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Effect of Organotin Compounds on Trout Hemoglobins

Anna Maria Santroni,* Donatella Fedeli,* Rosita Gabbianelli,*
Giovanna Zolese,† and Giancarlo Falcioni*,[1]

**Dipartimento di Biologia M.C.A., Università di Camerino, Camerino, Italy; and †Istituto di Biochimica, Facoltà di Medicina e Chirurgia, Università di Ancona, Ancona, Italy*

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The stability of trout hemoglobin was examined in the presence of some organotin compounds. Tributyltin chloride (TBTC) and triphenyltin chloride (TPTC) protect HbI most efficently from the oxidation. On the other hand, the same compounds accelerate the precipitation process in HbIV to a great extent. Parahydroxymercuribenzoate (PMB), an agent bloking free SH-groups of the protein, abolished the ability of TPTC to decrease the oxidation rate of HbI.

Key Words: **organotins; trout hemoglobins; oxygen binding; met-hemoglobin.**

The continuous use of organotin compounds in agriculture and industry brings about an increase of their concentration in the environment. Their presence in the air is due to agricultural spraying, volatilization from biocidal treatments etc., while in water it principally depends on their use in marine antifouling paint formulations and especially as stabiliser for PVC (1). Organotin compounds are known to exert toxic effect in humans and animals, but little is known about the molecular basis for these effects.

Organic derivatives of tin are much more toxic than their inorganic analogues; alkyltin compounds are generally more toxic than aryltin analogues. Toxicity passes through a maximum as the length of the n-alkyl group increases, and decreases thereafter (2,3). Maximum toxicities were observed for triorganotin compounds. In general, the toxicity of triorganotin compounds is believed to be due to their ability to bind to certain proteins, and the results obtained so far permit to propose that cysteine and histidine residues are involved. Rose and Aldridge (4) examined the interaction of these compounds with a variety of proteins and found that organotin compounds were bound to only a few. Included among these were cat and rat hemoglobins (5,6); other hemoglobins examined did not interact with these compounds, indicating that a highly specific binding site is required (7).

The aim of the present investigation is to study the effect that some organotin compounds have on trout hemoglobins and then on the oxygen transport of fresh water fish.

As the source of hemoglobin we used red blood cells from the rainbow trout *Salmo irideus*. It is known that these cells contain four hemoglobin components which have been extensively characterized and whose functional role is largely understood (8).

Hemoglobin components according to their anionic mobility have been called HbI, HbII, HbIII and HbIV. Two of these, HbI and HbIV, represent, respectively, about 20 and 60 % of the whole pigment and have very different oxygen-binding properties. HbI is characterized by the presence of cooperative phenomena and complete absence of the pH and organic phosphate effect while in HbIV oxygen affinity and cooperativity depend on pH and organic phosphates (Root effect). The structural and functional properties of HbI make it possible to satisfy the oxygen demand of the tissues; in contrast, HbIV permits to pump oxygen to the swim bladder.

In this study we used only HbI and HbIV and applied to them monobutyltin trichloride (MBTC), dibutyltin dichloride (DBTC), tributyltin chloride (TBTC) and triphenyltin chloride (TPTC) to examine the oxygen-binding properties and their stability.

MATERIALS AND METHODS

Organotin compounds were obtained from Aldrich. All reagents were of analytical grade.

Preparation of trout hemoglobin components was carried out as previously described (10). The desired amount of organotin compounds, dissolved in ethanol, was added to hemoglobin solution. Since all the organotin derivatives used here were dissolved in ethanol, control experiments were performed in this solvent. The rate of

[1] Correspondence to Prof. Giancarlo Falcioni, Department of M.C.A. Biology, University of Camerino, Via Camerini, 2, 62032 Camerino (MC), Italy. Fax: (39) 737 636216. E-mail: falcioni@cambio.unicam.it.

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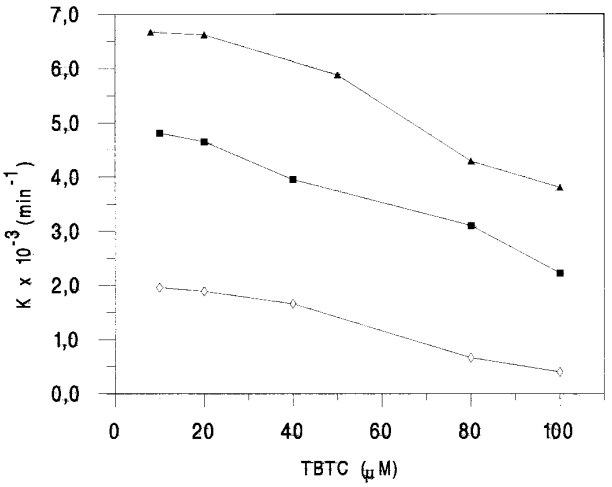

FIG. 1. Effect of TBTC concentration (μM) on the autooxidation rate constant of trout HbI at pH 6.7 (▲), pH 7.0 (■) and pH 7.7 (◇). Conditions: 0.1 M phosphate buffer; t = 30 °C. HbI = 0.8 mg/ml.

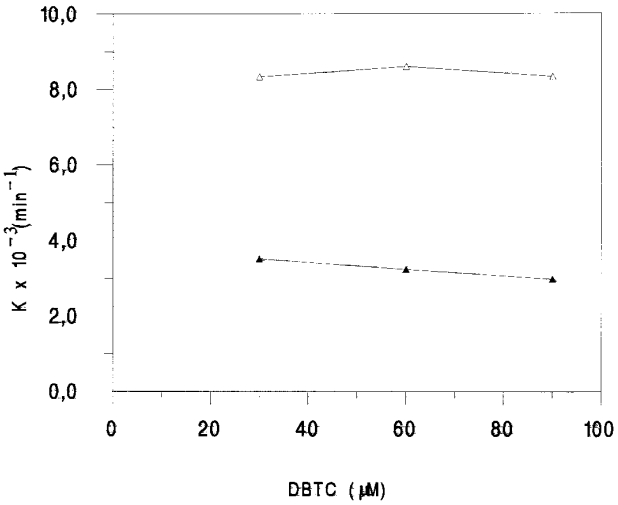

FIG. 2. Effect of DBTC concentration (μM) on the autooxidation rate constant of trout HbI at pH 6.7 (△) and pH 7.4 (▲). All other conditions as in Fig. 1.

met-Hb formation was followed in a Cary 219 spectrophotometer in the visible region; reference values (i.e. complete reduction and oxidation) were estimated by addition of, respectively, sodium dithionite and ferricyanide. Absorbance at 700 nm was followed as an index of turbidity to monitor the onset of hemoglobin precipitation. The concentration of PMB was determinated spectrophotometrically using $\epsilon_{230}^{1mM} = 16.9$.

Oxygen equilibrium curve were determined spectrophotometrically by the method of Rossi Fanelli and Antonini (11).

RESULTS

The oxygen equilibrium curve for HbI was practically unaffected by organotin compounds at concentrations up to 50 μM. Similarly, no influence was observed on the fractional saturation with oxygen of trout Hb IV in air (pO_2 = 155 mm Hg) as a function of pH (data not shown).

The effect of increasing concentrations of tributyltin chloride (TBTC) on the rate of HbI oxidation ($k=1/t_{1/2}$ where $t_{1/2}$ is the half-time of the process) measured at different pH values is reported in Fig. 1. The TBTC effect is very marked and it exhibits the same behavior at different pH. The presence of TBTC reduces the rate of oxidation of HbI by stabilizing the ferrous state (Fe^{2+}) of the protein. A similar protective effect, although less marked, was observed when the experiment was done in the presence of triphenyltin chloride (TPTC) (data not shown). When the experiment was done with 100 μM TBTC, the half-time of the process was more than doubled.

The effect of DBTC is reported in Fig. 2 it may be seen that this compound influences only slightly the rate of HbI oxidation. A completely different effect is observed when the oxidation rate is followed in the presence of MBTC. This organotin compound increases slightly the rate of oxidation (Fig. 3).

It was not possible to perform similar experiments with HbIV (the trout Hb component that is characterized by the presence of the Root effect) because the presence of organotin compounds in this case enhances protein denaturation. The influence that these organotin compounds have on the time of onset of HbIV precipitation, is shown in Fig. 4. TBTC and TPTC, the compounds most efficient to protect HbI from oxidation, are those that to a greater extent accelerate the precipitation process in HbIV. The precipitation begins immediately after adding the two monochloro derivatives while DBTC and MBTC, at least during the time of our experiment, do not modify the rate of precipitation.

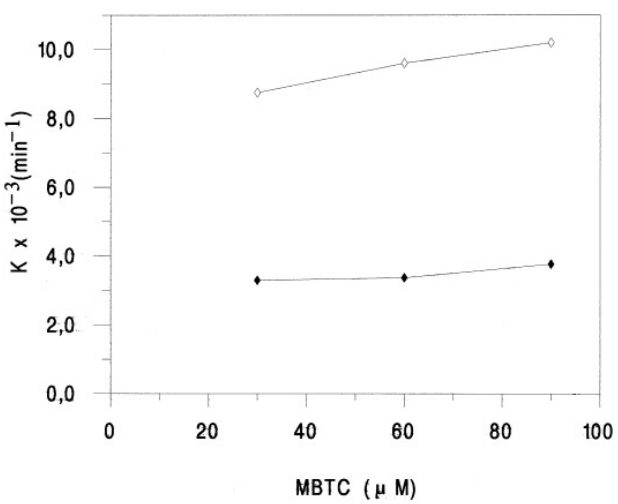

FIG. 3. Effect of MBTC concentration (μM) on the autooxidation rate constant of trout HbI at pH 6.7 (◇) and pH 7.4 (◆). All other conditions as in Fig. 1.

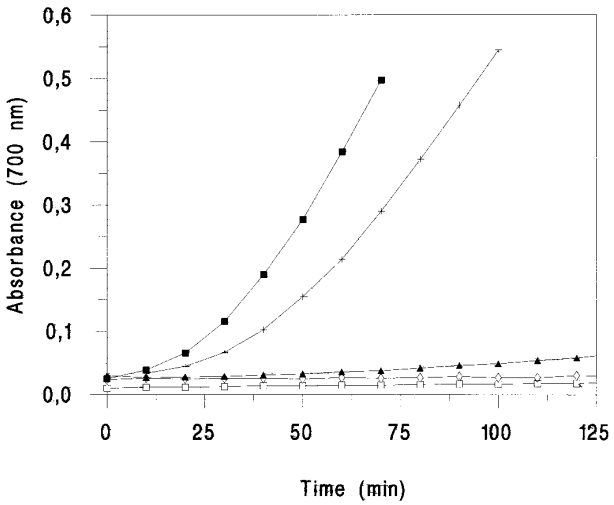

FIG. 4. Effect of some organotin compounds on the time of onset of HbIV precipitation. Control (□); MBTC (◇); DBTC (▲); TPTC (+); TBTC (■). Conditions: 0.1M phosphate buffer (pH 7.45); t 30°C; HbIV = 1mg/ml; organotin = 50μM.

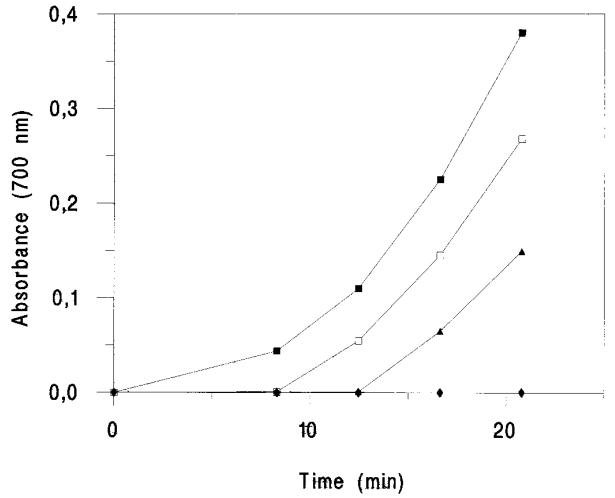

FIG. 6. Effect of TBTC and TPTC on the time of onset of HbIV precipitation. HbIV (♦); HbIV+PMB (▲); HbIV+PMB+TPTC (□); HbIV+PMB+TBTC (■). Same conditions as in Fig. 4.

(Fig. 4) After a certain time these compounds also influence the precipitation of HbIV (data not shown).

To establish a possible involvement in these processes of the free SH-groups of the two proteins, we blocked them with parahydroxymercuribenzoate (PMB). The presence alone of PMB does not influence the stability of HbI; the time course of hemoglobin oxidation does not change when its four free SH-groups are blocked (Fig. 5).

Now the oxidation rate of HbI with its SH-groups blocked with PMB. is still stabilized by TBTC while the presence of PMB deletes the capacity of TPTC to decrease the oxidation rate of HbI (Fig 5).

A different behavior was found with HbIV. By adding PMB to the hemoprotein solution, the molecule is destabilized and, as reported in Fig. 6, the presence of TBTC and TPTC increases further the precipitation rate similarly to what occurs in the absence of PMB.

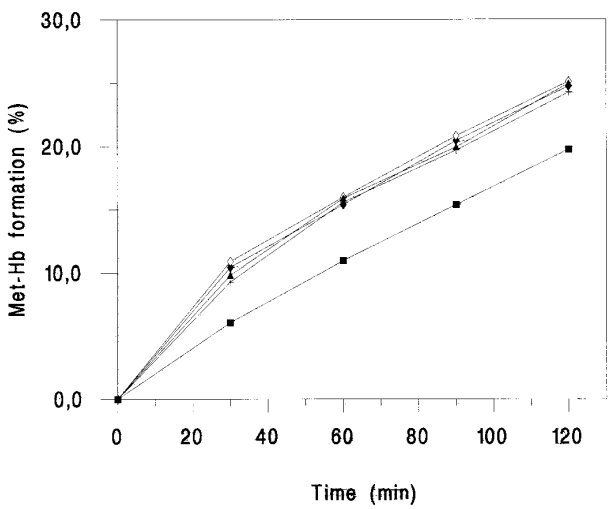

FIG. 5. Effect of TBTC and TPTC on the time course of oxidation of HbI with free SH-groups blocked by PMB. HbI (◇); HbI+PMB (♦); HbI+PMB+ethanol (▲); HbI+PMB+TPTC (+); HbI+PMB+TBTC (■). Same conditions as in Fig. 4.

DISCUSSION

Organotin compounds bind to a limited number of proteins which permits one to assume that the binding correlates with the thredimensional protein structure rather than with a single chemical group.

Studies performed by Taketa et al. (7,12) on hemoglobins have put in evidence that only cat and rat hemoglobins show the property of binding trialkyltin compounds. The possibility to form the trialkyltin-hemoglobin complex should be due to the capacity of these compounds to form pentacoordinated complexes with certain ligands (i.e. histidine and/or cysteine residues).

The above data permit to include in this group also trout (fish) hemoglobins. A peculiar characteristic of fish hemoglobins is their oxidation rate, they are less stable with respect to human hemoglobin and then it is possible to follow the process at relatively short time periods (9). In general, the rate of spontaneous oxidation of hemoglobin is highly dependent on the tertiary and quaternary structure of the molecule. Fish hemoglobins are highly stable as tetramers (the dissociation constants are much smaller than for human hemoglobin); therefore, modifications of the oxidation rate could be due only to tertiary events.

The results presented above reveal some unexpected properties of the two main hemoglobin components

from trout blood; these two hemoglobins display marked differences in the effect produced by interaction with organotin compounds.

Increased number of hydrophobic groups of the organotin compound enhances the stability of trout hemoglobin. The two mono-chloro derivatives (TBTC and TPTC) had a larger effect although it was completely different for the two proteins: while they increased HbI stability, they decreased it in HbIV.

Experiments performed in the presence of PMB showed a special role of cysteine residues on trout hemoglobin stability.

HbI was not influenced by the presence of PMB alone while HbIV was. This could be due to a different localization of cysteine residues in the two hemoglobin molecules.

Amino-acid sequence studies performed on these hemoglobins by the group in Rome (13-16) showed a very marked difference in their primary structure. Comparison of the amino-acid sequence of trout HbIV and trout HbI showed that cysteine residues were located in different helical regions. In HbI there is a cysteine in β 68, in helix E (E11), while in HbIV there are two cysteine residues located in β109 and 113, in helix G (G11 and G15). Another cysteine residue is present in HbIV and precisely in the α chain at position 69 (E18). The binding of PMB may then alter the protein conformation in a different manner, depending rather on the position than on the number of free SH-groups.

As to the involvment of free SH-groups only the protective effect of TPTC on HbI oxidation depends on the status, blocked or not, of the SH-groups. The effect of TBTC on HbI reduction and of both the triorganotins (TPTC and TBTC) on HbIV precipitation is always present also when the -SH groups are blocked by PMB. This behavior suggest an important role of the histidine residues.

Although the molecular mechanism by which organotin compounds influence the trout hemoglobin structures is still unknown, the different effect seen on HbI and HbIV (remember also the different role of these two proteins) could be important in evaluating the environmental risks deriving from the use of these molecules in marine paint formulations

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