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Tris: an allosteric effector of tarantula haemocyanin

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Abstract

The effect of the chemical buffering component Tris (hydroxy-methyl-amino-methane) and of chloride ions on the oxygen binding of tarantula hemocyanin was studied at constant pH. It revealed that Tris at micromolar concentrations decreases the oxygen pressure at half-saturation (p_{50}) by a factor of more than two, whereas chloride does not influence oxygen affinity. A thermodynamic analysis in terms of the nested model of allostery [(1987) Proc. Natl. Acad. Sci. 84, 1891–1895] indicated that Tris acts a an allosteric activator of oxygen binding by influencing the interaction between the 12-meric half-molecules of the 24-meric tarantula haemocyanin.

Key words: Allostery; Chloride effect; Haemocyanin; Nesting model; Oxygen binding; Tarantula; Tris

1. Introduction

Haemocyanins are giant extracellular respiratory proteins in the haemolymph of Arthropods and Molluscs. Their oxygen binding is highly cooperative and sensitive to allosteric effectors such as protons or Cl^- ions [1–4]. The influence of an allosteric effector is usually tested by recording oxygen binding curves in the presence of chemical buffering systems. However, these buffers may themselves change the oxygen binding properties of hemocyanins in a complicated manner [5,6]. Therefore the influence of a particular buffer such as Tris-HCl has to be elucitated, before oxygen binding curves can be used for a physiological interpretation of measured allosteric effects.

This study shows that Tris (hydroxy-methyl-amino-methane) at micromolar concentrations strongly increases the oxygen binding affinity of 24-meric tarantula (*Eurypelma californicum*) haemocyanin. A closer analysis reveals that Tris acts as an allosteric molecule whose effect on oxygen binding can be explained by the recently developed nesting model of allostery [7,8].

2. Materials and methods

2.1. Preparation of the hemocyanin

Haemolymph of the spider *Eurypelma californicum* was obtained by dorsal puncturing of the heart. All samples were pooled on ice and aliqots were immediately diluted 1:2 (v/v) with Ringer solution that contains 210 mmol/l NaCl, 2.6 mmol/l KCl, 0.4 mmol/l $MgCl_2$, 4.2 mmol/l $CaCl_2$ and 1 mmol Na_2HPO_4 [9] with a resulting pH value of 7.6. All samples were then centrifuged for 10 min to remove any cells. The supernatant was kept as a stock solution. It contained 30 g/l hemocyanin. An aliquot of this haemocyanin pool was extensively dialysed against 100 mmol/l Tris- HCl, pH 7.8.

2.2. Oxygen binding curves

Continuous oxygen equilibrium curves were recorded with the fluorimetric-polarographic method at 20°C [10]. In order to prepare haemocyanin for oxygen binding curves, aliquots of the stock solution without Tris were diluted about 300 fold with Ringer that contained different concentrations of Tris (0, 0.01, 0.1, 1 mmol/l). Aliquots of the haemocyanin pool that had been dialysed against 100 mmol/l Tris, were diluted with water in order to obtain different Tris concentrations (1, 10 and 100 mmol/l).

Before oxygen binding curves were performed, the haemocyanin sample was flushed with pure oxygen for 30 min in order to space out all dissolved CO_2. Then the pH value of the sample was measured in the cuvette within 15 s. If necessary, the pH value of the samples was readjusted to pH 7.8 with 0.1 N HCl and checked with a pH-electrode purchased from Eschweiler (Ingold) changing the concentration of chloride ions by not more than 1 mmol/l. The concentration of chloride ions was calculated from the amount of the added HCl for each experiment and is given in the legend of Fig. 1. The temperature was kept at 20°C. After the oxygen binding curves were completed, the pH-value of the sample was immediately rechecked. The difference of the pH-value before and after the experiment was always less than 0.05 units. The error range of the half-saturation p_{50} was determined to ± 0.5 Torr.

2.3. Data analysis

The binding curves were digitalized using a digitalizing tablett (Cybernetic Research and Production, Freiburg) and a computer system from ATARI 1040 ST. The data were analyzed according to Decker and Sterner [11] by optimizing the parameter values by a non-linear least-squares method based on the Marquardt algorithm.

3. Results and discussion

Oxygen binding curves of haemocyanin from the tarantula *Eurypelma californicum* were recorded in solu-

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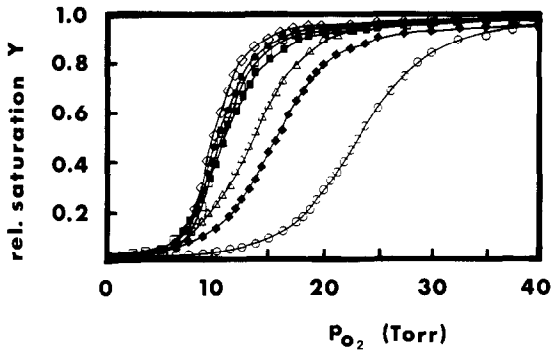

Fig. 1. Oxygen binding curves of tarantula haemocyanin recorded in presence of various concentrations of Tris. The solute was either Ringer (with no Tris (○), 0.01 mmol/l Tris (◆), 0.1 mmol/l Tris (△) and 1 mmol/l Tris (■) present) or water (with 1 mmol/l Tris (□), 10 mmol/lTris (●) and 100 mmol/l Tris (◇). The pH was adjusted to 7.8 at 20°C using a 0.1 N HCl. In presence of Ringer the concentration of chloride for each binding curve was calculated to 221.8 mmol/l (no Tris (○)), 221.8 mmmol/l (0.01 mmol/l Tris (◆)), 221.9 mmol/l (0.1 mmol/l Tris (△)) and 222.5 mmol/l (1 mmol/l Tris (■)), in the absence of Ringer the chloride concentration was 0.7 mmol/l (1 mmol/l Tris (□)), 7 mmol/l (10 mmol/l Tris (●)) and 70 mmol/l (100 mmol/l Tris (◇)). The concentration of haemocyanin was 0.15 g/l. The symbols represent the experimental values, the drawn lines show the result of the fits according to the nesting model (see Figs. 2 and 3)

tions containing various concentrations of Tris; two series of experiments were performed. In one series, Tris was dissolved in water, in the second series Tris was dissolved in tarantula Ringer containing 221.8 mmol/l chloride ions (210 mmol/l NaCl, 2.6 mmol/l KCl, 0.4 mmol/l $MgCl_2$, 4.2 mmol/l $CaCl_2$ and 1 mmol/l Na_2HPO_4). Fig. 1 shows that Tris increases the affinity of oxygen binding at micromolar concentrations. In the range between 0.01 and 1 mmol/l, a value for $\Delta\log p_{50}/\Delta\log[\text{Tris}]$ of −0.084 was determined. Almost no additional influence on oxygen binding was observed at concentrations higher than 1 mmol/l Tris. The cooperativity of oxygen binding expressed by the Hill coefficient was constant under all applied conditions. The average value was 6.9 ± 0.3 (n = 7). The oxygen binding curves being recorded in the absence of Tris are in full accordance with those obtained by Paul et al. [9]. Oxygen binding curves obtained in the presence of 1 mmol/l to 100 mmol/l Tris are identical to those determined previously for purified haemocyanins in presence of 100 mmol/l Tris [11].

Does Tris itself or does the counter-ion chloride influence the oxygen binding behaviour of tarantula haemocyanin? Although chloride ions act as allosteric activator of several haemocyanins [3,4,12–16], we can exclude that chloride is responsible for the observed increase in oxygen affinity of tarantula haemocyanin: In presence of Ringer, which contains a high chloride concentration of 221.8 mmol/l, and in the absence of Tris, the lowest oxygen affinity of all experiments was observed, with p_{50} = 24 Torr. In absence of Ringer, at a concentration of 1 mmol/l Tris, which corresponds to 0.7 mmol/l chloride, a higher affinity is observed, with p_{50} = 10 Torr.

On order to exclude that a low ionic strength could be responsible for the higher oxygen affinities in the presence of Tris, we performed oxygen binding curves in presence of 100 mmol/l Tris and 100 mmol/l NaCl. The ionic strength of this solution is comparable to that of the Ringer solution. The haemocyanin shows the same oxygen affinity as in solutions contaaining 1–100 mmol/l Tris without NaCl (data not shown).

It is noteworthy in this context, that an influence of Cl^- ions on the kinetics of oxygen binding of *Eurypelma californicum* haemocyanin subunits could also not be detected with stopped-flow techniques [17].

While we cannot detect any influence of chloride ions on the oxygen binding of tarantula haemocyanin, chloride ions are reported to be allosteric activators of oxygen binding of the closely related haemocyanin from *Limulus polyphemus* [16]. According to X-ray analysis, the binding site of the chloride ion in *Limulus* haemocyanin is located in a highly conserved area. Therefore chloride ions were considered to play a functional role in most arthropod hemocyanins [18]. However, a chloride-sensitive oxygen binding has up to now been reported for only a few arthropod haemocyanins but for many molluscan haemocyanins, which are structurally completely different [3,4,19]. Our results confirm the idea

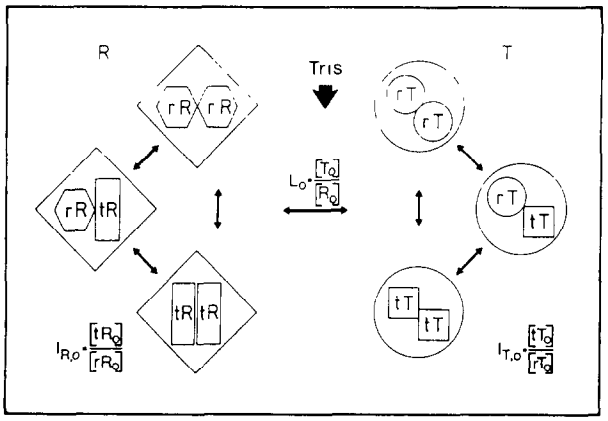

Fig. 2. Outline of nested allosteric interaction in the 24-meric tarantula haemocyanin according to Robert et al. [8]. In this model the two 12-meric half-molecules of the 24-meric tarantula haemocyanin are assumed to function as allosteric units in the sense of the MWC-model [22]. In contrast to assumptions of the MWC model, these allosteric units are interacting through two identical contacts. Depending on the strength of this interaction, the overall quaternary state of the 24-mer is designated T or R. Since in both states, R and T, the subunits of the allosteric units can adopt one conformation with high (r) and one conformation with low (t) oxygen affinity, altogether four different conformations (rR, tR, rT, tT) are possible for the subunits; the microscopic oxygen affinity constants of these four conformations are k_{rR}, k_{tR}, k_{rT} and k_{tT}. The three different allosteric equilibria are given by $l_{R,o} = [tR_o]/[rR_o]$ and $l_{T,o} = [tT_o]/[rT_o]$, which describe the interactions within the allosteric units and by $L_o = [T_o]/[R_o]$, which describes the interaction between the allosteric units (for further details see Decker and Sterner [11]).

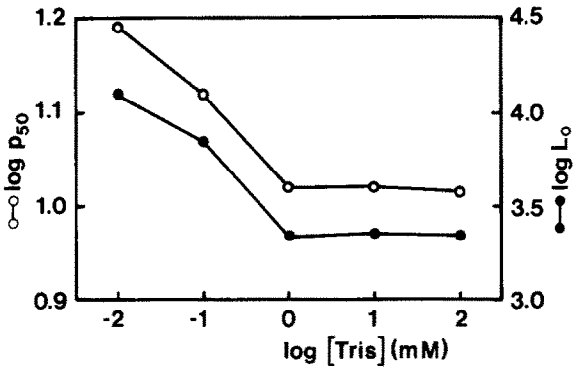

Fig. 3. Tris-dependence of the allosteric equilibrium constant $L_o = [T_o]/[R_o]$ and of the oxygen affinities p_{50} [Torr] as determined from the oxygen binding curves of Fig. 1. The value for L_o decreases with increasing Tris concentrations. Its dependence on Tris parallels the corresponding dependence of p_{50}.

that the influence of chloride ions on oxygen binding is limited to particular arthropod haemocyanins. Due to the lack of a highly resolved 3D-structure of tarantula haemocyanin, the structural basis of this functional discrepancy between closely related hemocyanins remains unclear.

Can we understand the mechanism with which Tris influences the oxygen binding of tarantula haemocyanin? Previous studies showed that the cooperative oxygen binding of tarantula hemocyanin and its regulation by allosteric effectors can be described by the recently developed nesting model of allostery, which is outlined in Fig. 2 [7,8,11,20]. When the oxygen binding curves obtained in the presence of various concentration of Tris were analysed in the framework of the nesting model, six of the seven parameters of the model are independent of Tris. On the basis of all oxygen binding curves of this study, the values for the four microscopic affinity constants and those for two allosteric equilibrium constants $l_{R,o}$ and $l_{T,o}$ were calculated to the following average values: $k_{rR} = 2.10 \pm 0.03$ Torr^{-1}, $k_{tR} = 0.052 \pm 0.003$ Torr^{-1}, $k_{rT} = 3.2 \pm 0.5$ Torr^{-1}, $k_{tT} = 0.006 \pm 0.001$ Torr^{-1}, log $l_{R,o} = [tR]/[rR] = 14.8 \pm 0.7$ and log $l_{T,o} = [tT]/[rT] = 20.0 \pm 0.7[n = 7]$. That the values of the affinity constants agree well with the corresponding values previously determined for oxygen binding curves of tarantula haemocyanin. In addition, the two allosteric equilibrium constants $l_{R,o}$ and $l_{T,o}$ are identical with the corresponding values obtained at pH 7.8 in a former study [11]. The allosteric equilibrium constant L_o, however, changes with increasing concentrations of Tris (Fig. 3). Obviously the dependence of L_o and the dependence of the oxygen affinity p_{50} on the concentration of Tris are strictly correlated (Fig. 3). According to the nesting model changes in L_o indicate that Tris influences the interaction between the two 12-meric half-molecules of the 24-meric tarantula haemocyanin [8,11,21].

Our results reveal that Tris is an allosteric effector of oxygen binding for tarantula haemocyanin such as for example protons. However, while protons mainly influence the interaction between the subunits within the two 12-meric half-molecules [11], Tris influences the interaction between the two 12-mers, most probably by interacting with the two identical inter 12-mer contacts mediated by subunits b and c [21]. These results demonstrate that the oxygen binding of complex allosteric haemocyanins can be regulated by different effectors at different levels of the quaternary structure. They also indicate that a physiological interpretation of oxygen binding curves constructed not in whole blood but in artificial solutions is only justified after a detailed investigation of the influence of the applied buffering components.

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