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Kinetic properties and heme pocket structure of two domains of the polymeric hemoglobin of *Artemia* in comparison with the native molecule

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ABSTRACT

In this project, we studied some physicochemical properties of two different globin domains of the polymeric hemoglobin of the brine shrimp *Artemia salina* and compared them with those of the native molecule. Two domains (*As*HbC1D1 and *As*HbC1D5) were cloned and expressed in BL21(DE3)pLysS strain of *Escherichia coli*. The recombinant proteins as well as the native hemoglobin (*Af*Hb) were purified from bacteria and frozen *Artemia*, respectively by standard chromatographic methods and assessed by SDS-PAGE. The heme environment of these proteins was studied by optical spectroscopy and ligand-binding kinetics (e.g. CO association and O_2 binding affinity) were measured for the two recombinant proteins and the native hemoglobin. This indicates that the CO association rate for *As*HbC1D1 is higher than that of *As*HbC1D5 and *Af*Hb, while the calculated P_{50} value for *As*HbC1D1 is lower than that of *As*HbC1D5 and *Af*Hb. The geminate and bimolecular rebinding parameters indicate a significant difference between both domains. Moreover, EPR results showed that the heme pocket in *Af*Hb is in a more closed conformation than the heme pocket in myoglobin. Finally, the reduction potential of −0.13 V versus the standard hydrogen electrode was determined for *Af*Hb by direct electrochemical measurements. It is about 0.06 V higher than the potential of the single domain *As*HbC1D5. This work shows that each domain in the hemoglobin of *Artemia* has different characteristics of ligand binding.

1. Introduction

Hemoglobins (Hbs) are a specific class of proteins consisting of a single or multiple globin chains. These globin chains display the specific globin fold consisting of 7 or 8 α-helical segments (indicated A to H) wrapped around a heme b moiety according to a 3-over-3 α-helical sandwich pattern [1,2]. The heme iron atom is penta- (F8His) or hexa-coordinate depending on the presence of an internal 6th ligand (usually E7His) [3,4]. Comparative studies demonstrate that Hbs occur in all kingdoms of life and that the canonical globin fold displays an extreme flexibility [4–7]. Due to the reactivity of the heme iron atom, globins are involved in a diversity of reactions varying e.g. from O_2 metabolism (O_2 sensing, carrying, storing) to redox chemistry (nitroso and oxidative stress metabolism) [8]. In some invertebrate classes (*Annelida*, *Mollusca*, *Crustacea*), Hbs occur as high M_r proteins dissolved in the extracellular fluid or hemolymph. Such high M_r is necessary e.g. to avoid pigment loss due to excretory filtration events. This high M_r is obtained either by disulfide bond based aggregation (e.g. in *Annelida*) or by the covalently concatenation of globin domains into polymeric globin chains (e.g. in *Mollusca*, *Crustacea*) [7].

An example of Hbs containing polymeric globin chains are those from the brine shrimp *Artemia*, a small branchiopod crustacean inhabiting worldwide diverse ponds with variations in O_2 partial pressure due to difference in salinity (e.g. up to 50% salinity) [9]. *Artemia* expresses genotypically four different globin chains (M_r ~160,000) namely C1, C2, T1 and T2 [10–12]. Structural analyses demonstrate that the T and C chains are ring-shaped polymers of nine genuine globin domains covalently joined by short inter-domain linkers (Fig. 1) [13]. All domains show different primary structures (identities: 23–39%)

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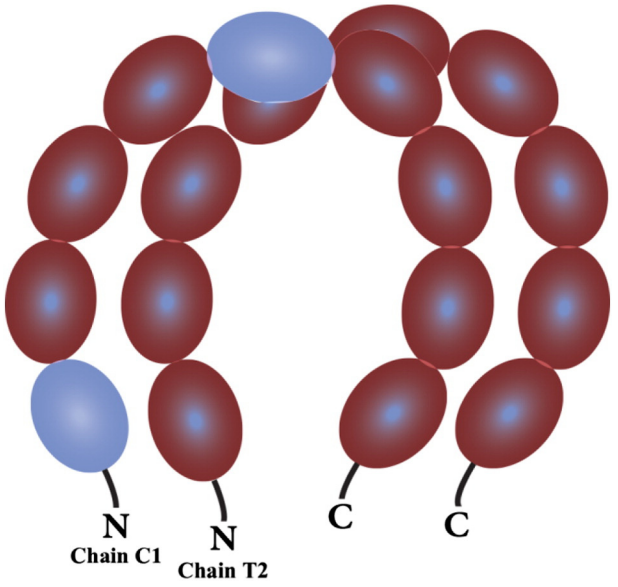


Fig. 1. Schematic view of *As*HbII. *As*HbC1D1 and *As*HbC1D5 are presented in blue.

and are presumed to be copied originally from a single-domain gene [11,14]. Phenotypically, two of these ring-shaped globin chains dimerize, by coaxially stacking, to produce three heterodimeric Hb isoforms (M_r ~320,000): HbI (C1C2), HbII (C1T2) and HbIII (T1T2) [12]. Analysis of the Hb quaternary structure demonstrates that in both globin chains the EF helices of all domains are in contact along the interpolymer surface, and that domain 1 of the T-polymer aligns with domain 1 of the C-polymer. Similar EF contacts are very common in cooperative Hbs [15].

The *Artemia* Hbs are definitively involved in O_2 storage/transport metabolism and serve e.g. to cope with the variable O_2 tension in the environment. All three Hbs bind O_2 cooperatively and with a different affinity [16]. Their biosynthesis is differentially controlled according to the species where they are expressed in, the ontogenetical stage and the temperature, pH and the O_2 tension of the habitat [10,17–20].

The role of the individual domains in the polymeric globin chain(s) and the native Hb(s) is unclear. However, single or multi-domain fragments of *Artemia salina* (*As*HbII), obtained by limited proteolysis, bind O_2 non-cooperatively [21,22].

This paper aims to answer the following questions; firstly, whether the eighteen domains have the same role in the ligand binding or not; secondly, whether the general structure of the heme pocket in the native Hb of *Artemia* is the same as that of myoglobin, and finally, whether Hb of *Artemia*, with 18 heme centers, has a higher redox potential than the normal reference globins.

To provide an answer to these questions, we studied the physicochemical characteristics (electron paramagnetic resonance (EPR), laser-flash photolysis and redox chemistry) of two recombinant globin domains [*A. salina* chain C1, domains 1 (*As*HbC1D1) and 5 (*As*HbC1D5) (Fig. 1)] of *Artemia urmiana* and *Artemia franciscana* from Urmia salt Lake, Iran and compared them with those of native Hbs of *A. franciscana* (*Af*Hb).

2. Materials and methods

2.1. Purification of *A. franciscana* Hbs (*Af*Hb)

Native *A. franciscana* Hbs were purified from frozen material (a gift from the Laboratory of Aquaculture and *Artemia* Reference Center, University of Ghent, Belgium) mainly as described previously [23]. Shortly, crude *Af*Hb (50% ammonium sulfate precipitate) was further purified on a HiTrap DEAE column by step elution at 225 mM NaCl. Hb tracing was spectrophotometric at 412 nm.

2.2. RNA extraction and amplification

A. urmiana and *A. franciscana* were collected from Urmia Salt Lake, Urmia, Iran. Total RNA was prepared using the combination of TriZol method and PureLink RNA Mini Kit. cDNA was synthesized as described elsewhere [24]. The cDNA fragments encoding globin domains 1 (*As*HbC1D1) and 5 (*As*HbC1D5) of chain C1 were amplified by PCR [25].

2.3. Cloning, expression, and purification of recombinant proteins

The PCR products were cloned into the TOPO-TA vector (Invitrogen) followed by subcloning into pET23a vector. *As*HbC1D1 and *As*HbC1D5 were expressed in *Escherichia coli* strain BL21(DE3)pLysS. Cells were grown at 37 °C in Terrific Broth (TB) medium (1.2% bactotryptone, 2.4% yeast extract, 0.4% glycerol, 72 mM potassium phosphate buffer, pH 7.5) containing 200 μg/ml ampicillin, 30 μg/ml chloramphenicol, and 2.5 mM δ-amino-levulinic acid. The culture was induced at $A_{550} = 1.2$ by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.4 mM, and expression was continued overnight (at 25 °C). The cells were harvested and resuspended in lysis buffer, 50 mM Tris–HCl (pH 7.5) containing 300 mM NaCl.

The cells were exposed to three freeze–thaw steps and sonication till completely lysed. The lysate was clarified by low speed (10 min at 10,000 ×*g*) centrifugation. Then, imidazole was added (final buffer composition of 50 mM Tris–HCl pH 7.5, 300 mM NaCl, and 20 mM imidazole) and the extract loaded on a Ni-affinity His 60 super flow column (Clontech), equilibrated with the same buffer. After washing of the unbound material, the His-tagged recombinant protein was eluted by 50 mM Tris–HCl pH 7.5, 300 mM NaCl and 500 mM imidazole. The fractions containing the proteins of interest were pooled and dialyzed against 50 mM Tris–HCl pH 7.5 containing 150 mM NaCl and 0.5 mM EDTA. After concentration by ultra-filtration (Amicon PM 10), the samples were loaded on Superdex Gf75, 15 × 1800 tricorn column (GE Healthcare) for gel filtration chromatography. All purification steps were assessed by SDS-PAGE.

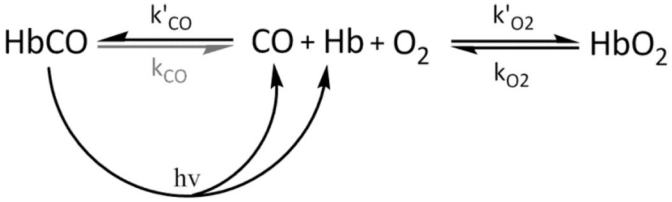
2.4. UV–visible spectroscopy

Optical measurements were done with a Varian Cary-5 UV–visible near-infrared spectrophotometer (Varian, Palo Alto, California). All UV–visible spectra were measured in the range from 250 to 700 nm.

2.5. Continuous wave EPR of the native protein

X-band continuous wave (CW) EPR measurements were performed on a Bruker ESP300E spectrometer with a microwave frequency of 9.45 GHz equipped with a gas-flow cryogenic system (Oxford Inc.), allowing for operation from room temperature down to 2.5 K. The magnetic field was measured with a Bruker ER035M NMR Gauss meter. During the experiments, a vacuum pump was attached to the EPR tube in order to remove O_2 from the frozen sample. The spectra are measured with modulation amplitude of 0.8 mT, a modulation frequency of 100 kHz and a microwave power of 0.1 mW.

For the EPR measurements, 20% glycerol was added as a cryoprotectant. All spectra were simulated using EasySpin, a toolbox for MATLAB (Mathworks, Natick, Mass., USA).



Scheme 1.

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