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Binding of Oxygen and Carbon Monoxide to Arthropod Hemocyanin: An Allosteric Analysis†

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ABSTRACT: The binding of oxygen and carbon monoxide to hemocyanin from the mangrove crab *Scylla serrata* and the lobster *Homarus americanus* has been studied by thin-layer optical absorption and front face fluorescence techniques. Three types of experiments were performed on subunit and oligomeric preparations of each hemocyanin: oxygen binding, carbon monoxide binding, and oxygen–carbon monoxide competition studies. The results obtained from the subunit preparations of dissociated oligomers from both hemocyanins show that the binding site can be ligated by either one oxygen or one carbon monoxide. The binding results obtained with the oligomeric samples of hemocyanin from both species cannot be described by the two-state MWC model [Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88–118] since the data from the three types of binding experiments cannot be fit with a single set of binding constants. The MWC model has been extended by including a third allosteric form, and an analysis based on the three-state model is able to fit the data from the three types of experiments with the same set of binding constants. The comparison of the oxygen to carbon monoxide affinity ratios (κ_{O_2}/κ_{CO}) indicates that the structure around the binding site of subunits in the T form oligomer is similar to that of the free subunits. The oligomeric forms of both these hemocyanins bind carbon monoxide with a weak but definite positive cooperativity. An analysis of the affinity ratios for the T, S, and R forms suggests that the high affinity of the R form results from a specific interaction between oxygen and binding site.

Hemocyanins are large multisubunit proteins responsible for oxygen transport in many species of arthropods and mollusks. Arthropod hemocyanins are composed of aggregates of 6, 12, 24, or 48 subunits with each subunit containing one oxygen binding site (Van Holde & Van Bruggen, 1971; Van Holde & Miller, 1982; Ellerton et al., 1983). The binding site consists of a binuclear copper center which binds both oxygen and carbon monoxide reversibly with a stoichiometric ratio of one ligand per two copper atoms (Redfield et al., 1928; Root, 1934; Vanneste & Mason, 1966). However, these two

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ligands differ in the specific nature of the binding interaction with the active site. Bound oxygen bridges the two copper atoms forming a peroxo–$Cu(II)_2$ complex (Freedman et al., 1976) whereas carbon monoxide does not bridge and apparently binds to only one of the coppers (Alben et al., 1970; Fager & Alben, 1972). The ligand binding properties of native oligomeric hemocyanins reflect these differences in ligand–active site interactions. Hemocyanins bind oxygen with high positive cooperativity; they bind carbon monoxide with no or very low cooperativity (Bonaventura et al., 1974; Brunori et al., 1981). The connection seen between high cooperativity and oxygen bridging has led to a stereochemical mechanism that proposes that the change in the copper–copper distance occurring with oxygen binding is the structural signal responsible for the cooperative interactions between subunits (Brunori et al., 1982). The large homotropic and heterotropic binding interactions of hemocyanins, along with their abundance and ease of purification, make them useful as models for the study of ligand-mediated functional control in large multisubunit protein systems.

The Monod–Wyman–Changeux (MWC)[1] allosteric model (Monod et al., 1965) has been used extensively to describe the cooperativity of hemocyanins. In this model, cooperativity is a consequence of the difference in ligand affinity between two allosteric forms of the protein. Electron microscopy results indicate that there is a definite structural difference between oxygenated and deoxygenated hemocyanin (Van Breemen et al., 1979). Cross-linking experiments on oxygenated and deoxygenated hemocyanin have shown that the protein can be "locked" into forms similar to the high- and low-affinity forms of native hemocyanin (Van Driel & Van Bruggen, 1975). These results support the general application of the MWC model to hemocyanin systems. However, in certain instances the effects of heterotropic ligands on the oxygen binding of hemocyanin cannot be completely explained by the MWC model. The presence of a hybrid R–T state (Buc et al., 1973) has been included in the model in order to describe the influence of protons, divalent cations, and chloride ions on the oxygen binding of arthropod hemocyanin (Miller & Van Holde, 1974; Brouwer et al., 1978; Arisaka & Van Holde, 1979). Thus, although the MWC model has proven to be very useful in the general description of hemocyanin ligand binding, it has been necessary to extend it in some specific cases.

We have recently shown that hemocyanin from the mangrove crab, *Scylla serrata*, binds carbon monoxide with a weak but definite positive cooperativity (Decker et al., 1983a). The comparison of the allosteric equilibrium constant, L, obtained from the analysis of oxygen and carbon monoxide binding curves indicated that the normal two-state MWC model might not be adequate to describe the binding of both ligands. In this study we present detailed oxygen, carbon monoxide, and oxygen–carbon monoxide competition binding results on hemocyanin from *Scylla serrata* and the lobster *Homarus americanus*. While there have been many studies on the binding of these ligands alone, to our knowledge there have been no detailed investigations involving direct competition between them. The analysis of these three types of experiments yields a uniquely detailed view of hemocyanin cooperativity.

Materials and Methods

Hemocyanin Preparation. *S. serrata* hemocyanin was obtained and prepared as described previously (Decker et al., 1983a). The sample was dialyzed against a buffer of 0.1 M Tris-HCl, pH 8.0, with 20 mM $CaCl_2$ at 25 °C. Analysis by analytical ultracentrifugation with a protein concentration of 0.5 g/L showed that this sample had a single boundary sedimentation profile with a sedimentation coefficient of 21 S (apparent). This indicates that the protein is in a dodecameric aggregation state under these conditions. Dissociated monomer solutions of *S. serrata* hemocyanin were prepared by dialyzing samples in a buffer of 0.05 M glycine/NaOH at pH 9.6. The monomer solution had a sedimentation coefficient of 4.7 S (apparent).

H. americanus hemocyanin was obtained from lobsters purchased at the local fish market. The hemolymph was obtained by heart puncture and centrifuged at low speed (8000 rpm) for 30 min to remove particulate matter. The clotted serum was broken up and filtered with a buffer of 0.1 M Tris-HCl, pH 8.0, and 20 mM $CaCl_2$. The hemocyanin was then concentrated by pelleting in the preparative ultracentrifuge at 34 000 rpm for 6 h in a Beckman Ti-60 rotor. The sample was further purified by gel filtration on a 2 × 40 cm Sephadex G-200 column equilibrated with a buffer of 0.1 M Tris-HCl, pH 8.0, with 20 mM $CaCl_2$. Analytical ultracentrifugation of this sample at a protein concentration of 0.5 g/L showed a single component with a sedimentation coefficient of 21 S (apparent). Monomer solutions of *H. americanus* hemocyanin were prepared by dialyzing samples against a buffer of 0.05 M glycine/NaOH, pH 9.1, with 10 mM EDTA. The monomers were purified by gel filtration on a 2 × 40 cm Sephadex G-200 column equilibrated with the dialysis buffer. The monomer solutions sedimented uniformly with a sedimentation coefficient of 4.9 S.

Binding Studies. The protein concentrations of the oligomeric hemocyanin solutions used in the binding studies were 42 and 49 g/L for *S. serrata* and *H. americanus*, respectively. The monomeric hemocyanin solutions had protein concentrations of 5.0 and 1.6 g/L for *S. serrata* and *H. americanus*, respectively. Protein concentrations were calculated from the absorbance at 280 nm by using an extinction coefficient of 1.3 L g^{-1} cm^{-1} as determined for *H. americanus* hemocyanin[2] (Morimoto & Kegeles, 1971). All binding experiments were done at a temperature of 25 °C.

Oxygen binding curves were obtained by using the thin-layer optical absorbance cell described previously (Dolman & Gill, 1978). The absorbance changes due to oxygen binding were measured at 340 nm.

The oxygen–carbon monoxide competition experiments were performed with the same apparatus. In this experiment, the cell is initially loaded with pure oxygen, and then repeated dilutions with carbon monoxide are made. The presence of bound oxygen is monitored by observing the optical density change at 340 nm.

Carbon monoxide binding was measured in two ways. The binding of the monomer solutions from both hemocyanins was studied by use of a thin-layer fluorescence cell (Decker et al., 1983b). The excitation wavelength was 295 nm, and the observation wavelength was 540 nm. The low protein concentrations of the monomer solutions precluded accurate measurement of carbon monoxide binding by the thin-layer absorption technique since the 315-nm absorption band has a small extinction coefficient. Carbon monoxide binding to the more concentrated oligomeric hemocyanin samples was studied by use of the thin-layer absorbance cell. Binding was

[1] Abbreviations: MWC, Monod–Wyman–Changeux; *S. serrata*, *Scylla serrata*; *H. americanus*, *Homarus americanus*; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

[2] The value for the extinction coefficient was quoted with incorrect units in a previous paper (Decker et al., 1983a). The units given here are correct.

measured by monitoring the absorbance at 315 nm (Bonaventura et al., 1974).

Analysis. The binding curves were analyzed by a nonlinear least-squares curve fitting program based on the Marquardt algorithm (Marquardt, 1963). This program was also modified to allow several data sets to be fit simultaneously with common parameters (Barisas & Gill, 1979). Each data set was weighted according to its variance and then combined into a single large set. The best estimates for the common parameters were then obtained by use of the normal algorithm on this global set.

THEORY

In this section we develop the ligand binding expressions that will be used for the analysis of the oxygen, carbon monoxide, and oxygen–carbon monoxide replacement experiments. For the purpose of this discussion we make the following assumptions: (1) Each binding site may bind one oxygen or one carbon monoxide. No binding site may have both ligands bound. (2) The subunits are all functionally equivalent. (3) The oligomeric forms of these hemocyanins are stable dodecamers. (4) Each oligomer functions as two independent and identical hexamers; i.e., the size of the allosteric unit is six.

The binding polynomial for subunits of dissociated hemocyanin is quite simple. Since each subunit is assumed to bind only one oxygen or carbon monoxide, the binding polynomial, P_{sub}, is expressed as

$$P_{sub} = 1 + \kappa_{O_2}x + \kappa_{CO}y \tag{1}$$

where κ_{O_2} and κ_{CO} are the intrinsic association constants for oxygen and carbon monoxide binding and x and y are the activities of oxygen and carbon monoxide. The average amount of oxygen or carbon monoxide bound per macromolecule may be obtained by taking the partial derivative of the logarithm of P with respect to the logarithm of the ligand activity. This procedure yields

$$\bar{\nu}_{O_2} = \left(\frac{\partial \ln P_{sub}}{\partial \ln x}\right)_y = \frac{\kappa_{O_2}x}{1 + \kappa_{O_2}x + \kappa_{CO}y} \tag{2}$$

$$\bar{\nu}_{CO} = \left(\frac{\partial \ln P_{sub}}{\partial \ln y}\right)_x = \frac{\kappa_{CO}y}{1 + \kappa_{O_2}x + \kappa_{CO}y} \tag{3}$$

where $\bar{\nu}_{O_2}$ and $\bar{\nu}_{CO}$ are the average moles of ligand bound per mole of macromolecule. These equations allow the binding of oxygen and carbon monoxide to monomeric solutions of hemocyanin to be analyzed.

The analysis of ligand binding for oligomeric hemocyanin is based on the MWC model (Monod et al., 1965). The macromolecule is assumed to be in equilibrium between two different allosteric forms. We here include the additional fact that the binding site can bind either oxygen or carbon monoxide. The binding polynomial for this system is thus given as

$$P_{MWC} = [(1 + \kappa_{O_2}{}^{R}x + \kappa_{CO}{}^{R}y)^6 + L(1 + \kappa_{O_2}{}^{T}x + \kappa_{CO}{}^{T}y)^6]^2 \tag{4}$$

where R and T denote the two allosteric forms, $\kappa_{O_2}{}^{R}$, $\kappa_{CO}{}^{R}$, $\kappa_{O_2}{}^{T}$, and $\kappa_{CO}{}^{T}$ are the intrinsic association constants for oxygen and carbon monoxide, and the size of the allosteric unit is six. Since each hexamer is assumed to function independently, the binding polynomial for the dodecamer is the product of the hexamer binding polynomials. The allosteric constant, L, is the ratio of the concentration of the T form to the R form in the absence of both ligands. As before, the amount of ligand bound per mole of macromolecule is given as

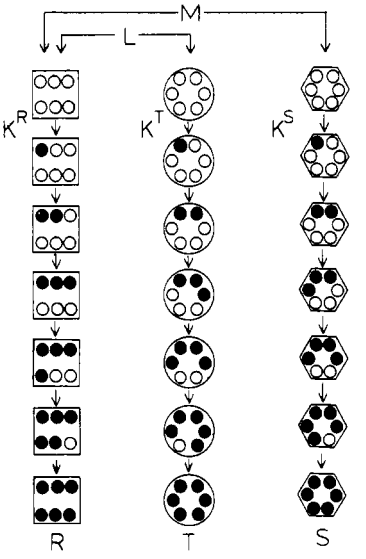

FIGURE 1: Schematic representation of the three-state allosteric model. The allosteric unit consists of six binding sites, and the protein can exist in three allosteric forms: R, T, and S. Each form binds the ligand independently with a characteristic affinity denoted as κ^R, κ^T, or κ^S. Ligated binding sites are denoted by filled circles; unligated binding sites are denoted by unfilled circles.

$$\bar{\nu}_{O_2} = \left(\frac{\partial \ln P_{MWC}}{\partial \ln x}\right)_y \qquad \bar{\nu}_{CO} = \left(\frac{\partial \ln P_{MWC}}{\partial \ln y}\right)_x \tag{5}$$

Our experimental results for both hemocyanins show that a two-state allosteric model is not adequate to describe the binding data, and we are thus led to consider the existence of a third allosteric form. A schematic representation of the three-state allosteric model for a hexameric allosteric unit is given in Figure 1. The binding polynomial for this three-state allosteric model is expressed as

$$P_{3\text{-state}} = [(1 + \kappa_{O_2}{}^{R}x + \kappa_{CO}{}^{R}y)^6 + L(1 + \kappa_{O_2}{}^{T}x + \kappa_{CO}{}^{T}y)^6 + M(1 + \kappa_{O_2}{}^{S}x + \kappa_{CO}{}^{S}y)^6]^2 \tag{6}$$

where S denotes the third allosteric form, $\kappa_{O_2}{}^{S}$ and $\kappa_{CO}{}^{S}$ are the intrinsic association constants for the binding of oxygen and carbon monoxide to the S form, and M is the allosteric constant which denotes the ratio of S to R forms in the absence of ligand. Equation 6 is then used to obtain expressions for the average amount of oxygen and carbon monoxide bound per mole of macromolecule, $\bar{\nu}_{O_2}$ and $\bar{\nu}_{CO}$, by taking the logarithmic partial derivatives described in equation 5. These binding expressions have been used to analyze the results from the oxygen, carbon monoxide, and oxygen–carbon monoxide competition experiments on oligomeric hemocyanin samples.

RESULTS AND DISCUSSION

Monomer Binding Studies. The binding of oxygen and carbon monoxide to monomer solutions of dissociated hemocyanin from *S. serrata* and *H. americanus* was studied in order to determine whether the binding polynomial given by eq 1 is adequate. Although the stoichiometry of oxygen and carbon monoxide binding has been known for some time (Redfield et al., 1928; Root, 1934), the existence of a mixed species with both ligands bound simultaneously has not been ruled out. If the data can be fit by eq 1, then mixed species are insignificant.

The results of the binding experiments done with subunit solutions of *H. americanus* hemocyanin are shown in Figure 2 and are given in terms of the change in optical absorbance

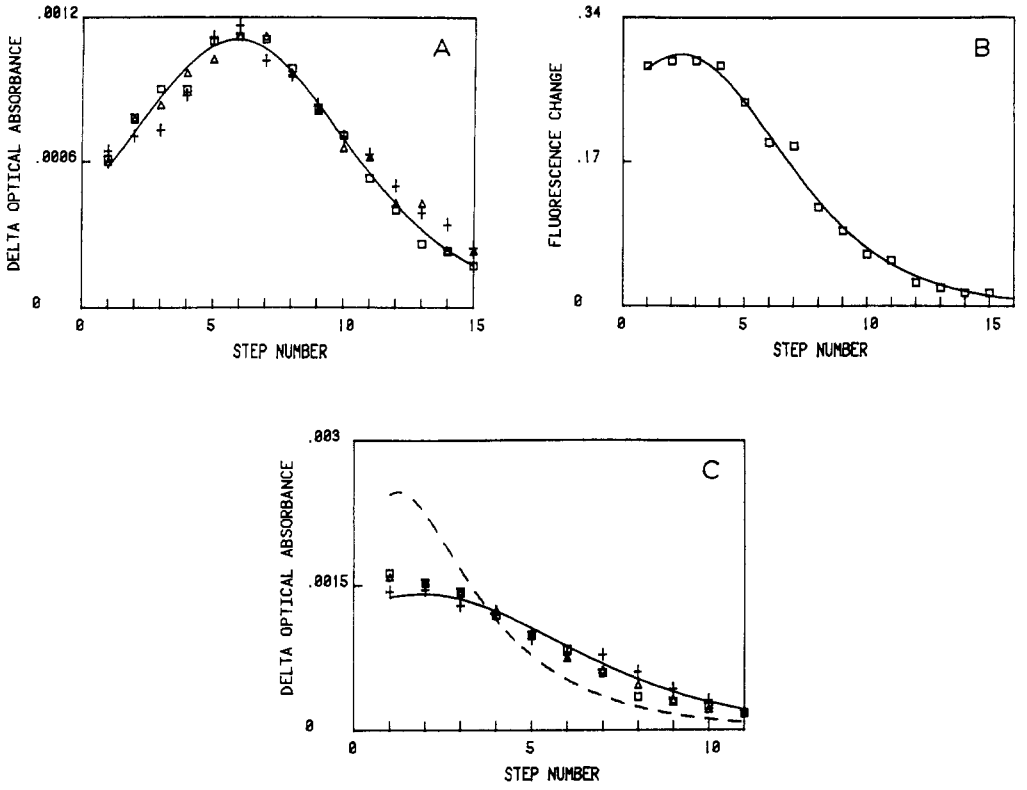

FIGURE 2: Ligand binding results for solutions of monomeric hemocyanin from *H. americanus*. Oxygen, carbon monoxide, and oxygen–carbon monoxide competition experiments are presented in panels A–C, respectively. The data are given in terms of the change in optical absorbance or fluorescence intensity caused by stepwise dilution of the ligand partial pressure. The initial partial pressure was 605 torr, and the stepwise dilution factor was 0.706 for the oxygen and oxygen–carbon monoxide competition experiments and 0.605 for the carbon monoxide binding experiments. The data points in each figure are denoted by the symbols (□), (△), and (+) and represent separate binding experiments done under the same conditions. The solid lines in (A) and (B) were obtained from a least-squares analysis based on eq 1. The solid line in (C) represents the result predicted for the competition experiment based on the binding constants obtained from the data in (A) and (B). The dashed line in (C) is the result predicted if there were two identical carbon monoxide binding sites per monomer.

Table I: Hemocyanin Monomer Binding Constants

species	oxygen binding, κ_{O_2}[c]	carbon monoxide binding, κ_{CO}	affinity ratio, κ_{O_2}/κ_{CO}	oxygen–carbon monoxide competition	
				κ_{O_2}	κ_{CO}
H. americanus[a]	0.011 ± 0.002[d]	0.0032 ± 0.0004	3.4 ± 0.7	0.009 ± 0.001	0.0033 ± 0.0006
S. serrata[b]	0.09 ± 0.01	0.021 ± 0.002	4.3 ± 0.6	0.11 ± 0.01	0.018 ± 0.002

[a] The solution conditions were 0.05 M glycine with 10 mM EDTA, pH 9.1, at 25 °C. [b] The solution conditions were 0.05 M glycine, pH 9.6, at 25 °C. [c] The association constants are given in units of $torr^{-1}$. [d] Errors are estimated from the range of parameter values obtained in fits to several experiments.

or fluorescence intensity resulting from stepwise reductions in ligand partial pressure. Similar results were obtained for *S. serrata* hemocyanin. The oxygen and carbon monoxide binding results were adequately fit with eq 2 and 3, respectively. The solid lines in Figure 2A,B represent the best fit curves calculated for each type of experiment. The values obtained for the binding constants are given in Table I. These results indicate that the binding sites in the subunit solutions of both hemocyanins are functionally equivalent since the oxygen and carbon monoxide results can each be fit with a single constant model.

The results obtained from the oxygen–carbon monoxide competition experiments on the subunit solutions verify that the binding polynomial of eq 1 is correct. The solid line in Figure 2C was calculated by using the binding constants obtained from the pure oxygen and carbon monoxide binding experiments. This predicted curve fits the competition results reasonably well and thus demonstrates that a mixed species, with both oxygen and carbon monoxide bound, is not necessary to fit the data. In addition, as seen in Table I, the best fit values obtained for κ_{O_2} and κ_{CO} from fits of the oxygen–carbon monoxide competition data agree within experimental error with those obtained from the pure ligand binding results.

The competition results also verify that there is only one carbon monoxide bound per binding site throughout the pressure range used in this study. The dashed line in Figure 2C was calculated by assuming that there were two equivalent carbon monoxide binding sites with affinities equal to that measured in the pure ligand binding experiment. The binding polynomial for this case would be $1 + \kappa_{O_2}x + 2\kappa_{CO}y + \kappa_{CO}^2y^2$. It is clear from Figure 2C that this model does not fit the data and thus indicates that there is only one carbon monoxide binding site per subunit.

The subunit binding experiments presented here, along with the stoichiometric results in the literature (Redfield et al., 1928; Root, 1934; Vanneste & Mason, 1966; Zolla & Brunori, 1983), all indicate that the binding polynomial of eq 1 is correct. We note, however, that there appears to be a small (15%) systematic difference between the carbon monoxide binding results obtained with the fluorescence and optical techniques (Decker et al., 1983a). This may be responsible for the slight differences seen between the predicted and actual

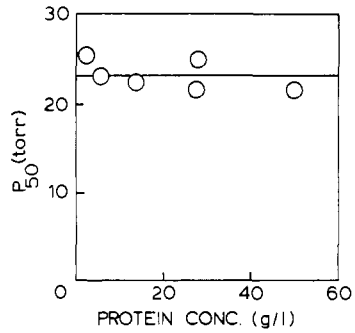

FIGURE 3: Dependence of the oxygen affinity of oligomeric *H. americanus* hemocyanin on protein concentration. The data are represented by open circles, and the solid line represents the average P_{50} value.

binding results shown in Figure 2C. In order to reduce this uncertainty, the optical absorbance method was used for all the subsequent binding studies on the oligomeric forms of hemocyanin.

Oligomer Binding Studies. The dependence of oxygen binding on protein concentration was investigated in order to determine if the dodecamer–hexamer equilibrium of *H. americanus* hemocyanin (Morimoto & Kegeles, 1971; Herskovitz et al., 1981) has any effect on the binding results obtained under the conditions of the present study. Figure 3 is a plot of the measured P_{50} values vs. protein concentration. It can be seen that there is no significant correlation between protein concentration and affinity. A simple mass law calculation using the value of the equilibrium constant given by Herskovitz and co-workers (Herskovitz et al., 1981) reveals that less than 1% of *H. americanus* dodecamers should be dissociated under the conditions used in the present study. These findings indicate that the effects of the dodecamer–hexamer equilibrium on the binding results of this study can be safely neglected. Equivalent information is not available for *S. serrata* hemocyanin, and we shall assume that the dodecameric form observed in the ultracentrifuge studies is maintained under the solution conditions used here.

The size of the allosteric unit (the number of subunits that change their allosteric form in a single concerted event) was determined for both hemocyanins by fitting oxygen binding results (the data are shown in Figures 4A and 5A) to the two-state allosteric model of eq 4 while allowing the allosteric unit size to float. These fits gave the allosteric unit size as 6 ± 1. Since arthropod hemocyanins are typically made up from hexamer building blocks, an allosteric unit size of six is quite reasonable. This value has also been observed with *Callinassa californiensis*, a large arthropod hemocyanin (Arisaka and Van holde, 1979) and is supported by the result of Pickett et al. (Pickett et al., 1966), who showed that hexamers and dodecamers of *H. americanus* hemocyanin can have identical oxygen binding curves under certain solution conditions. We shall assume that the allosteric unit size is six for carbon monoxide binding and that this value applies to the three-state allosteric model as well.

Oxygen and carbon monoxide binding studies and oxygen–carbon monoxide competition experiments were done on oligomeric hemocyanin samples from *H. americanus* and *S. serrata*. The results of these experiments are presented in Figures 4 and 5 in terms of the change in optical absorbance caused by stepwise changes in ligand partial pressure. All three types of experiments were done on samples from the same preparation over a period of 2 weeks. There was no detectable change in binding properties in this period.

The oligomeric binding results from the three types of experiments were initially analyzed with the two-state MWC model of eq 4. While the results from each type of experiment

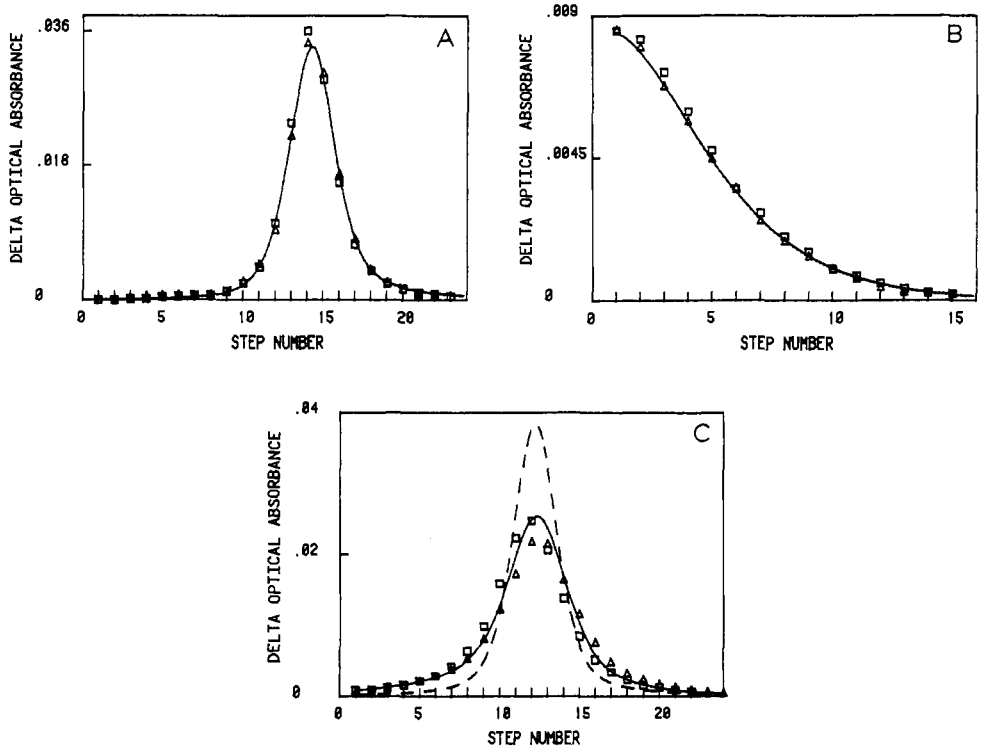

FIGURE 4: Ligand binding results for oligomeric *H. americanus* hemocyanin. Oxygen, carbon monoxide, and oxygen–carbon monoxide competition experiments are presented in panels A–C, respectively. The initial partial pressure of ligand was 609 torr, and the stepwise dilution factor was 0.706 for the carbon monoxide experiment and 0.789 for the oxygen and competition experiments. Experimental data for duplicate runs are denoted by the symbols (□) and (△). The solid curves are the best fit results obtained by using the three-state model with the constants given in Table II. The dashed line in (C) shows the results predicted for the competition experiment with a two-state model based on the results obtained from analyzing the data of (A) and (B).

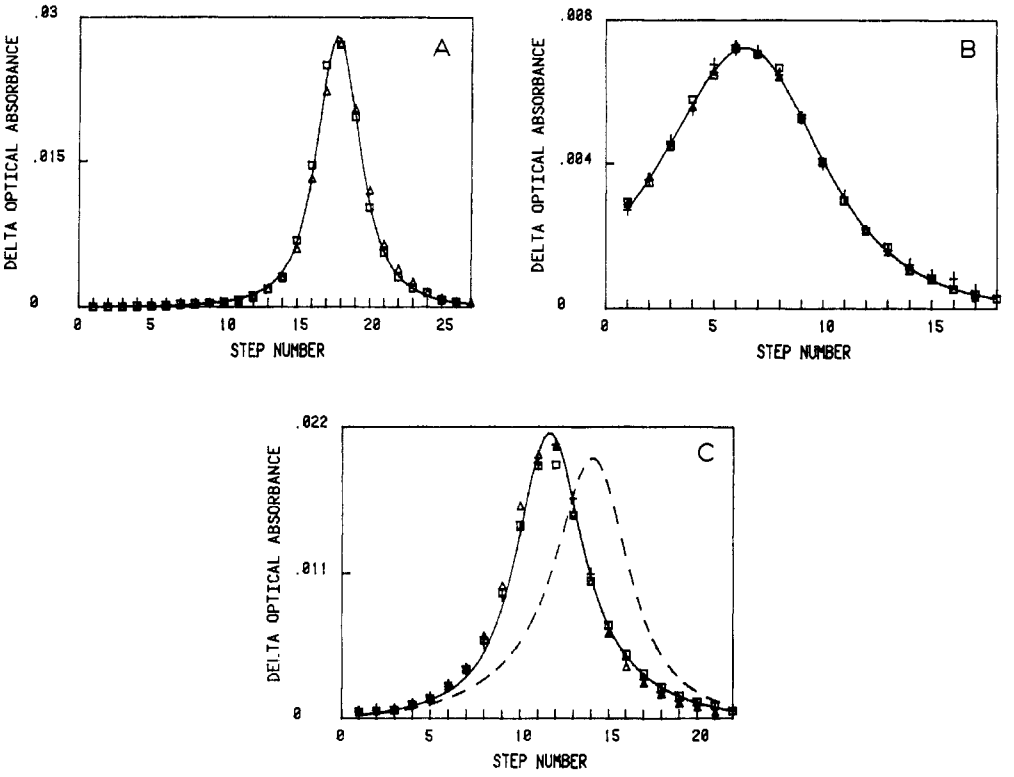

FIGURE 5: Ligand binding results for oligomeric *S. serrata* hemocyanin. The initial ligand partial pressure was 603 torr, and the stepwise dilution factor was 0.707. The designations of the data points and curves are analogous to those given in Figure 4.

can be fit with the MWC model, large systematic errors were observed in the fits when the parameters from one type of experiment were used in the analysis of another type of experiment. This is illustrated in Figures 4C and 5C where it is seen that the dashed curves, which were calculated with binding constants obtained from fitting the pure ligand binding experiments to the two-state model, do not fit the oxygen–carbon monoxide competition results. Also, the values of the constants obtained from fits to each type of experiment are not mutually consistent. In the case of *S. serrata* hemocyanin, the allosteric constant L is determined to be 6000 ± 2500 from oxygen binding results and 3.6 ± 1 from carbon monoxide binding results. These values should be equal if the two-state model is valid. In the case of *H. americanus*, the carbon monoxide binding parameters determined from the analysis of the pure carbon monoxide binding data were $\kappa_{CO}^{T} = (1.7 \pm 0.1) \times 10^{-3}$, $\kappa_{CO}^{R} = (3.8 \pm 0.3) \times 10^{-3}$, and $L = 30 \pm 10$. The carbon monoxide binding parameters obtained from fits to the oxygen–carbon monoxide competition data were $\kappa_{CO}^{T} = (6 \pm 3) \times 10^{-4}$, $\kappa_{CO}^{R} = 9 \pm 1.5 \times 10^{-3}$, and $L = (1.5 \pm 1) \times 10^{7}$. In addition, the value of L determined from the oxygen binding data, $L = (6 \pm 2) \times 10^{6}$, does not agree with the value of L determined from the carbon monoxide binding results. Thus, the two-state MWC model is not adequate to describe the binding data from either hemocyanin.

It should be emphasized that these oligomeric hemocyanin samples show no evidence of functional heterogeneity. The pure oxygen and carbon monoxide binding curves can be fit with the MWC model which is based on the assumption that the system is homogeneous. The functional homogeneity observed for the subunit hemocyanin preparations also supports the lack of permanent heterogeneity. Thus, the inconsistency seen in the binding constants obtained from the two-state MWC analysis cannot be attributed to functional heterogeneity caused by such things as damaged protein, mixtures of functionally different hemocyanins, etc.

Table II: Oligomer Binding Constants for the Three-State Allosteric Model[a]

	allosteric form		
	R	S	T
	H. americanus		
κ_{O_2}[b]	0.80 ± 0.07[c]	0.025 ± 0.007	0.0063 ± 0.0005
κ_{CO}	0.015 ± 0.001	0.0027 ± 0.0003	0.00145 ± 0.00005
ratio (κ_{O_2}/κ_{CO})	50	9	4
$L = (2.4 \pm 1) \times 10^{7}$			
$M = (8 \pm 4) \times 10^{5}$			
	S. serrata		
κ_{O_2}	3.7 ± 0.5	0.4 ± 0.1	0.03 ± 0.01
κ_{CO}	0.021 ± 0.003	0.0147 ± 0.0006	0.0060 ± 0.0004
ratio (κ_{O_2}/κ_{CO})	180	30	5
$L = (2.0 \pm 0.5) \times 10^{4}$			
$M = (6 \pm 2) \times 10^{3}$			

[a] Solution conditions: 0.1 M Tris, pH 8.0, 20 mM $CaCl_2$, 25 °C. [b] The association constants are given in units of $torr^{-1}$. [c] The errors are estimated from the range of parameter values obtained from duplicate experiments using the fitting procedure described in the text.

From these considerations, the next logical modification of the two-state model is the addition of a third allosteric state which we denote by S. The incorporation of a third form preserves the conceptual simplicity of the allosteric approach and, at the same time, expands its range of application.

The results of fitting the data from oxygen, carbon monoxide, and oxygen–carbon monoxide competition experiments to the three-state allosteric model of eq 6 are shown in Figures 4 and 5. The solid lines represent the fits obtained with the sets of constants given in Table II. The data of the three types of binding experiments were initially fit separately. The three-state analysis of the pure oxygen data yielded significant estimates for L, $\kappa_{O_2}^{T}$, and $\kappa_{O_2}^{R}$, the analysis of the carbon

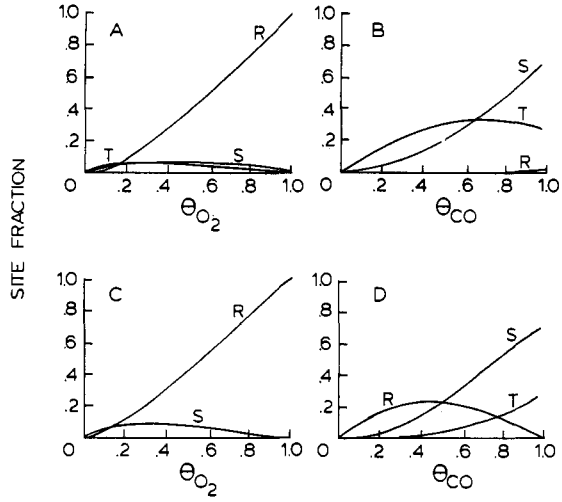

FIGURE 6: Plots of the fractions of binding sites which are ligated *and* in the R, T, or S allosteric form vs. fractional saturation for *H. americanus* hemocyanin. The sum of these fractions represents the total fractional saturation. The individual figures represent the species occurring in an oxygen binding experiment (A); a carbon monoxide binding experiment (B); a competition experiment (C and D). The curves were calculated with the constants given in Table II and thus cover higher carbon monoxide saturations than can be obtained under normal atmospheric conditions. Under the experimental conditions of this study, the maximum saturation of carbon monoxide binding only reaches a value of about 0.5.

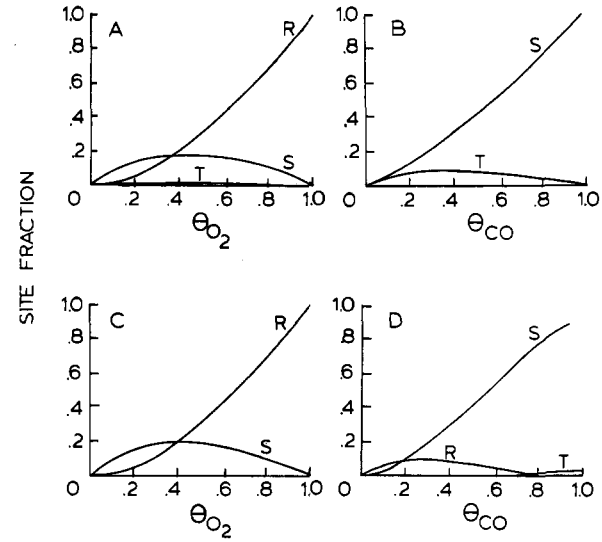

FIGURE 7: Plots of the fraction of binding sites which are ligated *and* in the R, T, or S allosteric form vs. fractional saturation for *S. serrata* hemocyanin. The figure designations are the same as those in Figure 6. Note that for this hemocyanin the total fractional saturation of carbon monoxide reached in an actual experiment has a value of about 0.85.

monoxide data yielded significant estimates for M, κ_{CO}^{T}, and κ_{CO}^{S}, and the oxygen–carbon monoxide competition data were used to estimate $\kappa_{O_2}^{S}$ and κ_{CO}^{R}. The final set of values was then checked by fitting all three types of experiments simultaneously. It can be seen from the figures that these constants allow reasonable fits to be made to the data from all three types of experiments.

As seen in Table II, the eight constants of the three-state allosteric model are evaluated with reasonable precision. It is only through combination of data from all three types of experiments that the parameters of the three-state model could be evaluated. However, certain binding constants, in particular $\kappa_{O_2}^{S}$ for *H. americanus* and $\kappa_{O_2}^{T}$ for *S. serrata*, are difficult to determine with high precision. Since each binding constant in the binding polynomial represents the relative concentration of a certain species in solution, that species must be present in a reasonable amount and its concentration must change in the course of the experiment in order for the constant to be evaluated with good precision. Figures 6 and 7 show the fractions of the ligated allosteric forms that occur in each type of binding experiment for *H. americanus* and *S. serrata* hemocyanin, respectively. Since the amounts of oxygen-ligated T form for *S. serrata* and oxygen-ligated S form for *H. americanus* hemocyanin are seen to be small in all three types of binding experiments, the evaluation the representative affinity constants is understandably difficult.

Several other studies involving arthropod hemocyanins have also found it necessary to extend the two-state MWC model by including an additional allosteric form (Miller & Van Holde, 1974; Brouwer et al., 1977; Arisaka & Van Holde, 1979). The additional form was suggested because the two-state model could not fit oxygen binding results obtained in the presence of varying amounts of ionic effectors such as protons, chloride ions, and divalent cations. The approach of these investigations was conceptually similar to that of the present study; the existence of an additional allosteric form is detected by studying the influence of heterotropic effectors on the oxygen binding properties of hemocyanin. The present study, however, takes the influence of the heterotropic effector into account directly. In particular, oxygen and carbon monoxide bind at the same site in a competitive manner with a 1:1 stoichiometry.[3] This leads to a specific binding polynomial for the binding site which applies to both isolated subunits and to subunits within the various oligomeric forms of the protein. In contrast to this situation, previous studies (Miller & Van Holde, 1974; Brouwer et al., 1978; Arisaka & Van Holde, 1979) have explored the allosteric properties of hemocyanin by use of heterotropic ligands which do not compete at the binding site and thus exert their influence in a less well-defined way. We feel that the present study allows the allosteric nature of hemocyanin to be investigated in a more precise manner.

The third allosteric form used in previous studies is an R–T hybrid made up of equal numbers of subunits in the R and T form. The intrinsic binding affinity of the hybrid form is thus defined by the properties of the R and T forms. This is a special case of the general three-state MWC model used here. The binding polynomial for the hybrid allosteric form may be written as

$$P_{\text{hybrid}} = [(1 + \kappa_{O_2}^{R}x + \kappa_{CO}^{R}y)^6 + L(1 + \kappa_{O_2}^{T}x + \kappa_{CO}^{T}y)^6 + M(1 + \kappa_{O_2}^{R}x + \kappa_{CO}^{R}y)^3(1 + \kappa_{O_2}^{T}x + \kappa_{CO}^{T}y)^3]^2 \quad (7)$$

where the third term of this equation represents the contribution of the hybrid form. This term may be compared to binding polynomial for the general third state, S, of eq 6 by expanding both polynomials and comparing the coefficients of terms with like powers of x and y. The suitability of the hybrid model can be judged by comparing the binding constants actually obtained for the general third form with those predicted by the hybrid formulation. This comparison is shown by Table III in terms of the ratios of the general S state binding constants to the comparable hybrid state constants. If these ratios are unity, then the hybrid model is an adequate representation of the allosteric system. It is seen the ratio values obtained for *H. americanus* hemocyanin are all sig-

[3] This special type of heterotropic effect has been described by Wyman as "identical linkage" (Wyman, 1948).

Table III: Comparison between the Generalized Three-State Model and the Hybrid Three-State Model: Ratios of Equivalent Binding Constant Representations for the Three-State and Hybrid Models[a]

	$2\kappa_{O_2}^S/(\kappa_{O_2}^R + \kappa_{O_2}^T)$	$2\kappa_{CO}^S/(\kappa_{CO}^R + \kappa_{CO}^T)$	$(\kappa_{O_2}^S)^2/(\kappa_{O_2}^R\kappa_{O_2}^T)$	$(\kappa_{CO}^S)^2/(\kappa_{CO}^R\kappa_{CO}^T)$	$2\kappa_{O_2}^S\kappa_{CO}^S/(\kappa_{CO}^R\kappa_{O_2}^T + \kappa_{O_2}^R\kappa_{CO}^T)$
H. americanus	0.06 ± 0.02	0.33 ± 0.06	0.13 ± 0.09	0.26 ± 0.10	0.11 ± 0.06
S. serrata	0.21 ± 0.08	1.1 ± 0.2	1.5 ± 1	1.8 ± 1	0.5 ± 0.4

[a] The five ratios given in this table are obtained by expansion of the cube root of the sixth degree binding polynomials for the S and hybrid forms and by constructing ratios with the terms of identical powers in ligand concentration.

nificantly less than unity, thus indicating that the hybrid model is unsuitable for this hemocyanin. However, the values obtained for *S. serrata* hemocyanin are close to unity and thereby suggest that the hybrid model provides an adequate description for this hemocyanin system. Since the hybrid model is not suititable for both cases, it appears that the hybrid formulation is not generally valid for all hemocyanins.

Ideally, one would like to compare directly the properties of the binding site in the different allosteric forms in order to determine how the tertiary conformation of the subunit affects its binding properties. However, the comparison of the properties of isolated subunits to those of the subunits in the various oligomeric forms is complicated by the fact that it is difficult to obtain binding data for monomers and oligomers under the same solution conditions. Since the binding of a given ligand to hemocyanin is generally subject to effects caused by ionic strength, pH, and the presence of divalent cations, equivalent solution conditions are needed in order to make a direct comparison. These problems may be minimized, however, by comparing the intrinsic oxygen to carbon monoxide affinity ratios, κ_{O_2}/κ_{CO}, for the binding site in each form. Factors that are expected to affect the absolute affinity of these two small neutral ligands in a similar way such as channel accessibility to the binding site, the solubility of the ligand in the protein matrix, the internal dynamics of the protein, etc. are cancelled by taking the ratio of the affinities. Presumably, the only factors left behind are those that would be unique to the local structure of the binding site. The comparison of the oxygen to carbon monoxide affinity ratios is particularly advantageous in the case of hemocyanin since these two ligands interact with the binding site in a very different manner. In this way, the ratio of affinities allows the differences of structure in the vicinity of the binding site to be assessed.

As shown in Table I, the value of the affinity ratio (κ_{O_2}/κ_{CO}) is approximately four for the monomer forms of hemocyanin from both *S. serrata* and *H. americanus*. From the preceding arguments, this implies that the structural features of the binding site are similar for both of these hemocyanins. The affinity ratios for the three allosteric forms of the oligomer (R, S, and T) are given in Table II for the hemocyanins from both species. The most striking feature is that the value of the affinity ratio for the T allosteric form is the same as that determined for the monomeric hemocyanins. This implies that the local structure around the binding site in the oligomeric T form is similar to that of the free monomers. This is in contrast to the situation found in hemoglobin where the separated α and β monomers have binding properties which correspond most closely to those of the R form of the oligomer. For the hemocyanins studied here, the T form of the oligomer has functional properties that are most closely associated with the free subunits, and, in this sense, would be considered to be the "relaxed" state.

It is apparent from the oxygen to carbon monoxide affinity ratios, given in Table II, that the ratio for the R form is much larger than the ratios of T and S forms. This suggests that the high oxygen affinity of the R form results from specific interactions occurring at the binding site rather than from generalized structural effects due to protein conformational changes. Generalized effects would be expected to significantly alter the carbon monoxide affinity as well, and this is clearly not the case. These results indicate that the molecular mechanism of arthropod hemocyanin cooperativity involves specific changes in the binding site of the R allosteric form.

The cooperativity of carbon monoxide binding is much less than that of oxygen bining because carbon monoxide does not have the large differences in affinity for the various allosteric forms that oxygen does. However, even though the cooperative interaction involved in carbon monoxide binding is small (the Hill coefficient is about 1.2 for both hemocyanin samples), it is definitely present. Since carbon monoxide presumably does not bridge the copper atom pair of the binding site, its cooperativity must be due to some other type of interaction. The specific molecular cause of the differences in carbon monoxide affinities cannot be determined from thermodynamic binding experiments, and thus we can only speculate as to the underlying molecular reason. One possible explanation could involve differences in steric constraints at the binding site. If the accessibility of the binding sites were ordered as R > S > T, then a small cooperative effect would occur.

While a detailed molecular description of the allosteric mechanism of these two hemocyanins will probably have to wait until high-resolution structural results on hemocyanins in the R, T, and S allosteric forms are obtained, our data can be used to give broad limits to possible allosteric mechanisms. In summary, the allosteric mechanism for *H. americanus* and *S. serrata* hemocyanin should involve more than two allosteric forms. Our data were fit adequately by an allosteric model with three allosteric forms. The values obtained for the oxygen and carbon monoxide binding constants show that both oxygen and carbon monoxide bind cooperatively. The high cooperativity of oxygen binding is a consequence of the fact that there is a strong interaction between this ligand and the binding site of R form hemocyanin. The analysis of the ratios of oxygen to carbon monoxide affinities indicates that the strength of the oxygen–R form interaction results from specific molecular interactions at the binding site. These features should also be incorporated into possible allosteric mechanisms.

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Isolation and Partial Characterization of U1–U6 Small RNAs from *Bombyx mori*†

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ABSTRACT: We have used a variety of techniques to characterize the U-series small nuclear RNAs from the posterior silk gland of *Bombyx mori*. Six molecular species have been identified which correspond to the vertebrate U1–U6 RNAs by the following criteria: (a) presence of the RNAs in ribonucleoprotein particles which can be immunoprecipitated by lupus Sm antisera; (b) presence of a 2,2,7-trimethylguanosine cap, as assayed by immunoprecipitation with anti-2,2,7-trimethylguanosine IgG; (c) size, as assayed by acrylamide/urea gel electrophoresis using HeLa cell U-RNA markers; and (d) primary nucleotide sequence, as determined by chemical/enzymatic cleavage of end-labeled molecules. The high conservation of primary sequence (66–81% homology based on partial sequences) relative to the corresponding vertebrate U-RNAs has permitted unambiguous identification of each molecule. With the exception of two subspecies of U3 RNA, the U-snRNAs of *Bombyx* exhibit a striking conservation of secondary structure relative to the proposed structures of the U-RNAs of vertebrates. This conservation is best exemplified by several compensatory base alterations that result in the maintenance of hairpin structures. These are particularly evident in U1 and U5 RNAs. *Bombyx* U3 is interesting in that two subspecies (of a total of four that were sequenced) diverge considerably in sequence (and presumably in structure) relative to the U3 RNA of vertebrates. The most abundant U-RNAs in the posterior silk gland appear to be U1 and U2, while U3–U6 are present in relatively small amounts.

In the analysis of the control of gene expression, one area of current intense interest is the role played by small RNA molecules (sRNAs).[1] These molecules are low molecular weight, nonribosomal, nontransfer RNAs that have been found, variously represented, in every eukaryotic organism examined to date. Different types of sRNAs have been pro-

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[1] Abbreviations: snRNP, small nuclear ribonucleoprotein; sRNA, small RNA; snRNA, small nuclear RNA; $m_2^2m^7G$, N^2,N^2,7-trimethylguanosine; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PBS, 10 mM Na_2HPO_4 and 0.15 M NaCl, pH 7.5; NET, 150 mM NaCl, 5 mM Na_2EDTA, and 50 mM Tris-HCl, pH 7.5; SSC, 15 mM sodium citrate and 0.15 M NaCl, pH 7.5; kb, kilobase; TBE, 90 mM Tris base, 90 mM borate, and 2 mM Na_2EDTA; RNP, ribonucleoprotein; PSG, posterior silk gland; pCp, cytidine bisphosphate.

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