A C T A U N I V E R S I T A T I S L O D Z I E N S I S
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OXYGEN AFFINITY OF CHIRONOMUS THUMMI THUMMI HAEMOGLOBINS*

The insect haemoglobin has been isolated from the fourth larval instar of Chironomus thummi thummi (Insecta, Diptera). This haemoglobin showing high degree of polymorphism was separated into monomeric and dimeric components. Parameters of oxygen affinity of monomeric, dimeric component mixtures and a crude haemoglobins extract were examined. A dithionite - ascorbate solution as an antioxidant system was used during the experiments. High oxygen affinities, an alkaline Bohr effect and any co-operativity of the oxygen binding in all the systems investigated were found. These results were discussed in physiological terms as the adaptation of the larvae to their changeable eutrophicated water environment. The mechanism of the action of the antioxidant system was also proposed.

INTRODUCTION

Haemoglobin (Hb) is widely distributed oxygen carrier protein which one can find in almost all vertebrates. Contrary to it, its presence in invertebrates is restricted to only some groups or to some representatives of a group. Hb is also found in such a large taxonomic group as the Insecta but only in a few species. Among them this of Chironomus thummi thummi (Diptera, Chironomidae) belongs to the best examined so far. Twenty years of intense research works showed its high heterogeneity - twelve different Hb components dissolved in haemolymph of a single larva [2, 14], its

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structural organization - five monomeric (mol. wt. ca. 15 000) and seven dimeric (mol. wt. ca. 32 000) components [10, 14] the known spatial structure of the CTT III component in different ligand states [11, 17], and its functional properties [1, 3, 24].

The data indicate the high oxygen affinity, the presence of an alkaline Bohr effect and the lack of co-operativity of oxygen binding in these Hbs [2, 8, 9]. Unfortunately, the mentioned high oxygen affinity of the Hbs produces some important difficulties in its correct measurements. The main problem is a maintenance of the Hbs at the native, functional reduce state. There are many ways to solve this difficulty: the using of reductants following a gel chromatography, the using catalase or (and) other special precautions. In our work we decided to use other reducing agents systems dithionite-ascorbate which makes possibility under strictly defined conditions to measure all important oxygen affinity parameters. This paper deals with the making progress with investigations of these parameters in Hbs showing the high oxygen affinity and the resolving the ways of this system action.

MATERIALS AND METHODS

Larvae of Chironomus thummi thummi were collected in a summer period (July-August) as described previously [14]. Only the fourth instar larvae were taken into account. Hb was isolated using the method described by W e b e r et al. [24] and L e y k o and O s m u l s k i [14]. Oxygen equilibria of the Hbs were determined using a spectrophotometrical method [1]. The Hbs solution was dissolved to concentration 0.2% using 0.2 $mol.l^{-1}$ phosphate or Tris-HCl buffer. Absorbance measurements were made with a Specord UV-VIS or a Spectrophotometer VSU-2G (Carl-Zeiss Jena) at 560 and 575 nm. Equilibration times after each addition of air portion were 5 min. Deoxygenation of the Hb solution was performed by passing argon through a tonometer for about one hour. Then 0.1 ml of sodium dithionite solution of appropriate concentration was dropped into the measuring chamber. Next, 0.1 ml of ascorbic acid solution was dropped. After each addition of the reductant solution argon was passed through a tonometer for about 15 min. The concentration of sodium dithionite and ascorbic acid in the Hb sample were 3.85 mg% and 7.70 mg% respectively. The experiments were carried out at room temperature (21-25^{o}C).

RESULTS

Our results of oxygen affinity measurments are shown in Figs 1-4 and are summarized in Tab. 1 where the data of other authors

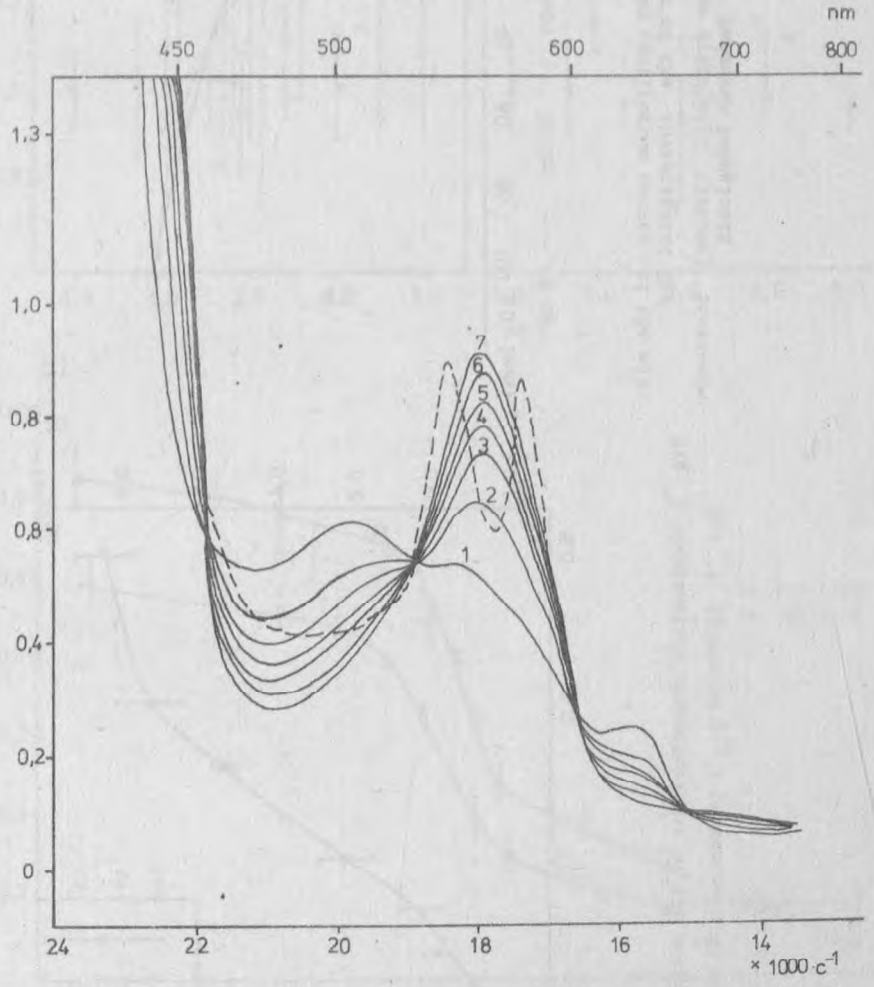

Fig. 1. Absorption spectra of a mixture of the monomeric components (-----) an oxy form spectrum in the presence of the reducing system; (———) 1 - MetHb, 2 - the same after ascorbate addition (5 min), 3-7 - the same after dithionite addition (2, 5, 7, 10, 15 min.)

Rys. 1. Widma absorpcyjne mieszaniny składników monomerycznych (-----) widmo formy utlenowanej w obecności układu redukującego; (———) 1 - MetHb, 2 - po dodaniu askorbinianu (5 min), 3-7 - po dodaniu podsiarczynu (2, 5, 7, 10, 15 min)

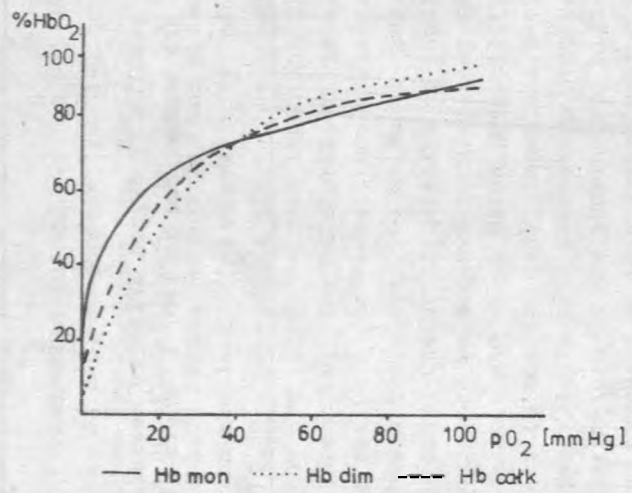

Fig. 2. Oxygen equilibrium curves of the mixtures of the investigated Hbs

Rys. 2. Krzywe dysocjacji tlenowej mieszanin badanych hemoglobin

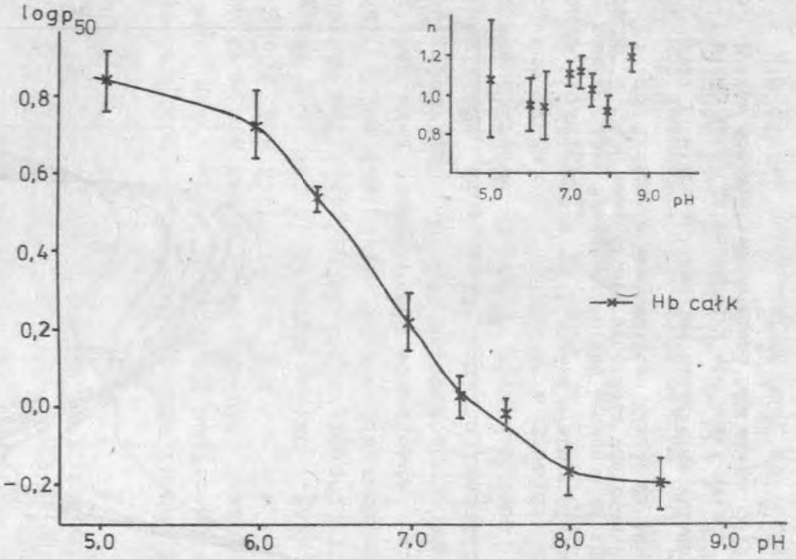

Fig. 3. Oxygenation characteristic of the mixture of the total Hbs

Rys. 3. Zależność p_{50} i parametru n Hb całkowitej od pH

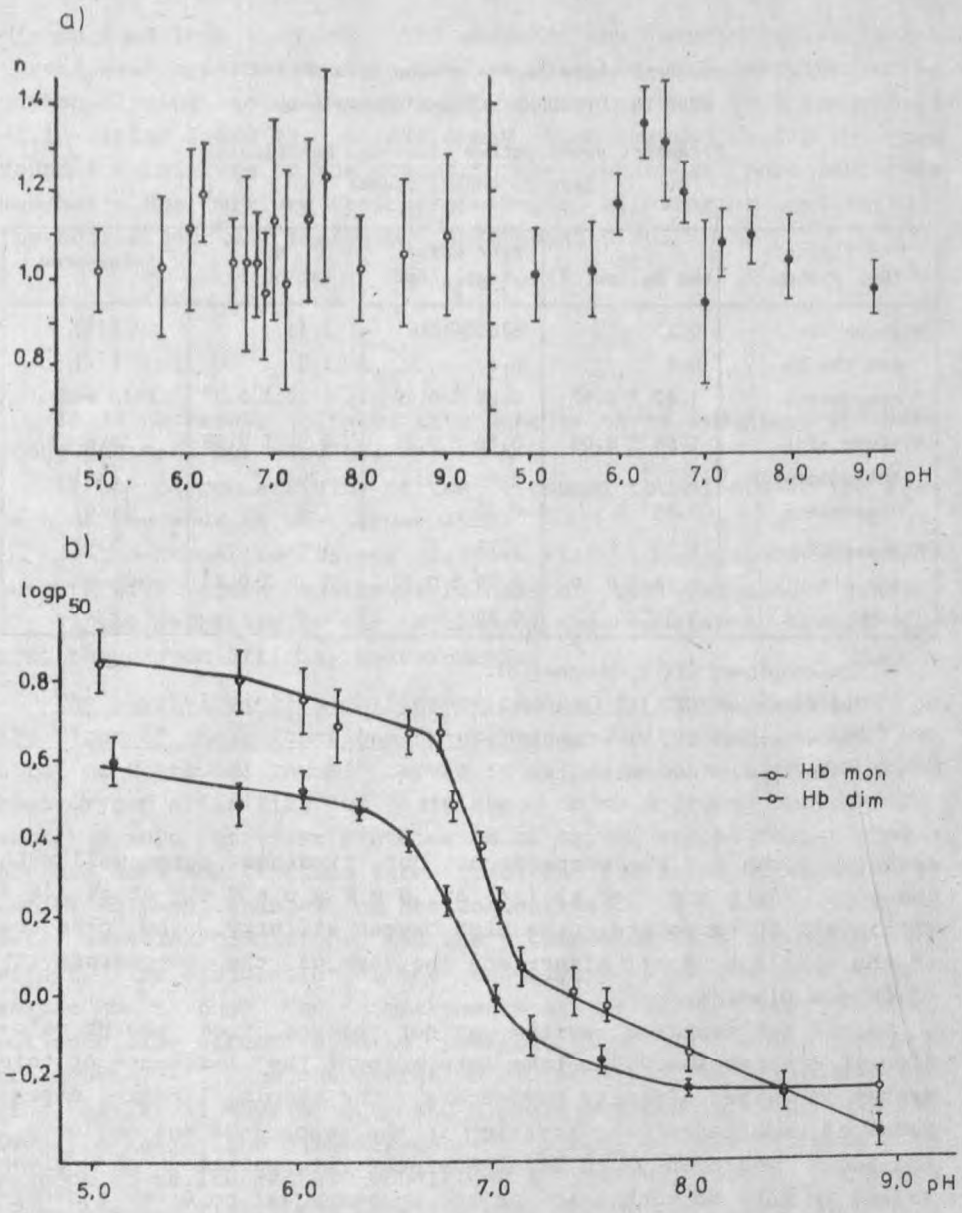

Fig. 4. Oxygenation characteristic of the mixtures of monomeric and dimeric Hbs
a) the pH dependence of the n parameter, b) the pH dependence of the p_{50} parameter

Rys. 4. Charakterystyka utlenowania mieszaniny Hb monomerycznych i mieszaniny Hb dimerycznych
a) zależność parametru n od pH, b) zależność parametru p_{50} od pH

Table 1

Parameters of the oxygen affinities of the C. thummi thummi haemoglobins

Parametry powinowactwa tlenowego hemoglobin larw C. thummi thummi

The kind of the system	P_{50} (mm Hg, pH 7)	Bohr effect $-\Delta \log p_{50}/\Delta pH$	n (at pH 7)	References
Mixture of	0.27	n.d.	1.15	[22]
all the Hb	0.5	n.d.	1.0	[7]
components	1.67 ± 0.37	0.70 ± 0.13	1.10 ± 0.07	this work
Mixture of	1.04 ± 0.09	0.80 ± 0.21	0.95 ± 0.19	this work
the monomeric	1.27[a]	0.0	1.0	[24]
components	0.63[b]	0.30	1.0	[24]
Mixture of	1.57	1.55	1.0	[9]
the dimeric	1.76 ± 0.19	1.29 ± 0.22	1.13 ± 0.23	this work
components	1.50[c]	0.79	1.0	[24]

[a] The component CTT I (monomeric).

[b] The component CTT III (monomeric).

[c] The component CTT VI (dimeric).

N o t e: n.d. - not determined.

are also given for the comparison. Our findings agree well with those of W e b e r et al. [24] or G e r s o n d e et al. [8,9] within all three points: the high oxygen affinity, the presence of the alkaline Bohr effect and the lack of the co-operativity of oxygen binding.

Since our reducing system was not removed from the Hb solution it was necessary to take into account the influence of this system on oxygen affinity parameters. The simple linear dependence of reductants concentration in the range 3-24 mg% on log p_{50} and log K was found, so after a linear interpolation of given values to the concentration of the system equal to 0 one can correct results.

During all the time of the experiment run the denaturation of the Hb was not observed although sometimes the full oxygen loading by the Hb was not possible (the α/β bands ratio decreased see also Fig. 1). The level of MetHb after the experiments fi-

nishing was less than 10%. The shape of the oxygen dissociation curves was hyperbolic (Fig. 2) showing non-cooperativity in the oxygen binding so values of a "h" parameter were in a range 0.95 -1.13 (Figs 3 and 4). An extremely high oxygen affinity was found for mixture of the monomeric Hbs, what is more, for this monomeric Hbs mixture the heterotropic allosterism was found - the Bohr effect was ($-\Delta \log p_{50}/\Delta pH$) equal 0.80.

DISCUSSION

It is necessary to take into account three important problems connected with our results:

1) the oxygen affinity of the C. thummi thummi Hbs in the respect of the mode of the larvae life;

2) the comparison of our results with those given by other authors with regard to the usefulness of our reducing system;

3) the mechanism of the action of the reductants system during the oxygen affinity measurements.

The physiological significance of the C. thummi thummi Hbs in the light of their functional properties. The hypoxic, benthic habitat of C. thummi thummi larvae is well connected with the found high oxygen affinities of their Hbs. On this ground one can suggest the role of these proteins as an oxygen store. This simple picture does not include three important facts: a circulation of the haemolymph, changes of Hbs concentration in the respect of water aeration conditions and the presence of the alkaline Bohr effect. The circulation of the haemolymph with the freely dissolved Hbs through the compartments of the larval body, though not specially effective makes possible the directional oxygen transport [24]. The concentration of the total Hb increases during the larval development and hypoxia periods in water [7, 14, 19-21]. Again, the dimeric/monomeric ratio of Hbs changes in a respect to environmental conditions so the best supply of oxygen is maintained [14]. The alkaline Bohr effect has two aspects: a site specific and a time specific [24].

The first view is a direct repercussion of the above mentioned compartmentation of the larvae and connected with it internal pH differentiation. The second is related to a periodic ventilatory behaviour of the larvae [19-21, 23]. The oxygen loading oc-

curs during the ventilation period followed by the intense oxygen consumption. Without the alkaline Bohr effect, the oxygen unloading would happen only at its extremely low tension in the tissues [22, 24]. Summarizing all these findings and conceptions we can solve the old question of the function of these Hbs: an oxygen store or a transporter? In the light of our present knowledge we can propose the following action of the Hbs system: it facilitates oxygen diffusion, transports it through the body segments, accumulates oxygen during the ventilation activity and releases it in the tissues of high metabolic activity and during the rest periods.

The oxygen affinity determination in the presence of the reducing system. Our results are consistent with those published previously by other authors, but generally show the lower oxygen affinities of C. thummi thummi Hbs [1, 8, 9]. This may be connected with the fact that some authors did not take into account the high autoxidation rate of these proteins. Other danger which can influence on the erroneously high values is an incomplete deoxygenation of a sample.

Our system have many possitive properties: 1) it does not create detectable amounts of hemo- and hemichromogenes; 2) it does not change the pH value of a medium (in the range ±0.05 of pH unit); 3) it excludes the difficult to define interactions of the Hb (or Mb) with other eventually added proteins (e.g. catalase) and 4) the stability of the reducing system in the pH range 6 - 9 makes possible the Bohr effect study. This system has also the negative properties of course: 1) it needs calculation of the special correction for the presence of the system in the investigated solution; 2) it makes possibility of the oxygen consumption by the system under specific experimental conditions so lower oxygen affinities may be received.

Reaction of the reducing system with C. thummi thummi Hbs. There are three aspects of the reaction of the reducing system with C. thummi thummi Hbs i.e. a reaction with dithionite anion, with ascorbate anion and a dithionite - ascorbate interaction. The mechanism of these reactions in the absence of oxygen is more simple and better known than those in its presence [18, 25]. Organic reducing agents can reduce an oxidized form of a haem protein in three fundamental ways: an electron transfer to the haem through an axial ligand of the porphyrin (it is suggested that dithio-

nite ion can react in this manner [5]), through the peripherical groups of the porphyrin or through the globin (the proposed way of the ascorbate ion reaction [18]).

T s u k a h a r a and Y a m a m o t o [18] reported that since the ascorbate anion is too large to enter into the haem pocket it must exist the globin-way of the electron transfer. Then the "strength" of the reducing properties of ascorbate may depend on a position and a kind of amino acid residues of the globin, particularly tryptophan or arginine residues. In fact, these suggestions were found confirmation on the basis of kinetic studies on the reduction of imidazolmetmyoglobin by ascorbic acid [18 and references cited therein]. The reaction of MetHb with ascorbate leads to reduced Hb and ascorbate radical. From two ascorbate radicals ascorbate anion and dehydroascorbate is formed. The mechanism of the anaerobic reduction of the haem proteins by dithionite is described by

$$S_2O_4^{2-} \rightleftharpoons 2SO_2^-$$

$$SO_2^- + \text{MetHb}(H_2O) \longrightarrow SO_3H^- + H^+ + \text{Hb}$$

so the effective reducing agent is a sulfur dioxide radical [25]. After an oxygen addition our system becomes more complicated and what is more not clearly defined. We can suppose on the basis of available literature the following scheme [4, 5, 6, 12, 15].

$$AH^- + O_2 \xrightarrow[\text{MetHb}]{H^+} A + H_2O_2 \quad (\text{Hb})$$

As an active intermediate superoxide radical is produced in the above process. Hydrogen peroxide reacts then with ferric and ferrous ion of the haem what gives hydroxyl radical and ferryl derivative of haemoglobin. The reaction of ferryl Hb with Hb and HbO_2 leads to ferri Hb generation [26]. In the presence of oxygen the radical anion monomer, SO_2^- may create hydrogen peroxide through a superoxide dismutation [25]. Thus MetHb will be formed if only the component of the system is used alone. The common addition of dithionite and ascorbate makes possible the interaction of sulfur dioxide radical with an ascorbate radical or with other intermediates.

Then ferryl derivative of Hb is not observed and a contribution of the ferric form is minimal. Unfortunately, details of these blocking reactions are not clear for us. We think that these

free radical reactions may be connected with the ease of the C. thummi thummi haemoglobins oxidation [14, 16]. Now, we try to clear up the reaction of ascorbic acid with different haem proteins (work in progress). There is still much to be learnt both about the well known reaction of dithionite anion as a reductor and about not so well understood reaction of ascorbate.

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POWINOWACTWO TLENOWE HEMOGLOBIN LARW CHIRONOMUS THUMMI THUMMI

Z larw czwartego stadium Chironomus thummi thummi wyizolowano białko oddechowe o właściwościach hemoglobiny. Białko to wykazuje wysoki stopień niejednorodności, gdyż metodami elektroforetycznymi i chromatografii jonowymiennej stwierdzono obecność 12 różnych składników w hemolimfie pojedynczej larwy. Zbadano parametry powinowactwa tlenowego mieszaniny składników monomerycznych, dimerycznych, a także mieszaniny wszystkich składników. Podczas wykonywania pomiarów zastosowano układ redukujący podsiarczyn sodu - kwas askorbinowy, ze względu na wysoką stałą szybkości samoutleniania badanych hemoglobin. We wszystkich zbadanych układach stwierdzono wysokie powinowactwo tlenowe, obecność alkalicznego efektu Bohra i brak kooperatywności wiązania tlenu. Wyniki te zostały przeanalizowane pod kątem fizjologicznego przystosowywania się larw do silnie zeutrofizowanego środowiska wodnego o zmiennych właściwościach tlenowych. Zaproponowano również mechanizm działania układu redukującego w warunkach tlenowych i beztlenowych.