The oxygen-binding properties of hemocyanin from the mollusk *Concholepas concholepas*

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A B S T R A C T

Hemocyanins have highly conserved copper-containing active sites that bind oxygen. However, structural differences among the hemocyanins of various mollusks may affect their physicochemical properties. Here, we studied the oxygen-binding cooperativity and affinity of *Concholepas concholepas* hemocyanin (CCH) and its two isolated subunits over a wide range of temperatures and pH values. Considering the differences in the quaternary structures of CCH and keyhole limpet hemocyanin (KLH), we hypothesized that the heterodidecameric CCH has different oxygen-binding parameters than the homodidecameric KLH. A novel modification of the polarographic method was applied in which rat liver submitochondrial particles containing cytochrome *c* oxidase were introduced to totally deplete oxygen of the test solution using ascorbate as the electron donor. This method was both sensitive and reproducible. The results showed that CCH, like other hemocyanins, exhibits cooperativity, showing an inverse relationship between the oxygen-binding parameters and temperature. According to their Hill coefficients, KLH has greater cooperativity than CCH at physiological pH; however, CCH is less sensitive to pH changes than KLH. Appreciable differences in binding behavior were found between the CCH subunits: the cooperativity of CCH-A was not only almost double that of CCH-B, but it was also slightly superior to that of CCH, thus suggesting that the oxygen-binding domains of the CCH subunits are different in their primary structure. Collectively, these data suggest that CCH-A is the main oxygen-binding domain in CCH; CCH-B may play a more structural role, perhaps utilizing its surprising predisposition to form tubular polymers, unlike CCH-A, as demonstrated here using electron microscopy.

1. Introduction

Hemocyanins function as large, multi-subunit oxygen carriers and storage glycoproteins in addition to their roles in the innate immune response in certain species of mollusks and arthropods [1,2]. These proteins are not contained in blood cells but are instead found freely dissolved in the hemolymph. Hemocyanins are polar and have negatively charged surfaces, which improves their solubility and hydration and prevents them from associating with other surfaces of similar charge, such as the glycocalyx of epithelial cells [3]. Hemocyanins belong to the type-3 copper protein superfamily, which includes functionally and structurally diverse proteins that are characterized by the presence of a di-copper center, where each copper atom (Cu-A and Cu-

B) is coordinated by three highly conserved histidine residues. In the hemocyanins and tyrosinases, these copper atoms reversibly capture O_2 molecules, which bind as a peroxide ion. In the deoxygenated form of the protein, the copper is in the cuprous state (Cu I state); in the oxygenated form, the copper is oxidized to the cupric state (Cu II state). This change accounts for the distinctive deep blue color and the name of these proteins. Hemocyanins are functional analogues of the hemoglobin present in vertebrates and, similarly, exhibit cooperativity in their oxygen-binding properties and allosteric regulation to efficiently uptake and deliver oxygen under physiological conditions. Significant differences have been described between the structures of mollusk and arthropod hemocyanins, especially on the quaternary level [4].

Specifically, mollusk hemocyanins have a complex oligomeric

structure that possesses up to 160 oxygen-binding sites [1]. These hemocyanins have a hollow cylindrical structure known as decamer, which forms a cylinder of approximately 35 nm in diameter and 18 nm in height [5]. In gastropods, the decamers may associate in pairs to forming didecamers and even tridecamers. The decamer is composed of ten dissociable subunits with molecular masses ranging from 350 kDa to 450 kDa each. The subunits include 7 to 8 paralogous globular folded domains known as functional units (FUs), which are covalently bound by a short, flexible linker region of amino acid residues [4]. Each FU is composed of two domains, the α-domain consisting of a four α-helix bundle that houses the di-copper center and the β-domain incorporating a seven-stranded anti-parallel barrel [6]. The interactions between these FUs in mollusk hemocyanins result in relatively low cooperativity as expressed by their Hill coefficients. In fact, at physiological pH (7.2), hemocyanins of the cephalopods *Nautilus pompilius* and *Octopus dofleini* showed cooperativity values of 2 and 4, respectively. For the hemocyanins of the gastropods *Archachatina marginata* and *Helix pomatia*, the cooperativity was 1 and 7, respectively, with the latter being the highest Hill coefficient known for any mollusk hemocyanin [1]. In the case of *Megathura crenulata*, known as keyhole limpet hemocyanin (KLH), the reported Hill coefficient is 3, but this value was determined at pH 7.9 [7]. While mollusk hemocyanins have been studied extensively, detailed information concerning the oxygen-binding parameters is still limited.

The most commonly used method to investigate the oxygen binding by carrier proteins has been electrochemical and optical systems [8]. Also, numerous publications have employed polarography, where a Clark-type electrode sensor detects a current flow caused by the chemical reduction of oxygen [9]. Alternatively, spectrometry using the absorbance properties of the intrinsic tryptophan fluorescence as a sensor of oxygen binding has also been extensively used [10]. More recently, new methods using fluorescent dyes [11] and flash photolysis [12] have been developed.

Herein, we report our findings on the oxygen-binding properties of hemocyanin from *Concholepas concholepas* (CCH) and its two isolated subunits (CCH-A and CCH-B). *Concholepas* is a marine gastropod distributed along the west coast of South America between Callao in Perú and Cape Horn in Chile [13]. We implemented an improved polarographic method [14], by introducing rat liver submitochondrial particles containing cytochrome oxidase, the terminal enzyme of the mitochondrial respiratory chain [15], to deplete the test solutions of oxygen using ascorbate as the electron donor.

The structural properties of CCH have been previously reported. We demonstrated that CCH has a quaternary structure that is distinct from that of the well-characterized KLH [16,17]. In KLH, both subunits, called KLH-1 and KLH-2 [18] or KLH-A and KLH-B, respectively [19], have a mass of 400 kDa and assemble to form homodecamers (i.e., the decamers are composed of either KLH-1 or KLH-2 subunits), which further interact in pairs to create homodidecamers. By contrast, in CCH, the subunits, named CCH-A (405 kDa) and CCH-B (350 kDa), are intermingled in each macromolecule to form heterodecamers; consequently, their association in pairs results in heterodidecamers [16]. Thus, considering the differences in the arrangement of subunits between CCH and KLH, we hypothesized that the heterodidecameric CCH has a different degree of cooperativity and oxygen-binding affinity ($K_{0.5}$) than the homodidecameric KLH.

2. Materials and methods

2.1. Materials

Ascorbate, hydrochloric acid, sodium chloride, sodium hydroxide and TRIS were from Merck (Darmstadt, Germany). Potassium chloride, magnesium chloride, calcium chloride, potassium cyanide, glycine, EDTA, D-mannitol, HEPES, EGTA, bovine serum albumin (BSA), ammonium persulfate, TEMED, bromophenol blue and uranyl acetate were

from Sigma-Aldrich (St. Louis, MO, USA). Coomassie Plus protein assay reagent and Tween-20 were from Thermo Scientific (Waltham, MA, USA). Q-Sepharose was from Pharmacia (Uppsala, Sweden). Formvar and Parlodion were from Polysciences (Warrington, PA, USA). All chemicals were analytical grade; reagents and solutions were prepared with bidistilled water.

2.2. Hemocyanin sources

Concholepas concholepas hemocyanin suspended in phosphate-buffered saline (PBS; 0.1 M sodium phosphate, 0.15 M NaCl; pH 7.2) purified according to methods previously described [16], from live adult individuals of *Concholepas concholepas* that were harvested from the Pacific Ocean (Quintay Bay; 33° 8′ S 71° 48′ W; 5th Region of Chile), was provided by Biosonda Corp. (Santiago, Chile). Lyophilized KLH in PBS containing a proprietary stabilizer was purchased from Thermo Scientific and reconstituted with ultrapure deionized water, as recommended by the manufacturer. The CCH subunits were purified according to Becker et al., [20]. In brief, CCH was dissociated into individual subunits by dialysis against 5 volumes of 130 mM glycine-NaOH, pH 9.6, 10 mM EDTA. The dissociated CCH was loaded onto a Q-Sepharose column (10 × 0.8 cm) equilibrated with the dissociation buffer and eluted along a linear gradient from 0.3 to 0.6 M NaCl (5 column volumes). Fractions were analyzed by SDS-PAGE, pooled and concentrated by centrifugation in an ultrafree centrifugal device (Millipore, Billerica, MA, USA). Finally, CCH-A and CCH-B subunits were dialyzed against PBS and filtered through a 0.22 μm filter. Protein concentration was determined using the Bradford method [21]. The purity and homogeneity of each CCH subunit were determined using electron microscopy, various electrophoretic methods [20] and Western blot analysis using *anti*-CCH subunits monoclonal antibodies [22]. Apo-CCH was prepared according the procedure described by Lommerse et al., [23] with some modifications. Briefly, CCH was dialyzed at 4 °C for 3 h against a buffer containing 10 mM KCN, 10 mM CaCl₂ and 50 mM TRIS at pH 7. To extract the cyanide, a second dialysis against PBS was performed. The protein concentration was then determined according to the Bradford method.

2.3. Gel electrophoresis

The SDS-PAGE of hemocyanin samples was performed according to the procedure described by De Ioannes et al. [16]. The native electrophoresis of proteins was carried out as described by Becker et al. [20] using a horizontal gel chamber and 1.5% agarose gels (AGE) prepared with 70 mM TRIS and 261 mM boric acid pH 7.4 (running buffer). Protein samples were diluted in the same buffer containing 50% glycerol and bromophenol blue. Electrophoresis was run at 20 V for 4 h at room temperature. Proteins were visualized by Coomassie blue staining.

2.4. Submitochondrial particles

Mitochondria from rat liver (female Sprague-Dawley rats weighing 250–300 g) were isolated by fractional centrifugation according to Ferreira [14] and Pedersen et al., [24] with modifications described by Plaza et al., [25]. In brief, each mitochondrial pellet was washed three times with the homogenization medium (pH, 7.2) in the absence of albumin to eliminate the adsorption of hydrophobic compounds. Mitochondrial pellets were suspended to give a concentration of 40–50 mg protein/ml. Then, mitochondria were treated with digitonin to obtain mitoplasts by the method described by Pedersen et al., [24] and Ferreira [14] which were frozen and thawed three times to obtain submitochondrial particles and were stored at − 20 °C until the time of their use. Protein concentrations were determined by the Biuret reaction and standardized with albumin [26].

2.5. Electron microscopy

The general procedure used here has been described by De Ioannes et al., [16]. Briefly, 20 μl aliquots of samples (approximately 100 μg/ml) were applied for 1 min to Formvar or Parlodion (Polysciences)-coated copper grids previously stabilized by vacuum evaporation on a carbon coat. The samples were stained for 1 to 5 min with 20 μl of a 1% to 2% aqueous uranyl acetate solution previously filtered through a 0.22 μm filter. The grids were air dried at room temperature and imaged under a Philips Tecnai 12 BioTWIN transmission electron microscope (Eindhoven, The Netherlands) at the Unidad de Microscopia Avanzada, Pontificia Universidad Católica de Chile.

2.6. Determination of oxygen binding by polarography

We used a method modified from Ferreira [14]. The oxygen content of the reaction medium (PBS) was first reduced to near-anaerobic levels (approximately 4–5% oxygen) by bubbling with nitrogen, where a precise working temperature of 25 °C was set using a thermoregulated bath. The medium was then made fully anaerobic ($\leq 1\%$ oxygen) using 0.9 mg of the submitochondrial particles containing endogenous cytochrome c, and 0.01 mmol ascorbate as an electron donor. The O_2 evolution was measured with a Clark-type polarographic oxygen electrode (Yellow Spring Instrument, Yellow Spring, OH, USA) covered with a high sensitivity membrane (Yellow Spring Instrument, Yellow Spring, OH, USA) mounted within a thermoregulated 2 ml glass chamber (Gilson Medical Electronics, Inc., Middleton, WI, USA) and connected to an YSI monitor (Yellow Spring Instrument, Yellow Spring, OH, USA), linked to a variable voltage input (0.5 mV–50 V) Linseis double channel L 200 E recorder and at a chart speed of 20 mm/s. Then, to determine the sensitivity of the method, a calibration curve was developed with pulses of oxygen. The amount of oxygen in the absence of hemocyanin was determined by measuring the distance between the baseline and the maximum obtained after adding the O_2 pulse (see Fig. 1A, asterisk) in PBS. Instead, the amount of oxygen in the presence of hemocyanin was determined by measuring the distance between the baseline and the maximum obtained after adding the O_2 pulse in the presence of hemocyanin, where equilibrium was also reached after some seconds. The solubility of oxygen in the air-saturated test medium used was 226 nmol O_2/ml at 25 °C and 718 mm Hg, which is the atmospheric pressure in Santiago (543 mt over the sea level) [27].

2.7. Determination of the oxygen-binding of hemocyanins by polarography

For measurements with the hemocyanins (either CCH or KLH), the procedure described above to achieve anaerobic conditions was performed. In each case, the protein samples were thermoregulated in a bath to reach the desired temperature. At low temperatures (≤ 20 °C), 1.81 mg of cytochrome oxidase and 0.015 mmol ascorbate were added, while at 25 °C, only 0.9 mg of submitochondrial particles and 0.01 mmol of ascorbate were added. Control pulses were added without hemocyanin either in medium saturated with air or medium saturated with oxygen. Subsequently, the following amounts of hemocyanin were added: 2.36 mg of CCH and KLH, 2.32 mg of Apo-CCH, 0.74 mg of CCH-A and 0.61 mg of CCH-B. The sample was then allowed to return to anaerobic conditions at a chart speed of 2 mm/min, and then oxygen was added to construct the binding curve. These pulses were provided using either medium saturated with air or saturated with oxygen, which was also dependent on temperature: 2.3 μM of O_2 to 12.04 μM of O_2 in the medium saturated with air and 4.5 μM of O_2 to 280 μM of O_2 in the medium saturated with oxygen. The values were obtained by adjusting the solubility of oxygen to the working pressure, 718 mm Hg [27].

2.8. Determining the Hill coefficient and $K_{0.5}$

These parameters were obtained from the linearization of the sigmoid curve obtained from the plot of the saturation fraction against the oxygen concentration using the logarithmic expression of the Hill equation ($\log Y/(1-Y) = n \log[O_2] - n \log K_{0.5}$), where whether $Y = 0$, then the hemocyanin is completely unbound of oxygen, but if $Y = 1$, it is completely saturated. Then, Y is the saturation fraction of the oxygen transporter protein, n is the Hill coefficient and $K_{0.5}$ is equal to the oxygen concentration at the half-saturation of the protein [28]. By plotting $\log Y/(1-Y)$ versus $\log O_2$ (μM), known as a Hill plot, we obtained a straight line with slope n and $K_{0.5}$. We consider the nmols of O_2 added taking the solubility of oxygen in the air-saturated test medium as 239.5 nmol O_2/ml at 25 °C and 760 mm Hg. Therefore, it was corrected to 718 mm Hg. After plotting the nmol of oxygen bound/mg protein versus the concentration of O_2 added in the SigmaPlot program (Jandel Scientific Corp, San Rafael, CA, USA), the maximum saturation fraction was obtained empirically and set to 1.

2.9. Evaluation of the effect of temperature and pH

To evaluate the effect of temperature on oxygen binding by the hemocyanins, the polarograph was adjusted to different temperatures, from 10 to 25 °C, using the cooling/heating circulating thermoregulated bath Haake D8-G (Karlsruhe, Germany).

2.10. Evaluation of the effect of pH

To evaluate the effect of pH on oxygen binding by the hemocyanins, the phosphate buffers in which the measurements were performed were adjusted from pH 5.5 to 8.9 by mixing the acidic and basic components properly. The pH was stabilized when the temperature changed using the following adjustment procedure. The desired pH values of the two standard buffer solutions were accurately achieved at the actual measurement temperature, according to the instructions from the buffer manufacturer. Likewise, all other solutions were used at the same temperature of these solutions, which was attained by a 30 min incubation in a thermoregulated bath. Then, the corresponding settings of the pH meter were adjusted accordingly.

2.11. Statistical analysis

The results of each experiment were represented individually in plots. The experiments were conducted in triplicate, except for those using the subunits and Apo-CCH, which were conducted in duplicate. In the plots of inserts, for linearizing data, the correlation coefficients for the data sets r (correlation for regression analysis in a linear curve) and r2 (square of the coefficient of correlation) coefficients were calculated. Comparisons between the parameters were made using Student's t-test. Statistical significance was defined at a p-value of < 0.05. Analyses were performed using the SigmaPlot program (Systat Software Inc., San José, CA, USA).

3. Results

3.1. Polarography using submitochondrial particles showed that CCH and KLH have different degrees of cooperativity and oxygen-binding affinity

Prior to working with the hemocyanins, a calibration curve was performed to sensitively monitor oxygen binding. Because anaerobic conditions are required to make accurate measurements of oxygen using polarography prior to the oxygen pulses, the reaction mixture was first bubbled with nitrogen to displace the oxygen dissolved in the medium. This treatment generated nearly anaerobic conditions ($\leq 4\%$ oxygen). Subsequently, we added an extract of rat liver submitochondrial particles containing endogenous cytochrome c and ascorbate to achieve fully anaerobic conditions for the measurements. Then, fixed concentrations of oxygen were added in the absence and in the presence of hemocyanin. The quantity of oxygen added in the
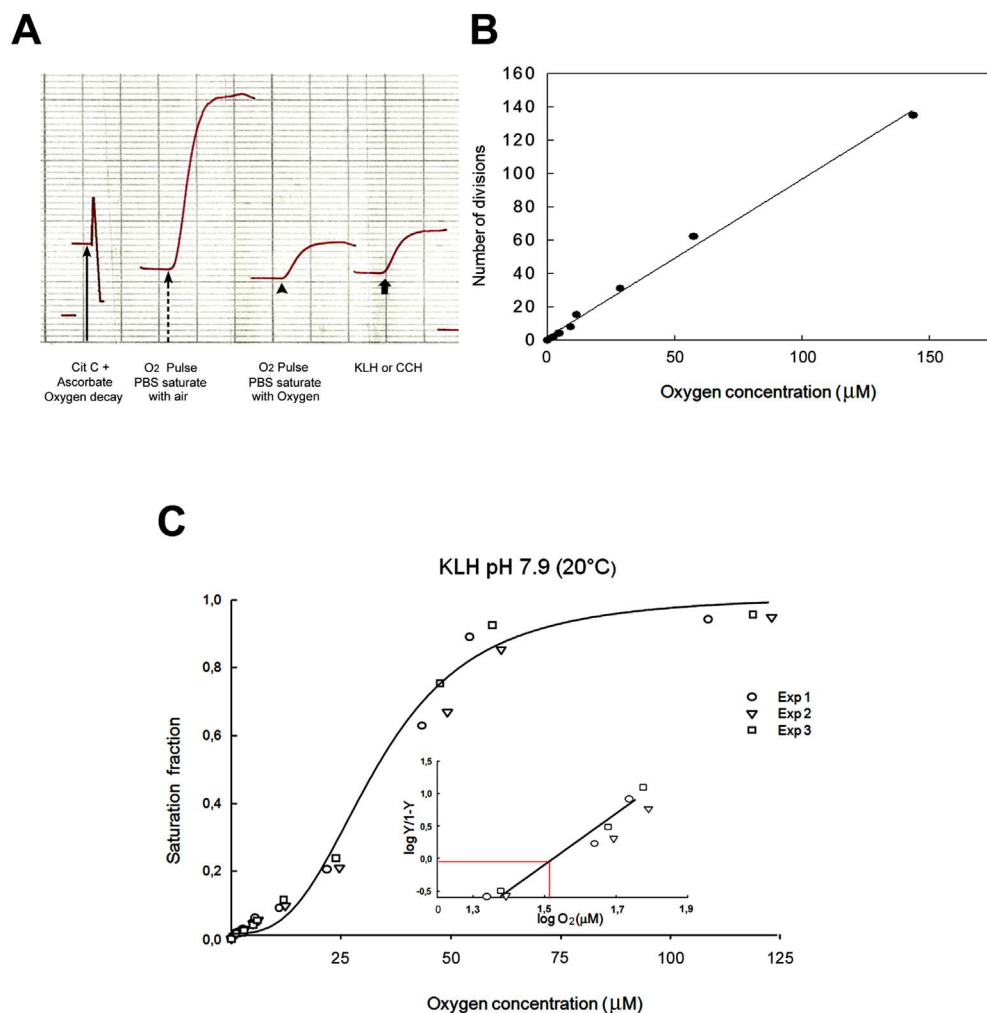
Fig. 1. Calibration of the polarographic method using submitochondrial particles. (A) Original record. The figure shows the typical polarograph register in which oxygen pulses are shown. In this case, oxygen decay took 30 min from the moment 50 μl submitochondrial particles and 15 μl of ascorbate were added to PBS at 4% of oxygen (arrow). The record was taken at 2 mm/min in the polarograph. Oxygen pulses were given with PBS saturated with air (dotted arrow) or oxygen (arrow head), and took 2–3 s to reach the maximum deflection and record was taken at 20 mm/min. After those control pulses were given, 50 μl of hemocyanin was added prior adding increasing pulses of oxygen (arrow). (B) Calibration curve to determine the oxygen concentration. The reaction was conducted at 25 °C in a thermoregulated water bath. Prior to the oxygen pulse, PBS (0.15 M NaCl, 0.1 M sodium phosphate, pH 7.2) was bubbled with nitrogen to reach nearly anaerobic conditions ($\leq$ 4% oxygen) and then 0.9 mg of an extract of rat submitochondrial particles and 0.01 mM ascorbate was added. The oxygen pulses were administered in a range between 0 and 570 nmol. The plot of number of divisions versus oxygen concentration showed a linear dose-response relationship from 1.13 μM O_2 ($r = 0.998$). This graphic shows a representative curve of several experiments. (C) Oxygen-binding properties of KLH. These experiments were carried out at 20 °C in 0.1 M TRIS-HCl and 10 mM CaCl at pH 7.9. The sigmoid curve obtained in the plot of saturation fraction versus oxygen concentration shows cooperative oxygen binding by KLH. The data are representative of three independent experiments ($\bigcirc$ Exp 1, ∇ Exp 2 and $\square$ Exp 3; $r = 0.993$). The inset shows the Hill plot with the linearization of the curve from which the Hill coefficient and $K_{0.5}$ (highlighted in red line) were obtained ($r = 0.967$).

absence of hemocyanin was determined by measuring the distance between the baseline and the maximum deflection reached by the outline recorded up to attain a constant slope (chart speed of 20 mm/s). An original record of the polarograph register showing the sequence of steps up to the oxygen pulse is shown in Fig. 1. By plotting the concentration of O_2 versus the number of recorded pulses, we obtained a linear dose-response curve from 1.15 μM O_2 added, a representative graph to shown this effect is presented in Fig. 1B. These results demonstrated that our method was suitable to detect free oxygen in solution with an adequate sensitivity and reproducibility.
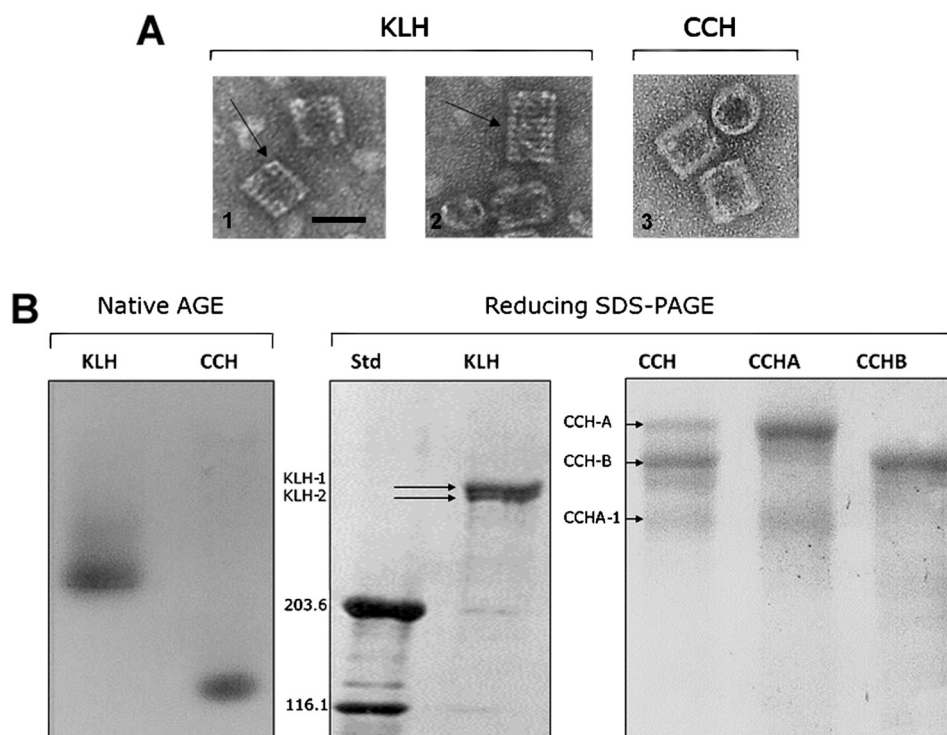
Furthermore, to verify the oxygen-binding parameter values obtained using the polarographic method, we used native KLH because its parameters have been well characterized using spectrophotometry by Senozan et al., [7]. The reported binding parameters at 20 °C and pH 7.9 were as follows: a Hill coefficient of 3 and a P_{50} of 2.4 mm Hg ($K_{0.5}$ of 3.36 μM O_2, where 5 mm Hg = 14 μM O). Under similar conditions, we obtained the results shown in Fig. 1C; a sigmoid curve was obtained, i.e., the oxygen affinity of the KLH was lower at low levels of saturation with oxygen than at high levels of saturation. This shape is indicative of cooperative binding of oxygen with the following parameters: a Hill coefficient of 3.5 and $K_{0.5}$ of 34.1 μM O_2 (P_{50} of 24.36 mm Hg).

In addition, we performed electron microscopy and an electrophoretic analysis of the KLH sample. KLH appeared as mostly didecameric in the electron microscope negatively stained micrographs (Fig. 2-A1), and in a few instances, multimers were observed (Fig. 2-A2). Although the native agarose gel showed a single band (Fig. 2B, left), the sample contained both KLH-1 and KLH-2, as shown in the

electrophoretic results (Fig. 2B, right). Usually, CCH appears as a didecameric species (Fig. 2-A3). The native agarose gel showed a single band, and in denaturing conditions, CCH-A and CCH-B subunits were observed (Fig. 2B, right).

Next, we studied the oxygen-binding parameters of *Concholepas* hemocyanins at different temperatures (Fig. 3). We obtained a Hill coefficient of 1.91 and $K_{0.5}$ of 11.83 μM of O_2 at pH 7.2 and 25 °C (Fig. 3A); these values are significantly different from those of KLH under similar experimental conditions (a Hill coefficient of 2.64 and $K_{0.5}$ of 23.47 μM of O_2, pH 7.4; $p < 0.001$ and $p < 0.01$, respectively, using Student's t-test). In addition, CCH at 10 °C was found to have higher cooperativity than at 25 °C (Fig. 3D and A, respectively), as shown by differences in the Hill coefficient and $K_{0.5}$ (2.96 and 36.07 μM of O_2 at 10 °C and 1.91 and 11.83 μM of O_2 at 25 °C, respectively). Apo-CCH was used as a negative control, and as expected, no oxygen binding was observed (Fig. 3E). Table 1 summarizes these results, showing that with increasing temperature, the Hill coefficient diminished. Additionally, at low temperatures, the oxygen affinity was lower (higher $K_{0.5}$).

Collectively, these results demonstrated that polarography using submitochondrial particles provides a controlled and reproducible method to determine oxygen-binding parameters. CCH, like other hemocyanins, exhibits cooperative behavior; however, KLH exhibits greater cooperativity than CCH in the physiological pH range, as is evident from their Hill coefficients, summarized in Table 2.

3.2. Oxygen-binding parameters of KLH and CCH are modulated by the pH

Because pH is a known modulator of oxygen binding by transport proteins, we investigated its effect on KLH and CCH in the pH range of 5.5 to 8.9 at 25 °C. At pH 5.5, 6.5, 7.4, 7.9, 8 and 8.9, the curves obtained for KLH exhibited different degrees of sigmoidicity in a graph of the saturation fraction versus the concentration of atomic oxygen (Fig. 4A, B, C, D, E and F, respectively). However, this sigmoidicity was lost at pH 8.9, in which the curve was hyperbolic (Fig. 4F). The maximum Hill coefficients of KLH were obtained at pH 7.9 (3.28) with a $K_{0.5}$ of 24.66 μM O_2 (Table 2). Regarding CCH, curves with different degrees of sigmoidicity were also obtained (Fig. 5). The data, summarized in the Table 3, showed that in response to low pH values (5.5) and high pH values (8), the Hill coefficient diminished (1.61 and 1.60, respectively) compared to the 1.91 obtained at pH 7.2.

In summary, these data reveal that KLH is more sensitive to low pH than CCH, which was more stable. Moreover, at high pH (7.9–8) KLH was more cooperative than CCH, a result that is consistent with the fact that the pH of fresh hemolymph obtained from *Concholepas concholepas* specimens is 7.2.

3.3. The subunits of CCH bind oxygen differently, and these differences are temperature dependent

Our previous studies demonstrated that isolated CCH subunits have profound structural differences, which are also reflected in the immune response that they induce in murine models [16,20]. For this reason, we evaluated the individual contribution of CCH-A and CCH-B to the oxygen-binding parameters at different temperatures. The first remarkable result was that the cooperativity of CCH-A was not only almost double that of CCH-B, but it was also slightly superior to that of CCH. In fact, CCH-A in a range of temperatures from 10 to 25 °C had Hill coefficients of 3.24 and 2.21 (Table 4), respectively; in contrast, CCH-B at the same conditions had Hill coefficients of 1.68 and 1.25, respectively (Table 4), and the values for CCH were 2.96 and 1.91, respectively (Table 1).

Cooperativity in CCH-A was higher at 10 °C, while for CCH-B,

cooperativity was higher at 15 °C (Fig. 6G and F, respectively), with a Hill coefficient of 3.24 and 1.74 and a $K_{0.5}$ of 31.46 μM O_2 and 23.52 μM O_2, respectively. At 25 °C, a Hill coefficient and $K_{0.5}$ of 2.21 and 24.1 μM O_2 for CCH-A and 1.68 and 21.43 μM O_2 for CCH-B, respectively, were obtained (Fig. 6A and B, respectively). As a negative control, Apo-CCH was used, and no oxygen binding was detected (data not shown).

In addition, the analysis of negatively stained preparations of previously isolated subunits showed that CCH-B subunits polymerized in the time to form tubular polymers, while CCH-A did not, as was the case for native CCH. Indeed, CCH molecules did not form aggregates and instead always existed as monomers (Fig. 7A), which is in contrast with the other hemocyanins, such as KLH, that can form multimers [17]. The tubules of CCH-B appeared hollow (Fig. 7B) with a variable length and a diameter that was smaller than the didecamers; they also had a clear zig-zag pattern (Fig. 7C–E). CCH-A in contrast adopted irregular forms (Fig. 7F).

Taken together, these results are consistent with our previous studies demonstrating the different properties of the CCH subunits. Here, the cooperativity of the CCH-A subunit is more pronounced than that of CCH-B and the native protein. Polymer formation similar to that observed by the isolated CCH-B subunit has been reported for KLH-B subunit, which can form elongated multimeric structures [19]. In addition, in the case of *Helix pomatia* hemocyanin, when the collar region is removed by mild trypsinolysis, multi-FU fragments are produced that spontaneously form hollow cylindrical tubes [29].

4. Discussion

In this study, the oxygen-binding properties of *Concholepas* hemocyanin and its two isolated subunits were investigated using sub-mitochondrial particles containing endogenous cytochrome *c* and polarography. The dependence of the oxygen-binding properties on temperature and pH was also monitored, as these are important factors and modulators of the cooperativity and binding affinity of oxygen-carrier proteins. We contrast some of our experimental results with the hemocyanin from *Megathura crenulata* because its oxygen-binding
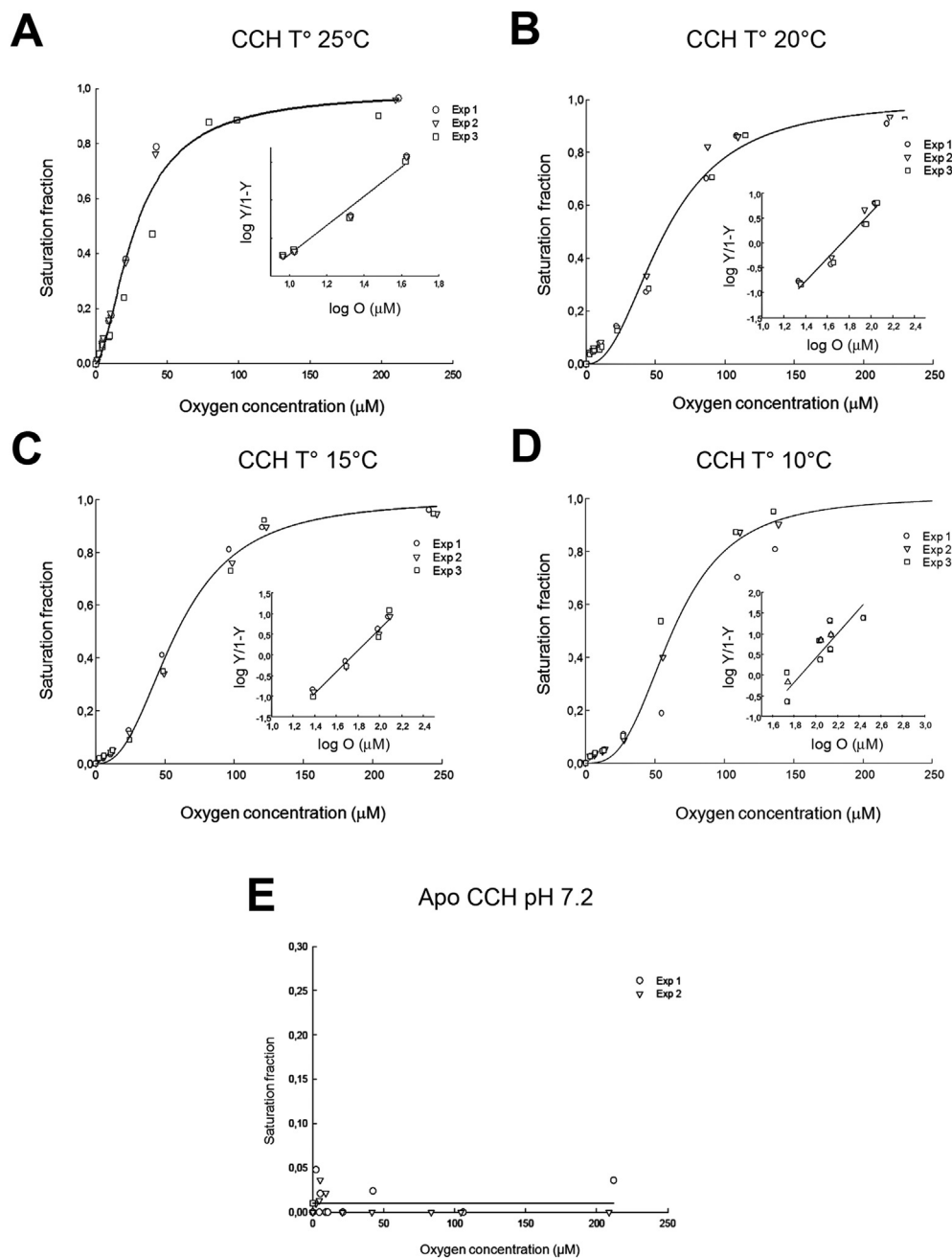
Fig. 3. Influence of temperature on the oxygen-binding parameters of CCH. The curves in each plot show a representative result of three independent experiments (○ Exp 1, ∇ Exp 2 and □ Exp 3) carried out in PBS (0.15 M NaCl, 0.1 M sodium phosphate at pH 7.2) and temperatures of (A) 25 °C, (B) 20 °C, (C) 15 °C, and (D) 10 °C. The plots show the temperature dependence of oxygen binding by CCH as the curve transitions from hyperbolic (A) to sigmoid (B, C and D). In the insets, the linearization of the curve is shown, from which the Hill coefficients and $K_{0.5}$ were determined. (E) The negative control with Apo-CCH. No oxygen binding was observed at different oxygen concentrations. The values of the Hill coefficients and $K_{0.5}$, as well r and r2 correlation coefficients, are summarized in Table 1.

Table 1
Oxygen binding parameters of CCH evaluated at pH 7.2 and at different temperatures.

Temperature	Hill coefficient	$K_{0.5}$ (μM O_2)	r	r2	P50 (mm Hg)
10 °C	2.96	36.07	0.990	0.886	8.59
15 °C	2.61	28.04	0.999	0.999	19.33
20 °C	2.30	27.15	0.991	0.979	20.35
25 °C	1.91	11.83	0.989	0.988	25.76

Note: Data were obtained from the curves of graphs contained in Fig. 2. The correlation coefficients for the data sets are r (correlation for regression analysis in a linear curve) and r2 (square of the coefficient of correlation).

Table 2
Oxygen binding parameters to KLH evaluated at 25 °C and at different pH.

pH	Hill coefficient	$K_{0.5}$ (μM O_2)	r	r2	P_{50} (mm Hg)
5.5	1.44	31.14	0.988	0.962	22.24
6.5	1.61	21.67	0.985	0.881	15.50
7.4	2.64	23.47	0.995	0.986	16.77
7.9	3.29	24.66	0.980	0.902	17.68
8	2.50	20.73	0.982	0.934	14.80
8.9	1.10	11.92	0.992	0.947	8.52

Note: Data were obtained from the curves of graphs contained in Fig. 3. The correlation coefficients for the data sets are r (correlation for regression analysis in a linear curve) and r2 (square of the coefficient of correlation).

parameters have been previously determined using spectrophotometry. In addition, CCH and KLH were chosen for study because they are used in biomedicine owing to their ability to potently activate the immune system of mammals; thus, they are used in the development of
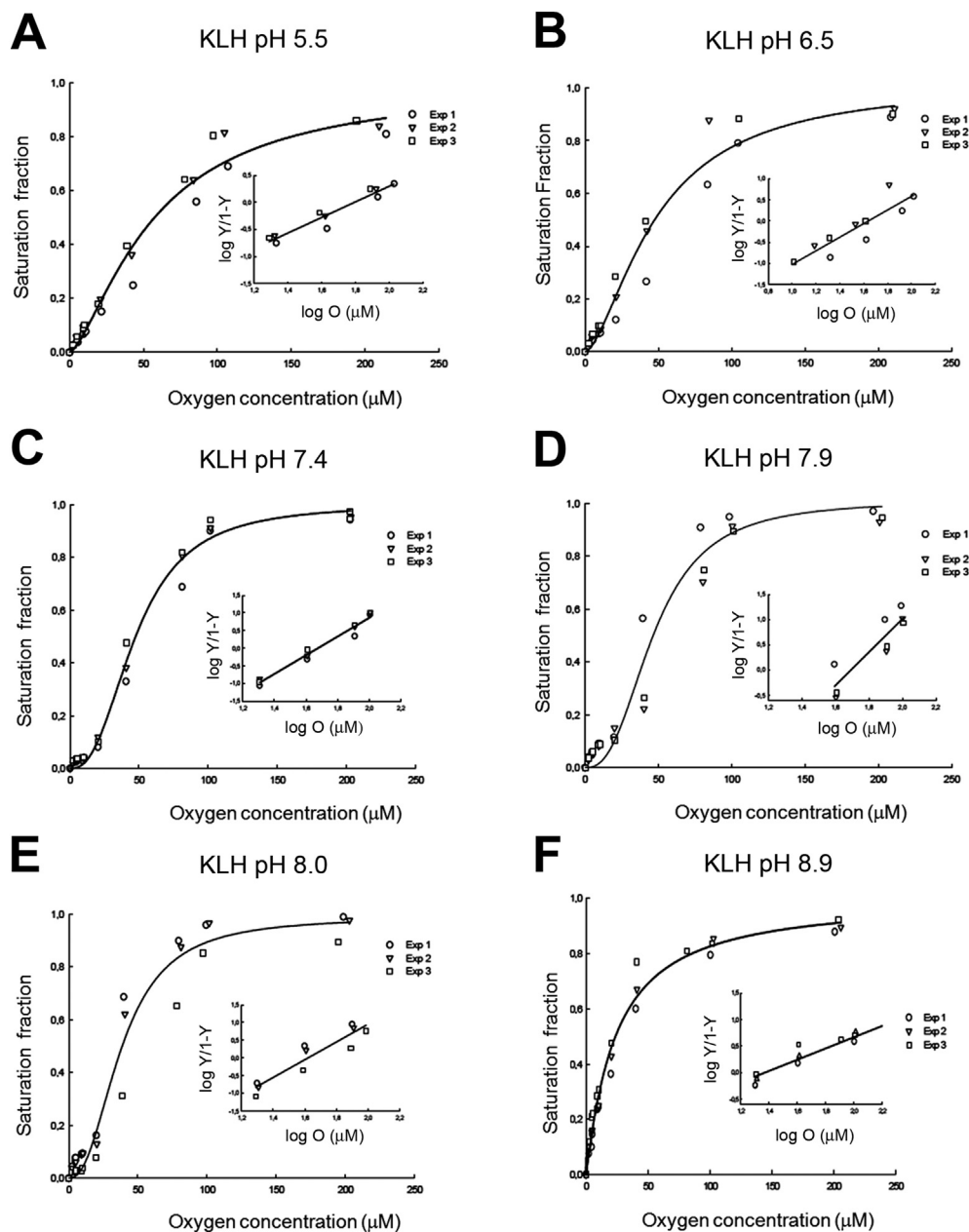
antibodies and vaccines and as immunomodulators in some cancers [17,30–34].

From the methodological point of view, one of the challenges with

Fig. 4. Influence of pH on the oxygen-binding parameters of KLH. The curves in each plot show representative results of three independent experiments (○ Exp 1, ∇ Exp 2 and □ Exp 3) carried out in PBS at temperatures of 25 °C, with the pH adjusted using 0.1 M HCl to (A) pH 5.5 and (B) pH 6.5 or using 0.1 M NaOH to (C) pH 7.4, (D) pH 7.9, (E) pH 8.0 and (F) 8.9. The plots show the pH dependence of oxygen binding by KLH, as the curves have different degrees of sigmoidicity between pH 5.5 and 8, while a hyperbolic curve is observed at pH 8.9. In the insets, the linearization of the curves is shown, from which the Hill coefficients and $K_{0.5}$ were determined. The values of the Hill coefficients and $K_{0.5}$, as well r and $r2$ correlation coefficients, are summarized in Table 2.

determining the oxygen-binding properties of a carrier protein is to obtain anaerobic conditions required to make accurate measurements. We circumvented this problem by introducing the submitochondrial particles containing cytochrome *c* oxidase, a transmembrane protein of the inner mitochondrial membrane. This protein is a part of complex IV of the electron transport chain, which reduces oxygen to water in the presence of a reducing agent [35], here, ascorbate through the redox reaction with cytochrome *c*. This allows a slow decrease of O_2 in the medium, reaching anaerobiosis, which is important for the measurement of the oxygen pulses, one of the reasons we use only ascorbate as substrate and not ascorbate plus TMPD. Cytochrome c oxidase is stable in the range of temperatures (10–25 °C) and pH values (5.48–8.9) used in the experiments reported here [36]. In addition, this enzyme possesses a Km value for oxygen of 1 μM [37] which is lower than the $K_{0.5}$ values of KLH and CCH. As a result, the enzyme is saturated with oxygen under conditions where the hemocyanins bind oxygen. Furthermore, the quantity added of submitochondrial particles without add cytochrome *c* extra was at least three times less than the quantity of hemocyanin (or subunit) added to the polarograph. For this reason, it

does not interfere with the measurements.

Because KLH subunits are homodecamers [18], the results obtained could be the superimposition of the oxygen binding curves of the two isoforms, KLH-1 and KLH-2, with very similar electrophoretic migration properties, as evidenced by native gel electrophoresis (Fig. 2B). Despite the presence of the two isoforms of KLH, we think that both isoforms exhibit very similar kinetic behavior. If the isoforms are different in cooperativity, they would show an abnormal sigmoidal shape, particularly at the beginning of the curve, which was not observed. In the work of Senozan et al., [7] who described the oxygen binding parameters for KLH using spectrophotometry, a KLH preparation with similar characteristics was used; i.e., the characterization of KLH by electrophoresis also revealed the presence of two bands corresponding to the two subunits of this protein. However, for CCH, this cannot be attributed since both subunits form a heterodidecameric structure and are not isoforms [16]. Thus, the CCH oxygen-binding curves should not correspond to a superimposition of curves from multiple species.

The data obtained here confirm our hypothesis that there are differences in oxygen-binding parameters between CCH and KLH. CCH
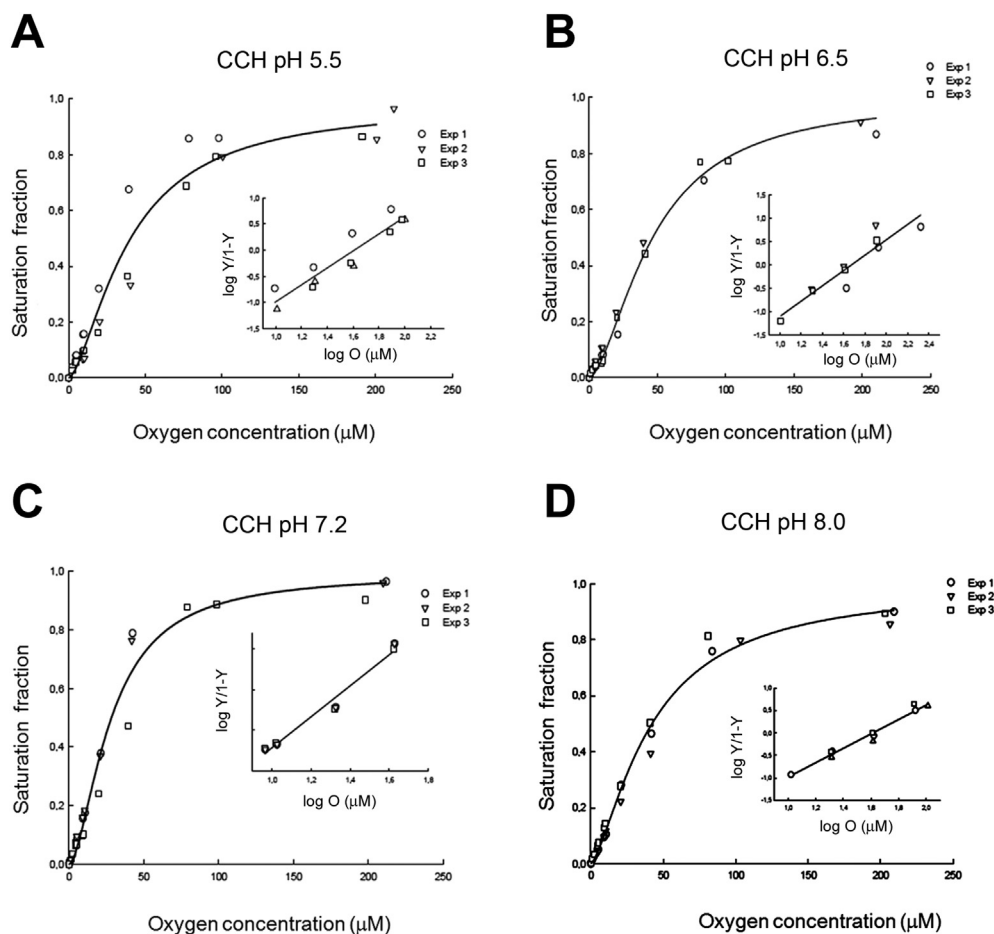
Fig. 5. Influence of pH on the oxygen-binding parameters of CCH. The curves in each plot show representative results of three independent experiments (○ Exp 1, ▽ Exp 2 and □ Exp 3) carried out in PBS at temperatures of 25 °C, with the pH adjusted using 0.1 M HCl to (A) pH 5.5 and (B) pH 6.5 or using 0.1 M NaOH to (C) pH 7.2 and (D) pH 8.0. The plots show the pH dependence of oxygen binding by CCH, as the curves have different degrees of sigmoidicity between pH 5.5 and 8. In the insets, the linearization of the curves is shown, from which the Hill coefficients and $K_{0.5}$ were determined. The values of the Hill coefficients and $K_{0.5}$, as well r and r2 correlation coefficients, are summarized in Table 3.

Table 3
Oxygen binding parameters to CCH evaluated at 25 °C and at different pH.

pH	Hill coefficient	$K_{0.5}$ (μM O_2)	r	r2	P_{50} (mm Hg)
5.5	1.61	22.17	0.978	0.934	15.07
6.5	1.86	22.39	0.983	0.998	14.65
7.2	1.91	12.01	0.989	0.988	8.58
8	1.60	21.86	0.995	0.983	14.73

Note: Data were obtained from the curves of graphs contained in Fig. 4. The correlation coefficients for the data sets are r (correlation for regression analysis in a linear curve) and r2 (square of the coefficient of correlation).

Table 4
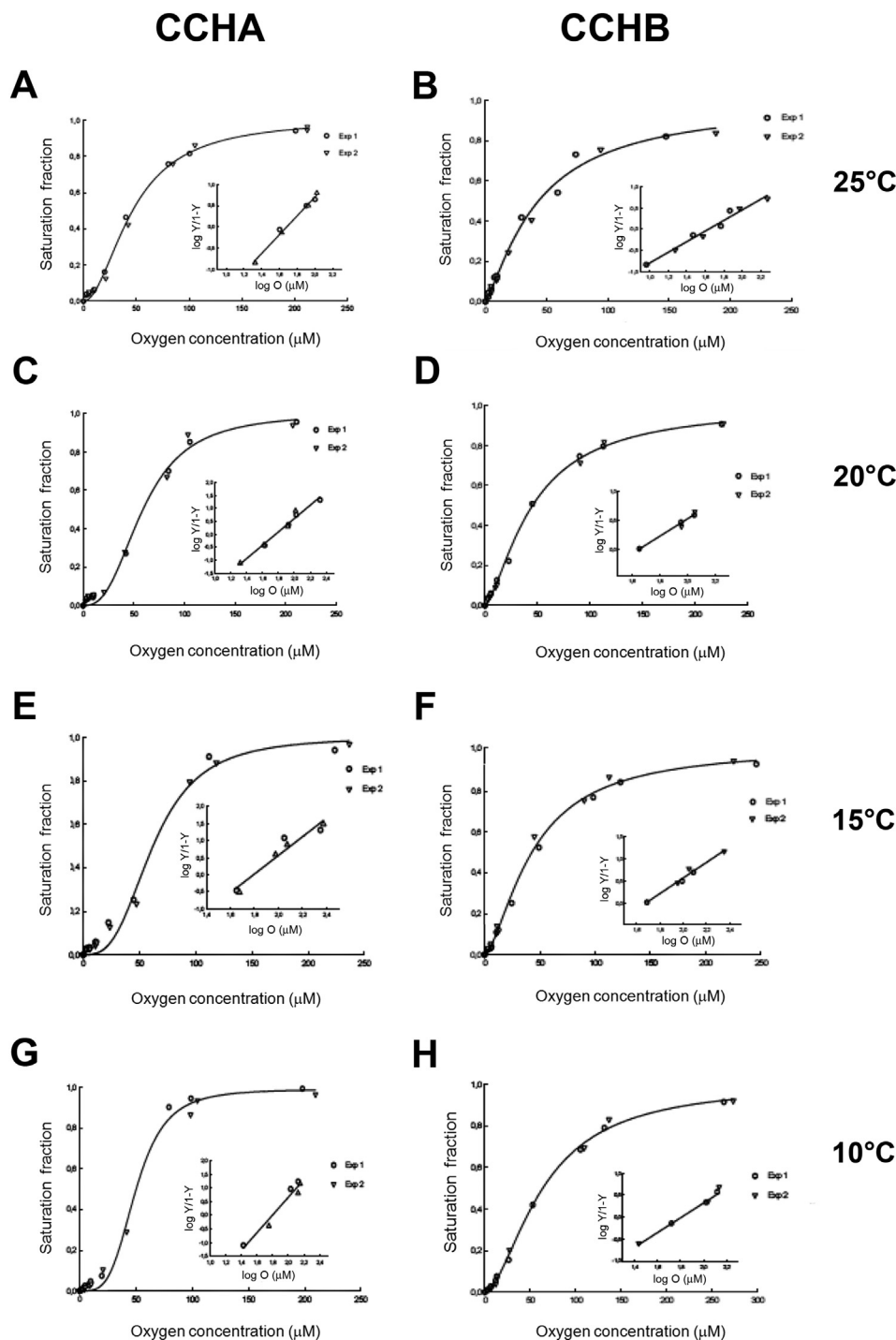Oxygen binding parameters of CCH subunits evaluated at pH 7.2 and at different temperatures.

Temperature	Subunit	Hill coefficient	$K_{0.5}$ (μM O_2)	r	r2
10 °C	CCHA	3.24	31.46	0.997	0.978
	CCHB	1.68	31.81	0.999	0.987
15 °C	CCHA	2.73	31.04	0.992	0.966
	CCHB	1.74	23.52	0.998	0.986
20 °C	CCHA	2.58	28.95	0.994	0.988
	CCHB	1.49	22.39	0.999	0.991
25 °C	CCHA	2.21	24.1	0.994	0.997
	CCHB	1.25	21.43	0.996	0.985

Note: Data were obtained from the curves of graphs contained in Fig. 5. The correlation coefficients for the data sets are r (correlation for regression analysis in a linear curve) and r2 (square of the coefficient of correlation).

exhibits cooperativity and has a greater affinity for oxygen than KLH. However, CCH has a lower Hill coefficient than KLH (1.91 and 2.64, pH 7.2 to 7.4, respectively), indicating that the heterodidecameric structure results in less cooperativity, probably owing to the two structurally and functionally different subunits (in an approximately 1:1 proportion as shown the chromatographic profiles) integrated into a single macromolecule [20]. In fact, this condition required tuning the delicate balance of conformational transfer of changes from one FU to another FU when the oxygen binds the copper in each subunit within the molecule. Note that, when we evaluate CCH-A and CCH-B subunits separately there are not quaternary interactions and that the whole is not equivalent to the sum of the parts. When compared the theoretical values, native CCH has higher Hills coefficient than the theoretical value, and remained between the values obtained for the separate subunits (Supplementary Fig. 1A). Thus, if we simulated the curves and sum, the Hill coefficient decreases markedly: CCH-A + CCH-B = 2.40 (theoretical) and CCH 3.24 (experimental). Oxygen affinity remained similar between 15 and 20 °C for CCH when compared to the theoretical values, and the differences observed at 10 and 25 °C are not so pronounced as the ones obtained for the Hill's coefficient (Supplementary Fig. 1B). Thus, affinities CCH-A + CCB $K_{0.5}$ = 31 μM O_2 (theoretical) and CCH 36 μM O_2 (experimental) at pH 7.2 and 20 °C. In contrast to KLH, the oxygen-binding parameters are not the result of interactions between the KLH-A and KLH-B subunits, as they form homodidecamers. Instead, they arise from the simple mixing of two independent isoforms of different oxygen affinities, thus representing an average result for two distinct proteins, as demonstrated by Swerdlow et al., [17].

With respect to the correlation of our results with data obtained for KLH by Senozan et al., [7], we found that the Hill coefficient is in good agreement, at 3.5 and 3, respectively (14.3% difference). However, the

Fig. 6. Influence of temperature on the oxygen-binding parameters of CCH-A and CCH-B subunits. The curves in each plot show a representative result of three independent experiments (○ Exp 1, ▽ Exp 2 and □ Exp 3) carried out in PBS (0.15 M NaCl, 0.1 M sodium phosphate at pH 7.2) and at temperatures of (A) 25 °C, (B) 20 °C (C), 15 °C and (D) 10 °C for each subunit. The plots show that the temperature dependence is affected differently between the two subunits, as CCH-A is more sigmoidal and CCH-B is more hyperbolic. In the insets, the linearization of the curve is shown, from which the Hill coefficients and $K_{0.5}$ were determined. The values of the Hill coefficients and $K_{0.5}$, as well r and r2 correlation coefficients, are summarized in the Table 4.

$K_{0.5}$ differs significantly, comparing 34.1 μM O_2 and 3.36 μM O_2, respectively (90.15% difference). One possible explanation is the experimental conditions of the spectrophotometric method, which does not approach the anaerobic conditions of our study; thus, the measurements of oxygen binding begin from approximately 30% of oxygen in the reaction medium up to 100%. This limitation translated into an underestimation of the value of $K_{0.5}$. In contrast, in the polarographic method used here, the conditions are practically fully anaerobic, which means the curve is obtained with a range of measurements between 0 and 100% oxygen. Furthermore, according to Senozan et al., [7], the affinities found for KLH at 20 °C are almost the same as those found at pH 8.9 with EDTA and without EDTA at pH 7.9. This result is questionable, as the protein in dissociating conditions is expected to have a

lower affinity because its quaternary structure is altered. For example, studies in *Helix pomatia* have demonstrated that interactions between the native subunits are essential for cooperative oxygen binding [38].

Other parameters such as pH were evaluated, demonstrating that both high and low pH alter the oxygen-binding parameters. These changes were most clear in the low Hill coefficient value, which was expected. When the outside medium is at a low pH, oxygen will bind to H^+ to form H_2O, and the kinetics of that reaction would be more energetically favorable than binding of oxygen to CCH. However, high pH values also diminished the affinity of CCH for oxygen. This result can be explained, at least in part, if we consider that CCH is not resistant to high pH the environment. Indeed, we have observed that CCH dissociates at pH values above 7.5. At low pH values, KLH and CCH were
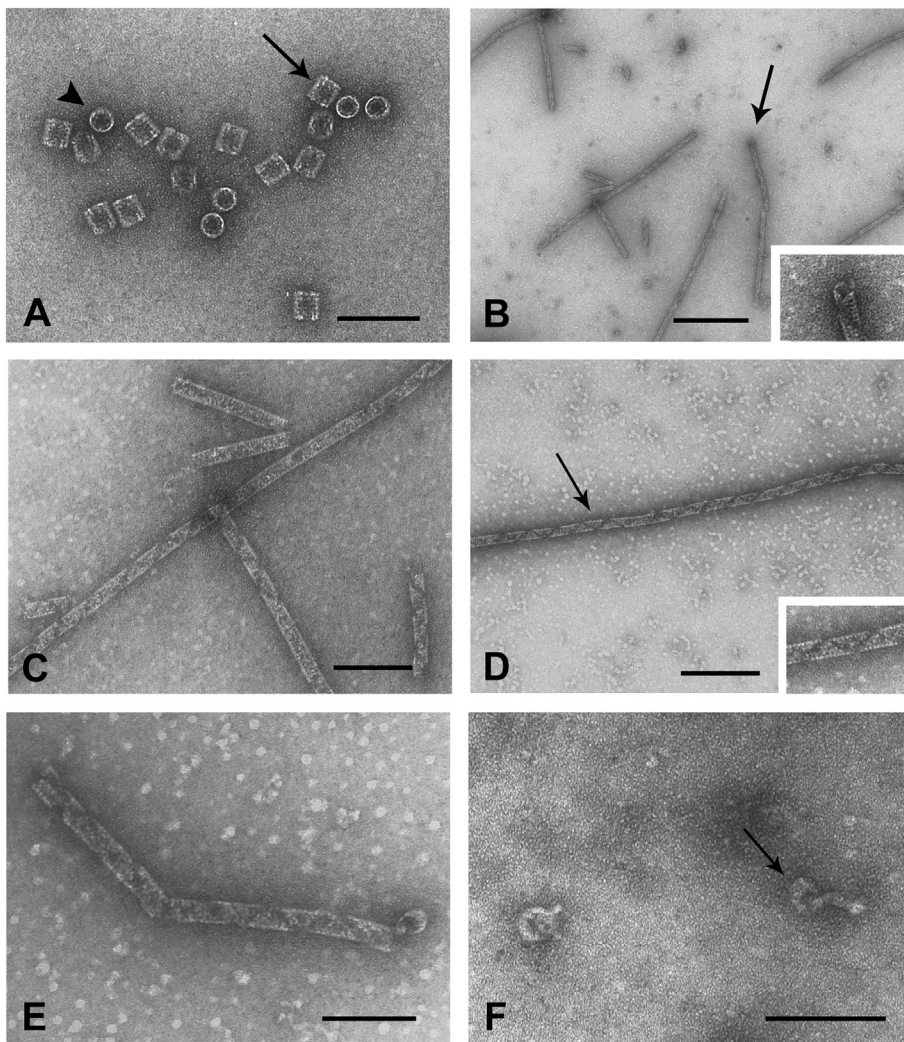
Fig. 7. CCH-B subunits show an unusual polymerization. (A) Electron microscopy of negatively stained purified *Concholepas* hemocyanin molecules showing their characteristic hollow cylinder form. The images show the top (arrowhead) and lateral (arrow) views of the molecules. The scale bar represents 100 nm. (B) CCH-B subunits form tubular polymers. Microphotograph at low magnification showing the tubules. The scale bar represents 500 nm. The inset shows a higher magnification (100,000 ×) of the end of the tubule, indicated with an arrow, which is apparently hollow. (C) Tubules of CCH-B have variable length. The image shows large and short tubules. The scale bar represents 100 nm. (D) Tubules formed by CCH-B subunits have a zig-zag organization. The scale bar represents 200 nm. Inset: higher magnification (110,000 ×) of the zig-zag organization of the zone indicated with an arrow. (E) Tubules of CCH-B. The image shows a tubule that is apparently broken or twisted in the elbow zone. The scale bar represents 100 nm. (F) Subunits of CCH-A do not form polymers. The images show the appearance of CCH-A subunits, which present aggregates with low frequency, as indicated by an arrow. The scale bar represents 250 nm.

both sensitive. However, KLH was more resistant to high pH values, as evaluated by their Hill coefficients. At low and high pH values, the $K_{0.5}$ increased, which could be explained by a dissociation of the native structure of the protein.

The subunits of CCH were also evaluated. CCH-A had oxygen-binding parameters that were surprisingly slightly superior to the native CCH. CCH-B, however, exhibited a Hill coefficient of approximately half that of CCH-A. These results are consistent with other aspects of CCH, as CCH-A and CCH-B have different structural and immunological properties [20]. Thus, the data obtained here suggest that the oxygen-binding domains of the CCH subunits are different in primary structure, which is in agreement with the electrophoretic analyses, the vibrational spectral analysis and the carbohydrate analyses, all of which showed differences among isolated subunits of CCH [20]. However, CCH is not unique; differences in the oxygen-binding properties of isolated KLH subunits, as measured with a tonometric method, showed differences in the Hill coefficients between the KLH subunits. Specifically, the cooperativity was lower for the native protein than for either isoform [19]. Unfortunately, in this report, neither the precise value of the Hill coefficient for native KLH nor the temperature at which the experiments were conducted is indicated. This information would have allowed us to compare the binding data for KLH obtained by spectro-photometry by Senozan et al., [7] and the polarographic method used in the present work. As previously noted, KLH-B subunits also have a tendency to form elongated multimeric structures in contrast to KLH-A, which forms random didecamer clusters [19]. However, it is unknown

if this phenomenon has a biological meaning. In this context, we propose the hypothesis that CCH-A subunits are the main O_2-binding domain in *Concholepas* hemocyanin, which is supported by their slightly superior oxygen-binding parameters to those of native CCH. Conversely, the CCH-B subunit could play a more structural role, as supported by the polymerization of this subunits. The experiments dedicated to test this hypothesis should be a matter of a new study, which would involve structural studies.

Finally, considering that CCH and KLH have limited bioavailability because their supply depends on natural resources, i.e., animal species, which are subject to the influence of physical or chemical fluctuations of environmental factors, the quantitative information obtained here can contribute to the knowledge of the upper and lower limits of the temperature-dependent oxygen limitations for these marine ectothermic animal species in a climate change situation [39,40]. In fact, ocean deoxygenation due to high temperatures leads to an increase in CO_2 and, consequently, to seawater acidification, a phenomenon that could influence the oxygen-binding properties of the hemocyanins of these species. These changes would directly affect aerobic ATP-producing pathways. In this context, it has been proposed that hemocyanins may play a role in thermal adaptation for different species of mollusks, such as the stenothermal Antarctic octopod *Megaleledone senoi* [41], the eurythermal cuttlefish *Sepia officinalis* [42,43] and the gastropod *Helix pomatia* [44], based on the available blood physiological, molecular and structural data [45,46]. The studies by Melzner [42] comparing the pH and temperature dependence of the hemocyanins in diverse species of

cephalopods showed that when the ambient temperature increased, a change in the cooperativity or a fall in the oxygen affinity of hemocyanin was observed; thus, some eurythermic cephalopods are able to decrease affinity with temperature at a reasonable rate and thereby support higher metabolic rates. Other, more stenothermal, species lack this flexibility in hemocyanin properties [42]. In this context, the Hill coefficients obtained here reflect the lifestyle of *Concholepas*, which live at water temperatures lower than 20 °C, because they show that CCH oxygen affinity decreases significantly when the temperature increases above 20 °C, suggesting that warming conditions could have a severe effect on *C. concholepas* because its hemocyanin is relatively inflexible and would be unable to liberate sufficient amounts of oxygen to satisfy the increased metabolic demand. In agreement with this last assertion are the current studies of Manríques et al., [47] in small juveniles of *C. concholepas* subjected to long-term exposure to CO_2 (ca. 500 and 1400 μatm) and moderate warming (temperatures of 15 °C and 19 °C) levels. Their results demonstrated a significant reduction in the metabolic rates of individuals breeding at high temperature and CO_2 levels.

In conclusion, this is the first study in which the oxygen-binding properties of *Concholepas* hemocyanin and its isolated subunits were evaluated using rat liver submitochondrial particles and polarography. From a biochemical point of view, our data add to the evidence that mollusk hemocyanins have a low Hill coefficient. Instead, hemocyanins have 160 site of oxygen binding, so it would be expected to observe higher Hill coefficient than small proteins (the Hill coefficient of hemoglobin is approximately 2.8), a phenomenon called a "Hill coefficient anomaly" by van Holde et al. [48]. From a physiological point of view, clearly, more detailed studies are needed that combine the *in vitro* biochemical data of oxygen-binding properties of CCH as studied here with in vivo physiological measurements using temperatures close to the upper thermal limit of *C. concholepas* to determine the role of hemocyanin in the thermal adaptation of this species.

5. Conclusion

The data presented in this report regarding the oxygen-binding properties of hemocyanin from the mollusk *Concholepas concholepas*, over a wide range of temperatures and pH, using an improved polarographic method, allowed us to determine that i) CCH, like KLH, has a cooperative oxygen-binding behavior; ii) temperature affects the Hill coefficients and the $K_{0.5}$; iii) temperature presents an inverse correlation with cooperativity; iv) both high and low pH alter the oxygen-binding parameters; and v) CCH subunits exhibit different oxygen-binding parameters, which, together with other results, allowed us to suggest that CCH-A has oxygen transport functions, while CCH-B plays rather a structural role in the molecule. Finally, in an effort of an integrative approach, we discuss the role of hemocyanins in thermal adaptation of mollusks, concluding that warming conditions could have a severe effect on this specie of mollusk, as oxygen affinity decreases significantly in CCH when the temperature increases above 20 °C.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbapap.2017.08.017.

Transparency document

The http://dx.doi.org/10.1016/j.bbapap.2017.08.017 associated with this article can be found, in online version.

Acknowledgments

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