Volume 247, number 2, 177–180 FEB 07018 April 1989

Endothermic oxygenation of hemocyanin in the krill *Meganyctiphanes norvegica*

O. Brix, S. Borgund, T. Barnung, A. Colosimo* and B. Giardina+

*Zoological Laboratory, University of Bergen, Allegaten 41, N-5007 Bergen, Norway, *Institute of Biochemical Sciences, University of Chieti, 31100 Chieti* and *+Department of Experimental Medicine and Biochemical Sciences, University of Rome (Tor Vergata), Via Orazio Raimondo, 00173 Roma, Italy*

Received 24 January 1989

The oxygen affinity of the hemolymph of the krill *Meganyctiphanes norvegica*, increases with temperature in the pH range 7.4 to 8.1 reflecting an endothermic overall heat of reaction. This striking feature may be of adaptive significance with respect to the feeding excursions of the animal, which at night reaches the warmer phytoplankton-rich surface layers, where the oxygen availability is reduced due to increased temperature and photorespiration.

Hemocyanin; Respiratory metabolism; Oxygen binding; (*Meganyctiphanes*)

1. INTRODUCTION

The Crustacean order Euphaciaoea constitutes the major part of the deep water zooplankton. Most species of Euphaciaoea migrate through a thermocline [1,2]. In particular, the epipelagic *Meganyctiphanes norvegica* descends at daytime down to 100–400 m, and ascends at night up to 100–0 m depth [2]. The species lives in the North Atlantic and Mediterranean waters [1] and is abundant throughout the year in Norwegian fjords [3,4]. The aim of the present study was to find a correlation between the vertical migration through the thermocline and the functional properties of the respiratory pigment. It has been reported, in fact, that the animal species facing large temperature variations often show an adaptive decrease in the temperature sensitivity of their blood [5–8], while the species living at more constant environmental temperatures may display a pronounced temperature sensitivity [7,9,10].

Correspondence address: O. Brix, Zoological Laboratory, University of Bergen, Allegaten 41, N-5007 Bergen, Norway

2. MATERIALS AND METHODS

Meganyctiphanes norvegica was collected during a four day cruise in the North Sea (approximately, 60°10′N 3°20′E) in the middle of August 1984. An IsaaKidd midwater trawl was used for trawling at night at a depth of 100–200 m (bottom depth: 260–280 m). The temperature was 9°C at 150 m depth, and the salinity was 35‰. At the surface the animals were carefully placed in a 200-l tank with aerated seawater at 10°C obtained from 30 m depth, and kept unfed in total darkness. Blood was sampled less than 48 h after capture. None of the krill were whitespotted, indicating their good health condition.

Immediately before blood sampling the animals were removed from seawater, blotted carefully dry and submerged in cold (4–8°C) mineral oil on ice. The blood was sampled in glass capillaries by micropuncture of the dorsal aorta after cutting posteriorly through the exoskeleton along the median line of the first abdominal segment [11]. The blood samples were pooled from 766 specimens (mean size 227–40 mm) into Eppendorf tubes and frozen. Prior to experiments, the frozen sample was thawed and centrifuged to remove remaining mineral oil and gelled blood. The blood was stored in iced water until use. It should be outlined that the small size of *Meganyctiphanes norvegica* sets natural limits to the type and the amount of experiments which can be performed. Thus only about 5 μl of blood could on average be drawn from each specimen.

Oxygen equilibrium was obtained spectrophotometrically at 364 nm at 5 and 10°C [12]. Serially connected Woesthoff gas mixing pumps delivered the equilibrating gas of constant carbon dioxide content of 0.01, 0.1, 0.2, 0.3, and 0.5% carbon dioxide. The Hill coefficient, $n_{1/2}$, and the oxygen tension at

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

half saturation, $p_{1/2}$, were calculated from the Hill plots. The change in enthalpy (ΔH) accompanying oxygenation was calculated from the integrated van 't Hoff equation:

$$\Delta H = -19.119\ T_1T_2/[T_1 - T_2]\ \Delta\log\ (P_{50})/1000,\ \text{kJ mol}^{-1}$$

The pH of oxygenated and deoxygenated blood was measured at 5 and 10°C equilibrated for 20 min with gas mixtures containing 0.01, 0.1 and 0.5% carbon dioxide. The pH values at 0.5 and 0.4% carbon dioxide were estimated from Astrup plots (log pCO_2 vs pH) in order to save material. The protein concentration was measured spectrophotometrically at 280 nm on diluted samples (10 μl blood + 10 ml of 0.9% NaCl) according to the method of Lowry [13] using the modification for microquantities of Rutter [14]. The oxygen-binding capacity (ctO_2) was estimated from the protein concentration assuming one oxygen-binding site per 75 kDa [15].

3. RESULTS AND DISCUSSION

The oxygen-binding curves of blood as obtained at pH 7.7 and at 5 and 10°C are shown in the form of Hill plots in fig.1. In the pH range 7.7–7.9, which has been reported to be the physiological pH range for crustacean blood [7,16], a relatively low [19] oxygen affinity (P_{50} = 50.1 and 18.2 mmHg, respectively, at 5 and 10°C, pH = 7.9) is coupled to a quite high cooperativity of oxygen binding, the Hill coefficient ranging between 2.5 and 3.0. As reported in fig.2 the Bohr shift is exceptionally large and not significantly different at the two temperatures [$\Delta\log(pO_2)/\Delta(pH)$ = −1.99 and −1.85, at 5 and 10°C respectively]. Both figs 1 and 2 show, at all pH values above 7.3, a marked increase in the oxygen affinity with increasing temperature, reflecting a strong endothermic overall heat of oxygenation (ΔH = 133.76 kJ mol^{-1}, pH 7.9).

To our knowledge this is a novel feature which has never been reported previously for respiratory pigments. Ligand binding, in fact, was reported as endothermic only in the case of the T states of trout Hb I [17] and of hemocyanin from *Levantina hierosolima* [18]. In the case of trout Hb I, binding of carbon monoxide to the T state of the molecule is characterized by a ΔH value of about 25.50 kJ mol^{-1} and, in the case of *Levantina hierosolima* hemocyanin, the ΔH value of oxygen binding to the T state was found to be about 12.54 kJ mol^{-1}. No example, however, is known of a fully endothermic oxygen binding to respiratory pigments. The ecophysiological significance of this effect may be related to the feeding excursions of the krill towards the warmer and phytoplankton-rich surface layers at night. The strong endothermic character of the oxygen binding, in fact, should be of great benefit for the krill when it ascends towards the upper layers, where the oxygen availability may be reduced due to the lower solubility at higher temperatures and to the photorespiratory activity of the phytoplankton, by safeguarding the postbranchial oxygen saturation. A similar behaviour has been observed in the blood from *Palaemon elegans* [19], while the temperature has very little influence on the oxygen affinity in the blood from the arctic krill, *Euphasia superba* [15], indicating a marked difference between the two krills. The oxygen affinity of the blood of *Meganyctiphanes norvegica* is very low compared to most other crustacean blood [20], but similar to that reported for *Euphasia superba* [15]. Such a low affinity coupled to a large Bohr shift and a marked cooperativity in crustacean hemocyanin has been interpreted in terms of adaptation to high levels of activity [20,21]. In this context it should be mentioned that ascending speeds up to 173 m/h have been reported for *Meganyctiphanes norvegica* [1], corresponding to about 1.4 bodylengths/s.

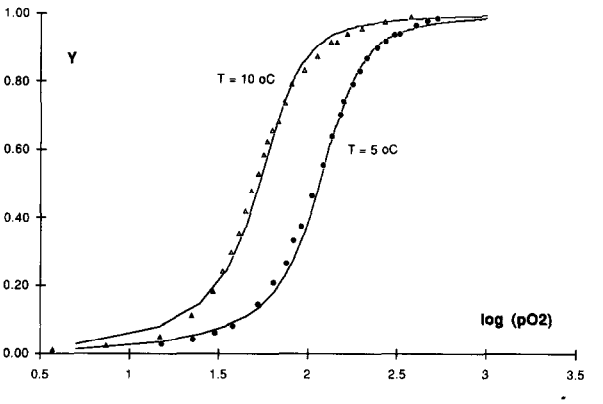

Fig.1. Oxygen binding isotherms of the hemolymph of *Meganyctiphanes norvegica* at 5 (closed symbols) and 10 (open symbols) °C, pH = 7.7.

It appears very informative and appropriate to try to correlate the observations above and the various contributions which, at a molecular level, are included in the overall thermal effects measured upon oxygen binding. These may be summarized as follows: (i) intrinsic heat of oxygenation; (ii) heat of ionization of oxygen-linked acid groups (endothermic) [22]; (iii) heat of solution of oxygen (exothermic: = 12.54 kJ mol^{-1})

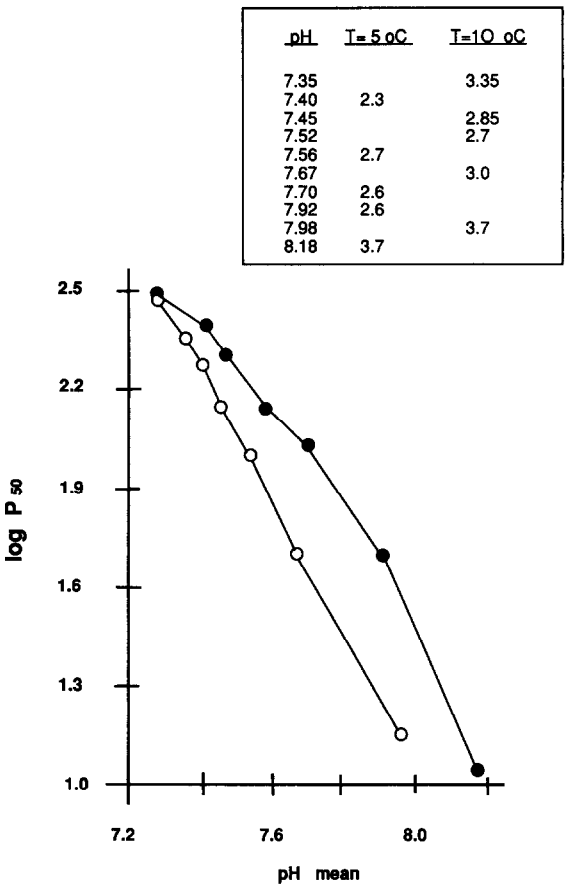

Fig.2. pH dependence of the Bohr effect of *Meganyctiphanes norvegica* hemolymph krill at two different temperatures (open circles, 5°C; filled circles, 10°C). The inset shows the Hill coefficients at the various pH values tested.

[22]; (iv) heat involved in the T⟶R allosteric transition (always endothermic in the cases examined so far) [22,23]; (v) heat of binding of other ions such as chloride and calcium.

Fig.3 reports the apparent overall dH of oxygen binding to *Meganyctiphanes norvegica* hemocyanin as a function of pH. The apparent ΔH decreases as the proton concentration is increased just in the range of pH in which the Bohr effect is operative and this behaviour is the opposite of what one would expect on the basis of the endothermic contribution of the Bohr protons. Therefore, leaving constant the (intrinsic) ΔH values of oxygen binding and solubilization, the only contribution which may be exothermic and thereby explaining the observed change of ΔH as a

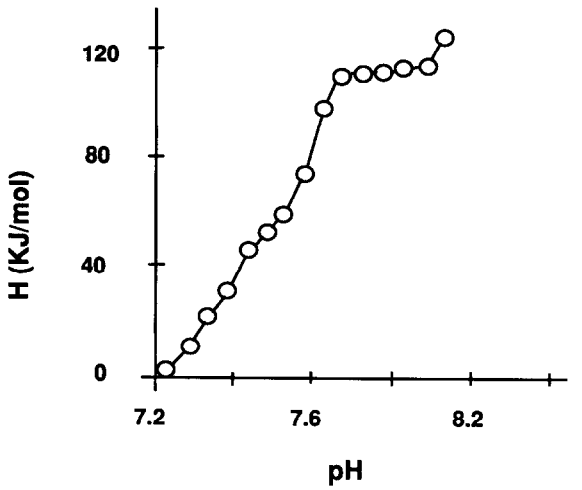

Fig.3. pH dependence of the apparent overall heat of oxygenation of *Meganyctiphanes norvegica* hemolymph.

function of pH, are the ones mentioned in points (iv) and (v). If we assume that the T⟶R conformational transition is endothermic as in most of the oxygen carriers tested so far, this implies an exothermic oxygen-linked binding of other ions whose relative weight becomes more and more important as the pH is decreased. It is worth recalling, in this respect, the case of *Panulirus interruptus* hemocyanin, in which the analysis of the temperature dependence of both ligand binding equilibria and kinetics within the context of the 2 state model [24], has provided an estimate of the thermodynamic parameters for the oxygen binding by the two states (T and R) and the ligand-linked conformational change. The most significant result emerging from that analysis is the strong exothermic character of the allosteric transition which amounts to a ΔH of -75.24 kJ per mol of hexamer. This is at variance with what is usually observed in vertebrate hemoglobins and namely in human Hb A, whose allosteric transition is characterized by a positive enthalpy change (ΔH = 71.06 kJ per mol of tetramer) [25]. On the basis of those results we cannot exclude the possibility that *Meganyctiphanes norvegica* hemocyanin is also characterized by an exothermic allosteric transition which might provide a basis for the observed pH dependence of the enthalpy changes. If this was the case, this hemocyanin would provide a unique example of thermodynamic properties exactly op-

posite to those normally observed, namely an endothermic oxygen binding coupled to an exothermic allosteric transition.

As a final conclusion, excluding any masking effect of the Bohr protons even in the presence of a large Bohr effect, the only possible explanation for the endothermic character of oxygen binding seems to be an intrinsic property of the macromolecule or a specific effect of other ions that still needs clarification. In any event, the unusual thermal properties of this hemocyanin remain per se a phenomenon of striking adaptive and evolutionary significance.

Acknowledgements: A.C. and B.G. acknowledge grants from the Italian MPI (40 and 60%).

REFERENCES

[1] Mauchlin, J. and Fisher, J.R. (1969) in: Advances in Marine Biology (Russel, F.S. and Younge, M. eds) vol.7, pp.1–454, Academic Press, London.
[2] Mauchlin, J. (1980) in: Advances in Marine Biology (Blaxter, J.H.S. et al. eds) vol.18, pp.3–677, Academic Press, London.
[3] Matthews, J.B.L. (1973) Proc. R. Soc. Edinburgh B67, 141–179.
[4] Wiborg, K.F. (1971) Fisk. Dir. Skr. Ser. Havunders 16, 10–35.
[5] Young, R.E. (1972) J. Exp. Mar. Biol. Ecol. 10, 193–206.
[6] Rutledge, S.C. (1981) Am. J. Physiol. 240, R93–R98.
[7] Jokumsen, A. and Weber, R.E. (1982) J. Exp. Zool. 221, 389–394.
[8] Brix, O., Bardgard, A., Cau, A., Colosimo, A., Condò, S. and Giardina, B. (1989) in press.
[9] Brix, O. (1983) Nature 303, 422–423.
[10] Brix, O. (1984) in: Comparative Physiology and Biochemistry. Section of International Union of Biological Sciences (Gilles, R. et al. eds) p.153, 27–31/8 1984, Liège, Belgium.
[11] Forward, R.B., jr and Fyhn, H.J. (1983) Comp. Biochem. Physiol. 74A, 301–305.
[12] Likkeboe, G., Johansen, K. and Maloy, G.M.O. (1975) J. Comp. Physiol. 104, 1–11.
[13] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[14] Rutter, W.J. (1967) in: Methods in Developmental Biology (Wilt, F.H. and Wessels, N.K. eds) pp.671–684, Academic Press, New York.
[15] Bridges, C.R., Savel, A., Stocker, W., Markl, J. and Linzen, B. (1983) in: Structure and Function of Invertebrate Respiratory Proteins (Wood, E.J. ed.) pp.363–356, Life Chemistry Reports, Harwood Academic Publishers, Chur, London, New York.
[16] McMahon, B.R. and Wilkes, J.C. (1983) in: The Biology of Crustacea (Mantel, L.H. ed.) vol.5, pp.289–372, Academic Press, New York.
[17] Wyman, J., Gill, S.J., Noll, L., Giardina, B., Colosimo, A. and Brunori, M. (1977) J. Mol. Biol. 109, 195–205.
[18] Er-El, Z., Shaklai, N. and Daniel, J. (1972) J. Mol. Biol. 64, 341–344.
[19] Morris, S., Taylor, A.C., Bridges, C.R. and Grieshaber, M.K. (1985) J. Exp. Zool. 233, 175–186.
[20] Weber, R.E. and Hagerman, L. (1981) J. Comp. Physiol. 145, 21–27.
[21] Redmond, J.R. (1968) in: Physiology and Biochemistry of Haemocyanins (Ghiretti, F. ed.) pp.5–23, Academic Press, London.
[22] Antonini, E. and Brunori, M. (1971) Hemoglobin and Myoglobin in their Reactions with Ligands, Elsevier/North-Holland, Amsterdam, London.
[23] Imai, K. and Tyuma, I. (1973) Biochem. Biophys. Res. Commun. 51, 52–58.
[24] Antonini, E., Brunori, M., Colosimo, A., Kuiper, H.A. and Zolla, L. (1983) Biophys. Chem. 18, 117–124.
[25] Imai, K. (1979) J. Mol. Biol. 133, 233.