Oxygen-Binding Characteristics of Three Extracellular Haemoglobins of *Artemia salina*

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The oxygen-binding characteristics of the three extracellular haemoglobins of brine shrimp (*Artemia salina*) were studied *in vitro* by using highly purified preparations. Haemoglobin I is induced last in the development of brine shrimps when functional gills are formed. It has the lowest oxygen affinity (p_{50} 5.34 mmHg), an intermediate Bohr effect (ϕ -0.09 at 20°C) above pH 8 and a temperature-sensitivity (ΔH -44.8 to -45.6 kJ/mol at pH 8–9) comparable with those observed with other invertebrate haemoglobins [Weber & Heidemann (1977) *Comp. Biochem. Physiol. A* 57, 151–155]. Haemoglobin II, which is the first to be induced, soon after hatching of nauplius larvae, persists generally throughout the whole adult life. It has an intermediate oxygen affinity (p_{50} 3.7 mmHg), the highest Bohr effect (ϕ -0.21 at 20°C) above pH 8 and a similar temperature-sensitivity (ΔH -46.0 to -54.8 kJ/mol at pH 8–9) as haemoglobin I. However, haemoglobin III, which is induced second several hours after the induction of haemoglobin II but disappearing from the haemolymph in the middle of adult life, has the highest oxygen affinity (p_{50} 1.8 mmHg), the lowest Bohr effect (ϕ -0.03 at 20°C) above pH 8.5 and a high resistance against temperature variation between 10 and 25°C at pH 8.5–9 (ΔH -22.6 to -23.0 kJ/mol). At pH 7.5–8, haemoglobin III exhibits a similar temperature-sensitivity under 30°C as do other haemoglobins. All three haemoglobins have a rather low co-operativity, with Hill coefficients (h 1.6–1.9 at pH 8.5), which are dependent on both pH and temperature. The highest co-operativity was observed at 20°C and pH 9 for haemoglobins I and II, whereas it was at 27°C and pH 8.5 for haemoglobin III. Thus the oxygen-binding behaviour of haemoglobin III *in vitro* is significantly different from those of haemoglobins I and II and indicates possibly its specific physiological role *in vivo* in the adaptive process in the natural environment.

The existence of haemoglobin in the brine shrimp *Artemia salina* was recognized by Claus (1886) and was later confirmed by Lockhead & Lockhead (1941). Three chemically distinct haemoglobins (I, II and III) are present in the haemolymph of adult *Artemia* (Bowen *et al.*, 1969; Moens & Kondo, 1976, 1977, 1978) and are induced sequentially during post-larval development (Heip *et al.*, 1977, 1978a). This ontogenetically controlled synthesis of *Artemia* haemoglobins occurs at any salinity (defined as a molarity of NaCl) between 0.08 and 1.53 M with oxygen concentrations above approx. 0.09 mm (Heip *et al.*, 1977, 1978a). Furthermore, Hb-III could be specifically re-induced in adults that normally lack this haemoglobin by lowering the

oxygen concentration to 0.04–0.06 mm and could be removed by increasing the oxygen concentration to 0.17–0.21 mm (Heip *et al.*, 1977, 1978b). These observations suggest that the three *Artemia* haemoglobins have physiologically different functions. Therefore we have examined *in vitro* various aspects of the oxygen-binding characteristics of three *Artemia* haemoglobins and the results are reported in the present paper. A preliminary report has been presented (Van den Branden *et al.*, 1978).

Experimental

Preparation of haemoglobin samples

Abbreviations used: Hb, haemoglobin; p_{50} , partial oxygen pressure at 50% oxygenation of haemoglobin; ϕ , Bohr factor defined as $\Delta \log p_{50} / \Delta \text{pH}$; ΔH , overall heat of oxygenation defined as $-2.303 R (\Delta \log p_{50} / \Delta T^{-1})$, where R and T are the gas constant and absolute temperature respectively.

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Adult *Artemia salina* (San Francisco race) grown for 5–6 weeks in 80-litre aquaria (Heip *et al.*, 1978a) were used to isolate the extracellular haemoglobins. Purification of individual haemoglobins was carried out as described previously (Moens & Kondo, 1977, 1978) and the purified samples were dialysed for 20 h at 3°C against 0.1 M-sodium borate buffer (pH 8, 8.5

or 9) or 0.05M-sodium phosphate buffer (pH 7, 7.5, or 8)/0.6M-NaCl/0.02M-KCl of appropriate pH. The dialysed samples were frozen at -70°C until use. The haemoglobin concentrations estimated from the absorption coefficient at 412nm ($A_{1\text{cm}}^{1\%}$ 47.7) (Moens & Kondo, 1977, 1978) were 1.5, 6.4 and 2.1 mg/ml for Hb-I, -II and -III respectively.

Oxygen equilibrium determination

The progress of the oxygenation of haemoglobin was followed by determining the A_{436} by using the oxygen-diffusion-chamber method developed by Sick & Gersonde (1969). Data in the present paper were based on at least five independent determinations for each point.

Materials

Encysted dormant gastrulae of *Artemia salina* were obtained from Metaframe (San Francisco Bay Brand, Newark, CA, U.S.A.). The diffusion chamber, made as described by Sick & Gersonde (1969), was constructed by Eschweiler Co. (Kiel, Germany). Pure oxygen and nitrogen gases were from Air Liquide (Deurne, Belgium). All other materials were the same as described previously (Moens & Kondo, 1976, 1977, 1978; Heip *et al.*, 1978a).

Results

Purification of *Artemia* haemoglobins

Three *Artemia* haemoglobins (I, II and III) were extensively purified from the adult haemolymph by the method described elsewhere (Moens & Kondo, 1977, 1978). Fig. 1 demonstrates the purified haemoglobin samples identified by cellulose acetate electrophoresis.

Oxygen equilibria and Hill coefficients

The oxygen-equilibrium curves determined for three *Artemia* haemoglobins in 0.1M-borate buffer (pH 8.5) containing 0.6M-NaCl and 0.02M-KCl at 25°C are shown in Fig. 2(a). These *Artemia* haemoglobins exhibited significantly different oxygen affinities, with respective p_{50} values for Hb-I, -II and -III of 5.34 (712), 3.7 (493) and 1.8 (240) mmHg (Pa) (Fig. 2a). Studies on the effects of pH and temperature on oxygen-equilibrium curves of Hb-II indicated that the pH had more influence on the oxygen equilibria at higher than at lower oxygen saturation of haemoglobin (Fig. 2b). This suggests that the degree of oxygenation of haem groups is an important factor in causing possible conformational changes, which lead to increased co-operativity. This view is supported by the Hill plots (Fig. 3). On the other hand,

Fig. 1. Electrophoretic analysis of *Artemia* haemoglobins on cellulose acetate strips

Adult haemolymph and purified individual haemoglobins were analysed by cellulose acetate electrophoresis, and protein bands were stained with 0.5% Amido Black (Moens & Kondo, 1976). Samples: 1, haemolymph; 2, Hb-I; 3, Hb-II; 4, Hb-III; 5, bovine serum albumin (BSA) and human adult haemoglobin (Hb A).

changes in the oxygen equilibria of Hb-II caused by varying the temperature were independent of the degree of oxygenation (Fig. 2c).

Hill plots for all three haemoglobins at pH 8.5 and 25°C (Fig. 3) were non-linear. For Hb-I and Hb-II Hill coefficients (h) greater than unity were obtained at high oxygen partial pressures, whereas at low oxygen partial pressures the slopes were nearly equal to unity. The curve for Hb-III was different from the other two. The Hill coefficients at high oxygen partial pressures were estimated to be 1.62, 1.92 and 1.58 for Hb-I, -II and -III respectively. Thus the degree of co-operativity in oxygen binding *in vitro* was not greatly different for the three *Artemia* haemoglobins, possibly owing to their nearly identical molecular structures (Moens & Kondo, 1976, 1977, 1978).

The effects of pH and temperature on Hill coefficients are illustrated in Fig. 4. For Hb-I and -II, the Hill coefficients increased as the pH changed from 7–7.5 to 9–9.5 without exhibiting any maximum (Figs. 4a and 4b). By contrast, Hb-III had maximal Hill coefficient at pH 8.5, and lower h values were

Fig. 2. Oxygen equilibria of *Artemia* haemoglobins

(a) Oxygen-equilibrium curves of three individual *Artemia* haemoglobins determined in 0.1 M-borate buffer (pH 8.5)/0.6 M-NaCl/0.02 M-KCl at 25°C. Δ , Hb-I; $\bullet$, Hb-II; $\circ$, Hb-III. Taken with permission from Van den Branden *et al.* (1978). (b) Effect of pH on oxygen-equilibrium curve of Hb-II determined as in (a) except that pH values of the buffer were changed to pH 8 (Δ), pH 8.5 ($\bullet$) and pH 9 ($\circ$). (c) Effect of temperature on oxygen equilibrium curve of Hb-II determined as in (a) except that temperature was changed to 20°C ($\circ$), 25°C ($\bullet$) and 30°C (Δ). p_{O_2} was expressed in mmHg.

Fig. 3. Hill plots of oxygen equilibrium of *Artemia* haemoglobins

The fractional saturation, Y , at each partial pressure of oxygen was calculated from the data of Fig. 2(a). Δ , Hb-I; $\bullet$, Hb-II; $\circ$, Hb-III.

observed on both sides of the pH range (Fig. 4c). Furthermore the maximal co-operativity was obtained at about 20°C for Hb-I and -II (Figs. 4d and

4e), whereas that for Hb-III was about 25–27°C (Fig. 4f).

Effects of pH and temperature on oxygen affinity

The effects of pH variation on the p_{50} of three *Artemia* haemoglobins at 10, 20 and 30°C are shown in Fig. 5. Both Hb-I and -II exhibited a Bohr effect at temperatures between 10 and 30°C in the range pH 8–9 (Figs. 5a and 5b), this pH value being close to the pH of *Artemia* haemolymph (D'hondt, 1977). Hb-III showed a slight Bohr effect at above pH 8.5 and below this pH a reversed Bohr effect at 10 and 20°C, whereas at higher temperature (30°C) it behaved like Hb-I and -II (Fig. 5c). The Bohr factors estimated at 20°C in the pH range 8–9 were -0.09 , -0.21 and -0.03 for Hb-I, -II and -III respectively. In general, the Bohr effect observed by *Artemia* haemoglobins seem to be rather small in absolute terms.

Fig. 6 illustrates the temperature effects on oxygen affinities of three *Artemia* haemoglobins. The temperature– P_{50} regressions (Fig. 6) were used to calculate the overall heat of oxygenation, which included a contribution of the heat of solution of oxygen (Weber, 1975). For Hb-I (15–35°C) and Hb-II (15–40°C), apparent ΔH values were found to be between -44.8 and -45.6 , and between -46.0 and -54.8 kJ/mol, respectively, in the pH range 8–9 (Figs. 6a and 6b). However, Hb-III exhibited a high insensitivity to temperature (10–25°C) in the pH

Fig. 4. Effects of pH and temperature on Hill coefficient

In each case, the Hill coefficient (h) was calculated from the steepest slope of individual Hill plots as shown in Fig. 3. Effects of pH on h values at 25°C of Hb-I (a), Hb-II (b) and Hb-III (c): 0.05M-phosphate buffer/0.6M-NaCl/0.02M-KCl was used for pH 7 and 7.5 and 0.1M-borate buffer (as in Fig. 2) for pH 8, 8.5, 9 and 9.5. Effects of temperature on h values at pH 8 (Δ), 8.5 ($\bullet$) and 9 ($\circ$) of Hb-I (d), Hb-II (e) and Hb-III (f).

range 8.5–9, having a ΔH value of -22.6 to -23.0 kJ/mol, whereas at lower pH (7.5–8), it behaved like Hb-I and -II in the temperature range 15–30°C, having a ΔH value of -53.6 to -54.8 kJ/mol (Fig. 6c).

Discussion

A few comments should be noted here on determination of the oxygen equilibrium by using the

oxygen-diffusion chamber of Sick & Gersonde (1969). Neither varying the NaCl concentration from 0.2 to 1.0M in 0.05M-sodium phosphate buffer (pH 7.5)/0.02M-KCl at 20°C nor decreasing the haemoglobin dilution from 4- to 8-fold significantly changed the oxygenation of *Artemia* haemoglobin (D'hondt, 1977).

The results described in the present paper demon-

Fig. 5. Effect of pH on p_{50} of *Artemia* haemoglobins. Bohr effects of Hb-I (a), Hb-II (b) and Hb-III (c) were illustrated at different temperatures, 10°C (Δ), 20°C (●) and 30°C (○). Experimental conditions were as in Figs. 2-4.

Fig. 6. Effect of temperature on p_{50} of *Artemia* haemoglobins.

Temperature effects on p_{50} of Hb-I (a), Hb-II (b) and Hb-III (c) were measured at pH 7.5 (▼), 8 (○), 8.5 (●) and 9 (Δ). Experimental conditions were as in Figs. 2-4.

strate clearly the distinct characteristics of the three *Artemia* haemoglobins with regard to both their oxygenation and their chemical nature (Moen & Kondo, 1977, 1978). Hb-I was found to be induced in the haemolymph of brine shrimps with functional gills to a detectable extent, as shown by cellulose acetate electrophoresis (Heip *et al.*, 1977, 1978a). This coincidence in development might be reflected by the characteristics of Hb-I, in that it has a low

oxygen affinity and a relative small Bohr effect at about the pH of *Artemia* haemolymph (i.e. 8.5). Transient variations in pH caused by acid metabolites in the gill may disturb the oxygenation of haemoglobin (McCutcheon, 1936). Further, Hb-I becomes a major haemoglobin in old females (2-3 months old) and could often be the single haemoglobin species present in the haemolymph of very old females; this contrasts with old males, where concentrations of Hb-I become negligible in the haemolymph (Heip *et al.*, 1977, 1978a). Further studies will be needed to clarify the possible correlation between Hb-I induction and gill formation as well as the observed sex-dependence of Hb-I content in old animals.

Hb-II has an intermediate oxygen affinity among the three *Artemia* haemoglobins, but exhibits the

highest Bohr effect at about the physiological pH of *Artemia* haemolymph, indicating a possible function in oxygen transport before the gills are formed in young animals. Hb-II was shown to be the first haemoglobin induced in *Artemia* and, except for very old females, it remains the major species present in the haemolymph during whole life cycles of both sexes (Heip *et al.*, 1977, 1978a). Thus Hb-II appears to play a significant role in respiration of brine shrimps.

By contrast, Hb-III is rather peculiar in that it was induced several hours after Hb-II, but generally it disappeared completely from the haemolymph during the 25th–35th days after hatching in both males and females (Heip *et al.*, 1977, 1978a). The concentration of Hb-III in the haemolymph was estimated to be approximately one-third of that of Hb-II. Furthermore, Hb-III could be specifically re-induced in the haemolymph of adult animals lacking Hb-III, to reach concentrations close to that of Hb-II, by decreasing oxygen concentrations in a culture medium from 0.17–0.21 mm (an optimal value) to 0.04–0.06 mm. The formation of Hb-III could then be reversed specifically by increasing the oxygen concentration (Heip *et al.*, 1977, 1978b). These earlier observations indicated that Hb-III may play specific roles in adaptive processes in the natural environment which are additional to other possible functions of this haemoglobin during the early developmental period. The oxygen-binding characteristics found here, that Hb-III has the highest oxygen affinity, the smallest Bohr effect and a much decreased temperature-sensitivity at around the physiological pH of *Artemia* haemolymph, appear to be compatible with the postulated function of this haemoglobin.

Finally, co-operativity in oxygenation of *Artemia* haemoglobin was dependent on the degree of oxygen saturation, the pH and the temperature, so agreeing with the properties of several other invertebrate haemoglobins (Waxman, 1971; Mangum & Carhart, 1972; Economides & Wells, 1975; Weber, 1975; Wood & Mosby, 1975; Bannister *et al.*, 1976; Wood *et al.*, 1976; Weber & Heidemann, 1977) and also in haemocyanins (Wood & Dalglish, 1973; Pearson & Wood, 1974).

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