Vol. 116, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1983 Pages 291-296

POSITIVE COOPERATIVITY IN BINDING CARBON MONOXIDE TO HEMOCYANIN

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Received August 29, 1983

The binding of carbon monoxide to hemocyanin from the crab Scylla serrata has been studied by thin layer optical absorption and front face fluorescence techniques. The binding to the monomeric form is completely noncooperative whereas the binding to the native oligomeric form is found to be weakly but definitely cooperative. An analysis based on the MWC model of the oxygen and carbon monoxide binding curves indicates that the allosteric constant, L, describing the equilibrium between the 2 unligated forms is different for each ligand. This implies that at least 3 allosteric forms are needed to characterize the binding of oxygen and carbon monoxide to this hemocyanin.

Hemocyanins are large copper containing respiratory proteins which are found freely dissolved in the hemolymph of many anthropods and molluscs (1,2,3). Each subunit has an active site which contains a binuclear copper center that binds oxygen and carbon monoxide reversibly. Bound oxygen is thought to bridge the two copper atoms, thereby inducing a conformational change which may be the allosteric signal responsible for the high homotropic cooperativity observed in these proteins (3,4,5). Carbon monoxide, like oxygen, binds to the active site in a ratio of one CO per two copper atoms (6,7,8,9). However, it appears that CO does not bridge the two copper atoms of the active site but rather binds to only one of the copper atoms (10,11). The difference in the way these two ligands bind to the active site has been used as the basis for explaining the difference in their cooperativity (12,13,14). The binding of oxygen to hemocyanin is strongly cooperative whereas the binding of carbon monoxide is regarded as noncooperative (12,15,16). However, there is some possibility that weak positive cooperativity exists with CO binding (15,16). A complete absence of cooperativity for CO binding implies that CO binds equivalently to all allosteric forms. The presence of cooperativity would imply that there is a

0006-291X/83 $1.50

difference in affinity between the different forms and that the binding of CO causes a shift in the distribution of allosteric forms. Establishing the precise nature of the CO binding curve will therefore add to our understanding of the allosteric properties of hemocyanin.

In this paper we present high precision measurements describing the binding of carbon monoxide to hemocyanin from the mangrove crab Scylla serrata. This hemocyanin has an unusually high affinity for CO, and allows a more complete determination of the binding curve at atmospheric pressure to be made.

Materials and Methods

Specimens of mangrove crab (Scylla serrata) were collected from the tidal waters about the Pacific island of Palau. The hemolymph was obtained by cutting several legs, collecting the fluid, and then centrifuging at low speed. The supernatant, containing mostly hemocyanin, was preserved for transportation by salting out in ammonium sulphate.

The hemocyanin was dialyzed extensively against a buffer of 0.1 M Tris/HCl, pH 8.0 with 20mM $CaCl_2$. The sedimentation coefficient was measured at 0.5 g/ℓ by analytical ultracentrifugation and was found to be 24S. This is consistent with a dodecameric state of aggregation. The sample was dissociated to the monomeric form by dialysis against a buffer of 0.05 M glycine NaOH, pH 9.6 with 10mM EDTA. The presence of the monomeric form was verified by observing a sedimentation coefficient of 6S. The protein concentration of the sample used for the oligomeric binding curve studies was 3.7 g/ℓ while the concentration of the sample used for the monomeric binding curve studies was 3.0 g/ℓ. Concentrations were calculated by assuming an extinction coefficient of 13 ℓ g^{-1} cm^{-1} which was determined for Homerus americanus (17).

Oxygen binding curves for S. serrata hemocyanin were obtained by using the thin layer optical absorbance cell described previously (18). The optical absorbance changes due to oxygen binding were measured at 340 nm.

Carbon monoxide binding was measured by use of a thin-layer front face fluorescence cell (19) and by use of the optical absorbance thin layer cell. The optical obsorbance changes due to CO binding were measured at 315 nm. The fluorescence studies were performed with an excitation wavelength of 295 nm and were observed at 550 nm. All oxygen and carbon monoxide binding studies were done at 25°C.

The thin layer techniques used in this study are advantageous for studying carbon monoxide binding because the degree of ligation is measured directly and the carbon monoxide partial pressure is precisely defined.

The binding curves were analyzed by a non-linear least squares fitting program based on the Marquardt algorithm (20).

Results

The binding of oxygen to the oligomeric form of S. serrata hemocyanin is characterized by a p_{50} of 0.9 torr and a Hill coefficient of 2.4. The experimental binding data were fit satisfactorily (standard error of a point =

Table 1
MWC Binding Constant for O_2 and CO Binding to Oligomeric S. serrata Hemocyanin[a]

Ligand	Method	$K_T(torr^{-1})$[d]	$K_R(torr^{-1})$[d]	L[d]	σ[b]
O_2	Absorbance	0.38 ± 0.01	4.2 ± 0.2	5000 ± 1000	1.6×10^{-3}
CO	Absorbance[c]	0.0069 ± .0007	0.016 ± 0.001	5 ± 2	2.2×10^{-3}
	Fluoresence[c]	0.0099 ± .0004	0.021 ± 0.001	11 ± 3	1.0×10^{-3}

a) Buffer conditions are: pH 8.0, 0.1 M Tris buffer, .020 M $CaCl_2$.
b) Units of σ (standard error of a point) are fractional saturation.
c) The values reported are the average of three experiments for each method.
d) K_T and K_R are intrinsic association constants and $L = [T]/[R]$. The size of the allosteric unit was chosen as 6.

2×10^{-3} in units of fractional saturation) by the MWC model (21). The values of the constants determined from the non-linear least squares analysis are given in Table I. Although the O_2 cooperativity is somewhat low, the oxygen binding properties of S. serrata hemocyanin are consistent with those of other anthropod hemocyanins (2,3) and demonstrate that the protein sample has normal functional properties under these conditions.

The binding of CO to the monomeric form of S. serrata hemocyanin was studied by the fluorescence method. A typical binding experiment is shown in Figure 1. The data are given in terms of the observed change in fluorescence intensity resulting from stepwise reductions in carbon monoxide partial pressure as designated by a given step number.[1] The solid curve in Figure 1 is the best fit line calculated by the non-linear least squares fitting program. The model used for this calculation assumes that the CO binding of the monomer solution can be described by a single binding constant, K. It can be seen that this model with $K = 0.021 \pm 0.001$ $torr^{-1}$ provides a good fit. This result shows that the monomeric hemocyanin solution is functionally homogeneous with respect to CO binding and does not have any cooperativity.[2]

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[1]Reference 19 gives a more detailed account of the procedure.
[2]Oxygen binding to the monomer solution can also be described by a single constant, $K = 0.091 \pm 0.002$ $torr^{-1}$, as determined by thin layer optical absorbance measurements.

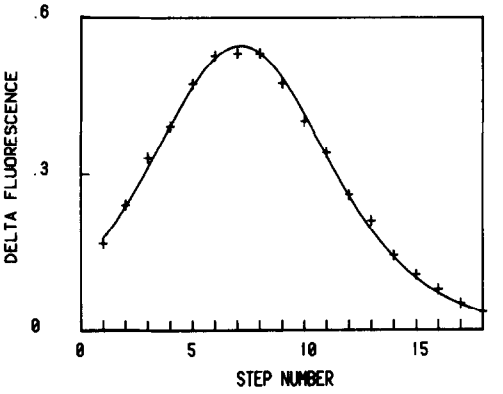

Figure 1: Carbon monoxide binding to S. serrata hemocyanin subunits as indicated by the observed fluorescence change (in arbitrary units) versus the stepwise reduction of CO partial pressure. The starting CO pressure was 596 torr and the temperature was 25°C. Each step represents a reduction in CO partial pressure by the factor 0.685. Data points are given by + and the solid line represents the theoretical best fit for a single binding constant model with K = 0.021 ± 0.001 $torr^{-1}$. The vertical length of the crosses is twice the standard error of the fit.

Typical results of the studies for the binding of CO to the oligomeric form of S. serrata hemocyanin, as determined by the fluorescence and optical absorbance thin-layer experiments, are presented in Figure 2. The data are

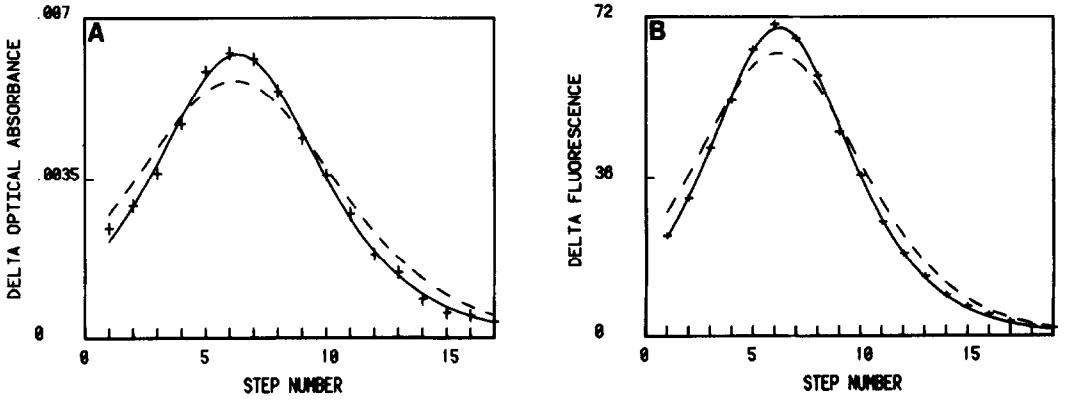

Figure 2: Carbon monoxide binding to S. serrata hemocyanin oligomers as followed by optical absorption (A) and fluorescence (B) measurements. The data are presented as in Figure 1. The experimental parameters for the optical absorbance and fluorescence experiments are: initial CO pressure = 601 torr, temperature = 25°C and stepwise CO dilution factors = 0.705 and 0.685, respectively. Data points are given by +. The dashed curves are the calculated best fit lines for the non-cooperative model. The solid curves are the best fit lines for the MWC binding model. The vertical length of the crosses is twice the standard error of the fit for the MWC model.

shown in terms of the change in fluorescence intensity (or optical absorbance) resulting from stepwise reductions in carbon monoxide partial pressure.

The CO binding of the oligomeric form was first analyzed by using a single constant noncooperative model. The dashed lines in Figure 2 are the calculated best fit curves determined by the fitting program. It is seen that the single constant curve does not fit the data. The narrower shape of the data indicate that the binding is cooperative. The MWC model was then used to fit the data. The size of the allosteric unit was determined to be six from the oxygen binding results and this value was used also for the CO analysis. The solid curves in Figure 2 are the best fit lines obtained from the non-linear least squares analysis. In this case the calculated lines fall within the scatter of the data and show that the MWC model adequately describes the CO binding process. Table 1 gives the average best fit values determined for K_R, K_T and L along with estimates of the standard deviations of these parameters as determined by several CO binding experiments.

Discussion

From our results on S. serrata hemocyanin we find clear evidence for the presence of positive cooperativity in CO binding. Other investigators (15,16) have reported results which led them to consider the possibility of weak cooperativity for CO binding by hemocyanin. However, the uncertainties in these experiments prevented a clear assessment of whether cooperativity was present. The two techniques used in the present study utilize direct methods which give results of higher precision than previously obtained for CO binding. Careful numerical analysis has showed that positive cooperativity definitely occurs.

These results have important consequences. It has been argued (13) that because CO does not bridge the binuclear copper binding site, no cooperativity should occur. The reduced cooperativity of CO binding as compared to oxygen binding is certainly consistent with this concept. However, the observed CO cooperativity indicates that some type of allosteric interaction must still be present. The value found for allosteric constant, L, depends on the

particular ligand, O_2 or CO. This means, within the MWC picture, the two allosteric forms which determine L must be different for each ligand. This requires the presence of at least three different allosteric forms. To characterize this situation more fully will require extensive binding studies with both ligands present.

The demonstration that CO as well as O_2 binding is cooperative leads to the conclusion that more than two allosteric forms are involved in describing the binding process of these two ligands to this oligomeric hemocyanin.

Acknowledgments. We wish to thank L. Fall for collecting the hemocyanin sample. This work was supported by NIH Grant 22325. We also wish to acknowledge support from NATO Grant RG132.82.

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