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pH dependence of the dissociation of multimeric hemoglobin probed by high hydrostatic pressure

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Abstract

We investigated the thermodynamic features of the classic alkaline dissociation of multimeric hemoglobin (3.1 MDa) from *Glossoscolex paulistus* (Annelidea) using high hydrostatic pressure. Light scattering measurements up to microscopic thermodynamic equilibrium indicated a high pH dependency of dissociation and association. Electron microscopy and gel filtration corroborated these findings. The volume change of dissociation decreased in absolute values from −48.0 mL/mol of subunit at pH 6.0 to −19.2 mL/mol at pH 9.0, suggesting a lack of protein interactions under alkaline conditions. Concomitantly, an increase in pH reduced the Gibbs free energy of dissociation from 37.7 to 27.5 kJ/mol of subunit. The stoichiometry of proton release calculated from the pressure-induced dissociation curves was +0.602 mol of H^+/mol of subunit. These results provide a direct quantification of proton participation in stabilizing the aggregated state of the hemoglobin, and contribute to our understanding of protein–protein interactions and of the surrounding conditions that modulate the process of aggregation.
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1. Introduction

Protein oligomers occur in several biological systems as allosteric enzymes, multienzymatic complexes, ribosomes and viral protein capsids. Studies of the properties of isolated monomers are difficult because of the high dilution needed to obtain adequate protein dissociation. Similarly, dissociation through alterations in temperature, pH or ionic strength, or the addition of external agents such as urea and calcium, may have direct adverse effects on the tertiary structure of the subunits. Since such structural changes are not seen with hydrostatic pressure up to 300 MPa [1], the use of this approach to study protein dissociation has allowed a better analysis of the thermodynamics of protein–protein interactions [1–5]. Furthermore, pressure affects protein–protein interactions solely by altering the distances (volumes) of the components while maintaining the total energy of the systems almost constant, with much less drastic effects than temperature [6].

Studies using hydrostatic pressure combined with factors such as subzero temperatures and urea have improved our understanding of the mechanisms involved in protein–protein interactions [5,7–10]. The analysis of virus dissociation by pressure has helped to explain the possible mechanisms involved in virus uncoating in host cells, and has provided important thermodynamic information about these systems [11]. In these cases, the dissociated protein subunits have a lower affinity for each other than do the subunits in the native form. The use of pressure to understand protein folding and aggregation could provide new approaches for studying several neurodegenerative diseases [12]. Another goal in such investigations involves biotechnological and medical applications such as sterilization and the development of antiviral vaccines [13].

The hemoglobin (Hb) of the annelid *Glossoscolex paulistus* is a giant extracellular Hb (erythrocruorin) that dissociates into low affinity subunits after incubation under pressure, with stabilization of the dissociated products. The behavior described by the corresponding pressure dissociation curve has general applications [3,14] that make this protein an appropriate model

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for studying protein–protein interactions. This Hb, which has a molecular mass of 3.1×10^6 Da [15] and a sedimentation coefficient of 58S, belongs to a class of multisubunit Hbs and has a two-tiered, hexagonal organization [16]. Experiments using extracellular Hb from several invertebrate species, especially *Lumbricus terrestris*, have led to a progressive understanding of the structural hierarchy in these proteins [17–21]. A recent crystallographic study of *L. terrestris* Hb at 5.5 Å resolution [19] has revealed the remarkable organization of this molecule composed of 144 oxygen-binding Hb subunits of four different types and 36 non-Hb linker subunits. *G. paulistus* Hb shares considerable structural similarity with other extracellular Hbs, particularly that of *L. terrestris*, with a large number of heme-containing subunits (12–16 kDa) and the presence of a trimer of non-heme subunits (26, 28 and 34 kDa) [22].

There is no evidence that high hydrostatic pressure affects disulfide bonds in proteins. The high energy involved in S–S bond formation (425 kJ/mol) [23] implies a very high ΔV (≈ -1400 mL/mol of disulfide bond, based on the relationship Energy$=p\Delta V$, where $\Delta V=-425$ kJ/300 MPa). The heme bound to Hb is also not significantly affected since the energy involved is likewise high (68–85 kJ/mol at pH 7) [24]. In addition, extracellular Hb retains its capacity to reversibly bind oxygen after incubation under high hydrostatic pressure, indicating that the heme groups remain bound to the protein [3,25].

High pressure studies have shown that oligomeric proteins with more than two subunits have a heterogenous free energy of dissociation since the dissociation pressure curves show little or no dependence on concentration [14]. Interestingly, the presence of a large number of subunits, such as in *G. paulistus* Hb and *Megalobulimulus ovatus* hemocyanin (the latter with 20 subunits), represents an extreme in this property since changes in the concentration of these proteins have no effect on the pressure dissociation curve [4,5].

Another important characteristic of extracellular Hb involves the properties of reassociation and stabilization of the associated form by the presence of effectors such as calcium and glycerol [4]. Protons are also important effectors in extracellular Hbs and have been studied by several groups. Although various reports have described the dissociation of oligochaete Hb at alkaline pH [26,27], including *L. terrestris* [28,29], *Eisenia foetida* [30,31], and two species of *Pheretima* [32], and of polychaetes such as *Arenicola* [33,34] and *Abarenicola affinis affinis* [35], very little is known about the process of association in these proteins and the role of protons. The main objective of this study was therefore to examine the thermodynamic features of the pressure dissociation of extracellular Hb at alkaline pH, with particular emphasis on quantification of the protons involved in the dissociation.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade. Distilled water was filtered and deionized through a Millipore water purification system (18 MΩ resistance). Unless stated otherwise, the experiments were done at 22 °C with 50 mM Bis–Tris-propane buffer for pH 6.0 to 6.8, and 50 mM Tris–HCl buffer from pH 7.4 to 9.0. These buffers show negligible change in pH over the pressure range studied [36].

2.2. Protein preparation

Specimens of *G. paulistus* were collected in Rio Claro, São Paulo, and were maintained in soil from the collection site for less than 1 week before blood extraction. The extracellular Hb was purified as previously described [3], except that the buffer for the last resuspension after ultracentrifugation was 50 mM Tris–HCl, pH 7.4. The purified protein was stored at 6 °C. The Hb concentration was determined as previously described [15].

2.3. Light scattering and fluorescence under pressure

The high pressure system used has been described elsewhere [37]. An ISS model HP high pressure cell with sapphire windows connected to a pressure generator (HIP) was used. Light scattering at 340 nm was recorded in an Edinburgh FL 900 spectrofluorometer and was measured at an angle of 90° relative to the incident light using the same wavelength for the excitation and emission monochromators.

The intensity of light scattering by native extracellular Hb under the conditions described here was positively correlated with Hb concentrations up to 1 mg/mL (data not shown). Since the experiments were done at Hb concentrations considerably less than 1 mg/mL, we estimated the average molecular mass based on the intensity of light scattering (equation 17–32 in Ref. [38]) at pressure p, S_p, and defined the degree of dissociation (α) as

$$\alpha_p = (S_i - S_p)/(S_i - S_f) \quad (1)$$

where S_f and S_i are the intensities of light scattering for the dissociated and associated forms, respectively. The fluorescence data were obtained at an excitation wavelength of 285 nm and an emission of 300–400 nm. The pressure system, including the spectrofluorometer, was automated and controlled as previously described [39].

2.4. Lifetime measurements

For these experiments, the samples were excited at 285 nm with an H_2-filled F900 flash lamp at a gas pressure of 0.4 bar and a pulse frequency of 40 kHz. The emission was analyzed at 320 nm in a model FS900 spectrofluorometer (Edinburgh Instruments). Time-resolved fluorescence decays were recorded and analyzed with software that uses up to four exponential adjustments to fit the data (Edinburgh Instruments).

2.5. Gel filtration

Size exclusion HPLC was done with a Shimadzu HPLC system. A prepacked SynChropack GPC300 column (250×4.6 mm, i.d.) (SynChrom, Inc., Linden, IN) was used for gel filtration. Elution of the samples was monitored by

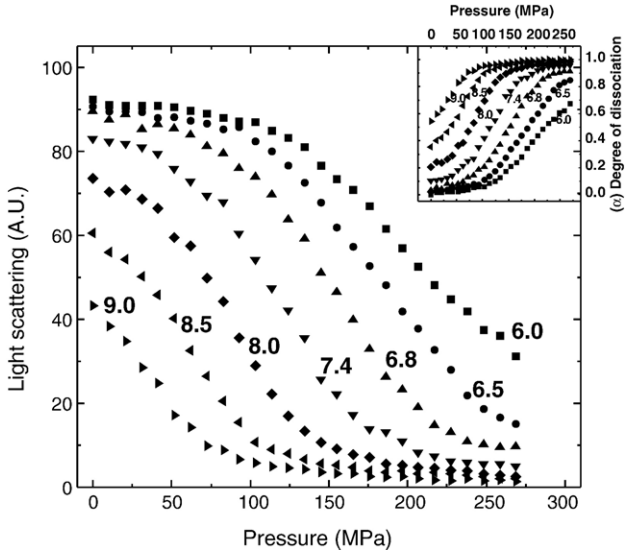

Fig. 1. Effect of pressure on erythrocruorin dissociation at different pH values. The erythrocruorin solution was incubated in Bis–Tris propane (pH 6.0 to 6.8) or Tris–HCl (pH 7.4 to 9.0) (both 50 mM). All measurements were done at a temperature of 295 K. (A) The light scattering was measured after a 13 min incubation at each pressure value (A.U.=arbitrary units). (A) Inset. Degree of dissociation based on the light scattering data and Eq. (1). The experiments were done in triplicate and the standard deviations were smaller than the symbols.

absorption at 280 nm. The void volume (V_0) of the column was measured with native tobacco mosaic virus (TMV) and the total volume (V_t) with a solution of human albumin.

2.6. Electron microscopy

Transmission electron microscopy was done in a Leo-902 microscope. Negative staining was done with 1% uranyl acetate. The samples were fixed with 0.5% glutaraldehyde prior to negative staining. The samples fixed under pressure were prepared as described previously [39,40].

2.7. Theoretical background

Since *G. paulistus* Hb has a molecular mass and subunit components similar to *L. terrestris* Hb [15,22,41] (see Introduction), for the thermodynamic analysis we considered the number of dissociating subunits to be similar to *L. terrestris* Hb, with 144 subunits arranged as 12 dodecamers and 36 linker subunits, with each dodecamer consisting of three disulfide-linked trimers (abc) and three monomeric d chains to give 108 dissociable subunits. As noted above, high pressure does not rupture covalent or disulfide bonds, or cause heme dissociation.

Even if the number of dissociable subunits were different from that adopted here, the use of higher or lower values would yield very similar results for the free energy, the volume change of dissociation and the proton release per mole of subunits. The protein dissociation into its subunits and the additional participation of protons in this process can be expressed as

$$P_{108} \overset{K^0_{atm}}{\longleftrightarrow} 108P + \upsilon_D H^+ \quad (2)$$

where P_{108} is the protein aggregate composed of 108 protein forms, including the linker subunits, $K^0{}_{atm}$ corresponds to the equilibrium constant of dissociation at reference conditions of atmospheric pressure, and υ_D is the total number of protons released or absorbed upon dissociation, such that when $\upsilon_D>0$ there is a net proton release while the opposite, $\upsilon_D<0$, corresponds to an absorption of protons; $\upsilon_D=0$ implies an apparently pH-independent process. This equilibrium corresponds to a global reaction since the dissociation most likely involves several intermediate steps. This equilibrium and its thermodynamic development are very similar to those described recently for TMV [39].

The corresponding equilibrium relationship for Eq. (2) is

$$K^0_{atm} = \frac{[P]^{108}[H^+]^{\upsilon_D}}{[P_{108}]} \quad (3)$$

and rewriting Eq. (3) gives the apparent equilibrium constant observed at a given pH. Thus,

$$K^{pH}_{atm} = \frac{K^0_{atm}}{[H^+]^{\upsilon_D}} = \frac{[P]^{108}_{pH}}{[P_{108}]_{pH}} \quad (4)$$

which gives

$$\Delta G^{pH}_{atm} = \Delta G^0_{atm} + \upsilon_D RT \ln[H^+] = \Delta G^0_{atm} - 2.303\upsilon_D RT\,pH \quad (5)$$

where K^{pH}_{atm} is the equilibrium constant of dissociation at a given pH and atmospheric pressure, ΔG^{pH}_{atm} is the respective Gibbs free energy change of dissociation, R is the gas constant, T is the absolute temperature and ΔG^0_{atm} is the corresponding energy change of dissociation at the reference pH (pH=0).

The relationship that correlates the energy change of dissociation of Eq. (5) with the experimental pressure can be

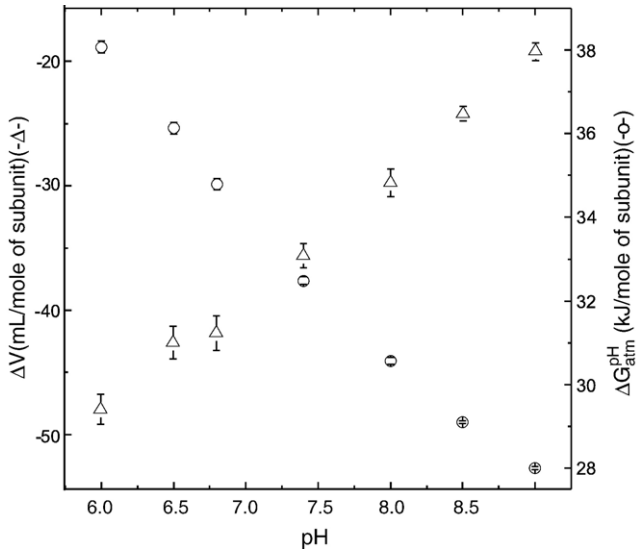

Fig. 2. $\Delta G^{pH}{}_{atm}$ and ΔV as function of pH. The open circles represent the results obtained from the intercept of the fits of a plot of $\ln K^{pH}{}_p$ *versus* pressure that corresponded to 108 ln108+107 ln$C$+108 lnα−ln(1−α) (data from the inset in Fig. 1); the open triangles represent data obtained from the slope of these fits. The bars correspond to the SD of triplicate experiments at each pH.

obtained based on the volume change of dissociation, ΔV, [42], which gives

$$\Delta G_p^{\mathrm{pH}} = \Delta G_{\mathrm{atm}}^{\mathrm{pH}} + p\Delta V \tag{6}$$

where is the Gibbs free energy of dissociation at a specified pH and pressure p. The equilibrium constant of dissociation derived from Eq. (6) corresponds to

$$K_p^{\mathrm{pH}} = K_{\mathrm{atm}}^{\mathrm{pH}} \exp\left(\frac{-p\Delta V}{RT}\right) = \frac{[P]_{\mathrm{pH},p}^{108}}{[P_{108}]_{\mathrm{pH},p}} \tag{7}$$

The presence of pH and p in the equilibrium constant of dissociation, K_p^{pH}, and in the concentrations of species on the right side of Eq. (7) indicates that these parameters are pH- and pressure-dependent. Eq. (7) rewritten in terms of the defined degree of dissociation, α, i.e., the ratio between the amount of protein subunits that dissociates relative to the total amount of associated forms, allows direct correlation with the experimental data as follows:

$$K_p^{\mathrm{pH}} = K_{\mathrm{atm}}^{\mathrm{pH}} \exp\left(\frac{-p\Delta V}{RT}\right) = \frac{108^{108} C^{107} \alpha^{108}}{(1-\alpha)} \tag{8}$$

where C is the total protein molar concentration and α is the degree of dissociation observed at each pH and pressure by using Eq. (1). The logarithmic form yields the linear relationship

$$\ln\left(K_p^{\mathrm{pH}}\right) = \ln\left(K_{\mathrm{atm}}^{\mathrm{pH}}\right) - \left(\frac{\Delta V}{RT}\right)\mathrm{p} \tag{9}$$

and a plot of $\ln(K_p^{\mathrm{pH}})$ *versus* p furnishes a straight line, the intercept and slope of which are given by Eq. (9) and yields the values for $K_{\mathrm{atm}}^{\mathrm{pH}}$ and ΔV.

3. Results

The effects of pH and pressure on the light scattering of Hb (0.5 mg/mL) are shown in Fig. 1. Increasing the pH and pressure reduced the intensity of light scattering as a result of Hb dissociation. The incubation time at each pressure before the light scattering measurements were done corresponded to more than 99% of the equilibration time, enough to validate the calculated thermodynamic parameters. Fig. 3A shows that at pH 6.5 the pressure curves for 10 and 100 min were very similar, and experimental data obtained at pH 7.4 (Fig. 2 from Ref. [3]) also showed stabilization over a similar time interval. Obviously a cyclic reversibility of the process (dissociation/association between subunits) should be considered irrelevant for validating the

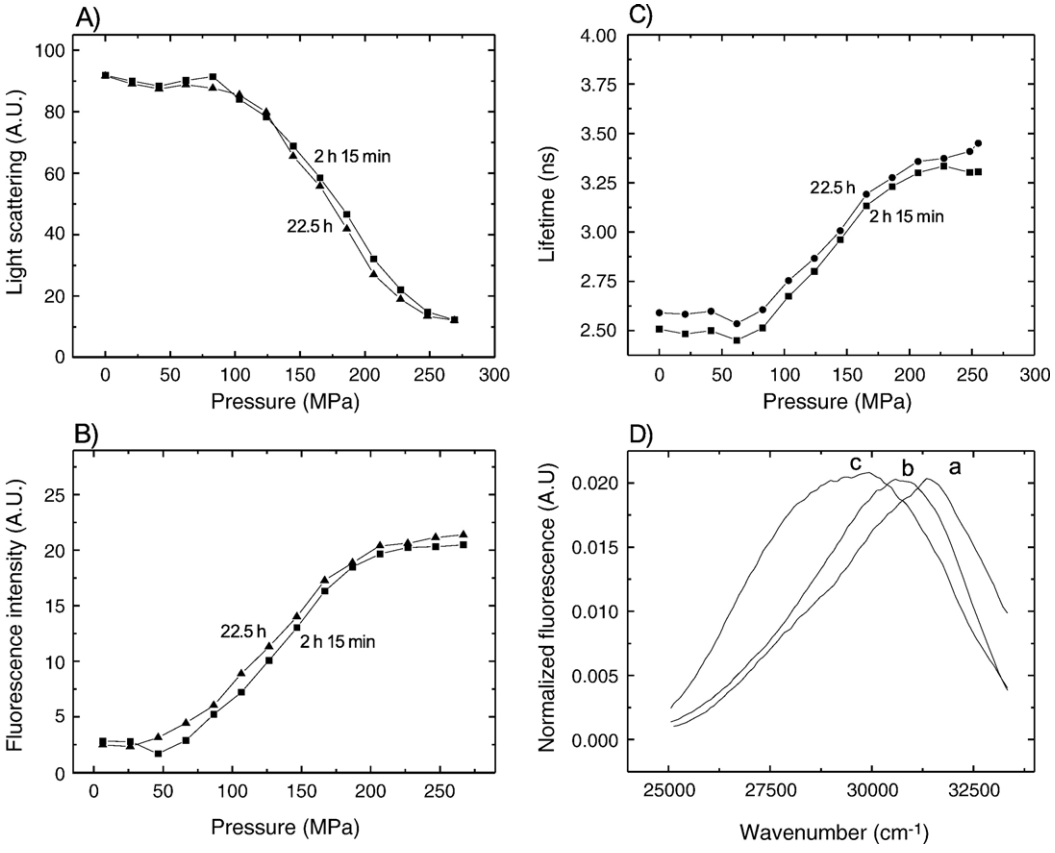

Fig. 3. Effect of pressure on the (A) light scattering intensities, (B) fluorescence emission spectrum intensities at pH 6.5, (C) lifetimes of erythrocruorin and (D) fluorescence emission spectrum. In (A), (B) and (C), the Hb (0.5 mg/mL) was maintained at 295 K. The total time for compression was 2 h and 15 min (squares and circles), and 22.5 h (upright triangles). (C) Spectrum at atmospheric pressure and pH 6.0 (a), 270 MPa and pH 9.0 (b) and in 4.0 M urea at atmospheric pressure and pH 7.5 (c). The spectra were normalized by the maximum intensity.

calculated parameters, and such "reversibility" has no relationship to and does not guarantee the microscopic reversibility, according with the basic principles of thermodynamics, particularly in the definition of entropy or Gibbs free energy [43].

To correlate the degree of dissociation with the respective light scattering intensities measured under each condition, Eq. (1) was applied as shown in the inset of Fig. 1A. The light scattering of the Hb solution at pH 6.0 and atmospheric pressure was considered as α = zero, whereas the light scattering at pH 9.0 and 270 MPa corresponded to $\alpha = 1$. Hence, at atmospheric pressure, an increase in pH enhanced the degree of protein dissociation so that at pH 9.0 almost 50% of the Hb was dissociated. The increased pressure caused Hb dissociation throughout the pH range studied. The dissociation at pH 6.0 at the highest pressure (270 MPa) was only 65%, indicating that protons caused significant protein stabilization. An increase in pH significantly facilitated the pressure dissociation such that at pH 9.0 total dissociation was reached at 100 MPa.

Plotting (K_p^{pH}) as a function of pressure (Eq. (9)) gave a linear relationship (data not shown) that allowed calculation of the free energy and volume change involved in dissociation, based on the intercept and slope (Fig. 2). The dissociation constants were considered as molar bases. Increasing the pH reduced the energy change needed for the dissociation of Hb from 37.7 kJ/mol of subunit at pH 6.0 to 27.5 kJ/mol of subunit at pH 9.0, which corresponded to 4.07 and 2.97 MJ/mol of Hb, respectively, assuming the number of dissociable subunits to be 108. The proton stoichiometry of the dissociation process was determined by Eq. (5) using the slope of the corresponding plot of ΔG *versus* pH (Fig. 2). The average number of protons released during dissociation (υ_D) was +0.602 mol of H^+/mol of subunit. The volume change of dissociation (Fig. 2) decreased (absolute value) from −48.0 mL at pH 6.0 to −19.2 mL at pH 9.0, suggesting that partial dissociation at atmospheric pressure caused a partial volume change, thereby decreasing the value observed with the application of pressure.

The effect of incubation time on the light scattering intensity during protein dissociation at pH 6.5 is shown in Fig. 3A. There were no significant differences in the dissociation curves obtained after incubation times of 2.25 h and 22.5 h over the range of pressures examined. This finding suggested that equilibrium was reached rapidly during pressure dissociation.

The fluorescence emission data (Fig. 3B) showed a pressure-induced increase in the quantum yield at pH 6.5 that probably reflected a decrease in the suppression of tryptophan by the heme groups during dissociation. Increasing the length of incubation to 22.5 h yielded results similar to the light scattering data, with little change in the pressure.

The lifetime measurements of the overall dynamics of the tryptophan residues at pH 6.5 showed a similar behavior (Fig. 3C). The increase in lifetime at elevated pressure indicated an overall decrease in the interaction of tryptophan residues with suppressors, and probably reflected a decrease in the proximity of heme as a consequence of protein dissociation. Again, increasing the incubation to 22.5 h did not significantly alter the data and suggested that equilibrium conditions had been reached in both curves.

The ability of Hb to reversibly transport oxygen at different conditions of pH and pressure with no significant formation of methemoglobin has been demonstrated [3,25]. To investigate the occurrence of denaturated protein states over the range of pH and pressure studied, we analyzed the fluorescence emission spectrum as shown in Fig. 3D. Significant denaturation leads to a marked red shift in the fluorescence emission spectrum [8]. As shown in Fig. 3D, the red shift of 554 cm^{-1} at extreme conditions of pH and pressure was compatible with the dissociation rather than with the denaturation of Hb [3]. Other conditions of pressure and pH yielded very similar and intermediate results (not shown). The presence of 4 M urea produced a very significant red shift (about 1850 cm^{-1}), indicating denaturation.

Gel filtration analysis by HPLC on a GPC300 column (Fig. 4) showed an increased reassociation at lower pH. Comparison of the results at pH 6.8 and 6.5 (Fig. 4A and B) revealed a higher concentration of the associated form of Hb at lower pH after

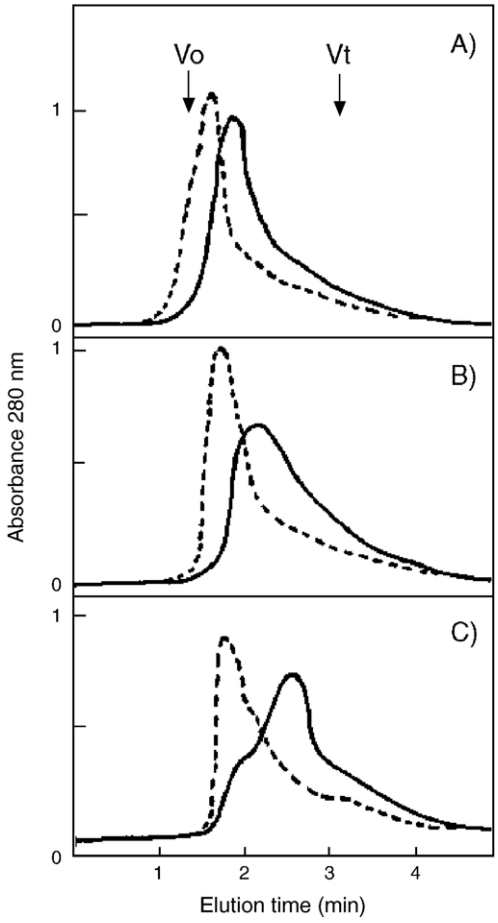

Fig. 4. Gel filtration HPLC analysis (GPC300 column) of Hb incubated at high pressure at different pH values. One hundred microliters of Hb (0.5 mg/mL) was injected and eluted at a rate flow of 0.3 mL/min. Dashed line: Hb at atmospheric pressure. Continuous line: Hb subjected to compression under the conditions described in Fig. 2. (A) pH 6.5 and (B) pH 6.8 in 50 mM Bis–Tris propane buffer containing 1 mM EDTA, and (C) pH 8.0 in 50 mM Tris–HCl buffer containing 1 mM EDTA. V_0: void volume, elution of TMV. V_t: final volume, elution of human albumin.

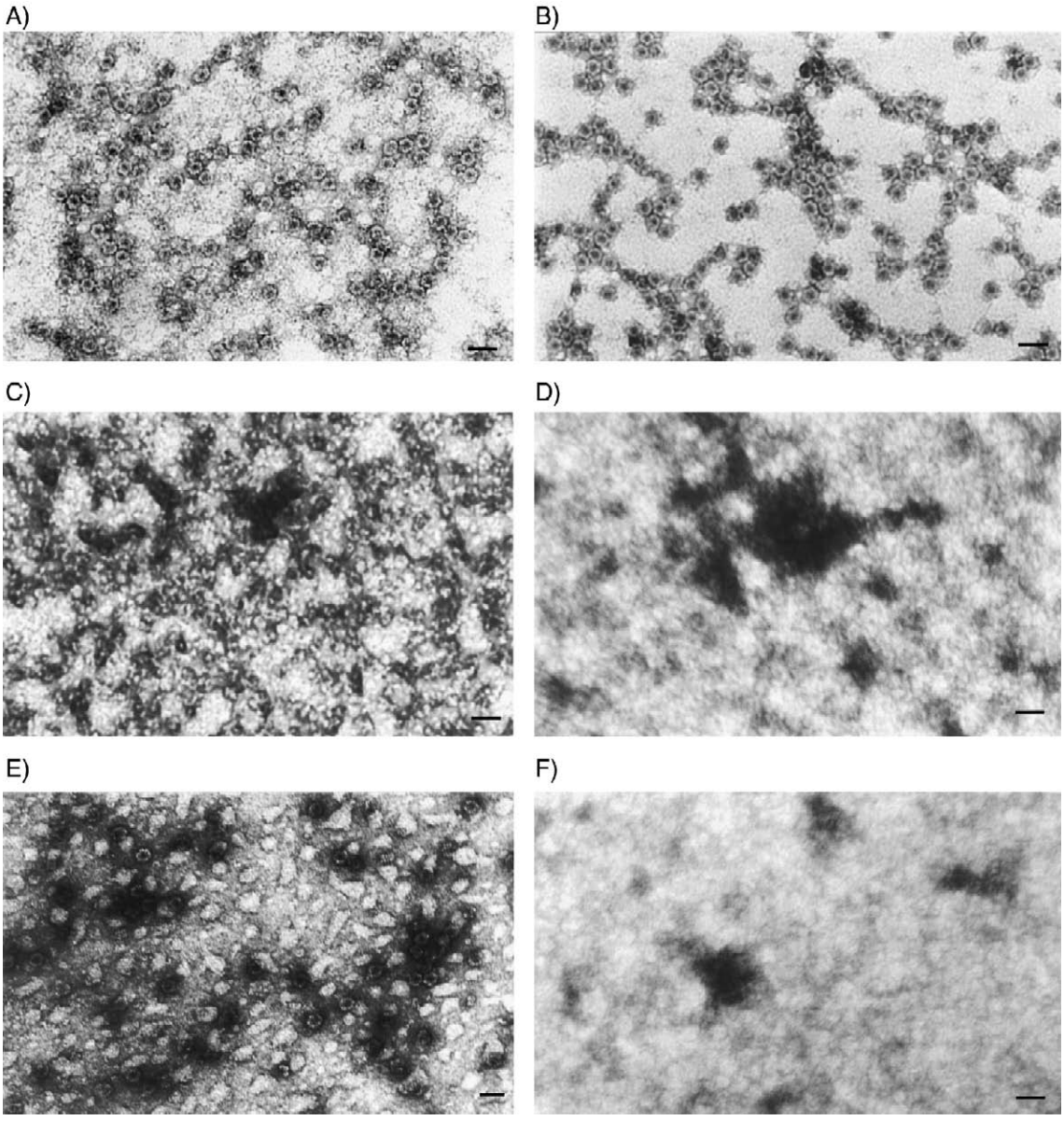

Fig. 5. Transmission electron microscopy of Hb incubated at different pH values and pressures. The Hb (0.5 mg/mL) was fixed at pH 6.5 (A, C and E) and pH 6.8 (B, D and F), at atmospheric pressure (A and B), at 270 MPa for 30 min (C and D) and after pressure incubation (E and F). Fixation was done in 0.5% glutaraldehyde solution. Negative staining was with 1% uranyl acetate. Bar = 50 nm.

compression. Under alkaline conditions at pH 8.0 (Fig. 4C), the gel filtration elution profile indicated that there was important dissociation after the pressure cycle and that reassociation depended significantly on the pH.

Electron microscopy provided additional information about the structural organization of extracellular Hb under the combinations of pH and high pressure studied here (Fig. 5). Associated Hb was observed at pH 6.5 and 6.8, with a lower number of particles at the latter pH (Fig. 5A and B). Pressure caused dissociation at pH 6.5 and 6.8 (Fig. 5C and D), with some images that suggested intermediate states of aggregation at pH 6.5. The complete pressure cycle led to significant reassociation at pH 6.5, but not at pH 6.8 (Fig. 5E and F), thus confirming the light scattering and gel filtration data, and the fast reassembly of Hb at a higher proton concentration.

4. Discussion

The results presented here provide further insights into the pH and pressure stability of a giant Hb. A careful quantitative analysis of this well-known effect of alkaline dissociation in this class of invertebrate Hbs is essential for understanding the dissociation process. Measurement of the light scattering intensity at distinct pH and pressure conditions made it possible to quantify the degree of dissociation (Fig. 1A) and to calculate several thermodynamic parameters of the Hb. Overall, increasing the pH reduced the protein–protein interactions and facilitated the dissociation by pressure. The free energy of stabilization per mole of subunit for a decrease in 3 pH units was 10.2 kJ. The stoichiometry of proton release was +0.602 proton/mol of dissociated subunit.

Pressure induces significant dissociation of Hb [3], with calcium or glycerol stabilizing the associated form of Hb [4]. The stabilization of Hb by calcium is maximal at at ~ 60 mM calcium, indicating a specific interaction with a free energy of stabilization of about 2.3 kJ/mol of subunit at pH 7.4, equivalent in effect to a decrease of 0.68 pH units, as shown here. In contrast, the effect of glycerol is proportional to its concentration, and 6 M glycerol corresponds to 5.2 kJ/mol of subunit, or a stabilization equivalent of about 1.5 pH units.

The addition of calcium to Hb dissociated by pressure leads to a significant recovery of the fully associated form over several weeks [4], a state reached here without calcium or glycerol, and with a greater efficiency at low pH (6.5). These findings confirm that the high degree of association and cooperativity seen in annelid Hbs is strongly dependent on the interplay of protons and divalent cations, as previously reported [44,45]. Moreover, the ferrous state of the heme sites in these Hbs, essential for oxygen transport, may be highly dependent on the associated form since, in the absence of divalent cations, an alkaline pH increases the susceptibility of Hb to oxidation [25].

The alkaline dissociation of TMV has also been extensively studied [46]. The pressure dissociation of this virus at different pH values is strongly H^+-dependent, as also observed here for annelid Hb, with a very similar proton release (0.584 mol/mol of dissociated subunit) and energy of stabilization (8.1 kJ/mol of dissociated subunit for a decrease of 3 pH units) [39].

The volume change of dissociation, which corresponds to the difference between the partial molar volume of the products and the reactants, was also affected by the surrounding pH, indicating that different structures were probably involved at the various pH values. Several factors contribute to the signal and magnitude of the volume change in a protein [47–49]. This parameter is related to the contributions of the intrinsic volume of all atoms, the void volume within the protein as a result of imperfect packing of the polypeptide chain, and the solvation volume resulting from the interaction of chemical groups with the solvent [50]. Other processes, such as the breaking of an ionic salt linkage, substrate dissociation, and the interaction of water molecules with the apolar domains also create a negative volume change, depending on the protein's susceptibility to compression [1,51–55]. In this context, the decrease in the absolute value of the volume change of dissociation from −48 mL to −19.2 mL/mol of subunit reflected the loss of protein interactions at high pH, with the occurrence of intermediate protein states that arose from the replacement of water molecules at the protein surface, including the interaction of a water molecule with apolar domains. Such behavior can produce populations of protein species in solution with distinct Gibbs free energies of dissociation, and can lead to the appearance of low affinity subunits that may represent intermediate states of protein aggregation. An increase in pH facilitates the process of dissociation and concomitantly increases the oxygen affinity by increasing the degree of exposure of the heme groups. Proteins and protons are thus linked to aggregation and oxygenation such that an increase in the proton concentration of the solution leads to protein association and a reduction in oxygen affinity that modifies the values of the Hill coefficient [56].

Recent work with TMV has also shown a decrease in the absolute values of the volume change of dissociation with increasing pH, from −50 mL/mol of subunit at pH 3.8 to −21 mL/mol of subunit at pH 9.0 [39]. This heterogeneous behavior could be attributed to the previously described intermediate forms of dissociated virus at alkaline pH [46]. The change in the Gibbs free energy of dissociation of TMV *versus* pH is biphasic, suggesting the existence of at least two viral species in solution [39]. Although the corresponding plot for the Hb studied here did not show a clear biphasic behavior (Fig. 2), the decrease in the values for ΔV with pH suggested that there were different species with different values for the volume change of dissociation. In any case, the different molecular populations seen over the pH range studied had a similar proton release (Fig. 2). The light scattering data provided the average size of the particles so that the thermodynamic parameters calculated were related to an average equilibrium value, as shown in Eq. (2).

The red shift in the fluorescence emission spectrum indicated that the dissociation process did not result in the formation of a significant amount of denaturated protein species in solution. This phenomenon has previously been observed for this Hb at pH 7.4, with the discrete red shift at high pressure being interpreted as the exposure of tryptophans at the protein–protein interface following dissociation [3]. The lifetime measurements at different pressures were done to understand the environment in which the intrinsic fluorophores of this protein were exposed.

Based on the results described here, we conclude that the high pH dependence of the aggregation and the fast recovery of the molecule promoted by pH were responsible for the reassociation seen within a few hours. This reassociation probably involved hydration of the dissociated form that remained after reassociation. In contrast to the many weeks required for calcium-induced reassembly [4], proton-mediated reassembly occurred within hours. These results suggest that protons have an important role in coordinating the normal assembly of this annelid Hb.

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