the Arenicola saline buffer. Flow rates were typically 0.5 mL·min⁻¹, and the absorbance of the eluate was monitored at 280 nm and 414 nm. The peaks were collected separately and concentrated by centrifugation on an Amicon Ultra-10 concentrator, cut-off molecular weight 10 kDa (Millipore, Billerica, MA, USA). One or two further purifications, using the same protocol, were performed to obtain pure fractions. Haem content and protein concentration were determined as described previously [4]. The samples were always kept at 4 °C except during chromatographic analyses, which were performed at ambient temperature (20–22 °C).

**Spectrophotometry**

The absorption spectra, as a function of pH and urea concentration over the 300–700 nm range, were obtained using a UV mc2 spectrophotometer (SAFAS, Monaco). The analyses were performed at room temperature at a concentration of Arenicola Hb of 0.5 mg·mL⁻¹.

**SDS/PAGE**

PAGE in the presence of 0.1% (w/v) SDS was carried out using the Laemmli discontinuous buffer system [49] and slab gels (0.75 mm × 10 cm × 8 cm) of 15% (v/v) acrylamide. The gels were electrophoresed for 1–2 h and stained in Coomassie Brilliant Blue R-250, as described previously [4].

**ESI-MS**

ESI-MS was performed under denaturing conditions to determine the molecular masses of the subunits of Arenicola Hb. Electrospray data were acquired on a Q-Tof II (MicroMass, Altrincham, UK), scanning over the m/z range 600–2500 at 10 s per scan, and 45 scans were averaged to produce the final spectrum. Samples were desalted by washing with MilliQ water, repeated 10 times on Amicon-3 kDa at 4 °C. Protein concentrations of 0.5 mg·mL⁻¹ in acetonitrile/water (1 : 1, v/v) containing 0.2% (v/v) formic acid, were introduced into the electrospray source at 5 μL·min⁻¹. The cone voltage (counter electrode to skimmer voltage) ramp was from 60 V at m/z 600 to 120 V at m/z 2500. The raw multicharged spectra were deconvoluted using maximum entropy-based software (MAXENT) supplied with the instrument [50]. Mass scale calibration was established using the multiply charged series from horse heart myoglobin (molecular mass 16 951.5 Da; M-1882; Sigma, St Louis, MO, USA). Molecular masses were based on the atomic weights of the elements given by the International Union of Pure and Applied Chemistry.

**MALLS**

MALLS measurements were performed using a DAWN EOS system (Wyatt Technology Corp., Santa Barbara, CA, USA) directly on-line with the HPLC system. The 18 discrete photodetectors are spaced around the flow cell and enable simultaneous measurements to be made over a range from 15° to 160°. The eluate was simultaneously monitored with a photodiode array detector (Waters 2996, Waters, Milford, MA, USA) and an RI detector (Waters 2414, Waters). The MALLS instrument was placed directly before the refractometer and after the Superose 6-C column and UV detector to avoid backpressure on the RI cell. Chromatographic data were collected and processed using the astra software (Wyatt Technology Corp.). The Zimm fit method was used for molecular mass determinations [51]. In the calculations, a dn/dc value of 0.19 mL·g⁻¹ was used, typical of nonglycosylated proteins. BSA monomer (Sigma) was used for normalizing various detectors’ signals relative to the 90° detector signal.

**Dissociation experiments**

The dissociating agent (urea; Sigma) was dissolved in 0.1 mM Tris/HCl/0.1 mM HCl buffer, pH 7.0, containing 1 mM EDTA, and Arenicola Hb stock solution was added to achieve a final concentration of 4 mg·mL⁻¹. The Hb was dissociated by exposure for up to 48 h to 0.1 mM Tris/HCl buffer at alkaline pH (from 7.0 to 12.0). For acidic pHs, Arenicola Hb was exposed, for up to 48 h, to 0.1 mM sodium formate/0.01 mM formic acid, pH 3.0–4.5, to 0.1 mM sodium acetate/0.01 mM acetic acid, pH 4.5–5.5 and to 0.1 mM Tris/HCl/0.1 mM HCl, pH 5.5–7.0. Further control dissociation experiments were carried out in the presence of protease inhibitor cocktail (Complete; Roche) or reducing agent.
(0.5 mM NADH and 2 mM butylhydroxyltoluene (BHT); Sigma). Dissociations of Arenicola Hb were followed by HPLC at neutral pH on the system described above. Each chromatogram was collected and processed using the millennium software (Waters) supplied with the instrument. All the dissociation experiments were carried out at 4 °C.

**Reassembly of HBL structure from dissociated Arenicola Hb**

The completeness of the dissociation was checked by HPLC. The Arenicola Hb exposed to either alkaline pH or urea, was dialysed overnight at 4 °C against 2 × 1 L of saline buffer with a salt composition similar to that of the physiological fluid of A. marina: 400 mM NaCl, 3 mM KCl, 32 mM MgSO4, 11 mM CaCl2 and 0.1 M Tris/HCl/0.1 M HCl at pH 7.0. The reassociation, achieved at 4 °C, was followed by HPLC and MALLS at neutral pH.

**TEM**

Arenicola Hb was diluted to 0.1 mg·mL−1 with 0.1 M Tris/HCl buffer, pH 7.0, and applied to a very thin carbon substrate supported on a microgrid, stained with 2% (w/v) uranyl acetate solution, as described previously [52]. The specimens were examined at 80 kV, using a Jeol JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan).

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