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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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 α to H) wrapped around a heme moiety according to a 3-over-3 helical sandwich pattern [1,2]. The heme iron atom is pentacoordinate (5His) or hexacoordinate depending on the presence of an internal 6th ligand [3,4]. Comparative studies demonstrate that Hbs occur in all kingdoms of life and that the canonical globin fold displays 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 O2 metabolism (O2 sensing, carrying, storing) to redox chemistry (nitroso and oxidative stress metabolism) [8]. In some invertebrate classes (Annelida, Mollusca, Crustacea), Hbs occur as high Mr proteins dissolved in the extracellular fluid or hemolymph. Such high Mr is necessary e.g. to avoid pigment loss due to excretory filtration events. This high Mr 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 O2 partial pressure due to difference in salinity (e.g. up to 50% salinity) [9]. Artemia expresses genotypically four different globin chains (M, ~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]. Due to the reactivity of the heme iron atom, globins are involved in a diversity of reactions varying e.g. from O2 metabolism (O2 sensing, carrying, storing) to redox chemistry (nitroso and oxidative stress metabolism) [8]. In some invertebrate classes (Annelida, Mollusca, Crustacea), Hbs occur as high Mr proteins dissolved in the extracellular fluid or hemolymph. Such high Mr is necessary e.g. to avoid pigment loss due to excretory filtration events. This high Mr 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].

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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 (Mr ~320,000): HbI (C1C2), HbII (C1T2) and HbII (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 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 (AsHbII), obtained by limited proteolysis, bind O2 non-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 O2 tension of the habitat [10,17–20].

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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 physico-chemical characteristics (electron paramagnetic resonance (EPR), laser-flash photolysis and redox chemistry) of two recombinant globin domains [A. salina chain C1, domains 1 (AsHbC1D1) and 5 (AsHbC1D5)] of Artemia urmiana and Artemia franciscana from Urmia salt Lake, Iran and compared them with those of native Hbs of A. franciscana (AfHb).

2. Materials and methods

2.1. Purification of a franciscana Hbs (AfHb)

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 AfHb (50% ammonium sulfate precipitate) was further purified on a HitTrap 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 (AsHbC1D1) and 5 (AsHbC1D5) 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. AsHbC1D1 and AsHbC1D5 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 L-amino-levulinic acid. The culture was induced at A550 = 1.2 by the addition of isopropyl-1-thio-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 G75, 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 O2 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).
were carried out in duplicate in 100 mM potassium phosphate, pH 7.0 at 20°C. The curve consists of four to five saturation steps. Experiments were carried out in duplicate in 100 mM potassium phosphate, pH 7.0 at 20°C.

2.6. Analytical gel filtration experiments

The apparent Mr of the recombinant AsHB1D1 and AsHB1D5 in solution was assessed by analytical gel filtration experiments using a Superdex 27 (2 × 30 cm; buffer 50 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.5 mM EDTA; 0.5 ml·min⁻¹) calibrated with human Hb (68 kD), cytoglobin (42 kD) and myoglobin (17 kD). Protein elution was monitored at 280 and 412 nm. Concentration dependent oligomerization was tested by loading different Hb concentrations (3.3 μM, 11.1 μM, 42.5 μM and 85.1 μM).

2.7. O₂ equilibria

The O₂ equilibrium curve for AfHb was determined using a modified diffusion chamber technique described previously [26,27]. Shortly, water-saturated gaseous mixtures of O₂ and ultrapure (99.998%) N₂ were used to equilibrate a thin smear (190 μm) created by Wösthoff (Bochum, Germany) gas mixing pumps were switched Nd:YAG laser (Spectra Physics Quanta-Ray). The absorbance changes were recorded with a Tektronix TDS220 digitizing oscilloscope fitted to a simple exponential expression. CO association rate value (k CO) was calculated as the slope of plots of kobs versus [CO]. For all ligand-binding kinetic studies, measurements were done at least in triplicate and averaged.

2.8. CO binding kinetics

2.8.1. CO binding kinetics

To measure the rates of CO association, the Fe²⁺ Hb stock solution was diluted with a 100 mM potassium phosphate buffer, 1 mM EDTA, pH 7.0, at 20°C to a final concentration of ~5 μM, equilibrated in different concentrations (200–800 μM) of CO and anaerobically reduced with 1 mM sodium dithionite. The experimental setup was described in detail elsewhere [28,29]. Shortly, photolysis of HbCO samples was carried out on a laser photolysis system (Edinburgh Instrument LP920) at 20°C using the second harmonic (532 nm) of a frequency-doubled Q-switched Nd:YAG laser (Spectra Physics Quanta-Ray). The absorbance changes were recorded with a Tektronix TDS220 digitizing oscilloscope fitted to a simple exponential expression. CO association rate value (k CO) was calculated as the slope of plots of kobs versus [CO]. For all ligand-binding kinetic studies, measurements were done at least in triplicate and averaged.

2.8.2. Geminate recombination

The geminate recombination time courses were fitted to one exponential decay. The observed rates of geminate recombination (k gem) were independent of ligand concentration. The fraction of geminate recombination (F gem) was calculated using Eq. (1):

\[ F_{\text{gem}} = \frac{\Delta A_{\text{gem}}}{\Delta A_{\text{gem}} + \Delta y_0} \]

where \( \Delta A_{\text{gem}} \) represents the absorbance change associated with the internal geminate rebinding and \( \Delta y_0 \) (the offset) is the difference between the absorbance after complete geminate recombination and the absorbance of the original ground state, which is observed prior to the photolysis or at the long times after the complete rebinding of bimolecular from the solvent. As the concluded rates for CO entry and exit appeared to be the...
same for those of O₂ and NO due to the almost same size and polarity of three diatomic gases [30], the geminate rebinding parameters were measured for CO.

2.8.3. O₂ binding kinetics

Time courses for O₂ association and dissociation were measured after complete laser photolysis of Hb samples containing various mixtures of O₂/CO. The mixed atmosphere allows measurement of the O₂ dissociation rate. In these experiments, Hb samples were prepared in five different buffers with 100 mM potassium phosphate buffer, pH 7.0, at 20 °C containing various concentration of CO and O₂ (e.g. 892 μM of CO, 750 μM of CO and 312.5 μM of O₂, 500 μM of CO and 625 μM of O₂, 250 μM of CO and 937.5 μM of O₂, and 1250 μM of O₂).

The sample is essentially bound to CO at equilibrium because the partition coefficient between both ligands is displaced toward the CO form as occurs for most globins.

As shown in Scheme 1, the photolysis of HbCO by a laser beam generates a deoxyHb species which react rapidly with either ligand and two distinct phases were observed. The rate of bimolecular rebinding phase (k₂ₕₒ₀) that was dominated by O₂ rebinding due to its larger association constant (k₀/O₂), was monitored by a large decrease in the absorbance of unliganded Hb at 436 nm [31]. The value of k₂ₕₒ₀ is given by Eq. (2),

\[ k_{\text{fast}} = k_{0}[O_2] + k_{O_2} + k_{CO}[CO] \]  (2)

Generally, the CO dissociation occurs on a timescale slower compared to the O₂ and then can be treated as an irreversible process.

The slow replacement phase represents the displacement of transiently bound O₂ by CO, which, although kinetically less reactive, has a higher affinity for heme. The time course of this replacement reaction is best monitored by a large increase in the difference of absorbance at 422–424 nm. The rate of this process, k₂ₕₒ₀, is given by Eq. (3).

\[ k_{\text{slow}} = \frac{k_{O_2}}{1 + k_{O_2}[O_2] + k_{CO}[CO]} \]  (3)

An iterative, nonlinear, least squares fitting routine (Solver in Excel) was used to optimize the values of k₂ₕₒ₀, k₀/O₂, and k₂ₕৎ to give the best fit to the dependence of k₂ₕₒ₀ and k₂ₕₒ₀ on [O₂]/[O₂]CO, including conditions of [O₂]= 0 and [CO] ≈ 0 [31].

2.9. Reduction potential

Electrochemical measurements were conducted in a conventional three-electrode cell using a µAutolab III interface controlled by Nova 1.10 software (Metrohm-Autolab BV, Netherlands). A saturated calomel electrode (SCE, 0.248 V versus standard hydrogen electrode (SHE) at 20 °C) and a glassy carbon rod were used as the reference and auxiliary electrodes, respectively. Prior to modification, the gold electrodes (1.6 mm in diameter, BASI, USA) were polished with 1 and 0.05 μm alumina slurry, washed, and sonicated in ultrapure water for about 5 min. Next, the electrodes were electrochemically cleaned by cyclic voltammetry (CV) in 0.5 M NaOH at the potential range from −0.35 to −1.35 V and in 0.5 M H₂SO₄ at the potential range from 0.2 to 1.5 V vs. SCE until repeatable cyclic voltammograms. Then the electrodes were incubated overnight in 10 mM 6-mercapto-1-hexanol (97%, Sigma-Aldrich) solution in water. Finally, the electrodes were thoroughly washed with water and dried in air stream. Electrochemistry of the proteins was studied using membrane electrodes as described [32,33]. Briefly, 2–3 μL of a protein solution was entrapped between the electrode and dialysis membrane (MWCO 12 kD). Differential pulse voltammograms (DPV) was recorded before and after placing the samples on the electrodes.

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td>The absorption maxima of AₜHbC₁D₁, AₜHbC₁D₅ and AₜHb in different forms.</td>
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<td>Soret band (nm)</td>
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<td>AₜHbC₁D₁</td>
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<td>AₜHbC₁D₅</td>
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<td>AₜHb</td>
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Fig. 4. X-band CW-EPR spectra of ferric native AₜHb (solid) and simulation (dashed). The asterisk shows the contribution to the signal of non-heme iron. All spectra were recorded at 10 K.

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3. Results

3.1. Cloning, expression and purification

The obtained cDNA sequences for domains 1 and 5 from both species were compared with the reported sequence of *Artemia* by Manning et al. [10]. For cDNA sequences of domain 1 from *A. urmiana* and *A. franciscana* two differences (positions 102 and 393) and for that of domain 5 one difference (position 391) were noted. The corresponding protein sequences for both domains were exactly the same as reported in literature. Therefore, AsHbCD1 and AsHbCD5 were used as abbreviations to refer to the recombinant proteins of both species. AsHbCD1 and AsHbCD5 are composed of 141 and 145 residues, respectively. The primary structure comparison indicated 26.1% identity and 41.8% similarity between the domains. Moreover, AsHbCD1 sequence is more similar to the non-symbiotic Hb1 of *Oryza sativa*, rice (38.8%) and to the globin C of *Caudina arenicola* (41.0%) whereas AsHbCD5 is similar to the mouse neuroglobin (40.6%) and the globin 5 of *Petromyzon marinus*, sea lamprey (39.1%) (Fig. 2). Expression and purification of the recombinant AsHbCD1 and AsHbCD5 yielded two recombinant proteins with a molecular mass ~16 kD, as verified by SDS-PAGE and gel filtration. The recombinant proteins were, however, not that stable and hard to store. A/Hb was successfully purified from the brine shrimp and showed a molecular mass of 160 kD by SDS-PAGE.

3.2. UV–visible spectroscopy

Fig. 3 shows the UV–visible absorption spectra of expressed/purified species of AsHbCD1, AsHbCD5 and A/Hb in the oxy-, deoxy- and carbon monoxy-forms. The optical absorption spectra of the oxy-form of AsHbCD1 and AsHbCD5 show the Soret maximum at 412 nm and α and β maxima at 579 nm and 540 nm, respectively (Fig. 3, Table 1). This is a typical absorbance spectrum for oxygenated globins, such as sperm whale myoglobin. Moreover, the absorption spectrum of the freshly purified A/Hb exhibits the Soret maximum at 413 nm and α and β maxima at 579 nm and 540 nm, respectively.

Upon CO binding to ferrous AsHbCD1, AsHbCD5 and A/Hb, the Soret maxima are shifted to 424, 423 and 423 nm, respectively. The α and β maxima are observed at 571–573 nm and 540–545 nm (Table 1).

3.3. Continuous wave EPR of the native Hb

The EPR spectrum of ferric native A/Hb is shown in Fig. 4 and indicates that the system is in a high-spin (HS) state. In this state, the distal side of the central heme iron is known to bind weak ligands like H₂O or have no distal ligand. Simulation of this spectrum gives the following g-values: gₓ = 5.92, gᵧ = 5.91 and gₑ = 1.996. After addition of imidazole (1:1 ratio imidazole:heme), which is a strong base, we expect imidazole to bind to the distal side of the heme iron because of the competition with the weak ligand, as is reported for metmyoglobin [34]. The EPR spectrum after addition of imidazole however shows no appearance of a low-spin state typical for an imidazole-ligated globin. This indicates that the imidazole is not able to enter the heme-pocket, and thus native A/Hb has a more closed heme-pocket structure than myoglobin.

3.4. O₂ equilibria

Analytical gel filtration experiments were performed to assess the quaternary structure of AsHbCD1 and AsHbCD5 in solution (expected molecular mass = 16.5 and 17.2 kD, respectively, as calculated from sequence analysis). Elution time for AsHbCD1 (Fig. 5) corresponds to two peaks with molecular mass around 50 kD and 16 kD, values consistent with a tetramer and a monomer assembly, respectively. The elution time for AsHbCD5 correspond to a molecular mass of 15 kD, which indicates a monomeric structure (Fig. 5). The elution times for both proteins were the same for all tested concentrations, which indicates that the oligomerization of both proteins is independent of protein concentration.

3.5. O₂ rebinding

A/Hb O₂ binding curve was hyperbolic (Fig. 6) with a P₅₀ value of 2.46 Torr, while the reported P₅₀ values for *Artemia* Hb I, Hb II and Hb III were 5.34, 3.70 and 1.80 mm Hg, respectively [17]. Moreover, the calculated P₅₀ value based on the association constant of O₂, Kₒ₂, was 33.31 1.51 Torr (Table 3). The difference can be explained by the different experimental conditions (e.g. pH and temperature). The native Hb showed a cooperativity value (nₒ₂) close to 1.04, which is different from the
376 whereas the native hemoglobin/ myoglobin.

377

378

379 3.6. Ligand binding kinetics

380 3.6.1. CO association rate constant

381 The CO association rates of AsHbC1D1, AsHbC1D5 and AfHb were

382 measured using Laser Flash Photolysis. Fig. 7 reports the fraction

383 of deoxy heme as a function of time after the photolysis for selected condi-

384 tions. Two distinct processes in the CO rebinding curves are clear: i) A

385 fast rebinding phase or geminate rebinding, which was completed in

386 less than 2000 ns, and shows no dependence on CO concentration;

387 and ii) a slower phase, whose apparent rate constant depends on CO

388 concentration, and is called bimolecular rebinding. The latter indicates

389 the rebinding of ligands to heme iron from the solvent phase.

390 As shown in Fig. 7, the ligand recombination in AsHbC1D1 and

391 AsHbC1D5 is remarkably different from each other. AsHbC1D1 showed

392 monophasic kinetics and the extracted association rate constant is

393 3.32 μM⁻¹ s⁻¹ (Table 2). Furthermore, AsHbC1D1 has slightly higher associa-

394 tion rate constant than AsHbC1D5, suggesting a greater accessibility of

395 this domain for CO. Bimolecular rebinding of both AsHbC1D5 and

396 AfHb, however, shows biphasic kinetics that could be fitted by a double

397 exponential relaxation. Therefore, as indicated in Table 3, the bimolecu-

398 lar CO association rate constants of AsHbC1D5 are 15.9 and 1.3 μM⁻¹ s⁻¹,

399 and that of AfHb are 1.7 and 0.17 μM⁻¹ s⁻¹. This kinetic heterogeneity for

400 AsHbC1D5 and AfHb suggests the existence of two different conforma-

401 tions at equilibrium. Moreover, it is noteworthy to mention that, as

402 there are 18 globin domains in AfHb, we cannot make a distinction be-

403 tween the rates of the separate domains when measuring on the native

404 Hb.

405

406 3.6.2. Geminate rebinding parameters

407 Internal CO geminate recombination was examined on nanosecond

408 time scales to first determine the fraction of geminate recombination

409 (F₉₀₉) and secondly to estimate rates of internal ligand bond formation

410 and escape. The F₉₀₉ values of the two recombinant proteins are 0.57

411 and 0.23, respectively, which indicate a different heme pocket structure

412 of this domain for CO. CO bimolecular rebinding of both AsHbC1D1 and

413 AfHb is slower than that of AsHbC1D5 (0.47), whereas F₉₀₉ of AsHbC1D5 (0.23) is lower than that of SWMb (Table 2).

414 However, it should be noted that the F₉₀₉ and other kinetic values of

415 SWMb in [35] were measured based on O₂ as ligand.

416 As shown in Fig. 8, the geminate CO recombination in all three sam-

417 ples appears to be a simple first order process. The k₉₀₉ values of

418 AsHbC1D1, AsHbC1D5 and AfHb are close (i.e. 55 and 61 μM⁻¹ s⁻¹, respectively) which is nearly 4 times greater than that of AfHb (i.e. 17 μM⁻¹ s⁻¹) (Table 2).

419 The bimolecular and geminate recombination parameters could be

420 used in calculating the internal binding and escaping rates of ligand to

421 or from the inside of the protein.

422 A two-step binding scheme was assumed for analysis that involved

423 internal bond formation between the ligand and iron atom (k₉₀ₑ), and

424 for ligand escape (kₑ₉₀) from or bimolecular return (kₑₑₑ) to the

425 native state (Hb–CO) in Scheme 2 [30]. The rate parameters which fit to these processes define the observed rates (kₑₑₑ, Eq. (5)) and the fractions (F₉₀ₑ, Eq. (4)) of geminate recombination and the overall bimolecular CO association rate constant (kₑₑₑ, Eq. (6)) [30,40]:

426

427 Fₑₑₑ = kₑₑₑ / (kₑₑₑ + kₑₑₑ)

428

429

430 kₑₑₑ = kₑₑₑ + kₑₑₑ

431

432 kₑₑₑ = kₑₑₑ + kₑₑₑ + kₑₑₑ = kₑₑₑ Fₑₑₑ.

433

434

435 The rate constant for the ligand entry, kₑₑₑ entry into the individual

436 proteins is calculated empirically as the observed bimolecular rate con-

437 stant divided by the fraction of geminate recombination (kₑₑₑ entry = kₑₑₑ / Fₑₑₑ). Therefore, kₑₑₑ entry is calculated independently [30,40].

438 The values of internal binding, ligand entry and escaping parameters

439 for studied proteins are listed in Table 3. As it is clear from Table 3, the

440 kₑₑₑ values for AsHbC1D1, AsHbC1D5 and AfHb are 31.35, 14.03 and

441 3.23 μM⁻¹ s⁻¹, respectively, while the kₑₑₑ values are 23.65, 46.27 and

442 13.77 μM⁻¹ s⁻¹, respectively. It must be noted that the kₑₑₑ entry for AsHbC1D1

443

444

445 Table 2

446 Kinetic values for O₂ and CO reactions of AsHbC1D1, AsHbC1D5 and AfHb at pH 7, 20 °C with some selected hemoglobin/myoglobin.

447

448 |        | kₒ₀ | kₒ₂ | kₒ₃ | Pₒₒ | kₒₛ | kₑₑₑ | kₑₑₑ entry | kₑₑₑ escape | kₑₑₑ bond | kₑₑₑ Ref. |
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<tr>
<td></td>
<td>μM⁻¹</td>
<td>μM⁻¹</td>
<td>μM⁻¹</td>
<td>Torr</td>
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<td>μM⁻¹ s⁻¹</td>
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<tr>
<td>AsHbC1D1</td>
<td>10.5 ± 1.6</td>
<td>8.1 ± 0.8</td>
<td>1.30 ± 0.08</td>
<td>0.42a</td>
<td>3.32 ± 0.18</td>
<td>55 ± 3</td>
<td>0.57 ± 0.01</td>
<td>31.35 ± 3</td>
<td>23.65 ± 4.2</td>
<td>5.82 ± 0.18</td>
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<tr>
<td>AsHbC1D5</td>
<td>11.2 ± 1.2</td>
<td>15.3 ± 2.1</td>
<td>0.86 ± 0.05</td>
<td>0.64a</td>
<td>15.9 ± 1.6</td>
<td>61.07 ± 0.23 ± 0.01</td>
<td>14.03 ± 0.7</td>
<td>49.67 ± 1</td>
<td>69.13 ± 0.12</td>
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<tr>
<td>AfHb</td>
<td>8.1 ± 0.7</td>
<td>35.4 ± 4.9</td>
<td>0.23 ± 0.05</td>
<td>2.39b (2.46b)</td>
<td>17 ± 0.6</td>
<td>0.19 ± 0.01</td>
<td>3.23 ± 0.6</td>
<td>13.77 ± 0.84</td>
<td>8.95 ± 0.35</td>
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<td>Carp Mb</td>
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<td>1</td>
<td>3.2</td>
<td>0.9</td>
<td>11</td>
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<td>76</td>
<td>640</td>
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<tr>
<td>Chironomus Hb</td>
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</tbody>
</table>

449 a These values are calculated based on O₂ as ligand, whereas those of the recombinant proteins are measured based on CO.

450 b Calculated in 22 °C.

451 c Calculated in 22 °C.

452 d Calculated in 22 °C.

453 e Calculated in 22 °C.

454 f These values are calculated based on O₂ as ligand, whereas those of the recombinant proteins are measured based on CO.

455 g Calculated in 22 °C.
and differs noticeably among the proteins. As shown in Table 4, recombinant proteins. The redox potential is found from the peak position in Fig. 9 shows differential pulse voltammograms of the native and recombinant proteins. The redox potential is found from the peak position in Fig. 9, which shows differential pulse voltammograms of the native and recombinant proteins. The redox potential is found from the peak position.

3.7. Reduction potential

Fig. 9 shows differential pulse voltammograms of the native and recombinant proteins. The redox potential is found from the peak position in Fig. 9, which shows differential pulse voltammograms of the native and recombinant proteins. The redox potential is found from the peak position.

Fig. 8. Time course of CO geminate rebinding to AfHb, AsHbC1D1 and AsHbC1D5.

As shown in Table 4, AfHb has the highest reduction potential $E_{1/2}$ ($-0.13$ V versus SHE) compared to AsHbC1D1 ($-0.16$ V) and AsHbC1D5 ($-0.19$ V).

4. Discussion

This present study was conducted to examine the role of the different domains in ligand binding of Artemia Hb by comparing the characteristics of the isolated domains to the native molecule. By cloning and expressing the two individual domains (AsHbC1D1: A N-terminal located domain; and AsHbC1D5: an internally located domain) separately, we have created one-domain, single-heme globin-like proteins, which mediate ligand binding.

When comparing the C1D1 domain of A. franciscana and A. urmiana, there is no difference in the sequence at the protein level (Fig. 2) and only few differences at the DNA level. The same conclusion can be taken for the C1D5 domain. This suggest that strain specific sequence differences are negligible.

Although there is a low identity between both recombinant domains and SWMb (~25%) virtually all key hydrophobic and heme-binding residues are conserved suggesting that both domains display the genuine globin fold (Fig. 2); A first indication that confirms this, is the UV–Vis spectroscopy, which shows that both recombinant domains as well as the native AfHb, are pentacoordinated like SWMb (Fig. 3). EPR spectroscopy, however, demonstrates that ferric native AfHb is unable to bind imidazole into the heme pocket suggesting a more closed structure than seen in SWMb (Fig. 4).

The quaternary structure of both recombinant globin domains in solution was studied by gel filtration experiments. They show that AsHbC1D1 occurs predominantly as tetramer and monomer whereas AsHbC1D5 occurs only as monomer. This suggests that the function of both domains in the assembly of the native AsHb molecule is different (Fig. 5). Furthermore, as each globin domain separately is not stable in physiological buffers, the polymeric structure is essential to stabilize the HB structure in Artemia. Hence, this confirms that the concatenation of globin domains into polymeric globin chains, as mentioned by Weber et al., is indispensable for stability [7].

The ligand binding kinetics and equilibrium studies of the native AfHb and the recombinant domains C1D1 and C1D5 also indicate slight differences in properties. The $P_{50}$ value of 2.46 Torr obtained by equilibrium measurements is very close to the $P_{50}$ calculated from the kinetic rate constants (2.39 Torr). They are, however, different from the published values (17) probably due to the fact that the AfHb we used is a mixture of the three Hb types and the difference in the experimental conditions (e.g. pH and temperature) used (Fig. 6; Table 3). Calculated $P_{50}$ values of AsHbC1D1 and AsHbC1D5 were 0.42 and 0.64 Torr, respectively. The difference in $P_{50}$ value for the native protein is 2.39 Torr, which is very close to the measured $P_{50}$ by O2 equilibrium measurements (2.46 Torr). Therefore, the $P_{50}$ value for native Hb is significantly higher and O2 affinity is lower in comparison with the recombinantly expressed domains. O2 binding constant of AsHbC1D1 (1.30 µM$^{-1}$) is similar to Chironomus thummi thummi Hb [39] and the values of AsHbC1D5 (0.86 µM$^{-1}$) is more like to Mb2 of carp [26] (Table 2).

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When comparing the CO-ligand binding properties of the recombinant domains we see differences between themselves as well as with the native Ahb molecule. Indeed AhbC1D1 shows monophasic CO binding kinetics, while AhbC1D5 shows a double exponential relaxation. This points to a heterogeneity of the AhbC1D5 domain and may point to the existence of two conformations. It is noteworthy that the CO association rate of AhbC1D1 is 1.3-fold higher than AhbC1D5 (Table 2). Furthermore, from the geminate rebinding, we can conclude that there is a different fraction of geminate recombination for the proteins measured (Fig. 8).

Scott et al. studied the effect of point mutations in different positions of SWMb and compared the kinetic values of wild type and mutated proteins [35]. Some mutations in SWMb are in the same position as in AhbC1D1 or AhbC1D5 and are listed in Table 4. As an example, in position 65, the residue is Gly in SWMb and Ile in AhbC1D1. In Table 4, the kinetic values of mutation Gly to Ile in SWMb are compared with that of AhbC1D1. The comparison of the kinetic values of the recombinant domains with SWMb shows that $K_O$ and $F_{gem}$ of AhbC1D1 are more close to the wild type myoglobin than those of AhbC1D5. Mutation in the second shell of distal residue, such as Val66Gly (smaller residue) increases the $F_{gem}$ from 0.47 to 0.58 which is finally similar to $F_{gem}$ of AhbC1D1 (0.57). This also affects the $K_O$ value and increases it from 1.14 to 1.79 $\mu$M$^{-1}$ while the other mutation in this shell (Thr69Arg) does not vary the $F_{gem}$. Moreover, the point mutation Gly65Ile in the wild type SWMb changes the $K_O$ of SWMb (from 1.14 to 0.52 $\mu$M$^{-1}$) closer to the $K_O$ of AhbC1D5 (0.86 $\mu$M$^{-1}$) while the $F_{gem}$ does not change significantly (from 0.47 to 0.49). Hence, it shows that the position 65 may have a significant role in $K_O$. In other words, changing a small residue to a bigger one decreases the $K_O$ value by 0.62 $\mu$M$^{-1}$. The other mutation in the distant place of heme such as Gln8Val modifies the $K_O$ of SWMb from 1.14 to 0.49 $\mu$M$^{-1}$ and closer to $K_O$ of AhbC1D5 (0.86 $\mu$M$^{-1}$) (Table 4). It should be considered that as the tertiary structure of the domains is not available, the implementation of the functional and structural relationship may not be totally accurate.

Furthermore, as it was mentioned above, the value of $k_{entry}$ is defined experimentally as the rate constant for the ligand entry into the Hb. It is calculated as the observed bimolecular rate constant ($k_{CO}$) divided by the observed total fraction of the internal rebinding ($F_{gem}$) (Eq. (6)). As it was also shown in Table 4, $k_{entry}$ for AhbC1D1 (5.84 $\mu$M$^{-1}$ s$^{-1}$) is 6-times less than the value of SWMb, whereas the value for AhbC1D5 (69.13 $\mu$M$^{-1}$ s$^{-1}$) is twice as big as the value of SWMb (34 $\mu$M$^{-1}$ s$^{-1}$). This means that the two domains show variant ability for the ligand to enter the protein compared to SWMb. The rate of ligand entry in the AhbC1D5 is higher than that in the SWMb and shows that the ligand can more easily enter the heme pocket of the domains. The lower $k_{entry}$ of the native Ahb confirms the closer heme-pocket structure seen using EPR. It must be noted that the values for SWMb are measured based on O$_2$ as the ligand, whereas we have used CO as ligand.

### Table 4

<table>
<thead>
<tr>
<th>MUTATIONS</th>
<th>HbC1D1</th>
<th>heme pocket</th>
<th>HbC1D5</th>
<th>heme pocket</th>
<th>HbC1D1</th>
<th>heme pocket</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1.14</td>
<td>12.12</td>
<td>0.47</td>
<td>5.7</td>
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<tr>
<td>AhbC1D1</td>
<td>10.5 ± 1.6</td>
<td>8.1 ± 0.8</td>
<td>1.30 ± 0.18</td>
<td>55 ± 0.07</td>
<td>0.57 ± 0.01</td>
<td>31.35 ± 3</td>
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<td>V66C</td>
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<td>14</td>
<td>1.79</td>
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<td>13.3</td>
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<td>6.3</td>
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<tr>
<td>AhbC1D5</td>
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<td>15.3 ± 2.1</td>
<td>0.86 ± 0.05</td>
<td>61.07</td>
<td>0.23 ± 0.01</td>
<td>14.03 ± 0.7</td>
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<td>32</td>
<td>0.52</td>
<td>13.5</td>
<td>0.49</td>
<td>6.6</td>
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<td>9.5</td>
<td>0.43</td>
<td>3.9</td>
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</table>

* The kinetic values of the SWMb and its mutants are measured based on O$_2$ as the ligand, whereas those of the recombinant protein are measured based on CO.
The rates of ligand exit from the interior of the protein or \( k_{\text{escape}} \) for AsHbC1D1 and AsHbC1D5 are 3.7- and 7.5-folds, respectively, higher than that of SWMb, which indicates that the ligand escapes faster from the inside of the domains than SWMb. To find the possible structural description for this behavior, we considered that ligands in myoglobin, are trapped in a “webbing” pocket of the distal site, and are surrounded by residues 28, 29, 32, 68, and 107 [35]. The size of this pocket determines the ligand entry equilibrium constant of the protein \( (k_{\text{entry}}) \), which is mathematically defined as \( k_{\text{entry}} / k_{\text{escape}} \). In Table 5, residues of these certain positions in AsHbC1D1, AsHbC1D5 and reference SWMb, as well as the corresponding values are summarized. As shown in the table, the smaller the residues, the lower the \( k_{\text{entry}} \) hence, less ligand entry into the protein. If we compare the residues, we encounter that the residue in position 68 is the same in all proteins, and the size of residues in positions 28 and 29 in the recombinant proteins (Val and Phe) are almost equal with the size of corresponding residues in SWMb (Ile and Leu). Therefore, the difference in \( k_{\text{entry}} \) values is related to the size of the residue in positions 32 and 107. More specifically, in AsHbC1D1 these positions are occupied by smaller residues (Val and Ser, respectively), where the ligand entry constant is the lowest (0.24 M\(^{-1} \)). In contrast, in SWMb due to the large size of residues in these two positions (Leu and Ile, respectively), the \( k_{\text{entry}} \) is the higher (5.39 M\(^{-1} \)). Moreover, in AsHbC1D5, the size of the residues and the \( k_{\text{entry}} \) Value (1.47 M\(^{-1} \)) are moderate. This is in accordance with Scott’s proposal [35]. In addition to the size, the nature and orientation of the residues play an important role in the ligand entry and binding to the proteins. To further elucidate this, more structural and mutational analysis is necessary.

As given in Fig. 9 and Table 3, the direct electrochemical measurements reveal variation in the reduction potential \( (E_{1/2}) \) of the studied proteins. Interestingly, the reduction potential for AsHb with 18 globin domains \( (E_{1/2} = -0.13 \, V \text{ versus SHE}) \) is the highest, whereas the value of AsHbC1D1 (−0.16 V), existing majorly in tetramer quaternary structure (Fig. 5), lies in between those of AsHb and the single domain AsHbC1D5 (−0.19 V). Thus, the higher number of globin domains stabilizes the iron in the heme in the Fe\(^{2+} \) state, i.e. 18 globin domains stabilize Fe\(^{2+} \) on about 1.5 kcal/mol \((\Delta_G^{\text{RT}} = -nF\Delta E)\) compared to the single domain. The latter can be important for minimization of metheglobin (Fe\(^{3+} \)) formation, which is unable to bind molecular O\(_2\).

### 5. Conclusion

To provide explicit answers to the above-mentioned questions, we cloned, expressed and purified two different recombinant globin domains in *E. coli* and purified the native Hb from the frozen animal. We utilized electron paramagnetic resonance, laser-flash photolysis and redox chemistry to investigate the physicochemical characteristics of two recombinant globin domains (AsHbC1D1 and AsHbC1D5) and the native one (AfHb). Particularly, we focused on the comparison of the ligand binding kinetics, heme pocket structure and redox potential. UV-visible spectroscopy and analytical gel filtration of the recombinant proteins indicated single-heme globin-like proteins, which mediate ligand binding. However, there were difficulties in stability of AsHbC1D5 in vitro. Moreover, the gel filtration indicated that there is equilibrium of tetramer and monomeric structure in AsHbC1D1, while the AsHbC1D5 just appears in monomeric structure.

Continuous Wave EPR showed that the heme pocket structure of the AfHb is more closed than the reference myoglobin. Furthermore, the results of difference pulse voltammetry indicated a difference between the redox potential for recombinants and the native protein, emphasizing that the more the globin domains, the higher the redox potential. In order to study the kinetics in more details, we calculated the association rate of CO binding together with geminate rebinding parameters in pseudo first order condition and the association and dissociation of O\(_2\) in double replacement condition. The results showed that the AsHbC1D1 has monophasic binding to CO, while the CO association to AsHbC1D1 and AfHb is biphasic. It is also indicated that AsHbC1D1 has a slightly higher oxygen affinity than AsHbC1D5. It shows that they have a diverse role in ligand binding. When studying the full native molecule using ligand binding kinetics, we are faced with a large heterogeneity due to the presence of at least 18 globin domains. However the overall measurements would support an average oxygen affinity.

Taken into account the fact that *Artemia* live in circumstances of variations in O\(_2\) partial pressure due to difference in salinity, we would have expected a larger oxygen affinity. For example, it was reported that Ascaris Hb [41-42], due to its unique organization of the B107yr and E7Gln, has a lower O\(_2\) dissociation rate and hence a higher affinity for O\(_2\) to support life under low oxygen pressures. Hence, our results demonstrate that the AfHb in contrast to Ascaris Hb supplies the O\(_2\) requirement just by increasing the number of the ligand binding domains not by increasing the O\(_2\) affinity. Indeed, the affinity is similar to SWMb.

### Translucency documents

The Transparency documents associated with this article can be found, in online version.

### Acknowledgments

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### References


J. Heip, L. Moens, M. Joniau, M. Kondo, Ontogenetical studies on extracellular hemoglobins of Artemia salina, Dev. Biol. 64 (1978) 73–81.


