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Crystallographic Analysis of Oxygenated and Deoxygenated States of Arthropod Hemocyanin Shows Unusual Differences

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ABSTRACT **The X-ray structure of an oxygenated hemocyanin molecule, subunit II of *Limulus polyphemus* hemocyanin, was determined at 2.4 Å resolution and refined to a crystallographic *R*-factor of 17.1%. The 73-kDa subunit crystallizes with the symmetry of the space group *R*32 with one subunit per asymmetric unit forming hexamers with 32 point group symmetry. Molecular oxygen is bound to a dinuclear copper center in the protein's second domain, symmetrically between and equidistant from the two copper atoms. The copper–copper distance in oxygenated *Limulus* hemocyanin is 3.6 ± 0.2 Å, which is surprisingly 1 Å less than that seen previously in deoxygenated *Limulus polyphemus* subunit II hemocyanin (Hazes et al., Protein Sci. 2:597, 1993). Away from the oxygen binding sites, the tertiary and quaternary structures of oxygenated and deoxygenated *Limulus* subunit II hemocyanins are quite similar. A major difference in tertiary structures is seen, however, when the *Limulus* structures are compared with deoxygenated *Panulirus interruptus* hemocyanin (Volbeda, A., Hol, W.G.J. J. Mol. Biol. 209:249, 1989) where the position of domain 1 is rotated by 8° with respect to domains 2 and 3. We postulate this rotation plays an important role in cooperativity and regulation of oxygen affinity in all arthropod hemocyanins.**

Key words: dinuclear copper site, hemocyanin, oxygen binding, allosteric regulation

INTRODUCTION

Arthropod hemocyanins are hexameric or multihexameric oxygen transport proteins that typically bind molecular oxygen cooperatively at dinuclear copper sites.[1,2] The horseshoe crab, *Limulus polyphemus,* has an octahexameric hemocyanin containing eight immunologically distinct subunit types.[3] *Limulus* subunit type II hemocyanin (*Limulus* II) comprises 628 residues and can form homohexamers similar to those observed in the native octahexameric complex.[4] This subunit forms crystals amenable to high resolution X-ray diffraction analysis[5] under conditions where its homohexamers are noncooperative in oxygen binding but are susceptible to allosteric regulation by chloride (data not shown). A low resolution analysis of the *Limulus* II hemocyanin structure indicated a hexameric arrangement of the subunits in the crystals.[6]

Crystallization, data collection, and refinement of oxygenated and deoxygenated structures of *Limulus* II hemocyanin were carried out independently in two of our laboratories.[7,8] Previous studies on the deoxygenated structure of *Panulirus interruptus* hemocyanin revealed a three domain architecture with the oxygen coordinating site centered in domain 2. The oxygen coordinating site consists of two copper atoms which are each coordinated by three histidine residues.[9]

MATERIALS AND METHODS

A brief description of the refinement of the oxygenated *Limulus* II structure is outlined below. A more detailed description of the refinement of the oxygenated *Limulus* II structure will be presented elsewhere.[7] The details of the deoxy *Limulus* II structure determination and refinement can be found in Hazes et al.[8]

Determination of the Oxygenated *Limulus* II Crystal Structure

Crystals were grown in 0.5 M NaCl, 0.16 M Bis-Tris, 0.03 M Tris-glycine, 0.006 M Na_2EDTA, 4%

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TABLE I. Summary of Crystallographic Information

Data sets		
Detector type	Xuong-Hamlin/UNC	R-Axis II/McMU
R-sym*	0.0864	0.054
Unique reflections	13387	27713
Percentage of observed reflections	86.9	71.2
Cell constants	$a=b=117.20$, $c=285.80$	$a=b=117.03$, $c=285.20$
Resolution	10.0–3.0 Å	10.0–2.4 Å
Model		
R-value†	28.2	17.1
Resolution	10.0–3.0 Å	6.0–2.4 Å
Number of nonhydrogen atoms	—	4656
Number of copper atoms	2	2
Number of ordered residues	559	573
Number of disordered residues	69	55
Number of ordered waters	0	323
Average B-value	Set to 20.0 Å^2	26.2 Å^2
Number of disulfide bonds	2	2
Deviations from ideality (rms)		
Bond distance	—	0.017 Å
Bond angle	—	3.523°

*R – Sym $= [(\Sigma|I - \langle I\rangle|)/(\Sigma \langle I\rangle)] \times 100$.
†R – value $= [(\Sigma||F_{obs}| - |F_{calc}||)/(\Sigma F_{obs})] \times 100$.

polyethylene glycol 8000 at pH 6.2.[5] There is one molecule of approximately 73 kDa per asymmetric unit. All crystals used for data collection of the oxygenated structure were distinctly blue, indicating the protein is in its oxygenated form. The crystallographic phase problem for this structure was solved at 4 Å resolution by molecular replacement as previously described.[6]

Two data sets were used in the refinement of the oxygenated *Limulus* II model (Table I). The first data set was collected at the University of North Carolina at Chapel Hill on a Xuong-Hamlin type multiwire area detector with an Elliott GX-13 rotating anode source operated at 2.4 kW. These data were merged using the program FS (Weissman, Stauffacher and Eisenberg, unpublished). A second data set was collected using a Rigaku R-axis II image plate system with a Rigaku RU-200 rotating anode source at 4 kW at McMaster University. The second data set was reduced and processed using the software PROCESS[10] and MOSFLM.[11]

Model building was done with an Evans and Sutherland PS390 using the programs FRODO[12] and O.[13] Initial refinement was accomplished with TNT[14] and completed using the simulated annealing procedure in X-PLOR.[15] Individual isotropic B-values have been refined for all atoms. The copper atoms and the bound oxygen molecule were placed using difference $(F_o - F_c)\alpha_c$ maps generated without these atoms. Atomic coordinates for the active site were verified using a simulated annealing omit map,[15] in which an 8-Å sphere centered at the active site was removed from the model before density calculation.

RESULTS

The Oxygenated *Limulus* Structure

The present *Limulus* II model is refined and is based on reflection data with resolutions between 6.0 to 2.4 Å. The model has an R-value of 17.1% with good stereochemistry and no disallowed contacts between atoms (Table I). Coordinate errors are estimated to be 0.2 Å by the program SIGMAA.[16] Of the 574 residues, 570 fall within the allowed regions of a Ramachandran plot[17] (Fig. 1). There are poorly-ordered residues found on the surface of the hemocyanin molecule which comprise residues 18–32, 132–150, 421–427, 522–530, and 570–573. The oxygen-binding site atoms are well-defined with B values of 19 and 20 Å^2 for CuA and CuB, 23 and 25 Å^2 for the two oxygen atoms, and from 16 to 19 Å^2 for the six histidine ligands. The overall B factor for all atoms of one subunit is 27.6 Å^2. The mean B factors for domains 1, 2, and 3 are 31.1, 22.1, and 29.9 Å^2, respectively.

Comparison of the Oxygenated and Deoxygenated *Limulus* Structures

The *Limulus* II oxygenated and deoxygenated structures are strikingly similar and superimpose with a root-mean-square deviation of 0.26 Å for the main chain atoms. Most differences are concentrated at the active site (Fig. 2). Table II presents

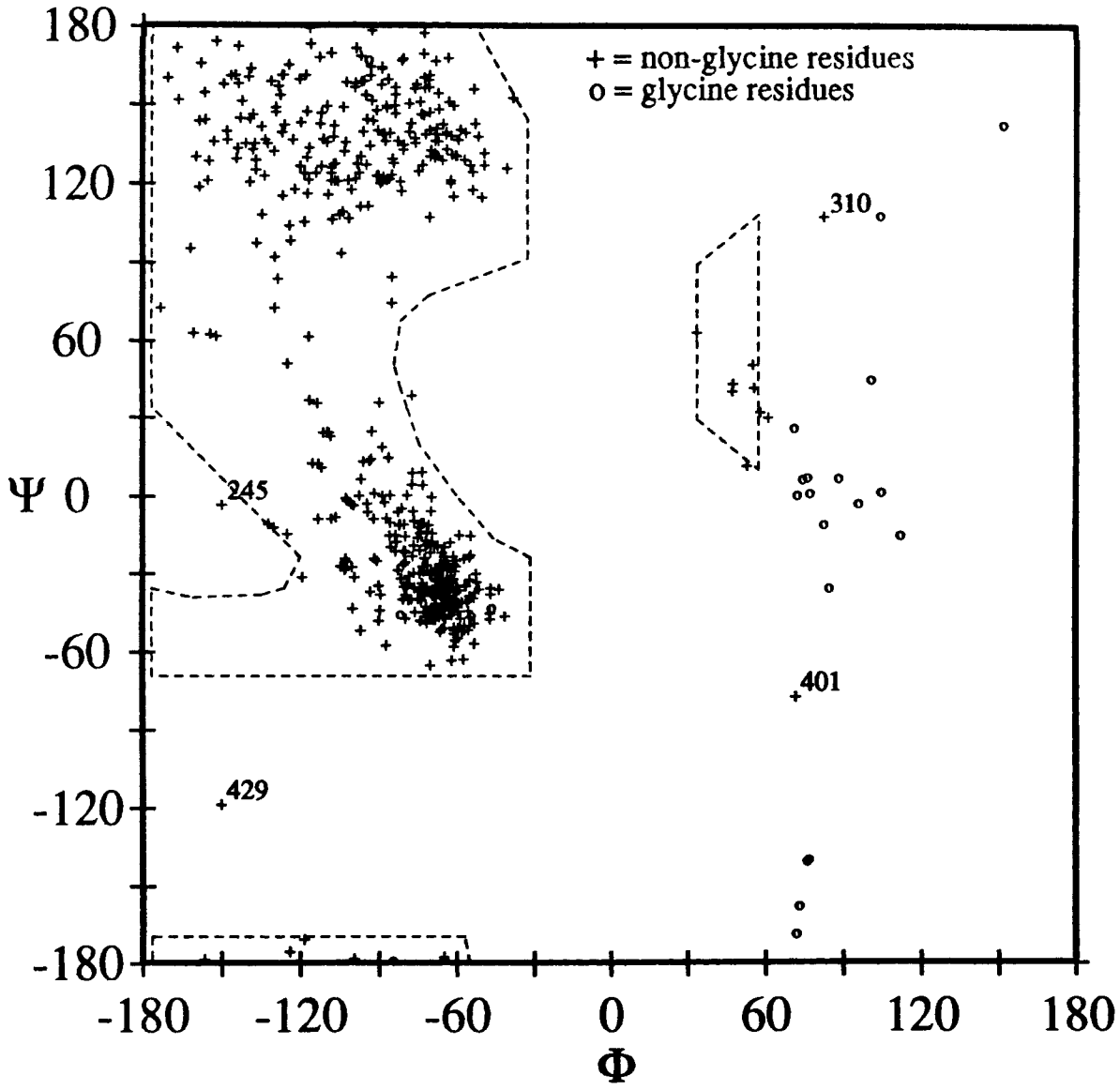

Fig. 1. Ramachandran plot[17] of the phi and psi angles of the oxygenated *Limulus* II structure. The most energetically favorable regions are outlined by the dotted lines. Four amino acids were modeled as adopting conformations that fall outside these regions. Residue 245 is involved in a tight turn which appears to require a high energy conformation. The residue at 310 is in the unusual *cis* peptide configuration. The amino acids at 401 and 429 are both on the border of structurally disordered regions of the protein.

data on the active site of oxygenated and deoxygenated *Limulus* II hemocyanin and from previously reported structures of *Panulirus* hemocyanin,[9] and a small peroxo-dinuclear copper(II) complex.[18] Other structural features of particular interest are described below.

It has been previously described that, in the deoxygenated *Limulus* II structure, the two coppers of the active site are each trigonally coordinated by three histidines (Fig. 2). The six histidine residues of the oxygen-binding site are conserved in all known arthropod hemocyanins.[8,19] The Cu–Cu distance in deoxygenated *Limulus* II hemocyanin is 4.6 ± 0.2 Å,[8] appreciably greater than found in deoxygenated *Panulirus* hemocyanin (3.5 Å),[8] as well as in oxidized ascorbate oxidase (3.7 Å),[20] a dinuclear copper-containing enzyme.

Our results show that, upon oxygenation, the coppers draw significantly closer to each other, resulting in a Cu–Cu distance of 3.6 ± 0.2 Å, while the histidines move only slightly. Molecular oxygen is bound symmetrically between the two coppers

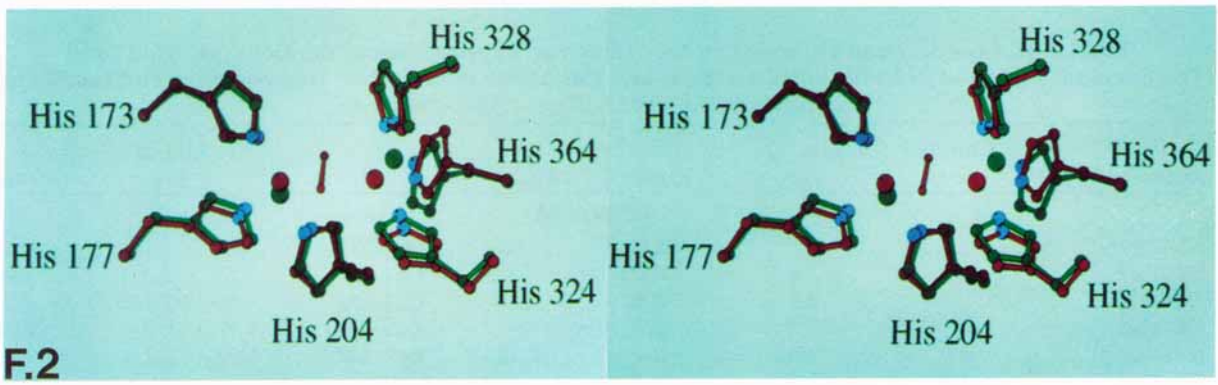

Fig. 2. Stereo view of the oxygen-binding site of deoxygenated (green) and oxygenated (red) *Limulus* II hemocyanin. Nitrogen atoms coordinate the copper ions (blue). With oxygen bound, the copper ions are significantly closer together, but the histidine residues barely move. Previous EXAFS data on hemocyanins are consistent with both the 3.6 Å Cu–Cu distance in the oxygenated form and the decrease in number of ligands around each copper from five to three.[25,26] However, the Cu–Cu distance of 4.6 Å in the deoxygenated state is much longer than the 3.4 Å indicated in the EXAFS experiments. Only the study by Co and Hodgson did not detect a Cu–Cu distance of less than 4 Å.[27] Histidine 364 may assume two conformations with its imidazole ring rotated ~180° apart.[8] The conformations have χ^2 values of −85 and 80° although the χ^2 = 80° conformation has been refined to low *B* value. The N^{δ} of histidine 364 can make hydrogen bonds with either a water molecule or the carbonyl oxygen of phenylalanine 360 in the standard and alternative rotamer, respectively. The change in coordination to basically square pyramidal is ascribed to an oxidation of the coppers to copper(II). The dioxygen molecule, presumably a peroxo (O_2^{2-}) state,[28] has no obvious close interactions with any other protein atoms, solvent molecules, or bridging ligands. In the deoxygenated form of the protein,[8] the coordination of each copper(I) ion is essentially trigonal planar with copper to histidine N^{ϵ} distances approximately equal. Viewed along the copper–copper axis, the histidines coordinating the coppers are approximately staggered in conformation, contrasting with the structure of oxidized ascorbate oxidase where they are eclipsed.[20] This figure was produced using the program MOLSCRIPT.[29]

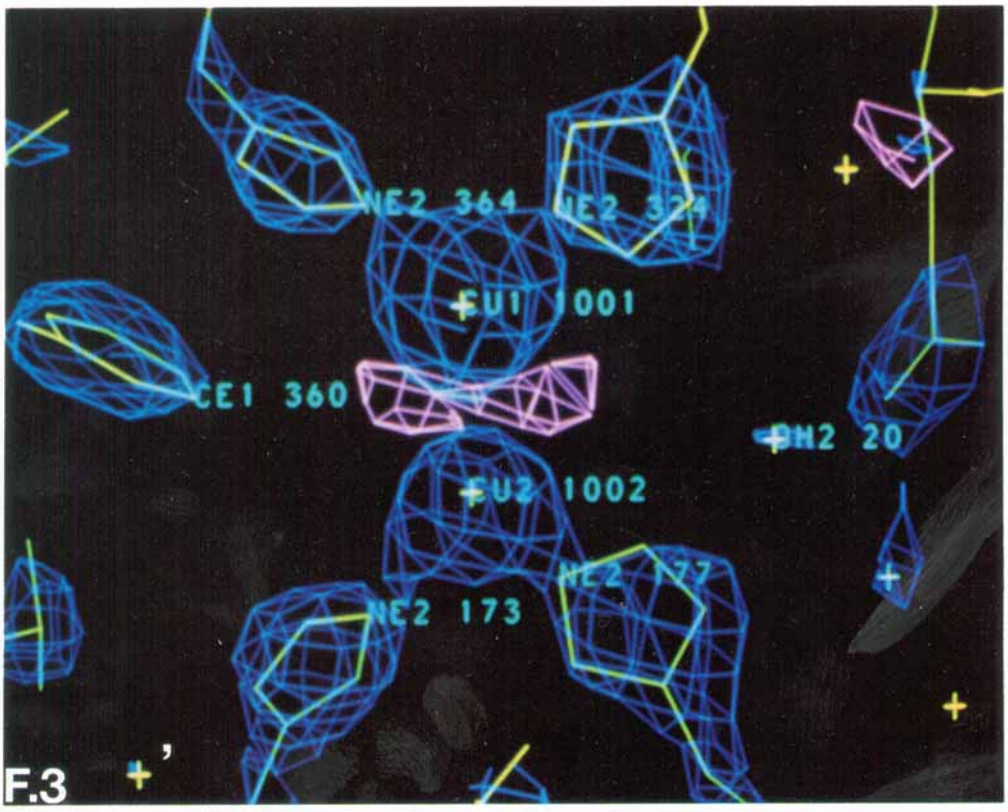

Fig. 3. The oxygen-binding site of *Limulus* II hemocyanin in its oxygenated form. Electron density (blue lines) is from a $(2F_o-F_c)\alpha_c$ Fourier synthesis contoured at 2σ. Protein atoms in the model used for phase calculations are shown connected by yellow lines. Features of the difference electron density map needing inclusion in the model (pink lines) are a difference Fourier synthesis, $(F_o-F_c)\alpha_c$ contoured at 2.5σ. Location of bound molecular oxygen is clearly visible in the oxygenated form. This figure was generated using the program FRODO.[12]

TABLE II. Coordination Geometry of the Dinuclear Copper Sites of the Deoxygenated* and Oxygenated† Forms of *Limulus polyphemus* II and *Panulirus interruptus*‡ Hemocyanins and the Model Compound§ [Cu(HB(3,5-iPr$_2$pz)3)]2(O_2)·6CH_2Cl_2

Atoms	Lim. II deoxy	Lim. II oxy	P. int.	Model	Atoms	Lim. II oxy	Model
			Distances (Å)				
N1–Cu1	2.1	2.2	2.0	2.02	O1–O2	1.4	1.43
N4–Cu2	2.2	1.9	2.0	—	Cu1–O1	2.0	1.96
N2–Cu1	2.0	2.1	2.0	2.17	Cu1–O2	1.7	—
N5–Cu2	2.1	2.4	2.7	—	Cu2–O1	2.2	1.94
N3–Cu1	1.9	2.4	2.7	2.43	Cu2–O2	2.1	—
N6–Cu2	1.9**	2.1**	2.1	—			
Cu1–Cu2	4.6	3.6	3.5	3.64			
			Angles (degrees)				
N1–Cu1–N2	99	99	94	89.2	Cu1–O1–Cu2	121	137.1
N4–Cu2–N5	97	106	93	—	Cu1–O2–Cu2	143	—
N2–Cu1–N3	126	104	100	97.1	O1–Cu1–O2	44	42.9
N5–Cu2–N6	142	113	105	—	O1–Cu2–O2	39	—
N3–Cu1–N1	131	121	105	91.6	N1–Cu1–O1	123	108.8
N6–Cu2–N4	108	94	99	—	N4–Cu2–O1	99	—
					N1–Cu1–O2	101	149.3
					N4–Cu2–O2	133	—
					N2–Cu1–O1	97	151.0
					N5–Cu2–O1	101	—
					N2–Cu1–O2	139	112.8
					N5–Cu2–O2	102	—
					N3–Cu1–O1	98	104.7
					N6–Cu2–O1	139	—
					N3–Cu1–O2	95	105.7
					N6–Cu2–O2	109	—

*N1 = His-173 N^ϵ, N2 = His-177 N^ϵ, N3—His-204 N^ϵ, Cu1 = CuA, N4 = His-324 N^ϵ, N5 = His-328 N^ϵ, N6 = His-364 N^ϵ, Cu2 = CuB.[8]

†N1 = His-173 N^ϵ, N2 = His-177 N^ϵ, N3 = His-204 N^ϵ, Cu1 = CuA, N4 = His-324 N^ϵ, N5 = His-328 N^ϵ, N6 = His-364 N^ϵ, Cu2 = CuB, O1 = O1, O2 = O2.

‡N1 = His-194 N^ϵ, N2 = His-198 N^ϵ, N3 = His-224 N^ϵ, Cu1 = CuA, N4 = His-344 N^ϵ, N5 = His-348 N^ϵ, N6 = His-384 N^ϵ, Cu2 = CuB. Average of the values of the six molecules in the crystallographic asymmetric unit.[9] The copper coordination in the *Panulirus* structure is rather unusual and may differ from a physiological deoxygenated conformation due to the low pH at which the crystals were obtained.

§N1 = N1, N2 = N3, N3 = N2, Cu1 = Cu, Cu2 = Cu′, O1 = O, O2 = O′. Compound is centrosymmetric. (The numbers shown were calculated directly from the coordinates in ref. 18.)

**The imidazole ring of histidine 364 coordinating CuB may be modeled as adopting two conformations. The distances here are for the predominant conformer (see Fig. 2 text).

and the two oxygen atoms lie in a plane orthogonal to the Cu–Cu axis (Fig. 3). This η^2:η^2 geometry of oxygen binding resembles that previously reported for two synthetic copper–dioxygen complexes.[18,21]

In oxygenated *Limulus* II hemocyanin, each copper is tightly coordinated in square planar geometry to both atoms of the oxygen molecule and to the N^ϵ atoms of two of the three histidine ligands (Figs. 2 and 3). These two copper, two oxygen and four N^ϵ atoms approximately form a single plane. Above and below this plane, the N^ϵ atoms of histidine 204 and histidine 328 complete the coordination spheres of CuA and CuB by occupying more distal axial square pyramidal positions.

Comparisons of *Limulus* and *Panulirus* Hemocyanin Structures

The oxygenation-linked changes described above allow us to propose a structural mechanism explaining allosteric regulation properties of oxygen binding by hemocyanins (Fig. 4). Analogous to hemoglobin, the basic oxygen binding behavior of hemocyanin can be described with a two-state model,[22] where the hemocyanin hexamer adopts conformational states of relatively high (R-state) or low (T-state) oxygen affinity that may be oxygenated or deoxygenated.

Under physiological conditions, deoxygenated hemocyanin is typically in the low affinity T-state

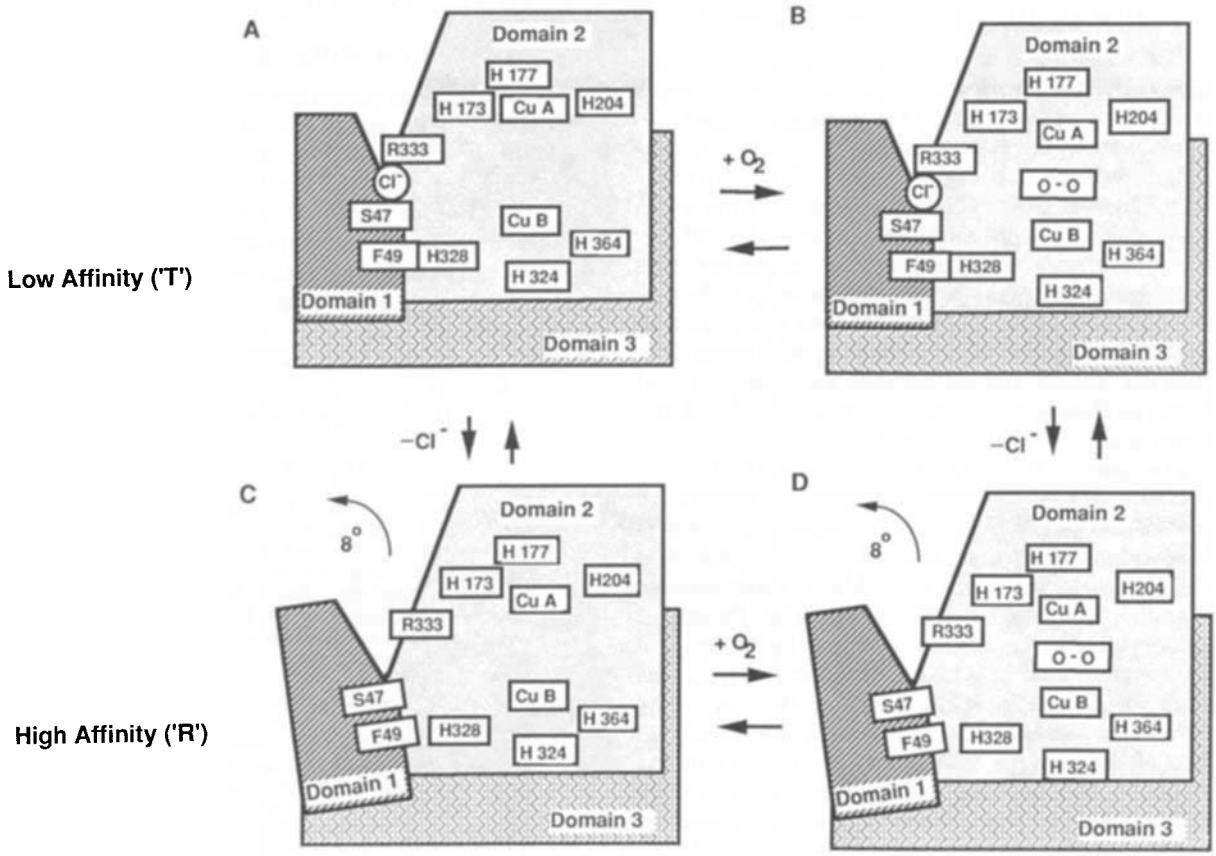

Fig. 4. Proposed mechanism for allosteric regulation of arthropod hemocyanins. (**A**) Deoxygenated T-state (deoxygenated *Limulus* II hemocyanin[8]). (**B**) Oxygenated T-state (oxygenated *Limulus* II hemocyanin). (**C**) Deoxygenated R-state (deoxygenated *Panulirus* hemocyanin[9]). (**D**) Oxygenated R-state (postulated).

conformation.[1] In this state, represented by the deoxygenated *Limulus* II structure shown schematically in Figure 4A, the phenyl ring of phenylalanine 49 and the imidazole ring of histidine 328 are tightly stacked, with their rings nearly parallel and 3.5 Å apart. A chloride ion is bound between serine 47 and arginine 333 in the interface between domain 1 and domain 2. The oxygen affinity of *Limulus* II hemocyanin is known to be greatly reduced by virtue of chloride binding,[4] possibly because of this interface position.

In the oxygenated state, hemocyanins typically assume a high affinity R-state conformation.[1] However, as in hemoglobin, the low affinity T-state of the hemocyanins can be maintained in oxygenated forms by structural constraints.[1] Either crystal packing or high chloride concentrations or both could contribute to maintenance of the T-state by the oxygenated crystals of *Limulus* II hemocyanin. To explain the strong structural similarity between the oxygenated and deoxygenated *Limulus* II structures, we presume that the oxygenated structure has maintained the T-state conformation, as shown schematically in Figure 4B.

Comparison of the two *Limulus* and the *Panulirus* structures revealed that the positions of the first domain in both the deoxygenated and oxygenated *Limulus* structures are essentially the same but differ from the *Panulirus* structure's first domain by a rotation of approximately 8°. The domain rotation affects (1) the oxygen binding site, since phenylalanine 49 in domain 1 moves away from histidine 328 in domain 2 by almost 3 Å releasing the steric constraint on this copper ligand, (2) the chloride binding site, since the two chloride ligands serine 47 in domain 1 and arginine 33 in domain 2 also move apart by approximately 3 Å, probably destroying the putative chloride binding site, and possibly (3) the domain 1–domain 1 interface between two subunits in opposite trimers of a hexamer, allowing the triggering of a change in hexameric quaternary structure. Exactly these three effects are required to explain the different oxygen affinity, chloride affinity and cooperative oxygen binding behavior

that is observed in *Limulus polyphemus* hemocyanin.

DISCUSSION AND CONCLUSIONS

The structure determination of oxygenated and deoxygenated *Limulus* II hemocyanin has revealed strikingly large changes of the dinuclear copper site conformation. Upon oxygenation, the copper coordination changes from trigonal planar to square pyramidal and the Cu–Cu distance decreases from 4.6 to 3.6 ± 0.2 Å. In spite of these changes, the tertiary and quaternary structures of the oxygenated and deoxygenated states are not significantly different. Oxygen binding studies indicate that this is caused by the stabilization of the T-state by the bound chloride ion. Hence, the oxygenated and deoxygenated *Limulus* II structures represent the oxygenated and deoxygenated T-states, respectively.

The domain 1–domain 2 interface in the *Panulirus* hemocyanin structure differs from those found in the oxygenated and deoxygenated *Limulus* II hemocyanin structures. We postulate therefore that the *Panulirus* structure resembles a deoxygenated R-state. The oxygen affinity state of *Panulirus* hemocyanin in the crystal could not be determined by oxygen binding studies at approximately the same conditions in solution due to the low pH of crystallization (pH 3.8),[24] which causes precipitation of the protein. Obviously, the low pH in the structure may have led to protonation of one or more of the histidines at the dicopper site. This would imply that, under physiological conditions, details of a deoxygenated R-state arthropod hemocyanin may appear differently.

At this moment, there is not yet a structure of an arthropod hemocyanin in an oxygenated R-state. It seems likely, however, that while this state would have a tertiary structure similar to the *Panulirus* structure, its oxygenated dinuclear copper center would most likely resemble that in the oxygenated *Limulus* II structure. It is also possible the copper coordination geometry in the oxygenated R-state hemocyanin may be closer to ideal than in the oxygenated T-state, especially around histidine 328, where phenylalanine 49 is further removed from the oxygen binding center in the T-state.

Considering the observed differences in the tertiary structures and dinuclear copper site conformations of R- and T-state arthropod hemocyanin, we propose that the trigger for cooperativity in these molecules is the 1 Å decrease in the Cu–Cu distance when oxygen is bound. To effect cooperativity, this shift is transmitted by histidine 328 to "sensor" phenylalanine 49, resulting in a rotation of domain 1, which is in turn in contact with other subunits in the hexamer of hemocyanin subunits. Comparison of the *Limulus* and *Panulirus* quaternary structures reveals a 3.2° rotation of the two trimers within the hexamer. Whether or not this represents the quaternary conformation change between R- and T-states will have to await the structure determination of a true R-state oxygenated hemocyanin.

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