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Oxygen Equilibria of *Octopus dofleini* Hemocyanin†

Karen I. Miller
Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331
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ABSTRACT: Oxygen binding by *Octopus dofleini* hemocyanin was examined under very nearly physiological conditions. The effects of pH, ionic composition, temperature, and aggregation were controlled so that the role each plays in modulating oxygen binding can be isolated. There is a very large effect of pH on affinity, the Bohr effect ($\Delta \log P_{50}/\Delta pH = -1.7$), which is the same at 10 and 20 °C. However, cooperativity is substantially altered over the same range of pHs at the two temperatures. The allosteric properties were examined by comparing the experimental data points to curves generated by use of the Monod-Wyman-Changeux model. A computer-fitting process was developed which allowed the individual allosteric parameters to be varied independently until the best fit could be determined. The relationship between k_R and k_T is responsible for the effect of pH on cooperativity. A change in the allosteric properties of the T form is primarily responsible for the differences due to temperature. Changing cation concentrations when the molecule is in the fully aggregated 51S form alters affinity without influencing cooperativity. The effect of Mg^{2+} is much greater than that of Na^+. If the 51S decamer is dissociated to 11S monomers by removing divalent cations, oxygen binding is noncooperative. There is evidence for negative cooperativity, indicating heterogeneity of function within the subunit which contains seven oxygen binding domains. Association into decamers generates conformational change which results in a much wider range of allosteric function.

Until now, our work on *Octopus dofleini* hemocyanin has been limited to physical studies. We have characterized the subunit structure (Miller & van Holde, 1982), and in the preceding paper, we have described the association behavior of this respiratory protein (van Holde & Miller, 1985). This paper describes oxygen binding by *Octopus* hemocyanin.

Oxygen equilibria have been examined in considerable detail for *Octopus dofleini* by Lenfant & Johansen (1965), and recently for *Octopus vulgaris* by Houlihan et al. (1982). In both cases, the Bohr effect was controlled by using CO_2, and oxygen binding curves were measured using whole blood. In neither case were Hill plots prepared, so the allosteric behavior of the protein could not be evaluated. At the other extreme, Salvato & Tallandini (1977) and Tallandini & Salvato (1981) have carried out extensive studies on allosteric modulations in oxygen binding of *Octopus vulgaris* hemocyanin. However, much of this work was done under conditions in which the aggregation state was unknown or in which mixtures of different aggregation states were present. No attempt was made to duplicate physiological conditions. For this reason, we decided to examine the allosteric behavior of *Octopus dofleini* hemocyanin in a series of oxygen binding equilibria under very nearly physiological conditions, but with knowledge of and control over pH, temperature, ionic composition, and aggregation state.

Materials and Methods

Hemocyanin was collected and purified by gel filtration on an A5-M column as described in the preceding paper (van Holde & Miller, 1985). The column buffer was $I = 0.1$ tris(hydroxymethyl)aminomethane (Tris),[1] pH 7.65, containing 50 mM $MgCl_2$ and 10 mM $CaCl_2$. Oxygen binding curve studies were performed by using Tris buffers [prepared according to Long (1961)] with added salts; however, we found the temperature sensitivity of Tris buffers to be a liability when we wished to control pH very precisely. In these cases, we used 0.1 M HEPES or 0.1 M PIPES buffers, titrated to the desired pH with NaOH. Hemocyanin purified by gel filtration was used for most oxygen binding experiments, but for the two Bohr effect series, we used a concentrated hemocyanin solution obtained by sedimenting the hemolymph and removing the serum. A small amount of the resulting soft pellet of highly concentrated hemocyanin was added to buffer to obtain the desired concentration. This did not compromise the precision of these binding curves, since there is only one hemocyanin species present in the hemolymph (Miller & van Holde, 1982). For oxygen binding experiments under close to physiological conditions, the buffers were made up in a physiological saline which we prepared from published values of the major ions present in *Octopus* blood (Potts & Todd, 1965) and from a physiological saline developed for squid (Prosser, 1973). The composition of this saline was as follows: 370 mM NaCl, 45 mM $MgCl_2$, 10 mM K_2SO_4, and 8 mM $CaCl_2$.

Oxygen equilibria were measured by the tonometric method as described previously (Miller & van Holde, 1974). In order

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; MWC, Monod-Wyman-Changeux.

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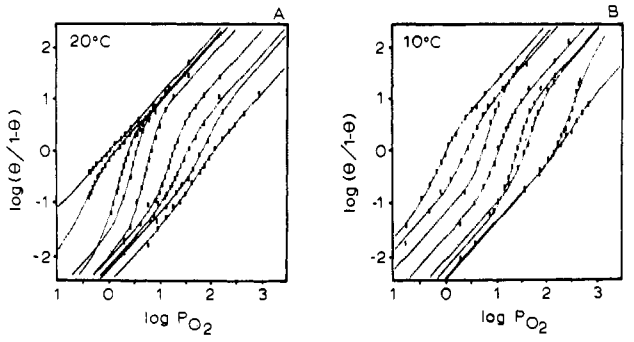

FIGURE 1: (A) Computer-fit oxygen equilibria (Hill plots) demonstrating the Bohr effect at 20 °C in 0.1 M HEPES, physiological saline. pH values (left to right) are 8.00, 7.70, 7.66, 7.50, 7.20, 7.07, 7.00, 6.78, and 6.60. These curves have been fitted to the MWC equation (eq 1) so that the rms statistic is minimized. (B) Computer-fit oxygen equilibria (Hill plots) demonstrating the Bohr effect at 10 °C in 0.1 M HEPES, physiological saline. pH values (left to right) are 8.30, 8.00, 7.78, 7.50, 7.38, 7.10, 7.00, and 6.81. These curves have been fitted to the MWC equation (eq 1) so that the rms statistic is minimized.

to obtain more precise binding curves, particularly at high pH, we developed a tonometer with a sidearm containing two gas bulbs in series, so that smaller increments of gas could be added for the initial part of the experiment. This greatly increased the resolution of the lower portion of the binding curve. At the lower pH values, the affinity is so low that oxygen at atmospheric pressure is necessary to obtain complete binding curves. In such experiments, we added the oxygen by attaching to the end of the tonometer sidearm a soft rubber reservoir filled with oxygen at atomspheric pressure. This was left attached for the duration of the binding curve. Temperature in the Cary 15 or Varian 2200 spectrophotometers was maintained with a thermostated cell holder. The binding curves were calculated by using the absorbance maximum at 345 nm, correcting for the scattering observed with the deoxygenated sample. At low pH, the ratio of the absorbance at 280 nm to that of 345 nm was monitored, and the maximum saturation of O_2 was corrected from 100% to the appropriate saturation level before the binding curve was calculated.

Results and Discussion

Oxygen Binding by Octopus Hemocyanin Is Cooperative and Shows a Pronounced Bohr Effect. We have conducted two series of oxygen binding equilibria, at 20 and 10 °C, as a function of pH in buffered physiological saline. These experiments, unlike those of Lenfant & Johansen (1965) and Houlihan et al. (1982), control pH with buffers rather than with CO_2. Hill plots of these oxygen equilibria are found in Figure 1A,B. The Bohr effect ($\Delta \log P_{50}/\Delta pH$) is very large and appears to be identical at both temperatures (Figure 2). The temperature and pH of the buffered solutions were somewhat more difficult to regulate precisely at 10 °C, leading to more scatter in the 10 °C binding data. Nevertheless, the points corresponding to 10 and 20 °C in Figure 2 fall on nearly the same curve. The slope of this curve is −1.7, one of the largest Bohr effects known (van Holde & Miller, 1982; Ellerton et al., 1983; Prosser, 1973; Mangum, 1980, 1981). Furthermore, the variation of log P_{50} wth pH is linear throughout the whole range of pH from 7.0 to 8.3, leveling out only when the low-affinity form of the protein is reached below pH 7.0. In this respect, the Bohr effect in *Octopus* is less complex than that of other hemocyanins which may shift from normal to reversed Bohr effect over the physiological range of pH or produce nonlinear curves, as in the case of *Callianassa* (Miller & van Holde, 1981).

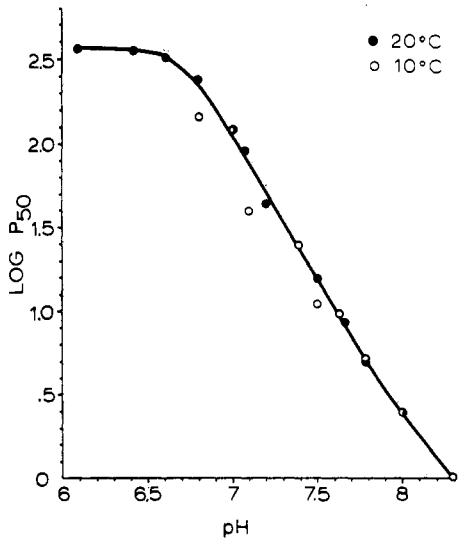

FIGURE 2: Effect of pH on oxygen affinity as measured by $p50$. Closed circles are 20 °C; open circles are 10 °C. Slope is −1.7.

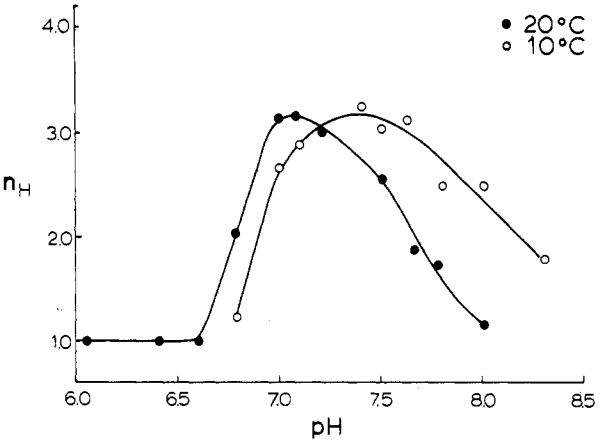

FIGURE 3: Effect of pH on cooperativity as measured by the Hill coefficient, n_H. Closed circles are 20 °C; open circles are 10 °C.

In contrast to the values for P_{50}, the Hill coefficients are quite different at the two temperatures over the pH range 6.6–8.3 (Figure 3). At 10 °C, the curve is nearly symmetrical with a broad peak of maximum cooperativity centering around pH 7.4. The physiological pH is close to 7.35 at this temperature (Lenfant & Johansen, 1965; Houlihan et al., 1982). The graph of n_H vs. pH is similar to curves described previously for *Callianassa* (Miller & van Holde, 1981). At 20 °C the curve is skewed sharply to the left, and below pH 6.8, the cooperativity never exceeds $n = 1$. The nonsymmetrical nature of this curve suggested to us a complexity in allosteric behavior that might be difficult to fit with simple mathematical models. We therefore developed a computer-fitting routine for analyzing oxygen binding behavior.

Computer-Fitting Oxygen Binding Curves Yields Allosteric Parameters. Figure 1A,B consists of our data points, but the curves have been drawn by the computer according to the Monod–Wyman–Changeux (Monod et al., 1965) model (MWC model) of allosteric behavior. According to this model, a Hill plot can be described by the equation:

$$\log\left(\frac{Y}{1-Y}\right) = \log\left[\alpha\frac{(1-\alpha)^{n-1} + cL'(1+c\alpha)^{n-1}}{(1-\alpha)^{n-1} + L'(1+c\alpha)^{n-1}}\right] \quad (1)$$

Y is the fraction saturation. The n-site protein is postulated to exist in two states, a weak binding (T) state and a strong binding (R) state. The quantity L' (which may itself be a

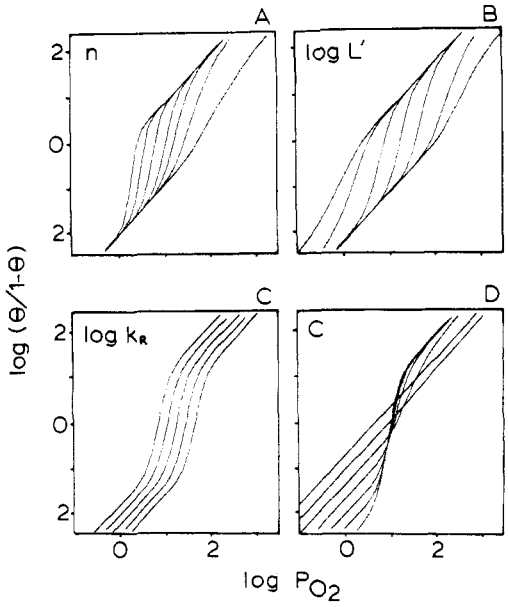

FIGURE 4: Computer-generated Hill plots illustrating the effect of modifying only one of four allosteric parameters. Our experimental data could be fit very precisely by altering one or more of the parameters. (A) Effect of varying n: n = 16, 12, 9, 7, 6, 5, and 4 (left to right); c = 0.02; log k_R = −0.2; log L' = 6. (b) Effect of varying log L': 1, 2, 4, 6, 8, 10, and 11 (left to right); n = 7; c = 0.02; log k_R = −0.4. (C) Effect of varying log k_R: log k_R = 0.0, −0.2, −0.4, −0.6, and −0.8 (left to right); n = 7; c = 0.02; log L' = 6. (D) Effect of varying c: c = 0.32, 0.16, 0.08, 0.04, 0.02, 0.01, and 0.005 (left to right); n = 7; log k_R = −0.2; log L' = 6.

function of *other* ligand concentrations) describes the equilibrium between T and R states: $L' = T/R$. α is related to the ligand concentration X by $\alpha = k_R X$, where k_R is the affinity constant in the R state. The ratio c ($=k_T/k_R$) measures the difference in affinity between the R and T states. For oxygen binding experiments, we define $\alpha = pO_2/p50_R$ where pO_2 is the partial pressure of oxygen and $p50_R$ is the half-saturation pressure for the R state. Thus, we express k_R and k_T is $1/p50_R$ and $1/p50_T$, respectively.

The MWC model has been used successfully, with the modification of Buc et al. (1973), to predict Bohr effect data for *Callianassa* (Miller & van Holde, 1974; Arisaka & van Holde, 1979) and *Penaeus* (Brouwer et al., 1978). The model was applied to single values for n, k_R, and k_T, derived from experimental data, with L' the only adjustable parameter. Altering L' was sufficient to produce curves which closely matched the experimental data points. As we began to fit our Bohr effect data, it became obvious that k_R and k_T could not be fixed and still generate the whole series of binding curves. At this point, we had two alternatives. We might be able to generate a new and more complex model which would describe the *Octopus* data. Alternatively, we could allow the parameters to vary freely until the best fit was obtained. We chose the second method, partly because the MWC model has been so successful for fitting oxygen binding data and partly because we saw no a priori reason why k_R and k_T should *not* vary with pH and/or temperature as they seemed to be doing.

The computer program was written so that it would generate a model binding curve when n, log k_R, c, and log L' were specified. Varying each one of the parameters independently allowed us to generate families of binding curves in which adjacent curves were related very differently to one another. Figure 4 shows what happens to binding curves if one changes a single parameter, leaving the other three constant. By systematically varying one or more of these parameters, a very accurate fit to our experimental data points was obtained. The

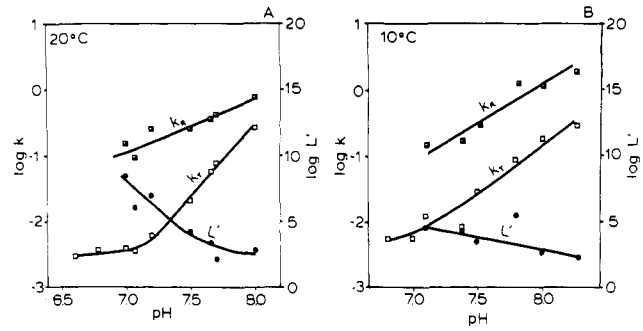

FIGURE 5: (A) Allosteric parameters used to fit the oxygen binding curves in Figure 1A. (B) Allosteric parameters used to fit the oxygen binding curves in Figure 1B.

value of n, the allosteric unit, was most critical for generating the fit. We found that using n = 7 generally gave the most accurate fits so we set n = 7 for all curves. Since our molecular weight per subunit was ~350 000 and the average molluscan oxygen binding site has a molecular weight of ~50 000, a value of n = 7 also seems reasonable from the molecular structure. The program calculated the root mean square (rms) deviation between the model curve and the data points, and fitting was continued until the minimum rms value was found. The average rms error is 0.033 ± 0.014 for the 20 °C binding data and 0.040 ± 0.016 for the 10 °C binding data.

When fitting was complete (Figure 1A,B), we were able to plot the alteration in the remaining three parameters with pH. Some very interesting relationships have emerged. Figure 5A,B shows what happens at 20 and 10 °C. Fitting the data was more difficult at lower pH because some of the binding curves were not complete enough to establish an accurate value for k_R. As a result, below pH 7.0 the values of L' and k_R became rather insensitive to the fitting process and indeed even meaningless. However, above pH 7.0, the relationship between k_R, k_T, and L' is extremely intriguing. At 10 °C, log k_R and log k_T increase in parallel fashion with increasing pH. log L' decreases slightly over this same range. At 20 °C, we see a very different pattern. The slope of the line for k_R is more or less the same, but k_T shows a much steeper slope, approaching the value of k_R at high pH. At 20 °C, the value of k_T seems to reach a steady minimum below pH 7.0, the point at which the limiting low-affinity form in the Hill plots is reached (Figure 1A). There is no alteration in the shape of the binding curves with lowered pH at this point. We did not reduce the pH of the 10 °C binding curves below 6.8, but it seems likely that they would show a similar leveling off at the lower limit. log L' decreases more sharply at 20 °C. The relationships between k_R and k_T thus determined can be seen in the Hill plots, if one examines them again. At 20 °C, the asymmetry of the relationship is most clearly seen in the way the upper ends of the Hill plots (corresponding to k_R) stay rather close together from one pH to the next compared to the lower ends (the k_T) which spread more evenly across the pH range. The Hill coefficients, therefore, *must* be largest at the low-pH end of the series, and indeed, that is what we have observed (Figure 3). At 10 °C where the k_R and k_T increase in parallel fashion, the ends of the Hill plots are more evenly spaced, and the Hill coefficients show a symmetrical distribution, with the maximum at the physiological pH. All of this suggests that the relationship between k_R and k_T determines the effect of pH. Alteration of the allosteric behavior of the T form is primarily responsible for the differences due to temperature.

Cations Influence Oxygen Affinity. One of our observations, mentioned in the preceding paper (van Holde & Miller, 1985),

Table I: Oxygen Binding Parameters under Conditions of Varying Sodium and Magnesium Concentration[a]

[NaCl] (mM)	[$MgCl_2$] (mM)	total ionic strength[b]	$p50$ (mmHg)	n_H
700	0	700	19.5	3.3
700	12.5	737.5	15.1	2.5
700	25	775	13.0	2.1
700	37.5	812.5	11.2	2.1
700	50	850	10.1	1.9
500	50	650	14.5	2.8
300	50	450	15.8	2.6
150	50	300	18.4	3.0
0	50	150	22.5	2.3

[a] Buffer conditions: 0.1 M HEPES, pH 7.6, at 10 °C. [b] Calculated according to the relationship $I = {}^1/_2\sum Z_i^2 C_i^2$.

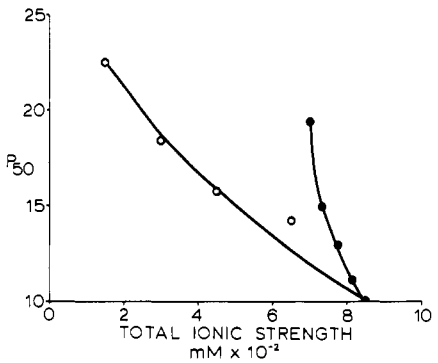

FIGURE 6: Effect of ionic strength on oxygen affinity as measured by $p50$. All buffers were $I = 0.1$ Tris, pH 7.5 at 20 °C. Open circles have 50 mM $MgCl_2$ and NaCl concentration is varied. Closed circles have 700 mM NaCl and $MgCl_2$ concentration is varied.

was that 0.7 M NaCl alone, in the absence of divalent cations, could stabilize the 51S form of the molecule. This allowed us to examine the effect of Mg^{2+} or Na^+ on oxygen binding behavior, independent of the effect of these ions on aggregation state. We fixed the NaCl concentration at 0.7 M and varied the $MgCl_2$ concentration, and conversely fixed the $MgCl_2$ concentration at 0.05 M and varied the NaCl concentration. We dialyzed whole molecules directly against the specific ion mixture rather than removing all Mg^{2+} with EDTA first, because we wished to be certain we were dealing with the native form of the 51S molecule rather than any possible alternative form reassembled in NaCl in the absence of Mg^{2+}. The fixed ion simply protected the whole molecule against dissociation. One consequence of this approach may have been to leave some Mg^{2+} tightly bound to the molecule. However, the Mg^{2+} concentration in the solution prepared in this way should have been defined by the dialysis. We checked all solutions in the ultracentrifuge to be certain that the 51S form was the only species present even under deoxygenated conditions. The results of these experiments are contained in Table I. The cooperativity, which was greatly altered by pH, was not affected significantly by either Mg^{2+} or Na^+. The oxygen affinity was decreased at decreasing cation concentrations. That this was not an effect of altering ionic strength is demonstrated in Figure 6. The two curves do not superimpose as one would expect if the effect were simply due to the ionic strength, but show clearly that the alteration in oxygen affinity due to changing Mg^{2+} concentration happens over a much smaller range of concentrations. The molecule is more sensitive to Mg^{2+}, both in oxygen binding affinity and in aggregation behavior (van Holde & Miller, 1985). These experiments were conducted in such a way that we cannot know precisely which ions were responsible for maintaining the fully aggregated form of the molecule, but the fact that

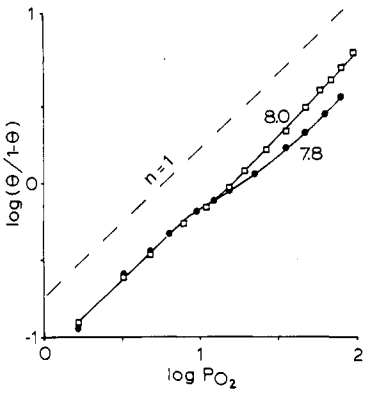

FIGURE 7: Hill plots for isolated subunits at pH 8.0 (□) and 7.8 (●) in $I = 0.1$ Tris and 10 mM EDTA.

it *was* fully aggregated means that the binding sites necessary for aggregation must have been filled. Therefore, we can conclude that there is a different set of ion binding sites which affect oxygen affinity and which are not involved in the association process.

Subunits Bind Oxygen Noncooperatively. J. Lamy (private communication) has demonstrated by immunoelectrophoresis and electron microscopy that *Octopus* hemocyanin contains a single homogeneous polypeptide chain with seven oxygen binding domains. Each subunit is thus potentially capable of cooperative binding. Therefore, the only way to know the source of allosteric behavior in *Octopus* hemocyanin is to examine oxygen binding of subunits. We prepared subunits by dialyzing whole molecules against buffers containing 0.01 M EDTA at pH 8.0 and 7.8. Oxygen binding curves are presented in Figure 7. The Hill plots clearly indicate noncooperative binding, but further, there is evidence of some heterogeneity of function. The upper and lower portions of the curve both have a slope of $n = 1$, but there is a shift to the right, with negative slope, at the region of the P_{50}. This shift is more exaggerated at lower pH, implying that some domains within the subunit have a Bohr effect and some do not. Bonaventura et al. (1977) in examining oxygen binding of several fragments of proteolytic cleavage of *Octopus* hemocyanin found evidence for heterogeneity of function as well but could not be certain the different functional units came from the same subunit. Our results provide fairly conclusive evidence for heterogeneity of function within the intact subunit. On the other hand, only when the subunits combine to form the decamer do we see cooperative oxygen binding. The associated molecule is clearly capable of a much wider range of allosteric function than the monomer despite its several domains. Thus, we can conclude that the conformational change produced by association into the quaternary structure forces allosterism.

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Registry No. O_2, 7782-44-7; Mg, 7439-95-4.

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Binding and Endocytosis of α_2-Macroglobulin–Plasmin Complexes†

Kathryn A. Ney, Susan Gidwitz, and Salvatore V. Pizzo*
Departments of Pathology and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710
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ABSTRACT: The clearance of ^{125}I-labeled α_2-macroglobulin–plasmin complexes (^{125}I-α_2M–PM) from mouse circulation is slower than that of ^{125}I-labeled α_2M–methylamine complexes (^{125}I-α_2M–CH_3NH_2). In addition, clearance of ^{125}I-α_2M–PM is biphasic, but that of ^{125}I-α_2M–CH_3NH_2 follows simple first-order kinetics. Treatment of α_2M–PM with trypsin yields a complex that clears like α_2M–CH_3NH_2. Complexes of α_2M with Val_{442}-plasmin (α_2M–Val_{442}-PM) were prepared; α_2M–Val_{442}-PM has a stoichiometry of 2 mol of Val_{442}-PM to 1 mol of α_2M and also clears like α_2M–CH_3NH_2. In vitro 4 °C binding inhibition studies with mouse peritoneal macrophages show that α_2M–CH_3NH_2, α_2M–PM, trypsin-treated α_2M–PM, and α_2M–Val_{442}-PM bind with the same affinity, apparent K_d = 0.4 nM. The binding isotherms at 4 °C are the same for ^{125}I-α_2M–CH_3NH_2, ^{125}I-α_2M–PM, and ^{125}I-trypsin-treated α_2M–PM in both mouse peritoneal macrophages and 3T3-L1 fibroblasts. The Scatchard plots for the binding isotherms in macrophages were curved; those in 3T3-L1 fibroblasts were linear with an apparent K_d of 0.48 nM and a receptor activity of 140 fmol/mg of cell protein for α_2M–CH_3NH_2, an apparent K_d of 0.29 nM and a receptor activity of 110 fmol/mg of cell protein for α_2M–PM, and an apparent K_d of 0.35 nM and a receptor activity of 210 fmol/mg of cell protein for trypsin-treated α_2M–PM. The time course of uptake and degradation and the concentration dependence of uptake at 37 °C are indistinguishable for ^{125}I-α_2M–PM and ^{125}I-α_2M–CH_3NH_2 in mouse peritoneal macrophages. The uptake of both complexes reaches a steady state after 1–2 h. Maximum cell-associated ligand at steady state is 2.0 pmol/mg of cell protein, and the concentration of half-maximal uptake is 50 nM for both complexes. These results suggest that the slower clearance of α_2M–PM from mouse circulation is not due to altered receptor recognition of the α_2M–PM complex but may be due to α_2M–PM interaction with some factor in mouse blood.

α_2-Macroglobulin (α_2M) is a large (M_r 718 000) plasma glycoprotein which binds to and inhibits endoproteases from each of the four classes serine, cysteine, metallo, and carboxyl [for reviews, see Starkey & Barrett (1977) and Barrett (1981)]. Protease binds to a "bait region" on α_2M (Barrett & Starkey, 1973; Sottrup-Jensen et al., 1981b) and then cleaves the bait region (Mortensen et al., 1981), causing a conformational change (Barrett et al., 1974; Nelles et al., 1980) in α_2M which sterically traps the protease (Barrett & Starkey, 1973) and exposes a reactive thiol ester (Sottrup-Jensen et al., 1981a). The exposed thiol ester is cleaved (Sottrup-Jensen et al., 1980) by nucleophilic attack by the bound protease or other nucleophiles in solution (Sottrup-Jensen et al., 1981c). Primary amines such as methylamine cleave the thiol ester (Sottrup-Jensen et al., 1980) and produce a conformational change without cleavage of the bait region (Steinbuch et al., 1968).

The conformational change and thiol ester cleavage following reaction of α_2M with protease or methylamine expose a receptor recognition site (Ohlsson, 1971a–c; Imber & Pizzo, 1981; Kaplan et al., 1981; Marynen et al., 1981). Once α_2M has reacted with protease or methylamine and becomes the "fast form" of α_2M, it is rapidly cleared from the circulation of dogs, humans, or mice (Ohlsson, 1971a–c; Blatrix et al.,

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* Address correspondence to this author at the Department of Pathology, Duke University Medical Center.

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