CO Binding by Hemocyanins of *Limulus polyphemus*, *Busycon carica*, and *Callinectes sapidus*†

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ABSTRACT: The equilibria and kinetics of carbon monoxide binding to the hemocyanins of *Limulus polyphemus*, *Callinectes sapidus*, and *Busycon carica* have been studied. The CO-binding properties of these hemocyanins show large differences relative to their oxygen-binding properties. The CO complex is colorless like deoxyhemocyanin but shows absorption differences in the ultraviolet region. Untreated *Limulus* hemocyanin, whose molecular weight is in the range of several million, shows the same low affinity for CO as stripped *Limulus* hemocyanin, whose molecular weight is about 66,000. The stripped hemocyanin has been fractionated into five components whose CO affinities do not parallel their respective O_2 affinities. The partial pressure of CO necessary for half-saturation of the components ranges from 54 to 290 mm. The CO pressure required for half-saturation of unfractionated *Limulus* hemocyanin is 100 mm. *Busycon* hemocyanin has a much higher CO affinity, and half-saturation is achieved at 3–4 mm. The higher CO affinity of *Busycon* hemocyanin relative to that of *Limulus* appears to be due to a much lower rate of CO dissociation. The untreated hemocyanins used in this study show large pH effects and cooperative interactions in O_2 binding. These features are shown to be reduced or absent in CO binding.

Hemocyanins are high molecular weight copper proteins found in the hemolymph of many molluscan and arthropod species. Their function is to bind and transport oxygen (Prosser, 1973). Little is known about the active site to which oxygen is bound. The copper–protein linkages are still undetermined, and the valency state of the copper in oxyhemocyanin remains a matter of speculation (van Holde and van Bruggen, 1971). Oxygen binds to hemocyanins with a stoichiometry of one oxygen per two copper atoms, and it is possible that molecular oxygen forms a bridge between the two coppers (Williams, 1966). Hemocyanin also reversibly binds carbon monoxide (Root, 1934; Kubowitz, 1938; Vanneste and Mason, 1966). CO binding has the stoichiometry of one carbon monoxide per two copper atoms. Results of infrared spectroscopy suggest a nonbridging structure for the carbon monoxide–copper complex with the oxygen of carbon monoxide coordinated to only one of the two copper atoms (Fager and Alben, 1972). This study concerns previously unexplored aspects of the kinetics and equilibria of CO binding by hemocyanins from both arthropods and molluscs.

The carbon monoxide form of hemocyanin is colorless and thus visibly indistinguishable from deoxyhemocyanin. Our results show, however, that as CO is bound there are changes in the ultraviolet absorption spectrum. The CO derivative lacks the strong absorption bands in the neighborhood of 340 and 400–800 nm which give oxyhemocyanin its characteristic blue color. Oxygen binding by untreated hemocyanin is cooperative and shows strong pH dependence. These properties are strongly influenced by inorganic ions, especially divalent cations (van Holde and van Bruggen, 1971). *Limulus* hemocyanin, which shows cooperative oxygen binding, is unusual in that it shows a reverse Bohr effect. The results presented here show that *Limulus* hemocyanin binds CO noncooperatively. Within the range examined, its CO affinity is not influenced by pH, ionic conditions, or state of aggregation of the protein. Studies with another arthropod hemocyanin and with a molluscan hemocyanin also show greatly reduced cooperativity and reduced pH effects in CO binding relative to oxygen binding.

Materials and Methods

Specimens of the horseshoe crab, *Limulus polyphemus*, the knobbed whelk, *Busycon carica*, and the blue crab, *Callinectes sapidus*, were obtained from waters in the vicinity of Pivers Island, N.C. Hemolymph was obtained from the cardiac hemocoel of *Limulus*, from the pedal hemocoel of *Busycon*, and from a pedal sinus of *Callinectes*. Untreated hemocyanin was obtained by allowing the hemolymph to clot and expressing the hemocyanin solution from the clot through 4–6 layers of cheese cloth. Solutions were centrifuged for 20 min at 20,000*g* prior to use and diluted with buffered, filtered seawater to concentrations appropriate for spectrophotometry. Extensive dialysis of *Limulus* hemocyanin *vs.* 0.052 M Tris-glycine–0.01 M EDTA (pH 8.9) does not remove copper but presumably removes most of the other ions which EDTA can chelate. Material so treated is referred to as stripped *Limulus* hemocyanin. Components of stripped *Limulus* hemocyanin were isolated as previously described (Sullivan *et al.*, 1974) by ion-exchange chromatography on DEAE-Sephadex A-50 (0.052 M Tris-glycine–0.01 M EDTA (pH 8.9); 0.0–0.5 M NaCl linear gradient). Absorption spectra were obtained with a Cary 14 uv recording spectrophotometer with the sample compartment jacketed at 20°. Oxygen equilibria were performed at 20° by a spectrophotometric method (Riggs and Wolbach, 1956). Indirect determinations of carbon monoxide equilibria were obtained by measuring oxygen binding in the presence of carbon monoxide. Values for the percentage saturation were determined at 340 and 360 nm for oxygen binding and at 290 and 310 nm for direct measurements of carbon monoxide binding. Rapid mixing experiments were performed with a Gibson–Durrum stopped-flow apparatus, equipped

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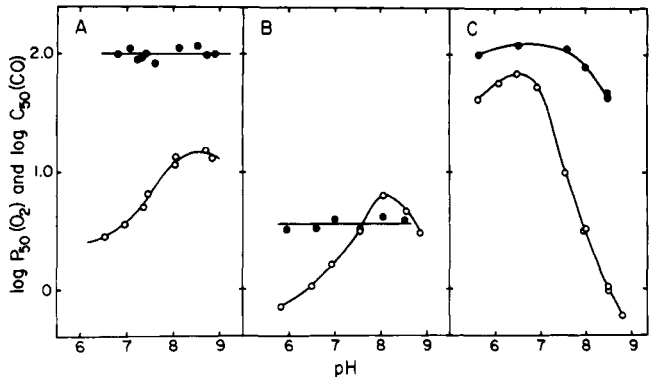

FIGURE 1: The effect of pH on the oxygen (○) and carbon monoxide (●) affinities of (A) *Limulus*, (B) *Busycon*, and (C) *Callinectes* untreated hemocyanins at 20°. Hemocyanins were at concentrations of 3–4 mg/ml. Solutions were buffered with bis-tris or Tris at a concentration of 0.05 M. Log P_{50} and log C_{50} correspond to the log of the O_2 and CO affinities expressed in mm Hg.

with a pneumatic drive, and having a measured dead time of 2.4 msec.

Results

Oxygen and Carbon Monoxide Equilibria. Carbon monoxide binding by hemocyanin was measured by both direct and indirect spectrophotometric methods. The direct method makes use of small changes in the protein absorption band which accompany CO binding. Because the absorbance changes are very small, the data obtained by the direct method show more scatter than data obtained by the indirect method. Carbon monoxide binding was determined indirectly by measuring the effect of known concentrations of CO on subsequent oxygen binding. This replacement method (as described below) was used to investigate the influence of pH on CO binding. P_{50} (O_2) values were determined as a function of pH in the presence and absence of *saturating* concentrations of CO. The partial pressure of CO necessary to achieve saturation varied from species to species. Values of the partition coefficient, M, were calculated as a function of the percentage saturation with oxygen (Y) according to

$$M_Y = (1 - Y)p_{O_2}/(Y)p_{CO} \quad (1)$$

where p_{O_2} and p_{CO} are the partial pressures of these gases. The partial pressure of CO necessary to produce a given degree of saturation with CO in the absence of oxygen can then be calculated according to

$$(p_{CO})_Y = (p_{O_2})_Y/M_Y \quad (2)$$

where $(p_{O_2})_Y$ and $(p_{CO})_Y$ are the partial pressures of O_2 and CO necessary to produce the degree of saturation denoted by Y, in the absence of the other ligand.

The results obtained with untreated hemocyanins are shown in Figure 1A–C. Oxygen binding by untreated preparations of *Limulus* and *Busycon* hemocyanins show reverse Bohr effects (Figure 1A and B) which may be physiologically advantageous for these species (Johansen and Petersen, 1974). Figure 1A and B shows that carbon monoxide binding by these hemocyanins is pH independent over the range examined. There is a large difference between the carbon monoxide affinities of *Limulus* and *Busycon* hemocyanins. The partial pressure necessary to give half-saturation with CO is 100 mm for *Limulus* hemocyanin and only 3.6 mm for *Busycon* hemocyanin.

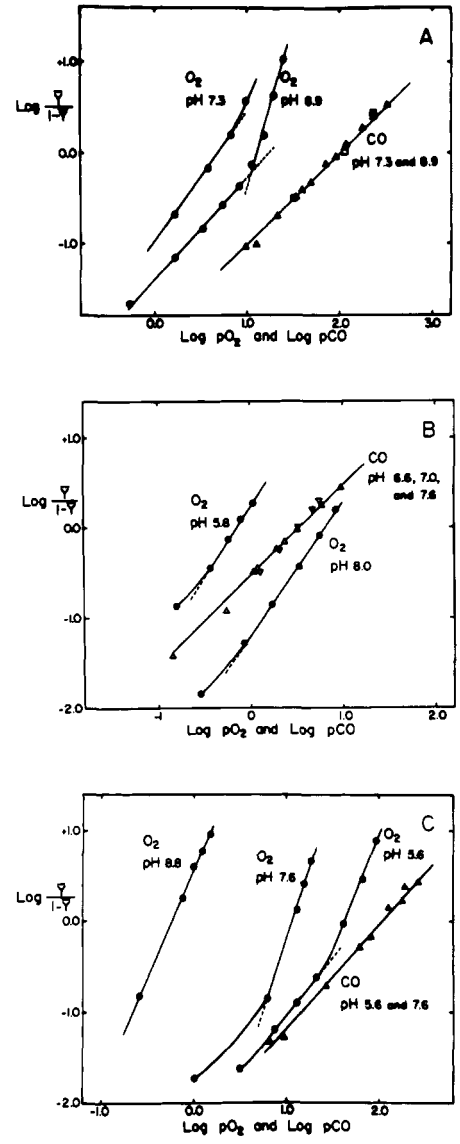

FIGURE 2: Hill plots for binding of oxygen (○) and carbon monoxide (other symbols) by (A) *Limulus*, (B) *Busycon*, and (C) *Callinectes* untreated hemocyanins. Conditions as in Figure 1. Carbon monoxide binding curves were determined indirectly (see text). In A, points from a direct determination of carbon monoxide binding are also shown (□). Lines of unit slope are drawn through the CO data points for *Limulus* and *Busycon* hemocyanins. The slope of the line through the CO data points for *Callinectes* hemocyanin is $n = 1.1$.

Oxygen binding by untreated *Callinectes* hemocyanin shows a very large positive Bohr effect (Figure 1C). This hemocyanin has a low affinity for carbon monoxide. A p_{CO} of about 110 mm is required for half-saturation between pH 5.5 and pH 7.5. *Callinectes* hemocyanin shows a pH dependent increase in CO affinity above pH 7.5. The pH effect for CO binding is, however, much reduced relative to the pH effect on oxygen binding.

The indirect method was also used to establish the shape of CO binding curves for various preparations of hemocyanin. Oxygen binding curves at a given pH were first carefully established in the absence of CO and then in the presence of *saturating* concentrations of CO. Values of M_Y and $(p_{CO})_Y$ were determined according to eq 1 and 2. In the untreated preparations of hemocyanin, which showed cooperative oxygen binding, values of M_Y varied with per cent saturation. Figure 2A–C shows Hill plots of oxygen and CO binding curves of untreated preparations of *Limulus*, *Busy-*

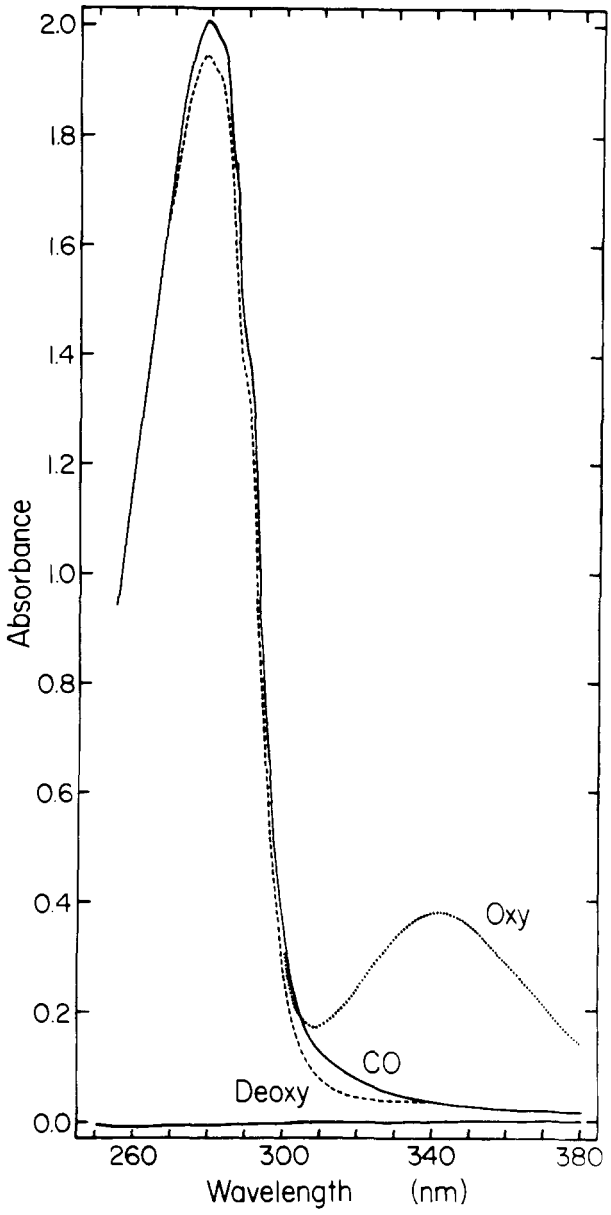

FIGURE 3: Absorption spectra of oxy (···), deoxy (– – –) and CO (—) forms of stripped *Limulus* hemocyanin in 0.052 M Tris-glycine (pH 8.9). The concentration can be estimated from $E_{1\,cm}(1\%)$ 2.23 at 340 nm as reported for oxygenated untreated *Limulus* hemocyanin (Nickerson and van Holde, 1971). The spectra were recorded in quartz tonometers at 20°.

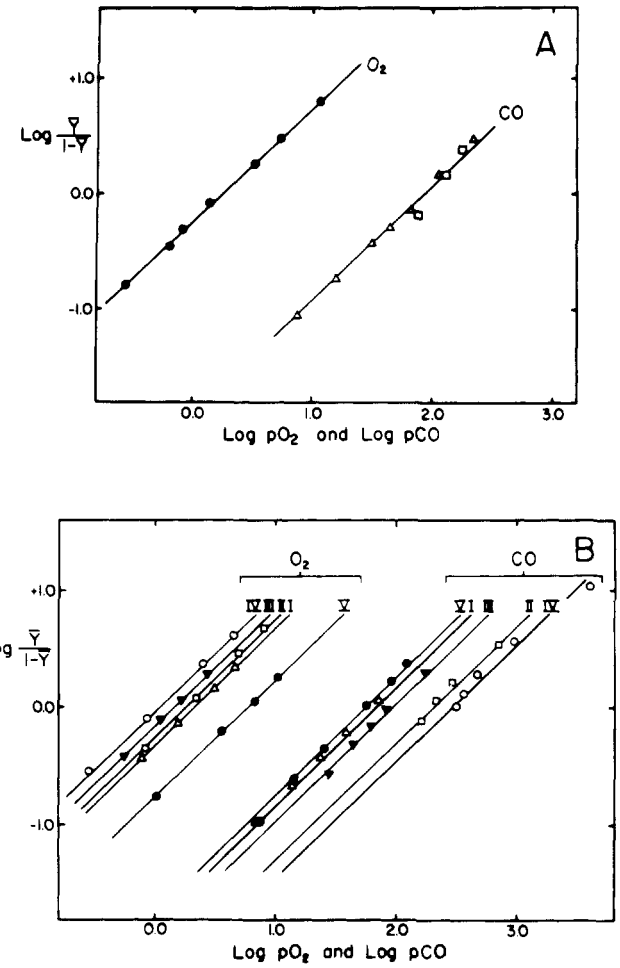

FIGURE 4: Hill plots for oxygen and carbon monoxide binding by (A) stripped *Limulus* hemocyanin and (B) isolated components I–V. Hemocyanins were in 0.05 M Tris (pH 8.9) at 20° at concentrations of about 2.4 mg/ml. Carbon monoxide binding curves were determined indirectly (see text). In A, points from a direct determination of carbon monoxide binding are also shown (□). Solid lines correspond to lines of unit slope drawn through the data points.

con, and *Callinectes* hemocyanin. Oxygen binding curves for these three hemocyanins show both cooperative interactions and pH-dependent changes in shape. These features are greatly reduced or absent in the CO binding curves.

Direct measurements of carbon monoxide binding by untreated *Limulus* hemocyanin were made, and the results are shown by the squares of Figure 2A. These direct measurements confirmed the low affinity and absence of cooperative interactions in CO binding by this hemocyanin. Direct measurements of carbon monoxide binding by *Busycon* hemocyanin confirmed its high CO affinity and the absence of cooperative interactions. With *Callinectes* hemocyanin, however, n_{50} values showed experimental variations from 1.1 to 1.5 and direct measurements also suggested that cooperative interactions might be present. While we cannot assert that cooperative interactions are absent in CO binding by this hemocyanin, it is clear that cooperativity in CO binding is greatly reduced compared to O_2 binding.

The binding of CO to the three hemocyanins investigated results in small increases in absorption in the ultraviolet region. Figure 3 shows the absorption spectra of the oxy, deoxy, and carbonmonoxy forms of stripped *Limulus* hemocyanin. Difference spectra (deoxy minus carbonmonoxy) show changes which suggest perturbations of tryptophan residues (Herskovits, 1967) upon binding of CO.

Untreated preparations of *Limulus* hemocyanin give complex elution patterns on Sephadex G-200 and show the presence of hemocyanin molecules of high molecular weight (greater than 200,000). In contrast, stripped *Limulus* hemocyanin is monodisperse and has a molecular weight of about 66,000 (Sullivan *et al.*, 1974). Figure 4A shows the results of direct measurements (squares) and indirect measurements of CO binding with stripped *Limulus* hemocyanin. Neither changes in aggregation nor in ionic conditions affect the affinity of *Limulus* hemocyanin for carbon monoxide. As in experiments with untreated *Limulus* hemocyanin, there is good agreement between the direct and indirect measurements of CO binding.

Stripped *Limulus* hemocyanin contains multiple electrophoretic components which show functional differences in

their oxygen binding behavior (Sullivan *et al.*, 1974). These components also show differences in their affinities for carbon monoxide, but not in parallel with their oxygen affinities. Figure 4B shows Hill plots of O_2 and CO binding curves for the isolated *Limulus* components. The weighted averages of O_2 and CO affinities of the isolated components are in reasonable agreement with the O_2 and CO affinities of the unfractionated mixture whose binding properties are shown in Figure 4A. This shows that the binding of O_2 or CO to unfractionated, stripped *Limulus* hemocyanin reflects the individual affinities of the five components.

Kinetics of Ligand Binding and Release. The preceding results show that *Busycon* hemocyanin and the isolated hemocyanin components from *Limulus* have very different affinities for carbon monoxide. Rapid-mixing experiments were undertaken with hemocyanin components I and IV from *Limulus* and untreated hemocyanin from *Busycon* to determine the kinetic basis for their differences in CO affinity. Rates of oxygen dissociation, k, were measured by rapid mixing of oxyhemocyanin solutions with degassed buffers containing sodium dithionite. Oxygen dissociation from *Busycon* hemocyanin was autocatalytic. At pH 9 the rate increases from about 6.5 to about 12 sec^{-1} as successive oxygen molecules are removed. This may reflect the cooperative interactions between its subunits. As we have previously reported (Sullivan *et al.*, 1974), the kinetics of oxygen dissociation from *Limulus* components I and IV can be described by single exponentials with rates of 7.5 and 2.4 sec^{-1}, respectively. Rates of CO dissociation were measured by a replacement reaction with oxygen. This was done by rapid mixing of CO-hemocyanin solutions with oxygenated buffers. In these replacement reactions no dithionite was used. The general expression for the replacement reaction under conditions where $l'CO \ll k'O_2$ is

$$R \text{ (rate)} = l + \frac{kl'CO}{k'O_2} \qquad (3)$$

where l represents the rate constant for CO dissociation and l' and k' are the combination velocity constants for CO and O_2, respectively. For the hemocyanins used in this study (under conditions of low CO and high O_2 concentrations), the second term in this expression is of the order of 0.1–0.5 sec^{-1} which is negligible with respect to l. CO concentrations were selected which were sufficiently high to saturate the degassed hemocyanins. High oxygen concentrations were obtained by equilibrating degassed buffers with pure oxygen at 1 atm. Under these conditions the observed rate is a measure of the rate of CO dissociation from the fully saturated carbonmonoxy hemocyanin. Results obtained with the isolated hemocyanin components from *Limulus* are shown in Figure 5. The kinetics show simple, first-order reactions with values of 38 and 75 sec^{-1} for the CO dissociation constant for components I and IV, respectively. The rates were not significantly altered by a tenfold variation in the CO/O_2 ratio.

The results of replacement reactions with unfractionated *Busycon* and *Limulus* hemocyanins are shown in Figure 6. The kinetics of CO dissociation from these unfractionated hemocyanins are heterogeneous, with overall rates of about 70 and 3.5 sec^{-1} for *Limulus* and *Busycon* hemocyanins, respectively. Heterogeneous kinetics of CO dissociation from unfractionated *Limulus* hemocyanin are attributable to the functional heterogeneity of its components. The heterogeneity in *Busycon* CO dissociation also may be due to functional heterogeneity within the unfractionated material. *Busycon* hemocyanin, like unfractionated *Limulus* hemocyanin, shows a complex pattern on disc gel electrophoresis.

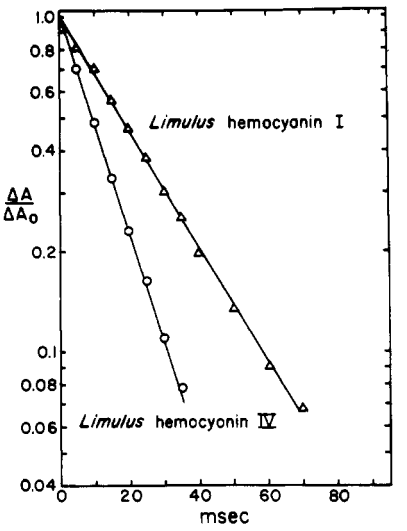

FIGURE 5: Time courses of carbon monoxide dissociation from *Limulus* hemocyanins I (△) and IV (○) determined by a replacement method described in the text; 2–4 mg/ml of carbonmonoxy hemocyanins in 10^{-3} M CO were rapidly mixed with 1.36×10^{-3} M O_2. The solutions were buffered with 0.05 M Tris (pH 8.9). The observation wavelength was 340 nm, and the temperature was 20°.

Knowledge of the equilibrium constants for oxygen (K) and CO (L), and the dissociation constants for these two ligands allows us to estimate values for the combination velocity constants for O_2 (k') and CO (l') by the relations

$$k' = kK \quad \text{and} \quad l' = lL \qquad (4)$$

These relations are approximations in complex systems, like *Busycon* hemocyanin, since subunit interactions may cause the rate constants to change with the per cent saturation. The measured values of k and l and the estimates of k' and l' for the two *Limulus* hemocyanins and *Busycon* hemocyanin are given in Table I.

The difference in CO affinity between *Limulus* and *Busycon* hemocyanins may be attributed primarily to the much smaller rate of dissociation of CO from *Busycon* hemocyanin. The lower CO affinity of component IV of

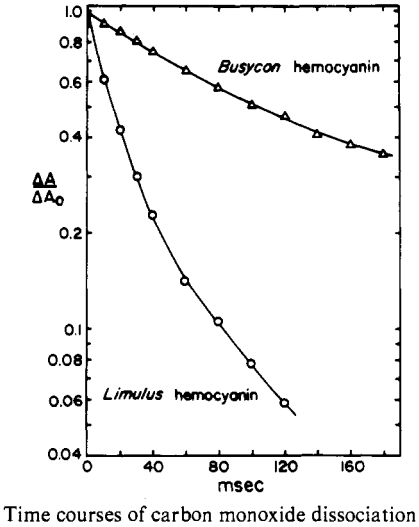

FIGURE 6: Time courses of carbon monoxide dissociation from unfractionated *Busycon* (△) and *Limulus* (○) hemocyanins. Conditions as in Figure 5.

TABLE I: Values of Equilibrium Constants K (for O_2) and L (for CO) and O_2 and CO Dissociation Rate Constants k and l Determined as Described in the Text. Values of O_2 and CO Combination Velocity Constants k' and l' Estimated from the Measured Parameters.[a]

Hemocyanin	Equilibrium Constants (M^{-1})		Dissociation Constants (sec^{-1})		Combination Constants (M^{-1} sec^{-1})	
	$K \times 10^{-5}$	$L \times 10^{-3}$	k	l	$k' \times 10^{-6}$	$l' \times 10^{-5}$
Limulus component I	2.6	11.2	7.5	38	1.9	4.3
Limulus component IV	5.4	2.7	2.4	75	1.3	2.0
Untreated *Busycon*	1.8	220	6.5–11.5[b]	3–4	1.1–2.2	7.7

[a] From experiments at 20° at pH 8.9. [b] Autocatalytic.

Lmulus hemocyanin relative to component I seems to be due to changes in both "on" and "off" constants.

Discussion

It has been known since 1919 that a hemocyanin solution will remain colorless for a prolonged interval after it is bubbled with CO (Craifaleanu, 1919). In 1934 *Limulus* hemocyanin was reported to form a colorless complex with CO in which the CO to copper ratio was 1:2 (Root, 1934). Surprisingly, the CO affinity was found to be much lower than the oxygen affinity. The CO affinities of heme proteins were known to be much higher than their oxygen affinities. In terms of kinetic parameters, this indicates that in hemocyanins the ratio of the rate of CO combination to the rate of CO dissociation is lower than for heme proteins. The CO combination velocity constants estimated for the hemocyanins used in this study are close to those reported for myoglobins from varied sources (Antonini and Brunori, 1971). The much lower CO affinity of these hemocyanins is due to very much higher rates of CO dissociation. CO dissociates from myoglobin at a rate of about 0.02 sec^{-1} (Antonini and Brunori, 1971), while for unfractionated *Limulus* hemocyanin a rate of about 70 sec^{-1} was observed. The higher CO affinity of *Busycon* hemocyanin relative to *Limulus* hemocyanin is associated with a lower rate of CO dissociation, about 3.5 sec^{-1}.

The binding of CO and oxygen to various heme proteins is analogous in many respects. For the hemocyanins used in this study, this is clearly not the case. The strong homotropic (cooperative) interactions and heterotropic (pH and specific ion) interactions so characteristic of oxygen binding by hemocyanin are reduced or absent when the ligand is carbon monoxide. The CO affinity of untreated *Limulus* hemocyanin is, moreover, the same as for stripped (unaggregated) *Limulus* hemocyanin.

In hemoglobin and myoglobin, CO and oxygen binding involve the interaction of the ferrous iron with the oxygen of either ligand (Fager and Alben, 1972; Alben and Caughey, 1968). From the available evidence it appears that carbon monoxide binding by hemocyanin involves only one copper ion while oxygen binding may require both coppers of the active site. This difference may be the principle reason for the diminished allosteric interactions in carbon monoxide binding. The diminution in allosteric interactions makes O_2 and CO binding to hemocyanin nonanalogous.

A two-step model of the oxygenation process has been proposed which involves addition of oxygen to one copper ion in the active site, followed by a conformational change of the protein which brings the second copper ion closer to the first and completes the oxygen bridge. The oxygenation of hemocyanin according to this model changes not only the Cu–Cu distance but changes the valency state of copper as well: from Cu(I) ions in deoxyhemocyanin to single-bridged Cu(II) ions in oxyhemocyanin (Schoot Uiterkamp, 1973). CO binding may then be like the initial stage of O_2 binding. The ligand-linked changes in protein conformation and valency state which are proposed to occur as a second step in O_2 binding appear to be reduced (or absent) in CO binding. This inference is based on the absence of a color change in CO binding and the insensitivity of CO binding to parameters which greatly influence O_2 binding. It is of interest that NO seems to bind to hemocyanin with an NO/Cu ratio of 1:1 (Schoot Uiterkamp, 1972). In light of this observation, it is possible that a second step in CO binding might occur if sufficiently high CO pressures were used. The CO affinity is very low, however, and a 1:2 stoichiometry of CO/Cu has been found with CO pressures up to 1 atm. Observation of the binding of a second CO molecule might therefore require much higher CO pressures.

According to current models, both homotropic and heterotropic interactions require ligand-linked changes of protein conformation. In both *Limulus* and *Busycon* untreated hemocyanins CO binding appears to be noncooperative and independent of pH. *Callinectes* hemocyanin, however, shows some increase in CO affinity above pH 7.6, and small cooperative interactions may also be present. The structural change which causes the increase in CO affinity is of considerable interest since no pH dependence was observed in CO binding by the other hemocyanins used in this study. In the pH range over which the CO affinity varies in *Callinectes* hemocyanin, there is a very large change in oxygen affinity. It is probable that the small change in CO affinity is due to the same pH dependent change that influences oxygen binding.

The hemocyanins from two molluscs (squid and limpet), two crustaceans (rock crab and crayfish), and the xiphosuran *Limulus* show distinct differences in infrared CO stretching vibrations. CO stretching vibrations near 2063 cm^{-1} were found for the molluscan hemocyanins, near 2043 cm^{-1} for the crustacean hemocyanins, and at 2054 cm^{-1} for *Limulus* hemocyanin (Fager and Alben, 1972). The higher frequency would imply weaker metal–CO bond strength (and possibly lower affinity) for the molluscan hemocyanins relative to *Limulus* hemocyanin. For the molluscan hemocyanin used in this study (*Busycon*) the CO af-

finity was higher than that of *Limulus* hemocyanin. Although the CO affinity depends on more than the metal–CO bond strength, positive correlations may be found when CO affinities and stretching vibrations of hemocyanins from the same species are compared. Our results, like those from the study on infrared stretching frequencies, strongly suggest that the various hemocyanins have different molecular environments at the active site where CO is bound. Differences in the active site of the hemocyanins must be due to differences in their primary structures. It is possible that the active sites are different due to different ligands binding the copper atoms to the protein. Alternatively, CO affinity might be affected by changes in amino acid sequence that are far removed from the active site. Results obtained with the isolated hemocyanin components of *Limulus* indicate that differences in CO affinities are not limited to hemocyanins from different phylogenetic groups.

The differences in ultraviolet absorption spectra between the CO and deoxy forms of hemocyanin indicate that binding of CO to *Limulus* hemocyanin alters the environment of some amino acids with aromatic side chains. Preliminary results suggest changes in the environment of tryptophan residues. The spectral changes which accompany ligand binding do not necessarily indicate large changes in tertiary structure, nor can they prove that aromatic amino acids are "part" of the active site. These spectral results, however, do provide evidence of changes in protein structure associated with ligand binding. Changes in absorption near 310 nm were used as direct measurements of CO binding to *Limulus, Busycon,* and *Callinectes* hemocyanins. It is unlikely that these are increases in scattered light due to increased aggregation as CO is bound. We argue that, at least for *Busycon canaliculatum* hemocyanin, the ligand-linked changes in state of aggregation are slow with respect to the period of an experiment (De Phillips *et al.,* 1970). Moreover, equivalent changes are observed with stripped (unaggregated) *Limulus* and with untreated *Limulus* hemocyanin.

It will be of interest to extend this study to determine the CO affinities and CO-deoxy difference spectra of other arthropod and molluscan hemocyanins. The fact that the CO complexes of copper lack the broad 340-nm band characteristic of oxyhemocyanin makes CO-deoxy difference spectroscopy a potentially useful tool for determining which aromatic amino acid residues are affected by ligand binding. Furthermore, due to the relative insensitivity of CO affinity to changes in pH, state of aggregation, etc., carbon monoxide may prove to be a useful probe of the molecular environment at the active site of hemocyanins.

Acknowledgments

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