The Allosteric Effector L-Lactate Induces a Conformational Change of 2×6-meric Lobster Hemocyanin in the Oxy State as Revealed by Small Angle X-ray Scattering*

Hermann Hartmann, Bernhard Lohkamp, Nadja Hellmann, and Heinz Decker‡

From the Institute of Molecular Biophysics, Johannes Gutenberg-University of Mainz, Welder-Weg 26, D-55128 Mainz, Germany

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Hemocyanins are multisubunit respiratory proteins found in many invertebrates. They bind oxygen highly cooperatively. However, not much is known about the structural basis of this behavior. We studied the influence of the physiological allosteric effector L-lactate on the oxygenated quaternary structure of the 2×6-meric hemocyanin from the lobster Homarus americanus employing small angle x-ray scattering (SAXS). The presence of 20 mM L-lactate resulted in different scattering curves compared with those obtained in the absence of L-lactate. The distance distribution functions $p(r)$ indicated a more compact molecule in presence of L-lactate, which is also reflected in a reduction of the radius of gyration by about 0.2 nm (3%). Thus, we show for the first time on a structural basis that a hemocyanin in the oxy state can adopt two different conformations. This is as predicted from the analysis of oxygen binding curves according to the “nesting” model. A comparison of the distance distribution functions $p(r)$ obtained from SAXS with those deduced from electron microscopy revealed large differences. The distance between the two hexamers as deduced from electron microscopy has to be shortened by up to 1.1 nm to agree well with the small angle x-ray curves.

Hemocyanins are the oxygen transport proteins of most mollusks and arthropods. They are enormous molecular structures, ranging in molecular mass from $4.5 \times 10^{6}$ to more than $10^{7}$ Da; some contain more than 100 oxygen binding sites (1–3). Hemocyanins exhibit cooperative oxygen binding and respond to a variety of allosteric effectors (4–8). As a consequence of their structural complexity, hemocyanins have proved important in extending our understanding of allostery. For example, explanation of the cooperative oxygen binding by arthropod hemocyanin has required extension of the classical MWC model to the “nesting” model (8, 9), which reflects the hierarchical structure of these proteins. The nesting model requires four states ($T, rT, Tr, rR$) instead of the two states ($T, R$) of the MWC model. It makes the prediction that even fully oxygenated hemocyanin can exist in two conformational states. Although the model has served well to explain oxygen binding and its dependence on effector (see Ref. 8, for example), it has never been tested directly. That is, no experiments have been performed to detect the two oxy conformations predicted by the nesting model. To be definitive, such experiments must be carried out in solution, under rigorously controlled oxygenation levels and with a sensitivity sufficient to detect even small conformational changes.

A suitable method to detect conformational changes of large proteins in solution is small angle x-ray scattering (SAXS). This technique has been applied successfully to monitor changes of the quaternary structure for several cooperative proteins such as hemoglobin and a number of allosteric enzymes (10–18). Different conformations have also been reported for the oxy- and deoxyhemocyanin from the tarantula Eurypelma californicum from SAXS measurements (19).

Here, we present SAXS data of fully oxygenated 2×6-meric hemocyanin from the lobster Homarus americanus in the absence and presence of the physiological effector L-lactate. We will show that the fully oxygenated 2×6-meric hemocyanin can adopt two clearly distinguishable conformations, which differ in structural details. This observation indicates that the allosteric influence of L-lactate on the oxygen binding behavior of this hemocyanin is based on a preferential binding of L-lactate to one of the two possible conformations present in the oxy state.

**MATERIALS AND METHODS**

Preparation of Hemocyanin—Lobsters (H. americanus) were obtained from a local fish supplier. Hemolymph was obtained from heart puncture as described elsewhere (8). The cellular content of the hemolymph was removed from the sample by centrifuging for 10 min at 20,000 × g. Hemocyanin (2×6-mer) was purified by size exclusion chromatography (Bio-Gel A1.5 m, Bio-Rad) in 0.1 M Tris/HCl buffer, pH 7.2 at 20 °C. Under these conditions the 12-meric lobster hemocyanin is stable at least for months. The degree of saturation with oxygen was determined by comparing the absorption at 280 and 340 nm. The ratio $A_{280}/A_{340}$ of about 4.5 shows that the hemocyanin was fully oxygenated under these conditions.

Protein concentrations were determined spectroscopically using $A_{280} = 1.34$ (20). Tris and MgCl$_2$ were purchased from Roth (Karlsruhe, Germany). CaCl$_2$ was obtained from Fluka (Deisenhofen, Germany), and L-lactate acid was from Sigma (Deisenhofen, Germany).

Small Angle X-ray Scattering—Small angle x-ray scattering experiments were performed at the SANS camera JUSIFA at the synchrotron beamline B1 at Hamburger Synchrotronstrahlungsabor, Hamburg, Germany (21). The scattered intensities were recorded by a two-dimensional multiwire proportional counter with 256 × 256 pixels. Two different distances between detector and sample (918 and 3618 mm)

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‡ To whom correspondence should be addressed. Tel.: 49-6131-3923570; E-mail: decker@biophysik.biologie.uni-mainz.de.

1. The abbreviations used are: MWC, Monod-Wyman-Changeux; SAXS, small angle x-ray scattering; TEM, transmission electron microscopy.
were used with a step in reciprocal space of $d_{q} = 0.017$ nm$^{-1}$ ($q = 4\pi \sin(\theta)/\lambda$) at a wavelength of $\lambda = 0.1553$ nm. Scattering curves were measured at two protein concentrations, 7.8 and 15.6 g/liter, both in the presence and absence of 20 mM L-lactate. A quartz capillary flow cell was used for all experiments. The scattered intensities were corrected for detector sensitivity and instrumental background and normalized for monitor counts and transmission. Scattering curves of the buffer were measured under identical conditions and subtracted from the protein scattering curves. The total measurement times for the protein samples and the buffer were divided into several time slices to monitor possible shifts. Since no differences of the scattered intensity between the individual measurements were detected, the time slices were added to decrease the statistical error.

Calculation of the distance distribution functions $p(r)$ and desmearing of the intensity were performed with the method of indirect Fourier transformation using the program GNOM, which also delivers error bars for the $p(r)$ curves (22, 23).

Model Calculations—For comparison with the experimental data, model distance distribution functions were calculated from atomic models. For this the x-ray structure of the hexameric crustacean hemocyanin from Panulirus interruptus was used (24). First, an atomic model of the dodecameric hemocyanin was constructed using the relative translations and rotations of the two hexamers as reported for the 2×6-meric hemocyanin from the closely related crab Cancer pagurus obtained by transmission electron microscopy (TEM) and three-dimensional reconstruction (25). From the atomic model a distance distribution function $p(r)$ was calculated by counting all distances between the atoms, each weighted by the atomic form factor. To fit the experimental $p(r)$, the center of mass distance between the two hexamers was varied in the direction of the $z$ axes in a second step. The rotational parameters and the other two translational parameters describing the relative position of the hexamers were adjusted to the results obtained from TEM experiments. The radius of gyration of the molecular models was calculated directly from the coordinates using the distances between the atoms and the center of mass.

RESULTS

Fig. 1a shows the original smeared scattering intensities of lobster hemocyanin in the presence and absence of 20 mM L-lactate. The difference curves between the scattered intensities without and with the effector were calculated for both protein concentrations, 7.8 and 15.6 g/liter (Fig. 1b). For both measurements with low and high protein concentration, the differences exceed the experimental errors up to a factor of five for $q$ values between 0.15 and 0.4 nm$^{-1}$. The shape of the two difference curves is very similar over the whole $q$ range.

The radius of gyration was determined from the distance distribution function $p(r)$ (Fig. 2). For the low concentration of hemocyanin it was found to be $7.21 \pm 0.05$ nm in absence of L-lactate and $7.02 \pm 0.07$ nm in presence of L-lactate. The corresponding values for the high protein concentration are $7.19 \pm 0.05$ and $6.99 \pm 0.04$ nm. These values agree excellently with the radii of gyration calculated from the desmeared Guinier plot (7.19 and 7.01 nm for the low protein concentration; 7.17 and 6.98 for the high protein concentration). Although small, the differences in the radii of gyration in the absence and presence of L-lactate are highly significant, given the precision of the data. The decrease of the radius of gyration by about 0.2 nm in the presence of L-lactate indicates that the hemocyanin molecule becomes more compact.

In addition, the maximum diameters of lobster hemocyanin were calculated from the $p(r)$ curves (Fig. 2). Although the value of $23.5 \pm 0.5$ nm in the presence of L-lactate is only slightly shorter than that observed in the absence of L-lactate ($24.0 \pm 0.5$ nm), the number of large distances in the range between 14 and 24 nm is significantly smaller, compensated by an increase of $p(r)$ at shorter distances (Fig. 2). Because the maximum diameter of a hemocyanin hexamer is about 13 nm as calculated from the x-ray structures (24, 25), only interhexameric distances can contribute to $p(r)$ above 13 nm. Thus, the observed differences in $p(r)$ indicate that the centers of mass of the two hexamers are shifted together by the binding of L-lactate.

These observations are supported by the following consideration: based on the subunit composition of the lobster hemocyanin, we can assume two identical hexamers (26). Therefore it is possible to calculate the distance between the centers of mass of the hexamers, $D_{hex-hex}$, from the $R_G$ values when the radius of gyration for the hexamer is known (27).

$$D_{hex-hex} = 2 \sqrt{R_G^2(2\times6-mer) - R_G^2(6-mer)} \tag{1}$$

From the x-ray structure of the related hexameric hemocyanin from another crustacean P. interruptus, the radius of gyration of 4.77 nm for $R_G(6$-mer) was calculated (24). The distances between the centers of hexamers were then calculated to be 10.8 nm in the absence of L-lactate and 10.3 nm in presence of L-lactate. Thus, the hexamers are shifted toward each other by 0.5 nm in the presence of L-lactate. This is consistent with the observed decrease of about 0.5 nm in the maximum diameter as determined above from the distance distribution function.

We compared data obtained for lobster hemocyanin in ab-
sence of L-lactate by SAXS with a model based on the three-dimensional reconstruction of the closely related hemocyanin from another crustacean, the crab *Cancer pagurus*, obtained by transmission electron microscopy TEM (25). The radius of gyration for this TEM model was calculated to be 7.44 nm. The SAXS experiments, however, yielded values that are smaller in both cases, by 0.23 nm in the absence of L-lactate and 0.42 nm in presence of L-lactate. In addition, the distance distribution function deduced from TEM showed an extremely pronounced shoulder (Fig. 3), which was not found in the SAXS experiments. The following consideration supports the hexamer-hexamer contacts in our SAXS measurements. Obviously, the TEM model does not fit our experimental data. The closest Cα-Cα distances between the hexamers in the TEM model are in the range of about 1.2 nm, which is too large for reasonable contacts between subunits. Usually values of about 0.4 nm for the Cα-Cα distances between subunits are found in x-ray structures. To obtain a good agreement between the experimental distance distribution function and the $p(r)$ calculated from the models, we reduced the center of mass distance between the two hexamers from 11.4 to 10.3 nm (with L-lactate). These two fitted values agree well with the distances (10.3 and 10.8 nm, see above) obtained experimentally. In addition, the closest Cα contacts between the hexamers were then determined to 0.3 nm in the presence of L-lactate and to 0.6 nm in the absence of L-lactate.

**DISCUSSION**

Small angle x-ray scattering demonstrates that oxygenated hemocyanin from *H. americanus* undergoes a conformational change in the presence of the allosteric effector L-lactate. Different SAXS intensities and values for the radius of gyration were obtained in the presence and absence of 20 mM L-lactate, a concentration that is higher than the values (5–10 mM) observed in crustacea after stress (1, 2, 6, 7). Concentrations higher than 20 mM do not effect the oxygen binding curve anymore (7). The effector L-lactate seems to bind preferentially to a more compact hemocyanin molecule based on the decrease of the radii of gyration and the distance between the two hexamers. The inter-hexamer distance decreases by 0.5 nm, which corresponds to 2% of the total molecular dimensions. Besides the change in the hexamer-hexamer distance, additional rearrangements within the hexamers may occur during the conformational transition but cannot be identified with this method. The observed differences seem to be small. However, even when the oxy and deoxy states of the 24-meric hemocyanin from the tarantula *E. californicum* were compared, differences of similar magnitude were obtained (19).

To our knowledge we have provided here the first direct evidence that an allosteric effector can induce changes in the quaternary structure of a hemocyanin even though it remains fully oxygenated. The existence of two distinct, albeit fully oxygenated, conformations unequivocally rules out the applicability of the simple MWC model (28), which in toto offers only two conformations: one high affinity (at high pO2) and one low affinity (at low pO2) conformation. The observed increase of the affinity upon addition of L-lactate for crustacean hemocyanin (6, 7) means that in the frame of a MWC model the R state is stabilized compared with the T state. Thus, under fully oxygenated conditions addition of L-lactate cannot, according to an MWC model, change the conformational distribution to an ob-

**FIG. 2.** Distance distribution functions $p(r)$. a, the $p(r)$ curves for lobster hemocyanin (7.8 g/liter) are shown in the absence (solid line) and presence of L-lactate (dashed line). b, the absolute differences between the $p(r)$ curves are shown for two protein concentrations, 7.8 g/liter (solid line) and 15.6 g/liter (dashed line). For calculating the distribution functions, the SAXS intensities from the two sample detector distances have been merged. Error bars are shown as given by GNOM (22, 25).

**FIG. 3.** $p(r)$ curves of experimental and model data. Dots, $p(r)$ obtained from experimental SAXS data (7.8 g/liter) in the presence of L-lactate; dashed line, $p(r)$ calculated from an electron microscopic model (25); solid line, improved model after decreasing the center of mass distance between the two hexamers from 11.4 to 10.3 nm.
servable amount, since the most favored conformation is already present to a large extent.

Thus, both oxygen binding curves obtained at different pH values (8) and the SAXS experiments presented here rule out the simple MWC model. In principle extensions of the MWC model may be considered to explain these two conformations in the oxy state (9, 29, 30). However, the dependence of the oxygen binding curves on pH can be better explained in terms of the nested MWC model (8, 31, 32). In addition, the binding curves of the half-molecules can be predicted and confirmed by this model (8, 31, 32). We could not find any other model that was in comparable agreement with all of these experimental data.

The structural interpretation of the observed change in $R_G$ values is independent of the model used for the interpretation of the oxygen binding behavior. The models for cooperativity are based on functionally different conformations, which are distinguished on the basis of their oxygen affinities. No structural constraints are imposed by the SAXS data. But any structural difference between the hemocyanin in the absence and presence of L-lactate is in accordance with the existence of two functionally different conformations in the oxy state as required in the nested MWC model.

For other hemocyanins, the existence of different conformations within both the oxy and deoxy state was also supported by analysis of the oxygen binding (8, 9, 30–33). Other methods also confirmed the applicability of the nested MWC model for arthropod hemocyanins. For the 2×12-meric tarantula hemocyanin two different conformations in the oxy state were found using a fluorescence tag 12-(-N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl))-chloride (34). This has also been confirmed by O2/C0 replacement experiments (35). An isothermic titration calorimetry study of effector binding to 2×6-meric hemocyanin from Homarus vulgaris also indicates the existence of two conformations in the oxy state, which differ in their affinities for the effectors urate and caffeine (36, 37). However, our small angle x-ray results provide the first direct structural evidence for two different conformations in the oxy state and reveal that structural changes are involved.

The simple MWC model describes well the oxygen binding properties of hexameric hemocyanins. For 1×6-meric hemocyanin of P. interruptus sophisticated O2/C0 replacement experiments confirmed that the simple MWC model is the appropriate model (38). Additionally, hexamers obtained from dissociation of 2×6-meric hemocyanin from H. americanus were also well described by the simple MWC model (8). The binding parameters obtained for this hexamer correspond well to the predicted binding parameters of the two states (rR, tR) in the R conformation according to the nested MWC model when applied to the native 2×6-meric molecule (8). The two additional states (rT, tT) of the T conformation as postulated by the nested MWC model for the 1×6-mers must have been created by the assembly of the two hexamers accompanied by a functional coupling. The two conformations observed by SAXS can be assigned to the two high affinity states, $R$ and $T$. On the basis of these considerations the nested MWC model seems the simplest to account for all the data. Therefore, other models were not considered for describing arthropod hemocyanins, especially from H. americanus.

Two questions arise: why do larger aggregates than hexamers exist, and is there any advantage for respiratory proteins such as the hemocyanins to function according to the nested MWC model? Obviously, for a number of species the hexameric hemocyanin serves well for the oxygen delivery (1–5). The reason to develop higher aggregates may be to be found in keeping the osmotic pressure low in the hemolymph with respect to the extracellular proteins (39). But no further advantage would result from increasing the size of the molecule or the allosteric unit while maintaining regulation by the simple MWC model. However, a more sophisticated regulation mechanism would also be established when the 1×6-meric allosteric units (each behaving according to the simple MWC model) will not only assemble structurally but will also establish a functional coupling according to the nested MWC model. As discussed in previous papers this offers the possibility to provide an influence of different allosteric effectors on the oxygen binding behavior at different levels of the quaternary structure (1, 8, 9, 40).

An additional advantage for multihexameric hemocyanins working according to the nested MWC model is the ability to create cooperative behavior over a broad range of saturation levels as discussed previously (8, 9, 40). Thus, extracellular hemocyanins working according to the nested MWC model posses a broader functional flexibility compared with a hypothetical molecule with a simple MWC mechanism. This might be a consequence of the fact that extracellular respiratory proteins in poikilothermic animals such as arthropods have to face more pronounced environmental fluctuations than do intracellular respiratory proteins in mammals. The larger functional flexibility may help to ensure the delivery of oxygen precisely to the tissues under a broad range of conditions.

The importance of applying different methods for structural investigations is demonstrated in our study by a comparison of hemocyanin structures obtained from TEM and SAXS. The interhexameric distance as obtained by TEM had to be short-ened to be in agreement with the SAXS data. Similar deviations of calculated distance distribution functions $p(r)$ based on TEM from those obtained by SAXS have been reported previously for the tarantula hemocyanin (19). As in the case of the 2×6-meric lobster hemocyanin, the half-molecules of the 2×12-meric tarantula hemocyanin had to be shifted together to yield contacts between the half-molecules and to be in reasonable agreement with the SAXS data (Fig. 4). The differences may arise from the fact that these TEM studies utilized negative staining. It may be that the hemocyanin molecules are slightly

![Fig. 4. Comparison of 2×6-meric hemocyanin as deduced from electron microscopy and after a decrease of the distance between the two hexamers by 1.1 nm. The structure of the closely related hexameric hemocyanin from P. interruptus is used (24). Two hexamers are arranged according to electron microscopical image analysis (25) as determined for the closely related 2×6-mer from Cancer pagurus (left side). At the right side the modified 2×6-mer is shown after a decrease of the hexamer-hexamer distance by 1.1 nm according to our SAXS data. The kidney-shaped subunits are shaded differently. Figures were produced using the programs MOLSCRIPT (41) and Raster3D (42, 43).](image)
distorted by the preparation of the negatively stained specimen resulting in larger differences between the two loosely connected half-molecules. Such an artifact cannot arise when SAXS is applied, since untreated proteins are investigated in solution. Thus, SAXS, which analyzes molecules in solution, may be considered as an important check for the three-dimensional reconstruction of macromolecules based on negatively stained specimens by TEM. This seems to be important especially for proteins with a large and complex quaternary structure.

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