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Oxygen Binding by *Callianassa californiensis* Hemocyanin†

Karen Miller and K. E. Van Holde*

ABSTRACT: The oxygen binding by the hemocyanin of the ghost shrimp, *Callianassa californiensis*, has been studied as a function of pH, divalent ion concentration, and temperature. Most of the experiments were performed with the hemocyanin C component, which is competent to undergo an association from a 17S form to a 39S tetramer near neutral pH. It is demonstrated that the effects of pH and divalent ions on oxygen binding can be described by the theory of Monod *et al.* (*J. Mol. Biol. 12*, 88 (1965)) for allosteric transitions as modified by Buc *et al.* (*J. Mol. Biol. 76*, 199 (1973)). The 17S hexamer of polypeptide chains appears to be the allosteric unit. The T and R states of this 17S particle exhibit differing tendencies to associate. The 17S particles of hemocyanin I, which are not capable of association, have a T state identical with that of hemocyanin C, but cannot adopt the same R state. Possible significance of this behavior to the physiology of the shrimp is discussed.

As has been described in the preceeding paper (Roxby *et al.*, 1974), the ghost shrimp *Callianassa californiensis* has a hemocyanin capable of a completely reversible monomer–tetramer association. There exist normally in the blood two components which can be completely separated on a A5m column. One sediments as a 17S component in the ultracentrifuge and the other as a 39S component.[1] The 39S component shows the reversible dissociation, breaking down into 17S subunits when Mg^{2+} is removed, and reassociating to 39S subunits when Mg^{2+} is replaced to a level of 0.05 M or greater. We have designated this hemocyanin C. The 17S component normally present in whole blood is incompetant to associate into 39S subunits regardless of the Mg^{2+} levels. This we call hemocyanin I. At high pH ($\geq$9.2) the hemocyanin dissociates further into 5S subunits, which apparently represent individual polypeptide chains. We have shown that the 17S component is a hexamer of these subunits. (Roxby *et al.*, 1974).

We wished to investigate oxygen binding by *Callianassa* hemocyanin for a number of reasons. The background respiratory physiology of this shrimp has been investigated (Thompson and Pritchard, 1969; K. Miller and Pritchard, in preparation) and in particular preliminary measurements were made of the oxygen binding of whole blood. It seemed logical in this case to attempt to duplicate the conditions of divalent ion composition, temperature, and pH normally found in whole blood under completely controlled conditions in an attempt to fully understand the effect that varying physiological states might have on the oxygen binding properties of the hemocyanin. The normal variations in physiological pH have been measured (K. Miller and Pritchard, in preparation). Several researchers report a pronounced effect of Ca^{2+} ions on oxygen binding in other hemocyanins (Hwang and Fung, 1970; Larimer and Riggs, 1964) and Mg^{2+} ions have been shown to have the same effect (Larimer and Riggs, 1964). The blood calcium levels in *Callianassa* have been measured by Thompson and Pritchard (1969) but it was necessary for us to measure the normal hemolymph Mg^{2+} levels before composing a buffer system for dilute samples.

Since with this hemocyanin one can obtain several different stable aggregation states, as well as some equilibrium mixtures of these, it makes an ideal system for the study of the interrelation between aggregation state and oxygen binding. In this paper we describe binding studies with both hemocyanin C and hemocyanin I, as a function of pH, divalent ion concentration, and temperature.

Experimental Section

Preparation of Solutions. Shrimp were dug at Yaquina Bay and bled, and the blood was purified on a Bio-Gel A5m column as described previously (Roxby *et al.*, 1974). The eluent for the column was 0.1 *I* Tris buffer (pH 7.65) with

† From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331. *Received September 6, 1973.* This work was supported by Public Health Service Grant HL-12326.

[1] The actual sedimentation coefficients at protein concentrations normally used with schlieren studies, and in the presence of 0.1 *I* Tris buffer, are about 16 and 35 S, respectively. However, since we have shown (Roxby *et al.*, 1974) that the $s^0_{20,w}$ values are 16.7 and 38.8 S, respectively, we shall refer to these as 17S and 39S components.

0.05 M $MgCl_2$. Unless otherwise specified all experiments were done with the purified 39S fractions, hemocyanin C. All solutions were made with 0.1 *I* buffers prepared according to the Biochemists Handbook (Long, 1961), using doubly distilled water.

For binding studies in which we wished to approximate the divalent ion composition of hemolymph, 0.05 M $MgCl_2$ and 0.01 M $CaCl_2$ were added, either directly or by dialysis, to the samples. In most cases dialysis was used, and was carried out overnight, with one replacement of the dialysate, using a volume ratio of dialysate to solution of about 100:1 in each case. The 17S form of hemocyanin C was prepared by dialyzing the 39S fraction against Tris buffer without added Mg^{2+}. The 5S subunits were prepared by dialyzing the 39S fraction against pH 9.2 borate or bicarbonate buffer.

Measurement of Mg^{2+}. Mg^{2+} levels in whole blood diluted 1:400 with distilled water were measured by atomic absorption spectroscopy using a *Perkin–Elmer Model 303* spectrometer. To resolve the Mg^{2+}, 1% $CaCl_2$ was added to the sample before flaming.

Oxygen-Binding Studies. Measurement of oxygenation of the hemocyanin was based on the 337-nm absorbance band. This band is absent in the deoxygenated hemocyanin which exhibits only scattering at this wavelength; the band increase in absorbance (A) with increasing oxygenation. The tonometer consisted of a glass bulb of 107.2-ml volume with a 1-cm fused-quartz cuvet attached. The ground glass stopper of the tonometer was equipped with straight sidearm consisting of a small capillary between two stopcocks. Depending on the oxygen affinity of the solution used the volume of the capillary used was either 1.1819 or 0.1718 ml. The volumes of the sidearms were determined by mercury weighing and that of the tonometer by weighing it filled with water.

A hemocyanin solution of $A_{337} \simeq 1$, corresponding to a protein concentration of 3–4 mg/ml, was placed in the tonometer and deoxygenated by alternate evacuation and nitrogen flushing until the peak at 337 nm was completely eliminated. Since it appeared likely that the oxygen binding might, under some circumstances, depend upon degree of association, and hence the concentration, all measurements were made with approximately the same hemocyanin concentration. Capillary increments of air or oxygen at 1 atm were added; the choice of gas depended on the oxygen affinity of the hemocyanin under the circumstances of the experiment. The tonometer was equilibrated in a temperature-controlled shaker bath for 10 min after each addition and the absorbance spectrum from 400 to 300 nm was quickly measured. Longer equilibrium did not increase the A_{337}. All binding curves, except for the temperature series, were made at 25 ± 0.1°. At low temperatures only the portion of the spectrum from 345 to 330 nm was measured, to prevent excessive changes in temperature from altering the absorbance readings. Gas was added until a pressure of 1 atm was reached.

The oxygen partial pressure was calculated according to the following equation used by Spoek *et al.* (1964).

$$pO_2 = F(B - W - pCO_2)\left\{1 - \left(\frac{V}{V + v}\right)^i\right\} \qquad (1)$$

F = volume fraction of O_2 in the gas added: 0.2045 for air, 1.000 for oxygen; B = barometric pressure; W = water vapor pressure; pCO_2 = CO_2 pressure negligible in our experiments; V = volume of tonometer; v = volume of sidearm; i = increments of gas added.

In cases where the blood was not fully saturated at 1 atm of air, or 1 atm of O_2, the curves were extrapolated to infinite oxygen pressure by plotting $1/A$ *vs.* $1/pO_2$. The absorbance value at the y intercept (A_∞) was used for calculations of fraction saturation of the hemocyanin ($\bar{Y}$)

$$\bar{Y} = (A - A_0)/(A_\infty - A_0) \qquad (2)$$

where A_0 is the value of the residual absorbance due to light scattering by the deoxygenated hemocyanin.

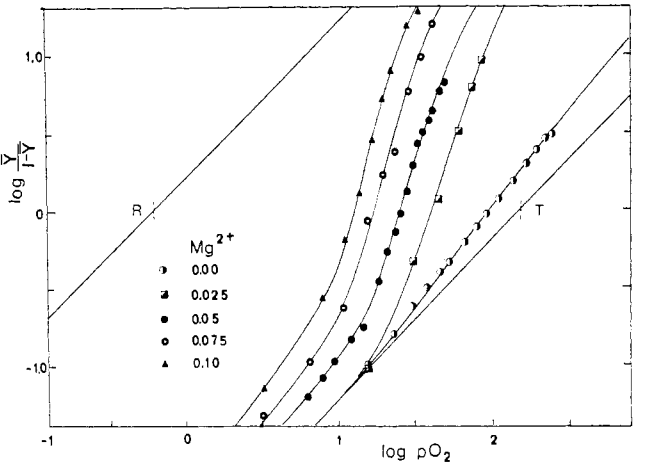

FIGURE 1: Hill plots of the binding of O_2 by hemocyanin C at 25°, pH 7.65. The numbers on the code indicate Mg^{2+} concentrations. The two straight lines with slope unity (T and R) are placed according to data in Figures 4 and 6.

In the following sections, we shall present data in several ways. In some cases we will give binding curves as $\bar{Y}$ *vs.* pO_2; in other cases Hill plots (log $[\bar{Y}/(1 - \bar{Y})]$ *vs.* log pO_2) will be used. In Tables we will present p50, the oxygen pressure at $\bar{Y} = 1/2$, and n_H, the maximum slope of the Hill plot.

Sedimentation Experiments. Sedimentation velocities were measured with the Spinco Model E analytical ultracentrifuge. Either the ultraviolet optical scanner or phase-plate schlieren optics were used. In experiments in which deoxygenated hemocyanin was used, the cell was loaded under nitrogen in a glove bag. Sedimentation coefficients were calculated either from the peak maximum (schlieren data) or from the half-maximum absorbance (scanner data). All data were corrected to $s_{20,w}$ values.

Results

We have investigated the effects of a number of parameters on the oxygen binding by *Callianassa* hemocyanin. These parameters include divalent ion concentration, pH, and temperature. These investigations were guided by two aims: (1) to elucidate the relationship (if any) between the state of aggregation of the hemocyanin and oxygen binding, and (2) to attempt to understand the significance of these parameters (pH, divalent ion concentration, and temperature) to the physiological function of the hemocyanin.

Effects of Divalent Ions on Oxygen Binding. Our investigations of divalent ion effects on binding were first prompted by our observations (Roxby *et al.*, 1974) that Mg^{2+} and Ca^{2+} markedly influenced the association equilibria. Others have noted divalent ion effects on both association and oxygen binding by hemocyanins (see, for example, Larimer and Riggs (1964) and Hwang and Fung (1970)) but to our knowledge no complete study of the interrelation of such effects has appeared. Accordingly, we carried out the series of experiments illustrated in Figure 1 and Table I, in which oxygen binding was measured at pH 7.65 in the presence of different concen-

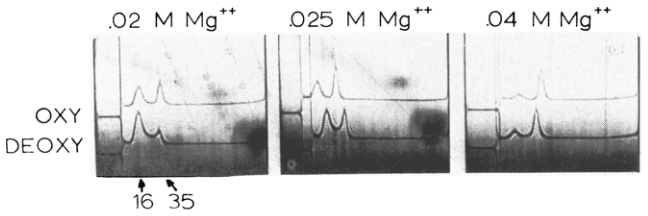

FIGURE 2: Schlieren patterns of oxygenated and deoxygenated hemocyanin C at different Mg^{2+} concentrations (as indicated). All data are at approximately 18°, pH 7.6. Each photograph was taken after about 16-min ultracentrifugation at 40,000 rpm. The boundaries correspond to approximately 16 and 35 S, respectively.

trations of Mg^{2+}. Solutions were prepared by dialysis of purified hemocyanin C *vs.* the appropriate buffers.

Hemocyanin that has been dialyzed against Mg^{2+}-free buffer is entirely in the 17S form (Roxby *et al.*, 1974) and exhibits a nearly noncooperative binding curve, and very weak binding overall (see Figure 1). As $[Mg^{2+}]$ is increased, the binding becomes stronger and the curve exhibits more and more cooperativity, as indicated by the maximum slope of the Hill plot. At Mg^{2+} concentration of 0.05 or greater, the protein is almost entirely tetrameric (almost completely in the 39S form) in both the oxygenated and deoxygenated states (see Figure 2). Raising $[Mg^{2+}]$ still higher increases the binding strength (lowers p50). This series of experiments was arbitrarily terminated at $[Mg^{2+}] = 0.1$ M; in retrospect (see Discussion) it would have been of interest to continue to higher $[Mg^{2+}]$. At low $[Mg^{2+}]$ (<0.05 M) a mixture of 39S and 17S components exists (Roxby *et al.*, 1974). Under these circumstances, the proportion of 39S material is sensitive to oxygenation, as shown in Figure 2, with the binding of O_2 shifting the equilibrium toward association. This matter will be taken up again in the Discussion section.

The effect of Mg^{2+} is not unique, for at least one other divalent ion (Ca^{2+}) acts in a similar way. We have already shown (Roxby *et al.*, 1974) that Ca^{2+} promotes association to the 39S form. Table II illustrates that Ca^{2+} also greatly increases the binding strength. In fact, a given concentration of Ca^{2+} appears to lower p50 more than an equal concentration of Mg^{2+}.

Variation of pH; the Bohr Effect. We wished to examine the Bohr effect of *Callianassa* hemocyanin under conditions as close as possible to those obtained *in vivo*. Consequently, we used ionic conditions similar to those found in the hemolymph. This information is available for *Callianassa* hemolymph. Atomic absorption analysis of Mg^{2+} gave levels of 0.048 (±0.003) M for samples of hemolymph from three shrimp. The blood Ca^{2+} levels for *Callianassa*, reported by Thompson and Pritchard (1969), are close to 0.01 M. As a consequence of these results, we adopted 0.05 M Mg^{2+} plus 0.01 M Ca^{2+} as divalent ion concentrations to be used in all of the Bohr effect studies. The results of these binding measure-

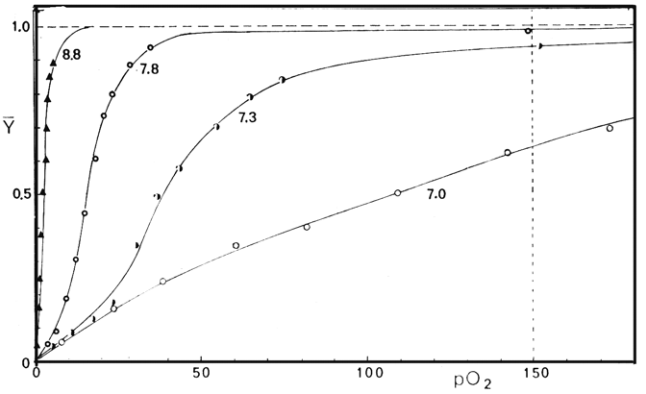

FIGURE 3: Some of the binding curves obtained in studies of the Bohr effect in *Callianassa* hemocyanin C. The pH values are indicated. All data at 25° in Tris buffers containing 0.05 M Mg^{2+}–0.01 M Ca^{2+}. Some curves have been omitted for clarity. The dotted vertical line corresponds approximately to the pO_2 in air.

ments are displayed in Figures 3 and 4, and in Table III. There is evidently a very strong Bohr effect, as can be seen from the variation of p50 with pH (Table III). The most illustrative graph, however, is Figure 4. Here we see that the curves change from an almost noncooperative form at low pH to strongly cooperative binding near pH 8.0, with the Hill coefficient decreasing again at higher pH. In all cases the curves seem to exhibit the expected form; the slope tends toward unity at either very low or high degrees of saturation. The two straight lines with slope of unity are our estimate for lines tangential to the set of curves. Their significance will be examined in the Discussion section.

Temperature Effects on O_2 Binding. Since *Callianassa* commonly lives at temperatures in the range 10–15°, rather than at 25°, the effects of temperature on O_2 binding were studied. These experiments utilized the same Tris buffer system, containing 0.05 M Mg^{2+} and 0.01 M Ca^{2+} that was used in the Bohr effect studies. Since the pH of Tris buffers is highly temperature sensitive, we were careful to prepare each solution in such a way that the pH *at the temperature of the O_2 binding study* would be 7.8, the value chosen for this series.

As can be seen from the data in Table IV, the temperature effect is complex; p50 decreases below 25°, but seems to level off, or even pass through a minimum at lower temperatures. We have not analyzed these data in detail, for we feel that the complexity of the behavior may well result from a combination of two factors: (1) variation of the intrinsic binding constant with temperature, and (2) shifts of the 17S–39S equilibrium with temperature. We have preliminary evidence that indicates the 39S form to preferentially dissociate at low temperature; this would result in an increase in p50, in opposition to the normally expected decrease in p50 as T is lowered.

Oxygen Binding by Hemocyanin I. Figure 5 shows binding curves obtained for purified hemocyanin I at both 0.0 M and

TABLE I: Oxygen-Binding Parameters for Hemocyanin C as a Function of $[Mg^{2+}]$, at pH 7.65, 25°.

$[Mg^{2+}]$ (M)	p50 (mm)	n_H
0.000	100	1.28
0.025	42	2.90
0.050	26	3.25
0.075	17	3.37
0.100	13	3.67

TABLE II: Effect of Mg^{2+}–Ca^{2+} Mixtures on Oxygen Binding by Hemocyanin C at pH 7.8, 25°.

$[Mg^{2+}]$ (M)	$[Ca^{2+}]$ (M)	p50 (mm)
0.050	0.000	26
0.000	0.050	14
0.050	0.0075	16
0.050	0.015	13
0.025	0.025	13

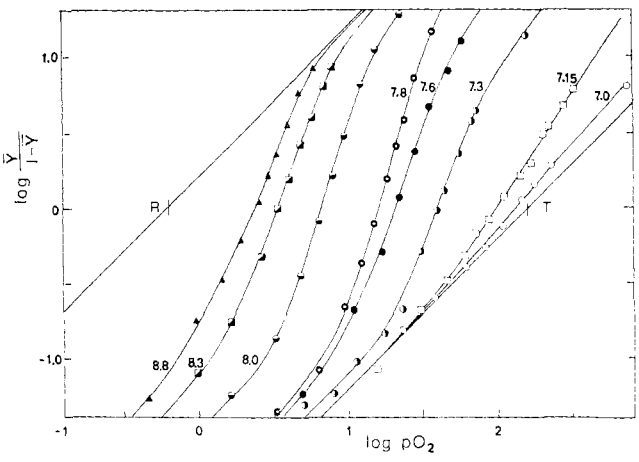

FIGURE 4: Hill plots for O_2 binding in 0.05 M Mg^{2+}–0.01 M Ca^{2+} at 25°. pH values are indicated. The two straight lines with slope unity (R + T) have p50 values corresponding to the intercepts in Figure 8.

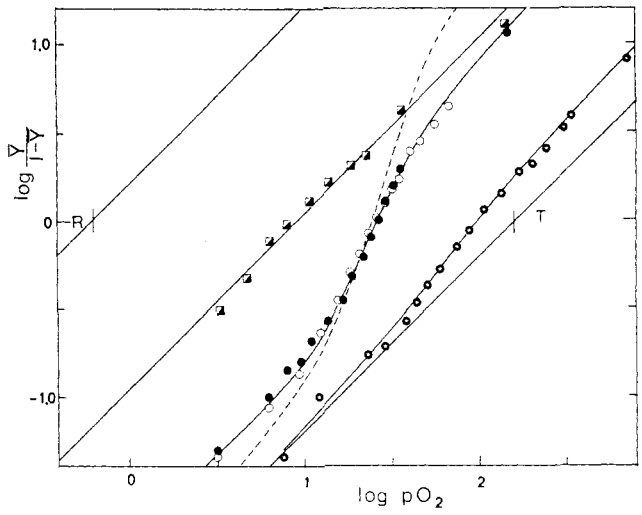

FIGURE 5: Hill plots for the hemocyanin subunits at pH 9.2 (squares) and for the incompetent hemocyanin I (circles). Starred circles, hemocyanin I at pH 7.65, $[Mg^{2+}] = 0.0$; open and filled circles, two experiments at pH 7.65, $[Mg^{2+}] = 0.05$ M. The dotted curve is for hemocyanin C under the latter conditions. Lines R and T from Figure 4.

0.05 M Mg^{2+}. While this form of the hemocyanin does not show appreciable association to 39S particles under any conditions tested, it clearly exhibited a degree of cooperativity in O_2 binding at the higher Mg^{2+} concentration. It is by no means as cooperative in its binding as is hemocyanin C at 0.05 M Mg^{2+}; the latter curve is drawn in for comparison. On the other hand, in the absence of Mg^{2+} the two components yield almost identical binding curves.

Oxygen Binding by the 5S Subunits. We wished to compare the O_2 binding of separated subunits of the hemocyanin with that of the aggregated forms. Ideally, one would prefer to do this under identical conditions of pH and ionic composition, but we know of no way of effecting dissociation to subunits at pH <9.2. At this pH dissociation is almost complete; if one goes to higher pH values a fairly rapid loss of binding capacity is observed in hours or minutes; at lower pH dissociation is only partial. At pH 9.2 the Tris buffers we have used are quite ineffective, so we were forced to turn to a borate buffer system.

The result is shown in Figure 5. While the data are not as precise as others we have obtained, they strongly indicate a noncooperative binding under these circumstances. The significance of this, and the p50 value found, will be discussed later.

Discussion

Analysis of the Binding Data in Terms of an Allosteric Model. It is evident from Figures 1, 3, and 4 that the binding of O_2 by *Callianassa* hemocyanin can exhibit wide ranges in p50 and considerable variation in cooperativity, depending on such parameters as the pH and the concentration of divalent cations. In addition, the dissociated 5S subunits of the hemocyanin and the association-incompetent 17S particles found in the hemolymph show quite different binding curves than the association-competent component (Figure 5).

While one may *a priori* expect the binding behavior of such large protein molecules to be complex, it is desirable to attempt to encompass as much of the behavior as possible within a single coherent scheme. In this section we shall attempt to demonstrate that many aspects of the behavior of *Callianassa* hemocyanin can be described by the theory of allosteric transitions, as first elucidated by Monod *et al.* (1965), and more recently extended and modified by Rubin and Changeux (1966), and Buc *et al.* (1973).

The key to this analysis is the observation, as illustrated in Figure 6, that the Hill coefficient, n_H, exhibits a systematic trend when graphed *vs.* log p50, passing through a maximum and approaching unity at certain limiting values of log p50. These limiting values correspond rather well to the p50 values of the limiting lines (R and T, Figures 1, 4, and 5) with slope $n_H = 1$ which represent the asymptotes of the curves in Figure 4. The simplest explanation is that these limiting lines represent the binding functions for molecules in R and T states, respectively, of an allosteric protein (see Monod *et al.* (1965) for a definition of these states). We are saying then, that Mg^{2+} and H^+ both act as allosteric effectors, low concentrations of Mg^{2+} and high concentrations of H^+ favoring the T state, and

TABLE III: Oxygen Binding Parameters for Hemocyanin C as a Function of pH at 0.05 M Mg^{2+}–0.01 M Ca^+, 25°.

pH	p50 (mm)	n_H
7.00	132	1.14
7.15	100	1.54
7.30	39	2.64
7.65	21	3.07
7.80	15	3.57
8.00	6.6	3.33
8.30	3.4	2.98
8.80	2.4	2.57

TABLE IV: Effect of Temperature on p50 of Hemocyanin C at pH 7.8, 0.05 M $[Mg^{2+}]$–0.01 M $[Ca^{2+}]$.

T (°C)	p50 (mm)
3.0	9.0
9.5	9.5
16.0	7.0
20.0	8.5
25.0	14.0

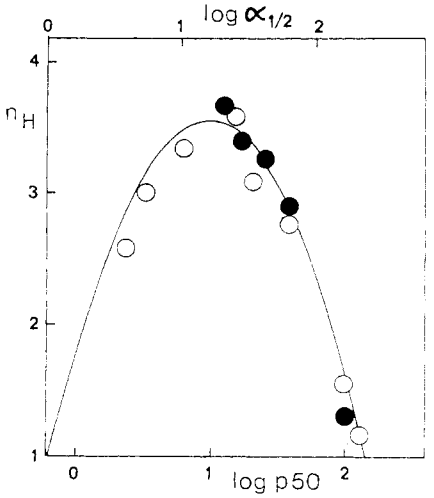

FIGURE 6: A graph of the maximum Hill coefficient, n_H, *vs.* log p50 or log $\alpha_{1/2}$. The open circles are points from data obtained in 0.05 M Mg^{2+}–0.01 M Ca^{2+}, at different pH values (Figure 4). The filled circles are from data at pH 7.65, with different Mg^{2+} concentration (Figure 1).

high concentrations of Mg^{2+} and low concentration of H^+ favoring the R state. At intermediate concentrations of both effectors, both states can be present, and the protein can exhibit cooperative oxygen binding. The fact that both R and T limits can be approached indicates that the protein exhibits nonexclusive binding for these effectors, as described by Rubin and Changeux (1966).

Recently Buc *et al.* (1973) have presented a modification of the Rubin and Changeux theory that takes into account the possible existance of hybrid states (in which some of the subunits are R, others T) in the binding. According to their analysis, the Hill coefficient will be given by

$$n_H = 1 + [n(\sigma^2/\sigma_m^2) - 1] \times [(\alpha_{1/2} - 1)(1 - c\alpha_{1/2})]/[(\alpha_{1/2} + 1)(1 + c\alpha_{1/2})] \quad (3)$$

where $\alpha_{1/2}$ is the ligand concentration at half-saturation normalized to the binding constant in the R state. In our case $\alpha_{1/2}$ = p50/p50$_R$. The constant c is the "nonexclusive binding coefficient," and n is the number of subunits in the cooperative unit. The value of factor σ^2/σ_m^2 depends upon the abundance of hybrid states; in a pure R–T system it will be unity. If $c > 0$, n_H will pass through a maximum at the point where $c\alpha_{1/2}^2 = 1$. The curve will go to unity at the extremes where p50 = p50$_R$, and p50 = p50$_T$, which corresponds to $\alpha = 1$, $\alpha = 1/C$, respectively. Thus, c can also be obtained from these limits.

Our data for the variation of n_H with *either* pH (at constant divalent ion concentration) *or* Mg^{2+} (at constant pH) conforms well to this model. Evaluation of c from either the value of $\alpha_{1/2}$ at maximum n_H, or from the p50 values of the limiting curves in Figure 5 leads to the same value, $c = 4.0 \times 10^{-3}$. From the height of the maximum, we obtain $n\sigma^2/\sigma_m^2 = 4.3$. Since σ^2/σ_m^2 may be somewhat less than unity (but would not likely be a very small number) we suggest that $n = 6$. This leads to the important conclusion that *the cooperative unit in this hemocyanin is the 17S hexamer*. This indicates a value of σ^2/σ_m^2 of about 0.72. Both this value and the value of c are in the range observed for other allosteric proteins; in fact they are (surely coincidentally) almost identical with values found for yeast pyruvate kinase by Johannes and Hess (1973).

The value of σ^2/σ_m^2, plus the symmetry of the curve of n_H *vs.* log p50 indicates that hybrid R–T states must be involved,

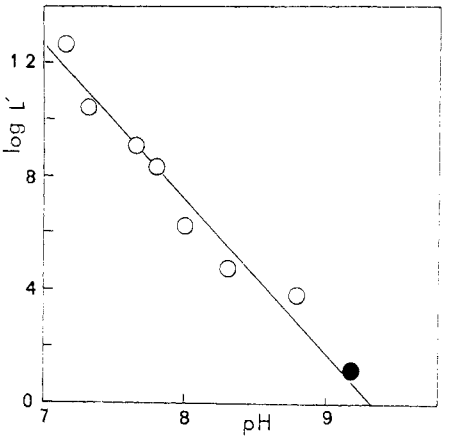

FIGURE 7: A graph of log L' *vs.* pH. Open circles are from data shown in Figure 4. Filled circle is for subunits at pH 9.2. See the text.

and that the symmetrical state R_3T_3 may predominate (Buc *et al.*, 1973). Accordingly, we have attempted to fit the n_H *vs.* log p50 curve to the model proposed by these authors, which involves the states R_n, T_n, and $R_{n/2}T_{n/2}$. Using their techniques, we obtain the curve shown by the solid line in Figure 6. Considering the uncertainty in the n_H values, the agreement may be considered satisfactory.

What we are saying, in effect, is that both the Bohr effect and the divalent ion effect result simply from the allosteric transition from T to R states. We may infer as a corollary that the association of the 17S particles into 39S particles is also a consequence, at least in part, of this transition. In situations in which the T state is favored (low divalent ion concentration, low pH, low O_2 binding) dissociation into 17S particles is favored. (See, for example, Figure 2, herein and Figures 6, 7, and 8 of the preceeding paper (Roxby *et al.*, 1974).) Conversely, situations that favor the R state of the subunits (high divalent ion concentration, high pH, O_2 saturation) favor association to the 39S form. Thus, we would conclude that 17S particles in the R state exhibit a greater association constant than those in the T state. It is quite possible, of course, that the association is more complex than this, and that both pH and divalent ion binding may influence the association above and beyond their influence on the R–T transition alone. The answer to this will depend upon more careful analysis of the association equilibrium than we have achieved to date.

A fundamental parameter of the Changeux–Rubin theory is the apparent allosteric contact L', which is the equilibrium constant for the R–T transition at given values of the allosteric effectors. This quantity can be calculated as a function of $\alpha_{1/2}$, c, and n

$$L' = \frac{(\alpha_{1/2} - 1)(1 + \alpha_{1/2})^{n-1}}{(1 - c\alpha_{1/2})(1 + c\alpha_{1/2})^{n-1}} \quad (4)$$

We have carried out this calculation for the variation of L' with pH, using $\alpha_{1/2}$ = p50/p50$_R$, and the values of c and n given above. The result is shown in Figure 7. These values pertain, of course, in circumstances where $[Mg^{2+}] = 0.05$ M, $[Ca^{2+}] = 0.01$ M.

It is of interest to attempt to explain the binding curve for the 5S subunits in terms of the model described above, and the parameters derived therefrom. While it was expected that the subunits would exhibit noncooperative binding, the relatively weak binding observed at this high pH (9.2) was unexpected to us. However, it can easily be shown that this behavior is in accord with the model. If one assumes that the same states

(R and T) with the properties described above, are present in the subunit solution, with an equilibrium constant given by L', it is easy to show from eq 2 of Monod *et al.* (1965) that the binding curve for the subunits should be given by

$$\bar{Y} = \frac{\left(\frac{1 + cL'}{1 + L'}\right)\alpha}{1 + \left(\frac{1 + cL'}{1 + L'}\right)\alpha} \quad (5)$$

or

$$\bar{Y} = \frac{pO_2/p50_m}{1 + pO_2/p50_m} \quad (6)$$

where $p50_m = \{(1 + L')/(1 + cL')\}p50_R$ is the p50 value expected from the monomer mixture. This result shows that $p50_R \leq p50_m \leq p50_T$, the exact value of $p50_m$ depending upon c and L'. If we use the observed values, $p50_R = 0.031$ mm, $p50_R = 9.5$ mm, and $c = 4.0 \times 10^{-3}$, we obtain a value of L' at the pH of these experiments (9.2) of approximately 14. This value of L' falls near reasonable extrapolations of the L' *vs.* pH curve shown in Figure 7. Exact comparison is hard to make, since the L' values are not very precise, and the buffer composition at pH 9.2 is somewhat different than in the other experiments. Nevertheless, we feel that these results indicate that the monomer binding data are at least roughly consistent with the allosteric model.

Finally, we consider the binding behavior of hemocyanin I. The results shown in Figure 5 indicate that this component possesses the same T state as hemocyanin C, but that it is in some fashion blocked from formation of the identical R state. Whether this corresponds to a modification of the R state, or to a limitation in the fraction of subunits that can achieve this state we cannot tell at this point. In either case, the result correlates qualitatively with the observation that these 17S particles do not tend to associate into tetramers.

Physiological Significance of the Binding Data. The effect of divalent ions on oxygen binding may have some physiological importance in *Callianassa*. The major divalent ions found in the blood are Ca^{2+} and Mg^{2+}, and both affect the oxygen binding of the hemocyanin. Of the two, only Ca^{2+} is likely to vary with the physiological state. It is resorbed from the exoskeleton and released into the bloodstream in significant quantities just prior to molting and would increase the oxygen affinity at this time of high metabolic demand. The Mg^{2+} level, which is almost equal to that of sea water, is regulated (Prosser and Brown, 1961) and thus remains stable. It would serve to maintain a highly cooperative basic oxygen binding pattern which could be modified by changes in Ca^{2+} or pH.

Callianassa should undergo very little, if any change in O_2 binding as a consequence of temperature changes. The animal spends much of its time buried in muddy sand, where the temperature range rarely exceeds (in Oregon, at any rate) 10–15°. Even in its active periods it is rarely exposed to higher temperatures. As Table IV shows, there is virtually no variation in p50 over this range; indeed, even a range of 3–20° would produce little effect.

The possible physiological significance of the Bohr effect is a much more complex matter. *Callianassa*, because of the nature of its habitat, will undergo periods in which the ambient oxygen pressure is very low. The blood pH may drop markedly in *Callianassa* when the animal is exposed to these anoxic conditions. Blood CO_2 and lactate levels will increase as the animal shifts to anaerobic metabolism (Hawkins, unpublished M.S. Thesis, Oregon State University.) Consequently the existence of a strong Bohr effect may have considerable physiological significance. The shrimp consumes oxygen at a constant rate down to a critical ambient oxygen pressure of 10 mm during sealed-jar respirometry (K. Miller and A. W. Pritchard, in preparation). Oxygen extraction at low pO_2 is more difficult. In all crustaceans a large gradient of oxygen pressure exists across the gills due to the high diffusion barrier created by the chitinous gill surfaces. As ambient oxygen pressure drops this gradient eventually becomes too small and diffusion of oxygen across the gill is no longer sufficient for the animal's needs. *Cancer magister* will die if exposed to low oxygen levels. *Callianassa*, on the other hand, shifts its metabolism to partial or total anaerobic glycolysis (Hawkins, M. S. thesis). As the tissue pH drops the hemocyanin will unload more of its stored oxygen and the size of the gradient will increase, facilitating the diffusion of oxygen. While *all* the oxygen needs might not be met, the continued oxygen gradient would allow the animal to maintain partial aerobic metabolism, at least for a short while. K. Miller and A. W. Pritchard (in preparation) report that animals which have been bled before oxygen consumption measurements lose their ability to maintain a constant rate of oxygen consumption, showing instead constantly dropping oxygen consumption with decreasing oxygen pressure. The presence of hemocyanin may be necessary for the maintenance of the normal oxygen gradient at both high and low oxygen tensions. While the hemocyanin appears to serve more of a storage function than a transport function in *Callianassa*, at least during inactive periods, it may still function in respiratory transport during periods of activity. Johansen *et al.* (1970) report for *Cancer magister* that the transport of oxygen by the blood is greatly enhanced during periods of activity. This could be the case for *Callianassa* as well.

In connection with this discussion, it is of interest to note that the normal blood pH range in active *Callianassa* is about 7.8–8.2. This corresponds with the range in which the Hill coefficient is maximal, under physiological levels of Mg^{2+} and Ca^{2+}.

In summary the pH response of O_2 binding appears to satisfy the rather diverse needs of this animal. In periods of high activity its respiratory pigment will show maximum cooperativity in binding. When the animal is inactive, the pH will drop and the Bohr effect will help maintain, at least for awhile, a continued supply of oxygen to the tissues.

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Tritium–Hydrogen Exchange of the Cyclic Peptide Polymyxin B_1†

Richard E. Galardy,‡ Lyman C. Craig,* and Morton P. Printz§

ABSTRACT: The exchange kinetics of the amide hydrogens of the basic polypeptide antibiotic polymyxin B_1 have been measured by a tritium–hydrogen exchange (THX) technique. The 11 amide hydrogens of polymyxin B_1 are observed near the pH of minimum exchange rate, while fewer hydrogens are observed away from pH_{min}. At all pH values studied, one amide proton exchanges significantly more slowly than the others. A single more slowly exchanging amide proton is also observed in a noncyclic heptapeptide derived from the ring portion of polymyxin B_1. However, comparison of the observed rate constants for specific acid and base catalyzed exchange in polymyxin B_1 with the theoretically calculated rate constants shows that in spite of one proton exchanging more slowly than the others no amide protons are involved in internal hydrogen bonding. Comparison of the exchange rates of the two slowest classes of amide protons of polymyxin B_1 and the acyclic ring peptide of polymyxin B_1 confirms an earlier conclusion that the cyclic portion of polymyxin B_1 is not stabilized by internal hydrogen bonding.

There now seems good reason to believe that the biological activity of many small natural polypeptides may be related to their solution conformations which are in turn determined by elements of secondary structure, *i.e.*, internal hydrogen bonding, hydrophobic interactions, electrostatic interactions, solvent effects, etc. The presence of strong hydrogen bonds places major restrictions on the number of conformational forms which need be considered for the polypeptide and thereby greatly simplifies conformational analysis. The exchange kinetics of the amide hydrogens of small polypeptides have proven to be sensitive probes of the secondary structure of these molecules (Laiken *et al.*, 1969; Galardy *et al.*, 1971; Printz *et al.*, 1972b). An amide proton exchanging sufficiently slowly under conditions of both specific acid and specific base catalysis probably indicates the presence of an intramolecular hydrogen bond or steric hindrance to access to the solvent in a small peptide.

The clearest example of such a relationship is found in gramicidin SA, a cyclic decapeptide. Our THX[1] data indicated the presence of four intramolecular hydrogen bonds in gramicidin SA (Laiken *et al.*, 1969) in agreement with the nuclear magnetic resonance (nmr) observations and the proposed model for gramicidin SA (Stern *et al.*, 1968). A single slowly exchanging amide proton was also found by THX in the cyclic peptide antibiotic bacitracin A (Galardy *et al.*, 1971). This amide proton is thought to be involved in an intramolecular hydrogen bond due to its slow exchange rate in native bacitracin A and its fast exchange rate in a bacitracin peptide with the cyclic portion of the molecule cleaved. THX of angiotensin II in aqueous solution containing 0.1 M NaCl showed two slowly exchanging amide protons which suggested two intramolecular hydrogen bonds (Printz *et al.*, 1972b). On the basis of the THX results and nmr data (Bleich *et al.*, 1973a,b), a solution conformation was proposed for angiotensin II (Printz *et al.*, 1972a) which was also found to be stable by potential energy calculations (Nemethy and Printz, 1972).

Although the cyclic portion of polymyxin B_1 (see Figure 1a) is topologically similar to the cyclic portion of bacitracin A, the results reported here suggest that, in contrast to bacitracin A, polymyxin B_1 contains no strong intramolecular hydrogen bonds. This lack of specific secondary structure is consistent with the nonspecific, detergent-like, antibacterial activity originally associated with the polymyxins (Goodman and Gilman, 1970). A solution conformation for polymyxin B_1 without internal hydrogen bonding is also consistent with the recently proposed mechanism of polymyxin B action based on the specific interaction between polymyxin B and certain phospholipids (Hsu Chen and Feingold, 1973).

Experimental Section

Materials. The structure of polymyxin B_1 is given in Figure 1 (I). The "B" refers to the presence of D-Phe in the variably substituted position in the ring of the polymyxin antibiotics. The subscript "1" refers to the particular fatty acid attached to the α-amino terminus, 3-methyloctanoic acid. The polymyxin antibiotics are products of *Bacillus polymyxa* and are bacteriostatic and bacteriocidal agents for a wide variety of gram-negative organisms. The peptide shown in Figure 1

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‡ Present address: Yale University School of Medicine, Section of Cell Biology, New Haven, Conn. 06510.

§ Present address: Division of Pharmacology, Department of Medicine, University of California at San Diego, La Jolla, Calif. 92037.

[1] Abbreviations used are: THX, tritium–hydrogen exchange; Dab, α,γ-diaminobutyric acid; pH_{min}, the pH of the minimum exchange rate; $t_{\frac{1}{2},min}$, the half-time for exchange at pH_{min}.