Conformational States of the *Rapana thomasiana* Hemocyanin and Its Substructures Studied by Dynamic Light Scattering and Time-Resolved Fluorescence Spectroscopy

Dessislava Georgieva,*† Daniel Schwark,* Peter Nikolov,† Krassimira Idakieva,† Katja Parvanova,† Karsten Dierks,‡ Nicolay Genov,† and Christian Betzel*

*Universitätsklinikum Hamburg-Eppendorf, Zentrum für Experimentelle Medizin, Institut für Biochemie und Molekularbiologie I, Hamburg, Germany; †Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria; and ‡Dierks und Partner, Hamburg, Germany

ABSTRACT Hemocyanins are dioxygen-transporting proteins freely dissolved in the hemolymph of mollusks and arthropods. Dynamic light scattering and time-resolved fluorescence measurements show that the oxygenated and apo-forms of the *Rapana thomasiana* hemocyanin, its structural subunits RtH1 and RtH2, and those of the functional unit RtH2e, exist in different conformations. The oxygenated respiratory proteins are less compact and more asymmetric than the respective apo-forms. Different conformational states were also observed for the *R. thomasiana* hemocyanin in the absence and presence of an allosteric regulator. The results are in agreement with a molecular mechanism for cooperative dioxygen binding in molluscan hemocyanins including transfer of conformational changes from one functional unit to another.

INTRODUCTION

The evolutionary development of respiratory proteins is a result of the accumulation of dioxygen in the terrestrial atmosphere. Three types of dioxygen carriers have been found in the aerobic organisms: hemoglobins/erithrocruorins, hemerithrins, and hemocyanins (Hcs). All of them bind dioxygen reversibly and transport it from the environment to the tissues of animal's body. Hcs are the most complex and sophisticated respiratory proteins freely dissolved in the hemolymph of invertebrates of two phyla, *Arthropoda* and *Mollusca* (Markl and Decker, 1992). Although identical in function, arthropod and mollusk Hcs have a completely different molecular architecture. There are many evidences that the two Hc families evolved independently (Burmester, 2002). Arthropod Hcs are hexamers (1×6) or multiples of hexamers of bean-shaped subunits with a molecular mass of the complex in the range $0.45 \times 10^6 - 3.9 \times 10^6$ Da (Herskovits, 1988; Markl and Decker, 1992). Each 75-kDa subunit carries one dioxygen-binding, binuclear copper-containing active site. Mollusk Hcs form hollow cylindrical decamers with a molecular mass of ~4 mDa, 18–20 nm in diameter and a height of ~20 nm (Orlova et al., 1997; Lieb et al., 2000). Hcs exist as decamers in the hemolymph of cephalopods, but in gastropods two decamers are assembled to form didecamers. In marine gastropods, two immunologically and physicochemically distinct hemocyanin isoforms usually occur. The isoforms consist of 400–450 kDa subunits, which represent a linear sequence of ~50 kDa functional units, each carrying a single binuclear copper active site (Lieb et al., 2000).

Rapana thomasiana grosse is a prosobranch gastropod widespread on the west coast of the Black Sea. The meat of this mollusk is used as food and is of industrial importance. The interest in gastropod Hcs is also due to their possible medical application, since a related Hc from keyhole limpet (KLH) is used in experimental immunology and clinically as an immunotherapeutic agent for the treatment of certain types of cancer including bladder carcinoma, for the diagnosis of Schistosomiasis (Harris and Markl, 1999), and as a hapten carrier for an AIDS vaccine (Naylor et al., 1991). For a number of years we have been investigating the respiratory protein of *R. thomasiana* and showed that the native Hc aggregates (RtH) are built of two isoforms, termed RtH1 and RtH2 (Idakieva et al., 2001). Also, the linear sequential arrangement of the subunits in both isoforms (Stoeva et al., 1997a; Idakieva et al., 2000) and the complete amino acid sequences of the functional units RtH2a and RtH2e (Stoeva et al., 1997b, 2002) have been determined. Recently, we have determined the x-ray structure of RtH2e and showed that it offers a mechanism for cooperative dioxygen binding (Perbandt et al., 2003).

Here, we describe the results of investigations on the conformational states of the oxy- and apo-forms of the *R. thomasiana* Hc and its substructures, the structural subunits RtH1 and RtH2, and the functional unit RtH2e, as well as the effect of an allosteric regulator on the conformation of the native, oxidized hemocyanin. Hydrodynamic parameters of the dioxygen binding proteins were determined by dynamic light scattering, which allowed characterization of the shape of the particles, and these were indicative of conformational changes. Time-resolved fluorescence measurements registered changes in the microenvironment of natural protein chromophores when the investigated proteins were in oxy- or apo-forms. The two forms of the respiratory protein and its

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Address reprint requests to Christian Betzel, Tel.: 49-40-8998-4744; Fax: 49-40-8998-4747; E-mail: betzel@unisgi1.desy.de.

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substructures differ in their conformations. We show that in the presence of an allosteric regulator, the oxidized *R. thomasiana* Hc adopts a different conformation. This is in line with the molecular mechanism for cooperative dioxygen binding in molluscan Hcs we have recently proposed (Perbandt et al., 2003).

MATERIALS AND METHODS

Hemocyanin and chemicals

Rapana thomasiana grosse (mollusk) specimens were caught on the west coast of the Black Sea, in the Golden Sands region near Varna. Hemolymph was collected from animals weighing ~20–25 g. The crude material was filtered on gauze and centrifuged for 30 min at 5000 rpm. The isolation of the hemocyanin was performed as described previously in Boteva et al. (1991) using a Spinco ultracentrifuge at 180,000 *g* (Spinco Biotech, Chennai, India). The obtained material was stored at −20°C in the presence of 20% sucrose until used. DEAE-Sepharose CL-6B was obtained from Fluka AG (Basel, Switzerland). The chemicals and reagents used were of analytical grade.

Isolation of the *Rapana thomasiana* structural subunits and the functional unit RtH2e

Native *R. thomasiana* hemocyanin was dissociated to subunits by dialysis against 0.05 M glycine/NaOH buffer containing 0.02 M EDTA, pH 9.6. The two structural subunits, RtH1 and RtH2, were separated and purified by ion-exchange chromatography on DEAE-Sepharose CL-6B according to the procedure described in Idakieva et al., (1993). Each of the two subunits contains eight functional units of ~50 kDa. The functional unit has a single copper-containing site reversibly binding the dioxygen molecule. FU RtH2e is the fifth unit from the amino-terminus of the RtH2 polypeptide chain. It was isolated after treatment of RtH2 with plasmin, separation of the products, and subsequent trypsinolysis of a fragment containing RtH2e, as described in Stoeva et al. (2002). The FU was purified to homogeneity by FPL chromatography on a Mono Q (HR 10/10) column (Amersham Biosciences, Freiburg, Germany). The apo-form of the Hc was prepared as described in Boteva et al. (1991).

Transmission electron microscopy

R. thomasiana hemocyanin samples were adsorbed on a glow-discharged Pioloform/carbon-coated support film, washed with distilled water and negatively stained with 1% (w/v) aqueous uranyl acetate, pH 4.5, or 5% (w/v) trehalose at pH 7.0 (Harris et al., 1995). Samples were viewed in a Philips CM 10 transmission electron microscope (Philips Medical Systems, Bothell, WA) at a 60-kV acceleration voltage and an instrument magnification of 52,000.

Dynamic light scattering

Dynamic light scattering (DLS) measurements were made using a RiNA GmbH system (Berlin, Germany) with a He-Ne laser providing a 690-nm light and an output power in the range of 10–50 mW. The data were measured under a scattering angle of 90°. An autopiloted run with 50 measurements at every 30 s, was applied. Measurements at nondissociating conditions were performed with protein solutions in 0.01 M Tris/HCl buffer, containing 0.02 M $CaCl_2$ and 0.02 M sodium azide, pH = 7.0, at a constant temperature of 20°C. The experiments with structural subunits were performed in 0.05 M glycine/NaOH buffer pH 9.6, containing 0.01 M EDTA, 0.02 M sodium azide, and 2 M urea to avoid association of the subunits. The samples to be analyzed were filtered directly to the cell. The functional unit RtH2e was investigated in the buffer with pH = 7.0 mentioned above, in the absence of urea.

Hydrodynamic parameters of the hemocyanin and its structural and functional (sub)units were determined as follows. The measured translational diffusion coefficient D_T is related to the frictional coefficient f by the Einstein-Sutherland equation,

$$D_T = k_B T/f(\mathrm{cm}^2/\mathrm{s}), \tag{1}$$

where k_B is the Boltzmann constant and T is the temperature in Kelvin.

The two structural subunits of the *R. thomasiana* Hc RtH1 and RtH2, as well as the functional unit RtH2e, are globular proteins and for them the hydrodynamic model for spherical proteins can be applied. The frictional coefficient of a spherical particle, f_{sph}, is a function of the fluid viscosity, η, and the radius of the particle, r_{sph}. It is defined by the Stokes law,

$$f_{sph} = 6\pi\eta r_{sph}. \tag{2}$$

The shape of the proteins was characterized using the so-called Perrin or shape factor F, which is informative for the shape of the molecule. This factor represents a ratio of the measured frictional coefficient f to the frictional coefficient f^{Theo} of a hypothetical sphere for which a hypothetical radius is calculated using the molecular mass,

$$F = f/f^{Theo}. \tag{3}$$

It can be shown that

$$f/f^{Theo} = R_H/R_H^{Theo}, \tag{4}$$

where R_H is the measured hydrodynamic radius and R_H^{Theo} is the radius of the hypothetical sphere, calculated from the molecular mass. The theoretical hydrodynamic radius was calculated from the formula

$$R_H^{Theo} = [(3M(V_s + h))/(4\pi N_A)]^{1/3}, \tag{5}$$

where M is the molecular mass, V_s is the particle specific volume, h is the hydration, and N_A is the Avogadro constant. The difference between the experimentally determined hydrodynamic radius and the theoretical value represents a deviation of the particle from the ideal spherical shape. The following molecular masses of the *R. thomasiana* hemocyanin structural components were used for the theoretical calculations: *R. thomasiana* Hc structural subunit RtH1, 420 kDa (Stoeva et al., 1997a); *R. thomasiana* Hc structural subunit RtH2, 420 kDa (Idakieva et al., 2000); and *R. thomasiana* Hc functional unit RtH2e, 50 kDa (Stoeva et al., 1997c). V_s has values between 0.69 and 0.75 $cm^3\ g^{-1}$ for proteins containing only amino acid residues (Cantor and Schimmel, 1980). A value of 0.73 $cm^3\ g^{-1}$ was used for the investigated proteins. According to the authors mentioned above, hydrations between 0.3 and 0.4 g H_2O (g protein)$^{-1}$ are needed to account for the hydrodynamic behavior of globular proteins. We have used a value of 0.35 g H_2O (g protein)$^{-1}$. The hydrodynamic parameters were corrected for changes in viscosity using values of 1.10 cPoise for 1 M NaCl and 1.08 cPoise for 2 M urea.

Molluscan Hcs are organized as cylinders with external diameter of ~370 Å and a height of the didecamer of ~400 Å (Orlova et al., 1997). For the native oxy- and for the apo-*R. thomasiana* Hc we have applied the hydrodynamic model for nonspherical molecules which can be modeled as rod-like particles (Varani, 2003). For such particles, the volume $V_{rod} = 2\pi ab^2$, where $2a$ is the length of the particle and b is the radius. The frictional coefficient for a rod-like molecule is defined as

$$f = f_o(2/3)^{1/3}P^{2/3}/((\ln(2P) - 0.30), \tag{6}$$

where P is defined as the ratio $P = a/b$ and $f_o = 6\pi\eta R_o$, where R_o is the radius of sphere with a volume equal to the volume of the rod with the ratio $P = a/b$. If $V_{sphere} = V_{rod}$, it can be shown that

$$R_o^3 = 3ab^2/2. \tag{7}$$

Time-resolved fluorescence spectroscopy

Time-resolved fluorescence studies were performed at 20°C using a nanosecond single-photon-counting spectrofluorimeter (system PRA 2000) and a nitrogen-filled flash lamp with a full width at half-maximum of ~2.5 ns. The protein samples were dissolved in 0.01 M Tris/HCl buffer containing 0.02 M $CaCl_2$ and 0.02 M sodium azide, pH 7.0. Then 0.05 M glycine/NaOH buffer containing 0.01 M EDTA and 2M urea, pH 9.6, was used for the measurements of the structural subunits. The data were analyzed by convoluting the instrument response function $L(t')$ with an assumed decay function $P(t)$, as

$$Y_c(t) = \int_0^t L(t')P(t-t')\mathrm{d}t', \qquad (8)$$

and comparing $Y_c(t)$ with the experimental time-dependence $R_m(t)$ using a nonlinear least-squares iterative convolution method based on the Marquardt algorithm. The decay curves contained 10^4 counts at the maxima and the time-resolution for these curves was 100 ps per channel. The goodness of the fit was assessed by the weighted residuals and the reduced χ^2-test.

Computer graphic studies

Computer graphic studies of the *R. thomasiana* hemocyanin functional unit RtH2e three-dimensional structure were carried out using our own coordinates (Perbandt et al., 2003; Protein Data Bank code 1LNL). The program TURBO Frodo (Roussel and Cambillau, 1991) was applied.

RESULTS AND DISCUSSION

Electron microscopy of the isolated hemocyanin

In Fig. 1, an electron micrograph of the native oxy-*R. thomasiana* Hc is shown. It is evident that the isolated material is homogeneous. Only didecameric cylindrical aggregates of RtH, formed by face-to-face association of two decamers, were identified in the micrograph. No dissociated material or tubular structures were observed. Fig. 2, *A* and *B*, show the lateral and axial projections of an individual Hc cylinder of D5 symmetry. This cylinder has a molecular mass of ~8.4 mDa, contains 20 structural subunits, and has 160 dioxygen-binding sites located in the same number of ~50 kDa functional units.

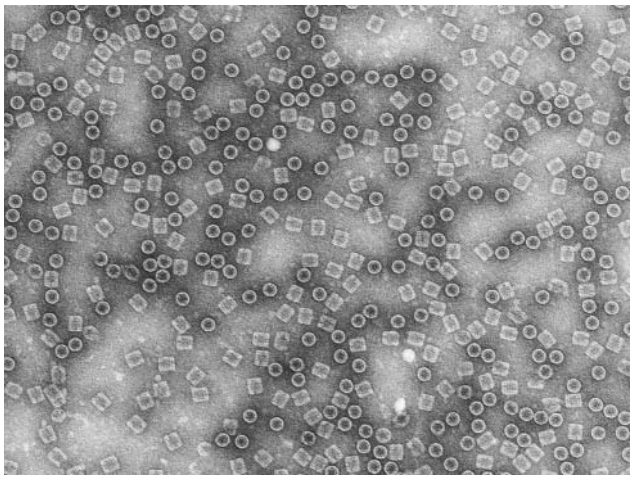

FIGURE 1 Electron micrograph of a specimen, negatively stained with 5% trehalose, of the native oxy-*R. thomasiana* hemocyanin at pH 7.0. Only didecameric cylindrical aggregates can be identified.

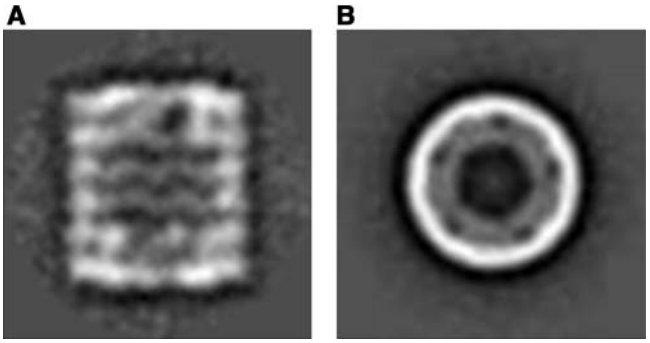

FIGURE 2 Lateral (*A*) and axial (*B*) projections of the native oxy-hemocyanin cylinder. The cylinder has a molecular mass of ~8.4 mDa, 20 structural subunits, and 160 dioxygen-binding sites.

Dynamic light scattering measurements of the active (oxy-) and inactive (apo-) *Rapana* hemocyanin, its structural subunits, and a functional unit

Fig. 3 shows DLS results from measurements of the native oxy-*R. thomasiana* Hc, structural subunit oxy-RtH1, and functional unit oxy-RtH2e. Only one single peak was observed for each protein, which is evidence for the monodispersity of the samples. Similar curves were obtained for the other protein solutions. The calculated hydrodynamic radii are summarized in Table 1. The R_H value for oxy-*R. thomasiana* hemocyanin is 23.6 ± 0.3 nm and that of the inactive apo-Hc is 22.2 ± 0.4 nm (Table 1), which indicates that, in the absence of the di-copper-dioxygen system, the hydrodynamic radius decreases and the hemocyanin becomes more compact. The two radii can be compared because they were measured in water solutions with identical viscosity and refractive index. The difference of 0.7 nm between the minimal value for the R_H of the oxy-form, 23.3 nm, and the maximal value for the apo-form, 22.6 nm, is outside the limits of the experimental error. For this reason the experimental data show small but real differences in the hydrodynamic radii of the active and inactive forms of *R. thomasiana* Hc. The differences in the long lifetimes (τ_2) of the tryptophyl residues in the two forms (Table 2) confirm conformational changes during the transition active-inactive form. The electronic micrograph (Fig. 1) shows that at neutral pH, RtH forms cylindrical structures in solution. This means that a hydrodynamic model of a spherical molecule is not realistic for theoretical calculations. For this reason a rod-like model of the investigated Hc was used. The estimated hydrodynamic radius of 21.7 nm is in a good agreement with the DLS measured value (Table 1). The difference between the experimental and theoretical values is due to the effect of the particular cylindrical shape of the hemocyanin molecule

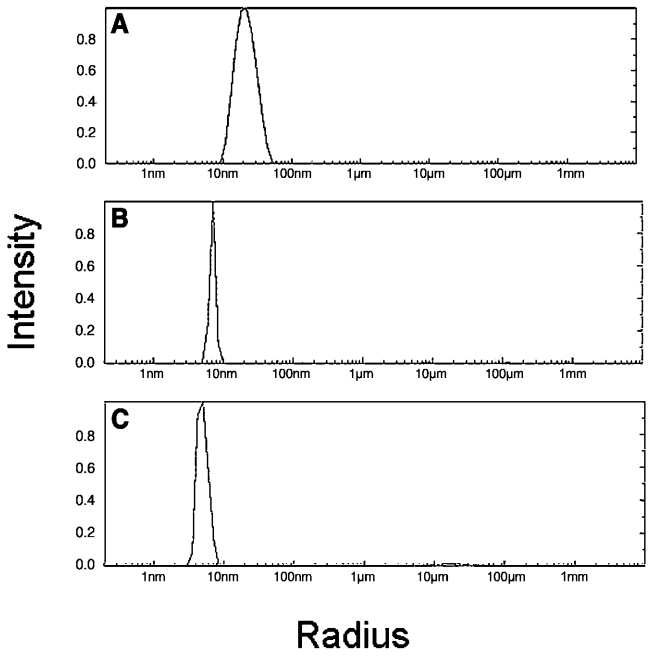

FIGURE 3 Dynamic light scattering measurements of the native oxy-*R. thomasiana* hemocyanin (*A*), structural subunit oxy-RtH1 (*B*), and functional unit RtH2e (*C*). Similar curves were obtained for the other protein samples.

on diffusion. In the case of oxy-RtH, the ratio of the frictional coefficients given in Table 1 compares the coefficient for a rod-like particle with that of a spherical particle of the same volume. The frictional coefficient of the nonspherical molecule is larger than that of the spherical one due to the larger surface in contact with the solvent. Calculations using a spherical model for the particles yielded 15.3 nm for R_H, which is quite different from the measured value. It was shown that in the case of the keyhole limpet Hc, the oxy-state of the protein molecule is slightly more compact than the deoxy-state, the difference in the radii of gyration being 0.3 Å

TABLE 1 Parameters calculated from dynamic light scattering measurements

Species	R_H (nm)	R_H^{Theo}(nm)	f/f^{Theo}
oxy-*R. thomasiana* Hc (pH 7.0)	23.6 ± 0.3	21.7*	1.95†
apo-*R. thomasiana* Hc (pH 7.0)	22.2 ± 0.4	n.c.‡	n.c.
oxy-*R. thomasiana* Hc + 1 M NaCl (pH 7.0)	24.6 ± 0.4	n.c.	n.c.
Substructures			
oxy-RtH1 (dissociated, pH 9.6)	7.8 ± 0.4	5.7	1.37
apo-RtH1 (dissociated, pH 9.6)	6.4 ± 0.4	5.7	1.12
oxy-RtH2 (dissociated, pH 9.6)	7.5 ± 0.3	5.7	1.32
apo-RtH2 (dissociated, pH 9.6)	7.4 ± 0.3	5.7	1.30
oxy-RtH2e (pH 7.0)	4.8 ± 0.3	2.8	1.71
apo-RtH2e (pH 7.0)	4.0 ± 0.2	2.8	1.43

*Calculated by the formula $R^3 = 3ab^2/2$ (see Materials and Methods).
†Calculated by the formula $f = f_o\,(2/3)^{1/3}P^{2/3}/((\ln(2P) - 0.30)$ (see Materials and Methods).
‡Not calculated (*n.c.*) because the axial ratio P is unknown.

(Hartmann et al., 2004). Our results do not oppose the data of these authors, because they compare two hemocyanin forms (oxy and deoxy), each containing the di-copper dioxygen-binding site whereas in the case of *R. thomasiana* Hc, we compare the active oxy-form (with dioxygen bound to the copper site) with the inactive form (without copper ions). It is not surprising that the binding of two copper ions to the apo-Hc leads to less compact and more asymmetrical form of the Hc molecule.

The two structural subunits of the *R. thomasiana* Hc, RtH1 and RtH2, have the same molecular mass, ~420 kDa, but differ in their carbohydrate content (Stoeva et al., 1995). The measured radii of the oxy-forms, 7.8 ± 0.4 nm and 7.5 ± 0.3 nm (Table 1), are practically the same because the difference is in the limit of the experimental error. The solutions of both structural subunits contained 2 M urea to avoid aggregation. The DLS measurements showed that the samples were monodisperse. 2 M urea is well known as a reagent which protects molluscan Hc subunits from aggregation without unfolding of the native structure and the subunits are monodisperse (Herskovits et al., 1986, 1991, 1992, and references therein). Circular dichroism (CD) and column chromatography measurements (not included because this is known for molluscan hemocyanins) showed that this is valid also for the subunits of the *R. thomasiana* Hc.

Here, we compare hydrodynamic parameters for protein solutions with identical refractive index. The hydrodynamic parameters were corrected for changes in viscosity in the presence of 2 M urea.

For the theoretical calculations both subunits were treated as globular proteins. The theoretical hydrodynamic radius R_H^{Theo} for each subunit represents the radius of a hypothetical hard sphere which diffuses with the same speed. The differences between the measured and theoretical radii of the subunits are not due to aggregation of subunits but are connected with the specific shape of these proteins. The Perrin factor $F = f/f^{Theo}$ is 1.37 for the oxy-RtH1 and 1.32 for the oxy-RtH2, which means that the DLS measured frictional coefficient is larger than the theoretical one. As f/f^{Theo} is equal to R_H/R_H^{Theo}, the measured radii are larger than that of the hypothetical sphere. It is evident that both subunits have an ellipsoidal shape because for equal volumes the surface area of an ellipsoid is greater than that of the sphere and the measured frictional coefficient/hydrodynamic radius are larger than the theoretical values for a hard sphere of the same volume. The measured R_H of the apo-form is smaller than that of the oxy-form and the Perrin ratio decreases, which shows that the two forms exist in different conformational states. For a constant mass, the decreased Perrin factor means less deviation from the spherical shape and the apo-form is more compact and more symmetric than the oxy one.

The *R. thomasiana* Hc functional unit RtH2e is a globular protein and for this reason the hydrodynamic model of a sphere can be applied for theoretical calculations. The molecular mass of the FU is ~50 kDa (Perbandt et al., 2003). It contains a single

TABLE 2 Fluorescence decay parameters of hemocyanins after excitation at 297 nm

Hemocyanin	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)	χ^2
oxy-*R. thomasiana* (pH 7.0)	0.11 ± 0.01	70	1.67 ± 0.14	30	1.2
oxy-*R. thomasiana* + 1 M NaCl (pH 7.0)	0.21 ± 0.02	74	2.47 ± 0.17	26	1.2
apo-*R. thomasiana* (pH 7.0)	0.13 ± 0.02	80	2.38 ± 0.14	20	1.3
oxy-RtH1 (dissociated, pH 9.6)	0.11 ± 0.02	74	2.59 ± 0.11	26	1.3
apo-RtH1 (dissociated, pH 9.6)	0.24 ± 0.2	70	2.86 ± 0.15	30	1.2
oxy-RtH2 (dissociated, pH 9.6)	0.27 ± 0.02	81	2.52 ± 0.13	19	1.2
apo-RtH2 (dissociated, pH 9.6)	0.26 ± 0.02	60	3.01 ± 0.15	40	1.2
oxy-RtH2e	0.19 ± 0.02	80	2.29 ± 0.12	20	1.2
apo-RtH2e	0.16 ± 0.02	78	2.79 ± 0.14	22	1.2

di-copper dioxygen-binding site. The hydrodynamic radius of the oxygenated functional unit is 4.8 ± 0.3 nm and that of the apo-form is 4.0 ± 0.2 nm (Table 1), which indicates that both forms exist in different conformations. The two radii can be compared because they were obtained in water solutions with identical viscosity and refractive index. The theoretical R_H of RtH2e was calculated to be 2.8 nm (Table 1) using the model of a hard sphere with the same volume. The difference between the experimental and theoretical values is not due to aggregation. In a previous article (Perbandt et al., 2003) we showed that at the conditions used, RtH2e exists as a monomer. Usually, globular proteins are nonspherical and a Perrin ratio >1.0 reflects the influence of the asymmetric shape of the particle. For a sphere, f/f^{Theo} is 1.00. The closer the ratio is to 1, the more spherical the shape is. In this way the Perrin factor provides information about the shape of the particle. As can be seen in Fig. 4, the shape of the functional unit is quite different from that of a sphere. Comparison of the values of the Perrin factors for the two forms of the *R. thomasiana* Hc functional unit shows that the apo-form is more symmetrical and more compact than the oxy-form. The ratio f/f^{Theo} for the oxy-RtH2e is 1.71 and the shape of the oxidized *R. thomasiana* Hc functional unit should be assigned as that of a prolate ellipsoid, because a disk shape with a Perrin factor >1.5 would have an unrealistically minor axis (Cantor and Schimmel, 1980).

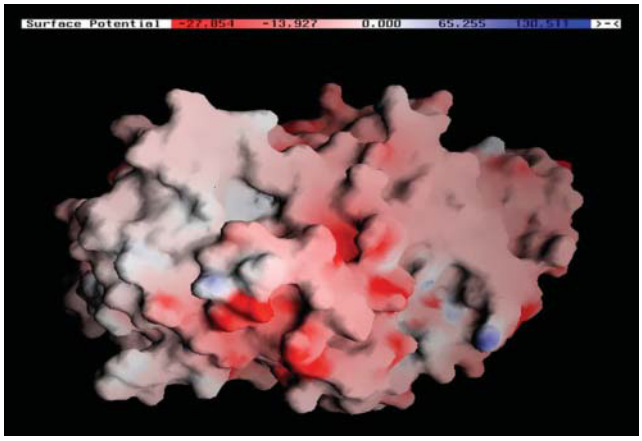

FIGURE 4 Conformation of the *R. thomasiana* hemocyanin functional unit RtH2e (PDB code 1LNL). The surface potentials are shown, the positive in blue and the negative in red.

Effect of an allosteric regulator on the conformation of the oxy-*Rapana* hemocyanin

Hcs bind dioxygen highly cooperatively (Hartmann et al., 2001). Chloride ions are allosteric regulators of the hemocyanin molecule, changing the affinity for dioxygen (Hazes et al., 1993). We studied the effect of these ions on the quaternary structure of the oxy-RtH. In the absence of added NaCl, the hydrodynamic radius of the oxy-Hc is 23.6 ± 0.3 nm, and in the presence of 1 M NaCl, the respective value is 24.6 ± 0.4 nm (Table 1); i.e., there is a difference of 3 Å, which is out of the standard error. The hydrodynamic parameters were corrected for changes in viscosity in the presence of 1 M NaCl. The changed lifetimes of the *R. thomasiana* Hc tryptophyl residues (Table 2) definitely conform to the conformational changes in the presence of the allosteric regulator (see below).

Time-resolved fluorescence data

For the further characterization of the conformational states of oxy- and apo-*R. thomasiana* Hc and its substructures, as well as to study the effect of the allosteric regulator Cl^-, we used time-resolved fluorescence spectroscopy. This is one of the most sensitive methods for studying protein conformation in solution and changes in conformation. We used the emission of the indole groups, which is dependent on, and very sensitive to changes in the microenvironment. *R. thomasiana* Hc contains eight tryptophans per functional unit (Boteva et al., 1991) and their location in the three-dimensional structure of one of the FUs building the hemocyanin molecule, RtH2e, is shown in Fig. 5. One of the tryptophyl residues, Trp-69 (Table 2), is in the immediate microenvironment of the dioxygen-binding site, in a distance of 5–6 Å from the copper atom Cu_1. Its emission should be very sensitive to conformational changes in the region of the active site. The other indole groups are more distant from this site, Trp-351 being at a distance of ~10–11 Å (Table 3). Most of the tryptophans are buried in the hydrophobic interior of the globule. The emission of the buried residues should respond to eventual conformational changes in the globule, which will change the polarity of their environment. The fluorescence decay of RtH, RtH1, RtH2, and RtH2e was

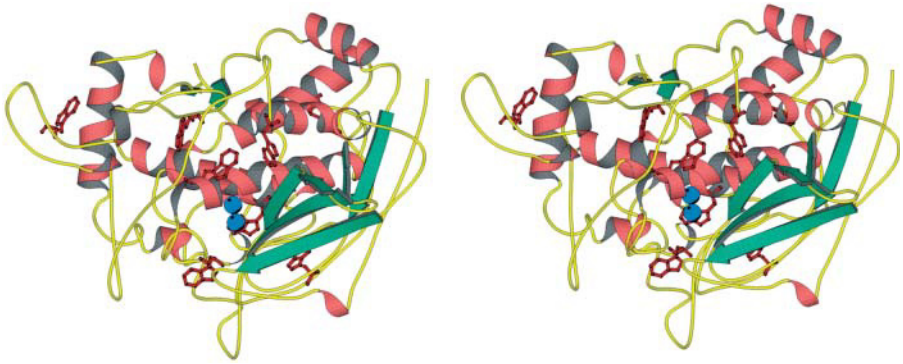

FIGURE 5 Stereo figure of the *R. thomasiana* hemocyanin functional unit RtH2e showing the location of the tryptophyl residues. (The α-helices are shown in *red*, β-sheets in *green*, and the two active site copper ions in *blue*.)

investigated upon excitation at 297 nm, where the light is selectively absorbed by the tryptophan chromophores, and was well fitted by two exponentials. With three exponentials the contribution of the third component was $<1\%$ and for this reason we present the data with two exponentials. The value of the parameter χ^2 was usually 1.2, and in two cases, 1.3 (Table 2); the residuals between the theoretical and experimental decay curves as well as autocorrelation plots were flat and random, which demonstrates a very good quality of the fit. Fig. 6 shows the fluorescence decay of oxy-*R. thomasiana* Hc. Similar curves were obtained for the hemocyanin substructures. The calculated lifetimes and relative amplitudes are shown in Table 2. In the presence of chloride ions both the short and the long lifetimes increase considerably, and the difference is far outside the standard error. This means changes in the microenvironment of the indole chromophores and could be explained with a new conformation adopted by the dioxygen-binding protein after the binding of the allosteric regulator. Comparison of the data for the oxy- and apo-forms of RtH shows that the shorter lifetime, τ_1, is the same for both forms because the difference is in the limit of the experimental error. The two forms differ in the longer lifetime, τ_2, which has a higher value for the apo-RtH. The difference in the excited states of the indole chromophores in oxy- and apo-RtH1 influences only the shorter lifetime τ_1, whereas the difference in τ_2 is in the limit of the experimental error. The two forms of RtH2 have practically the same shorter lifetime, and the same is valid for RtH2e. The oxy- and apo-states of the structural and functional units differ in the values of τ_2. The dynamic fluorescence parameters shown in Table 2 demonstrate the changed polarity of the tryptophan's microenvironment in the native Hc and its substructures upon the transition from the active to an inactive state in which these proteins adopt a different conformation.

TABLE 3 Intramolecular distances in the *R. thomasiana* hemocyanin functional unit RtH2e between atoms of the tryptophyl residues and the copper active site

Number of the tryptophyl residue	Atom	Distance to Cu_1, Å
69	CE3	5.74
	CZ3	5.39
	CH2	6.03
93	CE3	14.82
	CZ3	20.66
111	CD1	28.52
	CB	27.42
156	CH2	15.87
	CZ3	15.85
187	CD1	15.74
	CZ	13.28
222	CE2	19.16
	CZ2	19.47
351	CD1	10.74
	NE1	10.62
385	CD1	14.51
	CG	14.95

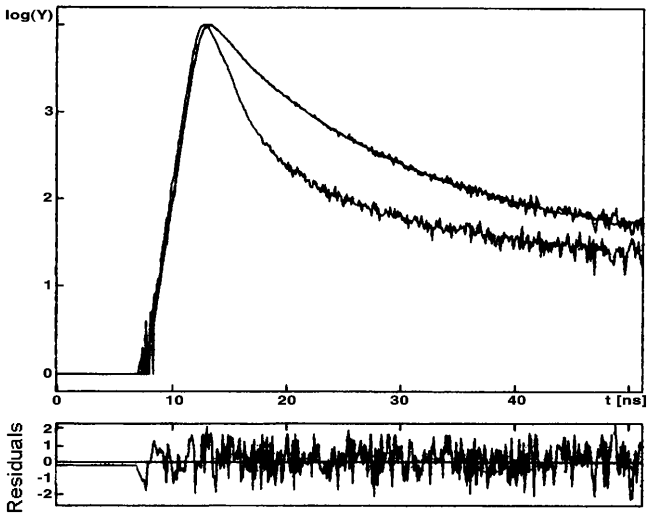

FIGURE 6 Fluorescence decay of oxy-*R. thomasiana* hemocyanin at pH 7.0 for excitation at 297 nm. The upper line denotes two superimposed curves; i.e., the experimental fluorescence data fitted with the convolution curve of a biexponential decay function, and the instrument response function. The last function is shown in the lower curve. The reduced χ^2-value of 1.2 and the residuals between the theoretical and experimental decay curves which are evenly distributed at ~ 0 demonstrate the good quality of the fit.

CONCLUSIONS

The results of the dynamic light scattering and time-resolved fluorescence measurements show that the physiologically active and inactive forms of the *R. thomasiana* hemocyanin, a representative of the molluscan respiratory proteins, and its substructures exist in different conformations. The transition active-inactive Hc is connected with changes in the shape of the molecule. Conformational dynamics play an important role in the reversible binding and transport of dioxygen by hemocyanins. In a previous article (Perbandt et al., 2003), we proposed a molecular mechanism for cooperative dioxygen binding in molluscan respiratory proteins which explains the modulation of the O_2 affinity. We supposed that structural changes in the region of the active site during the binding of dioxygen, which were evident from the respective x-ray models, are translated from one functional unit to the other, via structural changes concerning the whole hemocyanin. The results presented here demonstrate that changes in the region of the active site are really connected with conformational changes in the whole molecule and support the mechanism mentioned above.

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