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Inversion of the Bohr effect upon oxygen binding to 24-meric tarantula hemocyanin

(allostery/conformational transitions)

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ABSTRACT **The Bohr effect describes the usually negative coupling between the binding of oxygen and the binding of protons to respiratory proteins. It was first described for hemoglobin and provides for an optimal oxygen supply of the organism under changing physiological conditions. Our measurements of both oxygen and proton binding to the 24-meric tarantula hemocyanin establish the unusual case where a respiratory protein binds protons at low degrees of oxygenation but releases protons at high degrees of oxygenation. In contrast to what is observed with hemoglobin and other respiratory proteins, this phenomenon amounts to the inversion of the Bohr effect in the course of an oxygen-binding curve at a given pH value. Therefore, protons in spider blood can act either as allosteric activators or as allosteric inhibitors of oxygen binding, depending on the degree of oxygenation of hemocyanin. These functional properties of tarantula hemocyanin, which cannot be explained by classical allosteric models, require at least four different conformational states of the subunits. Inspection of the known x-ray structures of closely related hemocyanins suggests that salt bridges between completely conserved histidine and glutamate residues located at particular intersubunit interfaces are responsible for the observed phenomena.**

Hemocyanins are giant extracellular respiratory proteins in the blood of arthropods and molluscs, comparable in size to ribosomes or small viruses. Arthropod hemocyanins consist of up to 48 oxygen-binding subunits with molecular masses of about 72,000 each (1, 2). Interactions between these subunits result in extremely high cooperativity of oxygen binding (with Hill coefficients up to 9) as well as a pronounced linkage between the binding sites for oxygen and those for effector molecules such as protons. This interdependence of oxygen and proton binding is usually negative. It is generally very important for the efficient gas transport within the blood because it facilitates oxygen binding in the lungs and oxygen release in the periphery. This linkage was discovered first for hemoglobin and is known as the "Bohr effect." It also has been studied for a number of hemocyanins by recording oxygen-binding curves at various pH values. These were kept constant during the measurements by buffering systems such as Tris or Hepes (3, 4). However, the magnitude of the Bohr effect at different degrees of oxygen saturation can be estimated only very roughly from a comparison of these oxygen-binding curves. This is particularly true at both low and high degrees of oxygenation where the curves approach their respective minima and maxima asymptotically. In addition, the binding of nonphysiological buffer ions can modulate the functional properties of hemocyanins in a complicated manner (5–7).

We wanted to obtain a realistic picture of the Bohr effect of a giant arthropod hemocyanin *in vivo*. To this end, we measured directly and under physiological conditions the linkage between the binding of oxygen and protons to the 24-meric hemocyanin from the tarantula *Eurypelma californicum*. We found an unprecedented form of the Bohr effect in that oxygen binding to tarantula hemocyanin results either in the release or in the uptake of protons, depending on the number of oxygen molecules already bound to the hemocyanin. As a consequence, protons can act either as allosteric activators or as allosteric inhibitors of oxygenation of the hemocyanin, thus allowing a sophisticated regulation of the oxygen transport in this poicilothermic animal. Recently determined x-ray structures of very similar arthropod hemocyanins allowed us to identify completely conserved pairs of histidine–glutamate residues that are most probably responsible for the observed Bohr effect of tarantula hemocyanin.

EXPERIMENTAL PROCEDURES

Hemolymph from tarantula blood, which contains only hemocyanin as the respiratory protein, was obtained by heart puncture (8). As shown by analytical ultracentrifugation (4) and small-angle x-ray scattering (9), tarantula hemocyanin is a pure 24-mer, both in its oxygenated and deoxygenated form and over a wide concentration range of at least 0.15 to 40 g/liter. Hemocyanin with concentrations between 22 and 32 g/liter was dialyzed against a solution with physiological ion composition (210 mM NaCl/2.6 mM KCl/0.4 mM $MgCl_2$/4.2 mM $CaCl_2$) in the absence of any additional buffering system. The Bohr effect of the hemocyanin was directly investigated by using a computer-controlled instrument with which oxygen and proton binding to respiratory proteins can be measured simultaneously and with high precision (10). This apparatus allows equilibration of the dialyzed hemocyanin sample in a closed cuvette with a humidified mixture of nitrogen and oxygen, whose oxygen content was regulated by mass flow-controllers (Tylan, Eching, Germany). After each stepwise increase of oxygen content, the binding of oxygen was followed spectrophotometrically by recording the absorption of the hemocyanin at 334 nm. Proton release or uptake was followed by pH changes in the solution, which were measured with a pH minielectrode (Ingold, Eschweiler, Germany). Two self-constructed amplifiers of high precision that are coupled to the electrode allow pH changes of at least 0.001 unit to be measured reliably (10). By determining the buffering capacity of the solution before the experiment, the measured pH change can be converted into the number of released or bound protons. To determine the buffering capacity of the sample, it was purged with pure nitrogen until all dissolved carbon dioxide and oxygen had been removed and a stable pH value had been attained. Subsequently, the

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deoxygenated hemocyanin solution was equilibrated with oxygen. The ensuing drop of the pH value was readjusted by the addition of 20 mM NaOH. The buffering capacity of the solution in the appropriate pH range was calculated from the amount of NaOH that was necessary to restore the original pH value. After normalization to the hemocyanin concentration, the buffering capacity ($\beta = 8.87 \pm 0.57$ mM per pH unit) was found to be identical for the given pH values. That is, during the individual experiments, where the maximum pH change was 0.1 unit, the buffering capacity was constant.

RESULTS AND DISCUSSION

Simultaneous Recording of Oxygen and Proton Binding. The investigated tarantula hemocyanin contains 24 subunits, which belong to seven different but functionally equivalent types, designated *a* to *g*. Each subunit type has a specific position in the quaternary structure (Fig. 1) and can bind one molecule of oxygen at a binuclear copper-containing binding site. The tarantula hemocyanin was dissolved in an unbuffered solution, whose ionic composition was comparable to that of tarantula blood. This physiological solution was equilibrated in a closed cuvette with a humidified mixture of nitrogen and oxygen. The oxygen content of the solution was increased stepwise. For each level of oxygen concentration, the degree of oxygenation of hemocyanin and the concomittant change of the pH value of the solution was recorded by using newly designed experimental equipment that is consistently capable of measuring pH changes of 0.001 unit (10). By determining the buffering capacity of the solution before the beginning of the experiment, the number of released or bound protons was calculated from the measured pH change.

Fig. 2 shows the linkage between the relative amount of bound protons (Y_{H^+}) and the relative amount of bound oxygen (Y_{O_2}) at pH 7.37, 7.56, and 7.95. In each case proton release lags behind oxygen binding—for example, at pH 7.95 and at the relative oxygen saturations of 10%, 50% and 85%, about 0%, 30%, and 65% of all "Bohr protons" were released, respectively. At pH values of 7.56 and 7.37, which lie within the physiological range of tarantula blood (16), this effect is even more pronounced and culminates in proton binding at low degrees of oxygenation (Fig. 2). This inversion

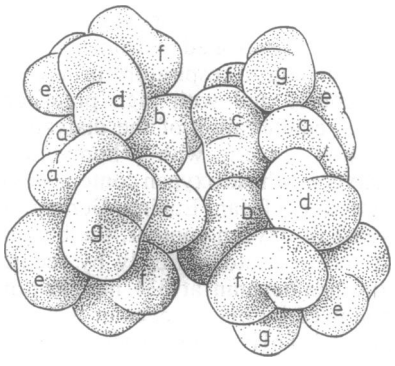

FIG. 1. Quaternary structure of the 24-meric tarantula hemocyanin. The oligomer is assembled from seven different but functionally equivalent subunit types (designated by letters, *a* to *g*). Each subunit reversibly binds one molecule of oxygen between two copper ions and has the same topology as the subunits of the hemocyanins from *Limulus polyphemus* and *Panulirus interruptus*, whose three-dimensional structures are known (8, 11–13). The subunits assemble to three special pairs of "tight dimers" (*a*–*b*(*c*), *d*–*e*, and *f*–*g*). These three "tight dimers" assemble to hexamers, which are the basic units of arthropod hemocyanins. Two hexamers—one carrying a *b* subunit and the other a *c* subunit—assemble to form identical 12-mers. These finally associate to the 24-mer by contacts between subunits *b* and *c*. (The figure is adapted from ref. 14.)

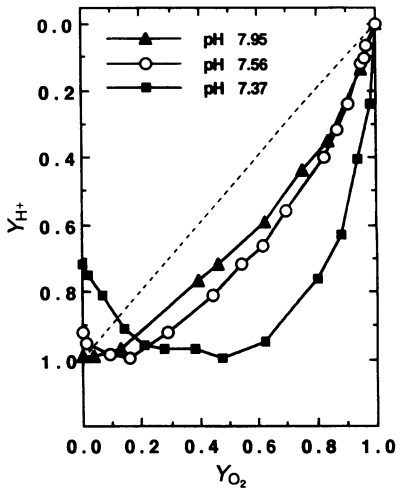

FIG. 2. Correlation of the relative amount of bound protons (Y_{H^+}) with the relative amount of bound oxygen (Y_{O_2}) for the tarantula hemocyanin at three different pH values (indicated pH values are measured for fully oxygenated hemocyanin). Each point represents the mean value of three individual experiments, and the error range is about twice the size of the symbols. For comparison a corresponding curve of human hemoglobin at pH 7.4 is shown (broken line; adapted from ref. 15).

of the Bohr effect in the course of an oxygen-binding curve is illustrated in Fig. 3 by an original recording in which both the pH value and the relative oxygen saturation of the hemocyanin were measured simultaneously. Fig. 2 shows that at pH values of 7.56 and 7.37, the maximum number of bound protons is not found in deoxy-hemocyanin, but rather at relative oxygen saturations of about 15% and 50%, respec-

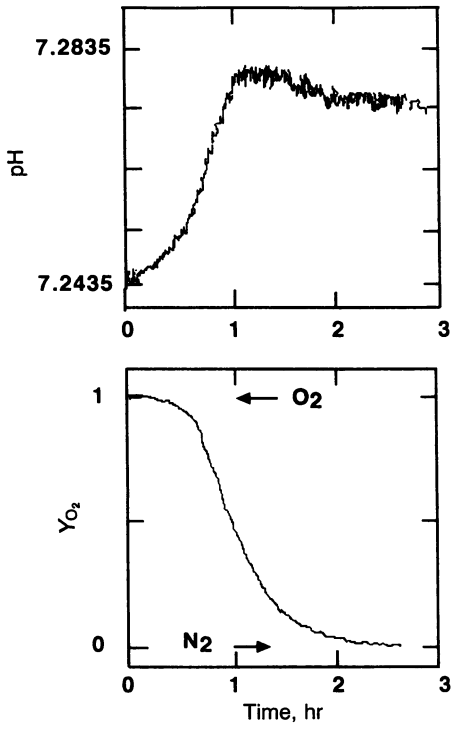

FIG. 3. Simultaneous recording of the pH value (*Upper*) and the relative amount of bound oxygen (Y_{O_2}) (*Lower*) during a continuous deoxygenation of the tarantula hemocyanin under physiological conditions. It is evident that the pH value first increases (because protons are bound to the hemocyanin) and later decreases (because protons are released). The observed pH change is fully reversible upon reoxygenation of the hemocyanin (data not shown).

tively. Oxygen binding beyond these limits results in a continuously increasing release of protons similar to hemoglobin.

Nonapplicability of Classical Allosteric Models. The binding of oxygen to hemocyanin is coupled to the binding of protons and vice versa. Thus, at low degrees of oxygenation and at physiological pH values, proton binding to tarantula hemocyanin promotes oxygen binding, whereas at high degrees of oxygenation, proton binding results in the release of oxygen. Therefore, protons in spider blood act either as allosteric activators or as allosteric inhibitors of oxygen binding, depending on the degree of oxygenation of the hemocyanin.

The release or binding of protons during oxygen binding is caused by pK changes of proteolytic groups (17–19). Since these pK changes are due to conformational transitions of subunits within the oligomer, our results place restrictions on models that can describe these allosteric interactions within the 24-meric spider hemocyanin. The simple sequential Koshland–Nemethy–Filmer (KNF) model (20) implies a continuous series of conformational transitions as oxygenation proceeds and thus a linear relationship between the degrees of oxygen binding and proton release. Fig. 2 shows that this expectation is not borne out by our experimental results. The concerted Monod–Wyman–Changeux (MWC) model (21) explicitly allows for a nonlinear relationship between oxygen binding and proton release. However, because it postulates only two conformations for the subunits, it cannot explain the observed inversion of the Bohr effect.

Recently, several novel and more complex thermodynamic models have been developed to describe the functional properties of hemocyanins. They all assume the existence of more than two different conformations of the subunits (22–27). However, up to now the postulated conformational transitions have been detected only indirectly—for example, by following changes in absorbance of bound dyes or changes in fluorescence of covalently attached labels (28–32). Our results demonstrate that hemocyanin undergoes conformational transitions under physiological conditions—that is, in the absence of nonphysiological buffers or covalent labels. Although our results do not allow us to discriminate unambiguously between the various complicated allosteric models that describe the function of hemocyanins, we can estimate the minimum number of conformations adopted by the subunits of the hemocyanin during oxygen binding in the following manner.

In Fig. 4 the number of protons that are released or taken up per molecule of bound oxygen is plotted against the relative oxygen saturation of the hemocyanin. At physiological pH values, one can discriminate between three different phases of the linkage relationship (a, b, and c). At oxygen saturation up to about 20%, oxygen binding is accompanied by proton uptake, indicating a transition from a conformation of low proton affinity to one of high proton affinity. Between 20% and 80% of oxygen saturation, a relatively constant release of protons per bound molecule of O_2 is observed. This is in agreement with the assumption that a new conformation with lower proton affinity accumulates continuously. Above 80% of oxygen saturation the relative proton release increases dramatically, suggesting that a transition to a conformation with very low proton affinity takes place. The detection of three different phases of proton binding and release indicates that the subunits of tarantula hemocyanin can adopt at least four different conformations. This result agrees with predictions of the nesting model (24), which assumes that the hierarchy in the quaternary structure of giant respiratory proteins such as the one from *E. californicum* (Fig. 1) is reflected in a functional hierarchy of allosteric interactions. When applied to fit oxygen binding curves of tarantula hemocyanin, the nesting model predicted four different quaternary states for the (12-meric) allosteric units and

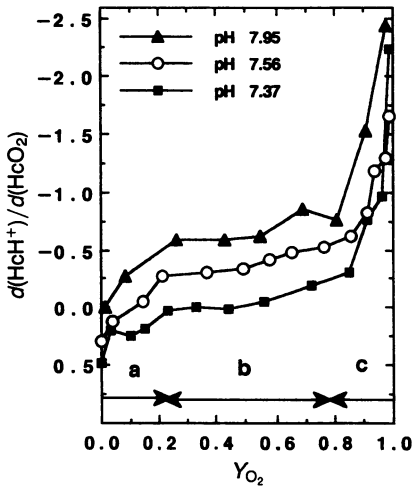

FIG. 4. Number of released or bound protons per molecule of oxygen bound to hemocyanin (Hc), $d(HcH^+)/d(HcO_2)$, as a function of relative amount of bound oxygen (Y_{O_2}) of the tarantula hemocyanin (secondary plot of the data in Fig. 2). Positive values indicate conformational transitions that lead to an uptake of protons during oxygen binding; negative values indicate conformational transitions that lead to proton release. Roughly three different phases of conformational transitions can be distinguished (a, b, and c). Each point represents the mean value of three individual experiments. The error ranges are between 2 and 5 times the size of the symbols and are omitted for the sake of clarity.

consequently four different conformations for the individual subunits (24, 33).

It is conceivable that the apparent inversion of the Bohr effect could be caused by a heterogeneous hemocyanin population characterized by different oxygen affinities and Bohr effects. However, as shown by Markl *et al.* (8), only one type of hemocyanin exists in the hemolymph of spiders. Furthermore, it is unlikely that heterogeneous oxygen affinities and Bohr effects of the individual subunit types can be responsible for the apparent inversion of the Bohr effect. It has been shown that, both in their isolated form and when incorporated in the oligomer, the different subunit types bind oxygen with very similar affinities (34, 35) and contribute equally to the Bohr effect of the 24-mer (35).

Structural Basis of the Bohr Effect. A comparison of the functional properties of different states of oligomerization (isolated subunits, 6-mers, 12-mers) with those of the native 24-meric tarantula hemocyanin indicates at which level of the quaternary structure "Bohr protons" may be triggered (14, 34). Whereas the isolated subunits exhibit hardly any pH dependence of oxygen binding, the Bohr effect is already fully established at the level of the hexamer. Therefore, "Bohr proton" donors should be located at the contact surface between the six subunits of the hexamer, rather than in the interior of subunits or at contact surfaces between the four hexamers of the tarantula hemocyanin (Fig. 1). Histidine residues are most probably responsible for the release and the uptake of "Bohr protons" of tarantula hemocyanin, since the pK values of their imidazole groups are close to the pH values of our study. To determine which of the >30 histidine residues per tarantula hemocyanin subunit might contribute to the Bohr effect, we made use of the known three-dimensional structures of the hexameric hemocyanins from the horseshoe crab *L. polyphemus* and from the spiny lobster *P. interruptus* (11, 12). Both, the topology of the individual subunits and their arrangement in the quaternary structure of the hexamers are conserved between these hemocyanins and the tarantula hemocyanin (11–13).

According to the structures of *L. polyphemus* and *P. interruptus* hemocyanins, the hexameric building block of

arthropod hemocyanins is composed of three pairs of "tight dimers," which in the case of tarantula hemocyanin are composed of the subunit pairs *a–b(c)*, *d–e*, and *f–g* (Fig. 1). When superimposing the amino acid sequences of the different subunits of tarantula hemocyanin onto the structure of *L. polyphemus* hemocyanin (11), it becomes obvious that one completely conserved histidine residue, His-239, is located at all "tight dimer" contacts of the hemocyanin from *E. californicum*. This histidine forms a salt bridge to the also completely conserved glutamate residue Glu-343 of the adjacent subunit within the "tight dimer" (Fig. 5).

It is known that the two subunits of a "tight dimer" change their distance along the threefold axis of the hexamers during conformational transitions of both *L. polyphemus* and *E. californicum* hemocyanins (9, 11). This movement leads to a change in the distances between the conserved histidine–glutamate pairs, which might well result in changes of the pK value of the imidazole ring of His-239, in analogy to the breaking of the salt bridge between His-146β and Asp-94β in hemoglobin (36, 37). In hemoglobin, His-146β adopts only two different pK values during oxygen binding. In contrast, it must be postulated that the conserved His-239 of tarantula hemocyanin subunits is able to adopt four different pK values during oxygen binding in order to explain the remarkable differences between the Bohr effects of the tarantula hemocyanin and the vertebrate hemoglobin (Fig. 2): for hemoglobin, oxygen binding and proton release are correlated negatively and almost linearily over a wide pH range between 6.9 and 8.0 (15, 38–41). A clear deviation from linearity is observed only at nonphysiological pH values >9 and <6.5 (39, 41). At acidic pH values a positive correlation between oxygen and proton binding has been observed for human hemoglobin A, which can be correlated with the appearance of an additional quaternary structure at pH ≤ 6 (42, 43) and attributed to a pK shift of His-143β upon oxygen binding (44). Such a reversed Bohr effect also has been found for some arthropod and molluscan hemocyanins (1).

However, to our knowledge, an inversion of the Bohr effect in the course of a single oxygen binding experiment at a given pH value has not been observed previously either for hemoglobin or for any other respiratory protein.

Biological Significance. The observed complex Bohr effect of tarantula hemocyanin might be an adaptation to particular conditions under which oxygen is transported within the poicilothermic spiders. Living in the deserts of Arizona, tarantulas face substantial changes of the ambient temperature between day and night. These temperature changes alter the pH value of the tarantula blood because of the temperature dependence of the degree of dissociation of water. Moreover, during short bursts of high activity, which are characteristic for the life style of *E. californicum*, the pH value of the tarantula blood drops by about 0.5 unit and requires a long period to recover (16). Thus, hemocyanin as an extracellular blood protein has to cover the oxygen need of the tarantula under widely different temperature and pH conditions, whereas hemoglobin as an intracellular protein transports oxygen under far more homeostatic conditions.

The complicated linkage between oxygen binding and proton release shown in this paper may also be an important reason why arthropod hemocyanins are much bigger than vertebrate hemoglobins. To establish the complex allosteric properties and the extremely high cooperativity of oxygen binding, the single subunits of tarantula hemocyanin must be able to adopt at least four different conformations as shown in Fig. 4. Comparison of functional properties of dissociation products of the hemocyanins from *Homarus americanus* and *E. californicum* shows that the number of possible conformations of the associated subunits increases with the aggregation state of these hemocyanins (4). In this context it will be interesting to see whether the observed inversion of the

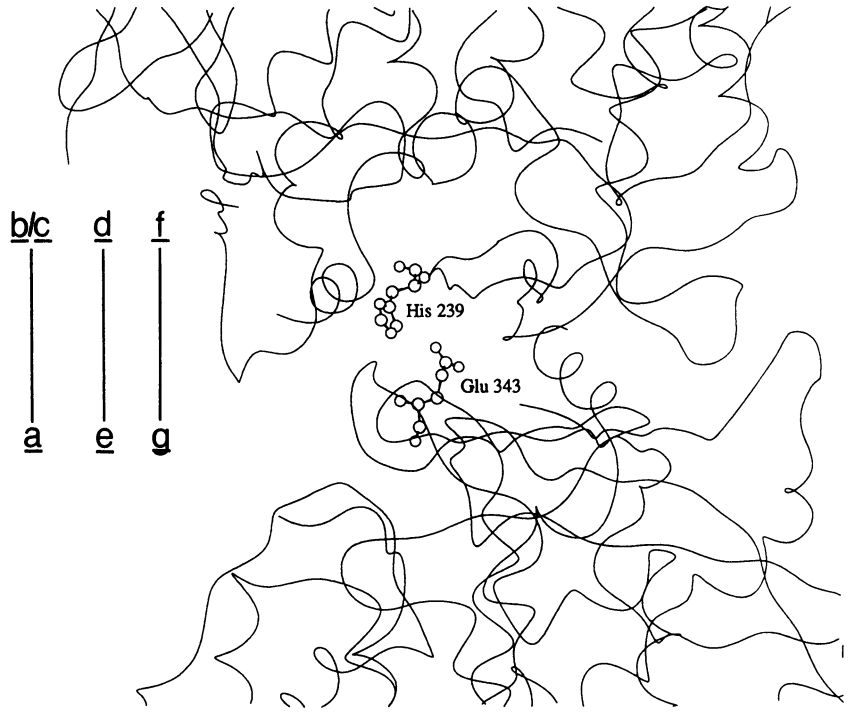

FIG. 5. Conserved salt bridges at the interfaces of "tight dimers" within the hexamers of 24-meric tarantula hemocyanin (Fig. 1). The amino acid sequences of tarantula hemocyanin subunits (*a* to *g*) were superimposed onto the structure of the subunits of the hexameric hemocyanin from *L. polyphemus* (11). Four conserved salt bridges at the contact sites within "tight dimers" were detected, which are formed between the residue pairs His-239 and Glu-343 as follows: His-239 (*b*)–Glu-343 (*a*), His-239 (*c*)–Glu-343 (*a*), His-239 (*d*)–Glu-343 (*e*), and His-239 (*f*)–Glu-343 (*g*). For the "tight dimer" *f–g*, also the corresponding salt bridge His-239 (*g*)–Glu-343 (*f*) is formed (not shown). During oxygenation, the two subunits of a "tight dimer" move apart along the threefold axis of the hexamer by 0.7 Å (9), which most probably results in changes of the pK value of His-239 and a concomittant release or binding of protons by the hemocyanin.

Bohr effect of 24-meric tarantula hemocyanin is also present in naturally occurring hexameric and 12-meric hemocyanins. The large number of subunits of hemocyanins could also enable the allosteric control of oxygen binding to be established by different effectors at different levels of the quaternary structure such as the hexamer, the 12-mer and the 24-mer (45). This situation might provide for a regulation of oxygen affinity of hemocyanin that is more sophisticated than that of vertebrate hemoglobin.

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