Gel Filtration Studies of Oxyhemerythrin. I. Effects of pH on the Association–Dissociation Equilibria†

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ABSTRACT: The pH dependency of the dissociation of oxyhemerythrin has been studied by frontal gel chromatography on Sephadex G-75. The extent of dissociation is markedly pH dependent increasing below pH 6.0 and above pH 8.8. In addition, the nature of the dissociation reaction undergoes dramatic change with pH. Below pH 6.4 the rate of equilibration between species is slow relative to their time of passage through the column and they are thus resolved on chromatography. Above pH 6.6 the rate of equilibration is rapid and the various forms of hemerythrin are not resolved on migration through the column. Below pH 7.4 the dissociation is an all-or-none process with no detectable intermediates. Above pH 8.0 several intermediate species can be detected. Values for K_{eq} and $\Delta G°$ are presented for various forms of oxyhemerythrin at the several pH's studied.

Hemerythrin, the oxygen-carrying protein of a number of marine organisms, has a molecular weight of 107,000 and is composed of eight identical subunits. Each subunit contains one cysteine residue and two atoms of non-heme iron, and can bind one molecule of oxygen. The protein can be dissociated into monomers by sulfhydryl modifying reagents, detergents, and urea (Keresztes-Nagy and Klotz, 1963; Klotz and Keresztes-Nagy, 1963; Manwell, 1963). It has been demonstrated that octameric hemerythrin is in equilibrium with monomers (Keresztes-Nagy et al., 1965; Klapper et al., 1966; Langerman and Klotz, 1969) and iron coordinating anions are known to promote dissociation (Klapper and Klotz, 1968; Klapper et al., 1966).

In this present work gel filtration is used to study the dissociation of hemerythrin. The utility of this technique for the study of interacting protein systems had earlier been suggested by the work of Winzor and Scheraga (1963) in which it was shown that the transport theories developed for the ultracentrifuge by Gilbert (1955) were qualitatively obeyed in Sephadex chromatography. Subsequently Ackers and Thompson (1965) formulated transport equations applicable to gel chromatography. More recently Ackers and his associates have refined these equations to include the effects of axial dispersion within the gel and have studied the effects of axial dispersion by computer simulation (Zimmerman and Ackers, 1971; Zimmerman et al., 1971). Previous results from our laboratory demonstrated that gel filtration is indeed comparable to ultracentrifugation in terms of the nature and accuracy of the information obtained. It was thus verified that octameric hemerythrin is in equilibrium with monomers and iron coordinating ligands promote dissociation (Rao and Keresztes-Nagy, 1972). Equilibrium constants calculated from the chromatographic data were found to be in reasonable agreement with those obtained previously in sedimentation velocity, sedimentation equilibrium, and ion binding studies (Rao and Keresztes-Nagy, 1972). Gel filtration has also been used successfully to compare the rate of reaction of hemerythrin with *p*-chloromercuribenzoate and the subsequent rate of dissociation of hemerythrin into monomers (Rao and Keresztes-Nagy, 1973a). More recently gel filtration has been used to study chloride ion binding to hemerythrin (Rao and Keresztes-Nagy, 1973b).

A major goal of investigations of hemerythrin has been to obtain information about the forces holding the subunits together (Langerman and Klotz, 1969; Langerman and Sturtevant, 1971). A recent finding has been the detection of carboxyl and imidazole groups at the subunit contacts of hemerythrin in chemical modification studies (Klippenstein, 1972; Fan and York, 1969). The importance of these groups in maintaining the quaternary structure of hemerythrin has not yet been elucidated. In this present work we have probed the nature of the subunit binding forces by studying the effects of pH, temperature, and ionic strength on the dissociation of oxyhemerythrin. In this paper it will be shown that both the nature and the degree of dissociation of oxyhemerythrin are pH dependent. The data will support the earlier conclusion that ionizable groups are present at the subunit contacts of hemerythrin and will indicate that the state of ionization of these groups strongly influences the stability of the octamer.

Experimental Section

Materials. Marker proteins used in the calibration of Sephadex columns were as follows: bovine serum albumin, ovalbumin, and horse myoglobin from Nutritional Biochemical Corporation; chymotrypsinogen from Schwarz Bio-Research, Inc.; and cytochrome *c* from Sigma Chemical Company. Blue Dextran and Sephadex G-75 were obtained from Pharmacia Fine Chemicals, Incorporated. The marine worm, *Golfingia gouldii,* was supplied alive by the Marine Biological Laboratories, Woods Hole, Mass. All buffers were prepared from deionized, distilled water and analytical grade chemicals. Buffer solutions were filtered through a 0.22-μ Millipore membrane and partially degassed prior to use.

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Table I: Sieve Coefficients for Various Species of Oxyhemerythrin.

i-mer	M_i	σ_i
1	13,500	0.485 ± 0.015[a]
2	27,000	0.305 ± 0.013[b]
4	54,000	0.124 ± 0.014[b]
6	81,000	0.047 ± 0.015[c]
8	107,000	0.039 ± 0.012[a]

[a] Measured experimentally. [b] Calculated according to the equation $\sigma_i = \sigma_1 - A \log i$ where $\sigma_1 = 0.485 \pm 0.015$ and $A = 0.599 \pm 0.010$. [c] Estimated by extrapolation of calibration curve.

Oxyhemerythrin was isolated from the coelemic fluid of the sipunculid worm, *Golfingia gouldii,* by the procedure of Klotz et al. (1957). The crystalline protein so obtained was stored wet at 3°. Stock solutions of oxyhemerythrin were prepared fresh each week and the protein concentration was determined by absorbancy measurements at 280 nm (Keresztes-Nagy and Klotz, 1965).

Frontal Elution Chromatography. A 1.5 × 60 cm jacketed chromatography column (Chromatronix), thermostated at a preselected temperature between 5 and 30°, was packed to a height of about 50 cm with swollen Sephadex G-75. To assure constant bed height and equilibration with eluent, columns were washed for 24 hr prior to each experiment with Tris-cacodylate buffer, 0.01 *M*, at the appropriate pH. A constant flow rate of 24 ml/hr was maintained with the aid of a Chromatronix, surgeless, positive displacement pump. The sample, equilibrated at the temperature of the column, was applied to the column through a Chromatronix sample injection valve without any change in the flow rate. The volume of sample was such as to yield a plateau in the elution diagram in which the protein concentration (C_0) was identical with that applied to the column. This necessitated sample volumes of 25–35 ml. The protein concentration was adjusted such that the experimentally determined weight average sieve coefficient ($\bar{\sigma}_w$) fell within the linear region of the column calibration curve. The column effluent was monitored essentially as described previously (Rao and Keresztes-Nagy, 1972) except that a "curve differentiator" and second recorder (Varicord Model 43, Photovolt Corp.) were included in the system (Tan, 1973). This permitted the simultaneous display of both the integral and differential elution profiles. A constant flow rate throughout sample application and elution made it possible to determine elution volumes directly from the recorded elution profiles with an error of less than 2%. The use of differential elution profiles obtained with the differentiator also appeared to improve the precision of the calculated values of the order (n) and equilibrium constant for oxyhemerythrin dissociation.

Calibration of Sephadex Columns. The exclusion volume (V_0) and the internal volume (V_i) were determined from elution patterns obtained with preparations of Blue Dextran and dilute solutions of potassium chromate. The internal volume was defined as $V_i = V_{K_2CrO_4} - V_0$ (Henn and Ackers, 1969). The molecular sieve coefficients (σ) of the calibrating standards were determined from their measured elution volume (V_e) according to the relationship $\sigma = (V_e - V_0)/V_i$. These sieve coefficients were found to obey the relationship $\sigma = -A \log M + B$ for values of M between 13,000 and 70,000. The values of A and B were found to be 0.599 ± 0.010 and 2.951 ± 0.018, respectively.

Treatment of the Data. The weight average partition coefficients ($\bar{\sigma}_w$) for oxyhemerythrin at the various protein concentrations corresponding to the plateau regions of the elution boundaries were obtained by measuring the centroid positions ($\bar{V}$) of the leading edges of the elution boundaries according to the relationships (Ackers and Thompson, 1965)

$$\bar{\sigma}_w = (\bar{V} - V_0)/V_i$$

where

$$\bar{V} = \frac{1}{C_0}\int_0^{C_0} V dC \qquad (1)$$

The centroid elution volume ($\bar{V}$) represents the first moment of the leading boundary (Longsworth, 1943). For symmetrical boundaries, this corresponds to the position of maximum in the differential elution diagram.

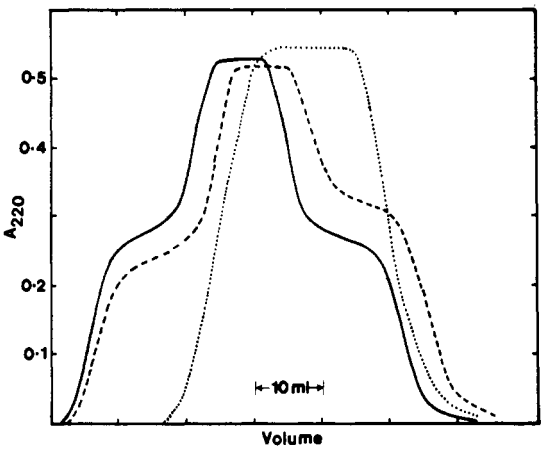

FIGURE 1: Elution profiles for 30-μg/ml solutions of oxyhemerythrin at 25°. Buffer is Tris-cacodylate, I = 0.01 M at pH 6.0 (—); pH 5.6 (- - -); and pH 5.2 (• • •). The pH 5.6 and pH 5.2 curves were offset to the right by 2.0 and 4.0 ml, respectively, for ease of viewing.

Determination of Sieve Coefficients for Oxyhemerythrin. Sieve coefficients for the monomeric and octameric forms of oxyhemerythrin were determined from frontal and zonal experiments under conditions where the protein was either fully associated or dissociated. The mean experimental values for σ_1 and σ_8 were 0.485 ± 0.015 and 0.039 ± 0.012. Sieve coefficients for species intermediate between monomer and octamer were calculated according to the equation $\sigma_i = \sigma_1 - A \log i$. For hexamer, the sieve coefficient fell outside the linear region of the σ vs. log M calibration curve. The sieve coefficient of hexamer (mol wt 81,000) was thus estimated by extrapolation of the calibration curve between the sieve coefficients for bovine serum albumin (67,000) and octameric hemerythrin (mol wt 107,000). The value for the sieve coefficients of various species of oxyhemerythrin are collected in Table I. Those sieve coefficients falling outside the linear region of the calibration curve for the Sephadex column (σ_6 and σ_8) were used only in the calculation of the order of the reaction, and the calculated values for n were in any case found to be largely independent of the exact magnitude of σ_6 and σ_8.

Results

Dissociation of Oxyhemerythrin below pH 6.4. Figure 1 illustrates several elution profiles obtained below pH 6.4. Both the leading and trailing boundaries of the elution profiles showed two well-defined plateau regions. Such elution profiles are characteristic of a two-component system in which complete resolution of the two species is attained.

Table II: pH Dependency of the Association Constant for Oxyhemerythrin in Tris-Cacodylate Buffer at 25° and $I = 0.01$ M.

pH	Protein Concn (g/l.) × 10^3	K_{eq} (M^{-7})	$-\Delta G^\circ$ (kcal/mol of Monomer)
4.80	180	7.59×10^{34}	5.95
4.90	180	9.75×10^{34}	5.97
5.00	180	1.64×10^{35}	6.00
5.20	180	3.93×10^{35}	6.07
5.30	185	2.96×10^{37}	6.39
5.40	30	8.74×10^{37}	6.47
5.60	30	2.31×10^{39}	6.71
5.70	30	4.68×10^{39}	6.77
5.80	30	1.97×10^{40}	6.87
6.00	30	2.93×10^{40}	6.90
6.60	30	5.83×10^{44}	7.63
7.00	30	5.83×10^{44}	7.63
7.20	30	4.38×10^{44}	7.61
7.40	30	4.81×10^{44}	7.62
7.60	30	5.83×10^{44}	7.63
7.81	30	7.85×10^{44}	7.66
pH	Protein Concn (g/l.) × 10^3	K_{eq} (M^{-3})	$-\Delta G^\circ$ (kcal/mol of Dimer)
9.00	30	8.67×10^{18}	6.45
9.18	30	1.63×10^{18}	6.21
9.40	30	9.76×10^{17}	6.14
9.70	30	1.88×10^{17}	5.89
10.0	30	1.57×10^{17}	5.86

Thus in the leading boundaries of curves a and b we observe a rise in protein concentration to a plateau corresponding to the concentration of the larger component, followed by a rise to a second plateau corresponding to total protein concentration. Following this second plateau the trailing edge shows a drop equal to the height of the first plateau in the leading edge. The height of the shoulder in the trailing edge thus corresponds to the concentration of the smaller species. The centroid elution volumes of the fast and slow moving components were measured and used to calculate sieve coefficients. The sieve coefficients corresponded to octamer and monomer, respectively. From Figure 1 it can be seen that dissociation increases with decreasing pH. At pH 5.2 and at a protein concentration of 30 μg/ml, oxyhemerythrin was completely dissociated into monomers (dotted line).

These elution profiles had two explanations. The first was the presence of a denatured or "crippled" component in the reaction mixture. This species would behave as a noninteracting and independent entity and would be separated from the rest of the interacting molecules during differential transport on the column. The second explanation was a slow equilibration between interacting species as compared with the time of their passage through the column, thus allowing their complete resolution. To verify the latter possibility the relative amounts of fast and slow moving components were measured as a function of protein concentration. Sample solutions containing 30, 15, and 5 μg/ml of oxyhemerythrin in 0.01 M Tris-cacodylate buffer (pH 6.0) were equilibrated for 6 hr and applied to column, and their elution profile was recorded. The relative amount of fast component was observed to decrease with decreasing protein concentration. From these data an association constant (K_{eq}) was calculated and found to be independent of protein concentration. This indicated a dynamic equilibrium despite a slow rate of equilibration. Association constants and calculated free energy changes are collected in Table II.

In a second series of experiments an elution profile was obtained for an oxyhemerythrin solution at pH 7.0. Identical hemerythrin solutions were then prepared at pH 6.0 and pH 5.0, and were refrigerated. After 6 and 24 hr aliquots were removed, adjusted to pH 7.0 with solid Tris, and chromatographed at pH 7.0. All elution patterns obtained at pH 7.0 were identical, and were thus independent of the prior history of the protein. This indicated that species of oxyhemerythrin generated at pH 5.0 and 6.0 (Figure 1) were able to participate in a rapid association–dissociation reaction (Figure 2) on readjustment of the pH to 7.0.

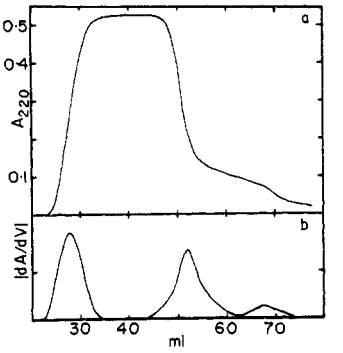

FIGURE 2: (a) Elution profile for a 30-μg/ml solution of oxyhemerythrin at 25°. Buffer is Tris-cacodylate, $I = 0.01$ M, pH 7.2. (b) Differential elution profile.

Dissociation of Oxyhemerythrin between pH 6.6 and 7.4. A typical elution profile for a 30-μg/ml solution of oxyhemerythrin between pH 6.6 and 7.4 at 25° is shown in Figure 2a. It is characterized by a single sharp leading edge, a plateau, and a broad bimodal trailing edge. Important details of this profile are illustrated by the differential diagram in Figure 2b. The centroid or elution volume of the leading boundary was determined by the position of the corresponding peak in the differential diagram. From the elution volume $\bar{v}_w$ was determined. It can also be seen that the differential elution profile of the trailing boundary shows the presence of two incompletely resolved peaks. Such integral and differential gradient curves are characteristic of rapidly polymerizing systems for which the order, n, is greater than 2 (Gilbert, 1955; Winzor and Scheraga, 1963; Zimmerman et al., 1971). From the position of the minimum in the bimodal trailing boundary ($\bar{V}_{min}$), it was possible to calculate the value of n according to the equation of Ackers and Thompson (1965):

$$n = \frac{(3\bar{v}_{min} - \sigma_m - 2\sigma_p)}{(3\bar{v}_{min} - 2\sigma_m - \sigma_p)} \quad (2)$$

In this equation $\bar{v}_{min} = (\bar{V}_{min} - S - V_0)/V_i$; V_0 and V_i have their usual meaning, S is the volume of the sample applied to the column, and σ_m and σ_p are the sieve coefficients of monomer and polymer, respectively. $\bar{V}_{min}$ was determined from the differential elution profile. The assumption implicit in the use of this equation is that the dissociation reaction is of an all-or-none type in which species intermediate between monomer and polymer are absent. The determination of n required selection of σ_m and σ_p values from Table I such that the calculated value of n equaled the ratio of molecular weights of the corresponding polymer and monomer. An additional restriction on the choice of σ_m and σ_p was that the ratio M_p/M_m had to be an integral number greater than 2. Between pH 6.8 and 7.4 only a monomer–octamer equilibrium satisified these criteria. The value of n calculated from V_{min}, σ_1, and σ_8 was 7.1 ± 0.8.

Equilibrium constants were calculated for the rapid association–dissociation reaction according to eq 3 for a two-

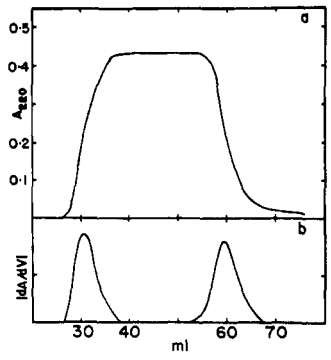

FIGURE 3: (a) Elution profile for a 30-μg/ml solution of oxyhemerythrin at 25°. Buffer is Tris-cacodylate, I = 0.01 M, pH 8.04. (b) Differential elution profile.

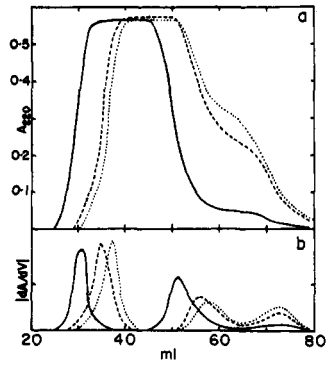

FIGURE 4: (a) Elution profiles for a 30-μg/ml solution of oxyhemerythrin at 25°. Buffer is Tris-cacodylate, I = 0.01 M at pH 9.0 (—); pH 9.5 (- - -); and pH 9.7 (· · ·). (b) Differential elution profiles, pH 9.0 (—); pH 9.5 (- - -); and pH 9.7 (· · ·).

component system (Ackers and Thompson, 1965):

$$K_{eq} = \frac{(1-\alpha)M_m^{n-1}}{n\alpha^n C_0^{n-1}} \quad (3)$$

In this equation C_0 is the concentration of protein in g/l. M_m is the molecular weight of monomer, and α is the weight fraction monomer and is determined by eq 4 for a two-component system:

$$\alpha = \frac{(\bar{\sigma}_w - \sigma_p)}{(\sigma_m - \sigma_p)} \quad (4)$$

In the pH region between 6.8 and 7.4 an unambiguous assignment of σ_m and σ_p equal to σ_1 and σ_8 was possible (vide supra). The calculated values of the association constants along with the corresponding standard free energy changes are collected in Table II.

Dissociation of Oxyhemerythrin between pH 8.0 and 8.8. Typical integral and differential elution diagrams for a 30-μg/ml solution of oxyhemerythrin between pH 8.0 and 8.8, and at 25° are shown in Figure 3a and b. These patterns are characterized by unimodal leading and trailing edges. This is more apparent in the differential gradient curve in Figure 3b. The observation of only a single peak in the differential diagram corresponding to the trailing boundary of the elution profile indicates a rapid association-dissociation reaction for which n is 2 (Winzor and Scheraga, 1963; Zimmerman and Ackers, 1971).

Additional elution profiles were obtained at pH 8.04 and at a number of protein concentrations between 3.8 and 180 μg/ml. The values of $\bar{M}_{app}$ obtained at pH 8.04 varied from 16,000 to 79,000. This range of values is too broad to be accounted for by a simple dimerization reaction. This suggests an association-dissociation reaction involving intermediate aggregation states such as consecutive or sequential dimerization.

Dissociation of Oxyhemerythrin above pH 9.0. Figures 4a and b depict several integral and differential elution profiles obtained between pH and 9.8 at 25°. These resemble curves observed at neutral pH indicating a rapid polymerization for which n is greater than 2. Figure 4b illustrates several additional features of the first derivative curves for the trailing boundary of rapidly polymerizing systems. These are the constancy of the position of the peak eluting at higher volumes (slower eluting peak) and the decrease in size and rate of elution of the faster peak with increasing extent of dissociation. The constancy of the position of the minimum ($\bar{V}_{min}$) indicates that the order, n, is constant in this region of pH and protein concentrations.

The order of the reaction was calculated from $\bar{V}_{min}$ as described previously. The assignment of σ_m equal to σ_2 was the only one compatible with the experimental value of $\bar{V}_{min}$. Values of 2.8 ± 1 and 2.7 ± 1 were then obtained for n on assigning σ_p equal to σ_6 and σ_8, respectively. These results are thus compatible with either a dimer-hexamer, or a dimer-octamer equilibrium between pH 9.0 and 9.8. The values of K_{eq} and $\Delta G°$ shown in Table II were calculated on the assumption of a dimer-octamer equilibrium.

Effect of pH on M_w and K_{eq} for Oxyhemerythrin. For a rapid association-dissociation reaction, $\bar{\sigma}_w$ may be obtained directly from the centroid position of the leading edge of the elution boundary. When only monomer and polymer are present, $\bar{\sigma}_w$ may be converted to a weight average molecular weight, $\bar{M}_w$, according to

$$\bar{M}_w = M_p + \left(\frac{\bar{\sigma}_w - \sigma_p}{\sigma_m - \sigma_p}\right)(M_m - M_p) \quad (5)$$

In this equation M_m, M_p, σ_m, and σ_p are molecular weights and sieve coefficients of monomer and polymer.

When the rapid association-dissociation reaction involves intermediate aggregation states, such as occurs between pH 8.0 and 8.8, $\bar{\sigma}_w$ may not be readily converted to $\bar{M}_w$. In this pH region $\bar{\sigma}_w$ was used to calculate an apparent average molecular weight, $\bar{M}_{app}$, according to the relationship

$$\bar{\sigma}_w = A \log \bar{M}_{app} + B \quad (6)$$

$\bar{M}_{app}$ in this equation is not equal to $\bar{M}_w$.

For a slow association-dissociation reaction, complete resolution of individual species may be obtained by chromatography. When only two species predominate, monomer and polymer, one can evaluate $\bar{M}_w$ according to the relationship $\bar{M}_w = \alpha M_1 + (1-\alpha)M_p$. In this equation α represents the weight fraction of monomer and is determined from the ratio of the heights of the plateau regions in the elution profile (Figure 1). Values for $\bar{M}_{app}$ may also be calculated with eq 4 and 6.

Figure 5 shows the variation in $\bar{M}_w$ and $\bar{M}_{app}$ of oxyhemerythrin with pH at 25° and at a protein concentration of 30 μg/ml. The pH-molecular weight profile is a flat-topped, bell-shaped curve suggesting the involvement of acidic and basic amino acid residues in the association-dissociation reaction. The native protein thus appears to be most stable in the neutral pH region.

The acid limb of the pH vs. molecular weight curve suggests the involvement of a group with a pK below 6.5 in the association-dissociation reaction of oxyhemerythrin. A plot of pH vs. the log of the association constant (from

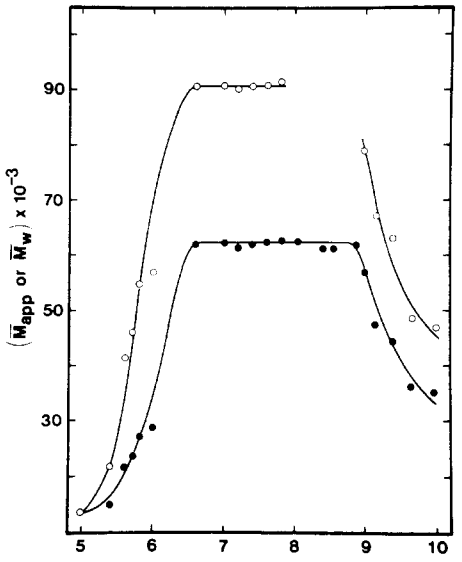

FIGURE 5: pH dependency of $\bar{M}_w$ (O) and $\bar{M}_{app}$ (●) for 30 μg/ml of oxyhemerythrin at 25°. Buffer is Tris-cacodylate, $I = 0.01$ M.

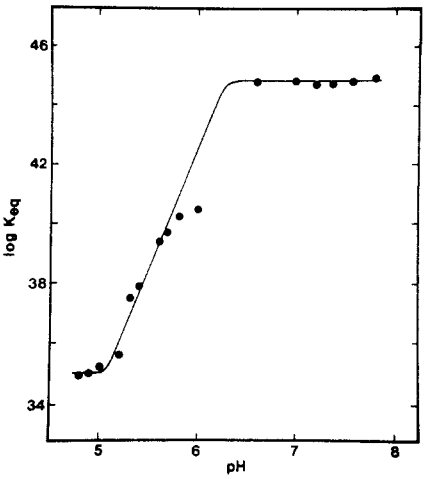

FIGURE 6: pH dependency of the association-dissociation reaction of oxyhemerythrin at 25° in Tris-cacodylate buffer, $I = 0.01$ M. In this pH range only monomer and octamer are present in equilibrium. The closed circles are the experimental points and the solid line was calculated (see text for details).

Table II) between pH 4.8 and 7.8 is shown in Figure 6. In this pH region only monomer and octamer are present in equilibrium. The curve contains two plateau regions which correspond to the association constants for the fully protonated and fully unprotonated oxyhemerythrin separated by a linear segment with a slope of 8. The circles represent the experimental points and the solid line was calculated with the aid of the empirical equation:

$$K_{obsd} = \frac{K_0 + K_H([H]/K_a)^n}{1 + ([H]/K_a)^n} \quad (7)$$

In this equation K_{obsd} is the observed pH dependent associated constant; K_0 is the pH independent association constant at neutral pH; K_H is the pH independent association constant at acidic pH; n is the number of protons involved in the reaction, in this case eight; and K_a is the acid dissociation constant of the amino acid side chain in monomeric hemerythrin controlling protein dissociation. We can also define an acid dissociation constant $K_a' = K_a(K_0/K_H)^{1/n}$ for the same amino acid side chain in octameric hemerythrin. The values of pK_a, pK_a', K_0, and K_H which gave the best fit to the experimental data were 6.30, 5.08, 6.03×10^{44} M^{-7}, and 1.0×10^{35} M^{-7}, respectively.

Discussion

All frontal elution profiles were interpreted according to the theory of Gilbert (1955) as applied to gel filtration by Ackers and Thompson (1965). The theory of Cann and Goad (1970), which relates to ligand mediated protein association reactions, does not apply in this present work. In order for elution profiles to be affected by ligands present in solution, it is necessary for a ligand concentration gradient to be established in the chromatography column as a consequence of differential binding to monomer and oligomer. One necessary condition for the establishment of such a gradient is that the concentration of ligand be of the same order as the total concentration of binding sites. In this work, with the exception of hydrogen ion, the concentrations of all ligands were in great excess of protein. Since the ratio of the concentrations of the various buffer components determined the pH of the protein solutions studied, and since as indicated, there could exist no concentration gradient in these buffer components, it must also follow that there could not exist a hydrogen ion gradient.

The work described in this paper demonstrates several previously unknown properties of the association-dissociation reaction of oxyhemerythrin. First, the dissociation of oxyhemerythrin is markedly pH dependent. From the variation of $\bar{M}_w$ or $\bar{M}_{app}$ with pH (Figure 5), it appears that the dissociation of oxyhemerythrin is controlled by two or more prototropic groups. Second, this work documents the existence above pH 8.0 of species of oxyhemerythrin that are intermediate between monomer and octamer. The dissociation of oxyhemerythrin can no longer be described as an all-or-none process as previously believed (Langerman and Klotz, 1969). Between pH 8.0 and 8.8 the data indicate the presence of aggregation states intermediate between monomer and octamer. Above pH 9.0 the predominant species in solution appear to be dimer and octamer. Finally, this work partially clarifies recent contradictory reports on the rate of the association-dissociation reaction of oxyhemerythrin (Klapper et al., 1966; Rao and Keresztes-Nagy, 1972). It indicates that the rate of equilibration of monomeric and octameric hemerythrin is markedly sensitive to experimental conditions, the reaction being slow below pH 6.4 and rapid above pH 6.6. In the second paper in this series (Tan et al., 1975), it will be shown that the rate of this reaction is also temperature and ionic strength dependent.

From the variation of K_{eq} with pH (Figure 6) it appears that a prototropic group with a pK between 5 and 6 is involved in the association-dissociation reaction of oxyhemerythrin. This may be either a carboxyl or an imidazole group. Evidence for the presence of both these amino acid side chains at the subunit contacts of hemerythrin has recently been obtained from chemical modification studies (Klippenstein, 1972; Fan and York, 1969).

The pK_a values of the group(s) responsible for the alkaline limb of the pH vs. $\bar{M}_w$ curve cannot be easily deduced from the present data. Although the pH vs. M_{app} curve (Figure 5) suggests the involvement of a single group with a $pK_a > 8.5$, such a conclusion is probably an over simplification. Between pH 6.6 and 8.5 the weight average molecular weight, $\bar{M}_{app}$, appears constant while the association-dissociation reaction changes from an all-or-none process to one

involving intermediate aggregation states. This transformation requires that there be a pH dependent change in the relative magnitudes of the individual association constants for the various *i*-meric forms of oxyhemerythrin. Thus in consecutive dimerization the relative magnitude of the association constants for each successive step is similar. For consecutive dimerization to appear as an all-or-none polymerization, the association constants for each successive step in the dimerization process must be larger than the corresponding constant for the preceding step (Klotz et al., 1970).

A second prototropic group is probably also involved in the association–dissociation reaction of oxyhemerythrin at alkaline pH. This group is responsible for the pH dependent transition from a consecutive dimerization to a dimer–octamer equilibrium between pH 8.8 and 9.2. Titration of this same group may also be responsible for the descending limb of the $\bar{M}_{app}$–pH profile observed above pH 8.5.

The present data do not allow precise determination of the pK_a's and identities of the amino acid side chains which influence the association–dissociation behavior of oxyhemerythrin at alkaline pH's. One may speculate that one of the two groups responsible for the pH dependency in the alkaline region is the sulfhydryl of cysteine. The importance of a cysteine residue in the dissociation of octameric hemerythrin has been well documented (Keresztes-Nagy and Klotz, 1963; Cress, 1972). The observation that the dissociation of oxyhemerythrin markedly increases at acidic and alkaline pH's suggests that ionic interactions and/or hydrogen bonding may play a role in stabilizing the octamer. The change in $\Delta G°$ for octamer formation between pH 4.8 and 7.0 is close to −2 kcal/mol of monomer and is in the range expected for an ionic–hydrogen bond (Scheraga, 1963).

The interpretation of the pH studies may be complicated by the presence of a minor variant in pooled preparations of monomeric hemerythrin from *Golfingia gouldii* (Klippenstein, 1972). The minor species comprises 15–20% of the total hemerythrin and migrates more rapidly on disc gel electrophoresis. The minor component has been shown to contain an asparagine residue in place of a histidine residue at position 82. Additional differences result from isologous replacements at positions 63, 78, 79, and 96. Octameric hemerythrin is likely to consist of hybrids of these two classes of subunits (Keresztes-Nagy et al., 1965). From the slope of the curve in Figure 6 it appears that dissociation of octameric hemerythrin at low pH results from the titration of eight amino acid residues, one per monomer. It is likely, therefore, that this group represents one of the invariant residues of the hemerythrin monomer.

The results reported here provide additional evidence of the validity of many of the qualitative and quantitative relationships which have been developed to describe the transport of rapidly interacting protein systems during gel filtration (Winzor and Scheraga, 1963; Ackers and Thompson, 1965; Zimmerman and Ackers, 1971; Zimmerman et al., 1971). Thus under appropriate conditions, oxyhemerythrin can display profiles characteristic of either rapid dimerization, rapid polymerization, or slow polymerization. Equilibrium constants calculated at pH 7.0 from the leading edge of the elution profiles are in reasonable agreement with those obtained by sedimentation velocity (Klapper et al., 1966), sedimentation equilibrium (Langerman and Klotz, 1969), and ion binding studies (Klapper and Klotz, 1968). The values of K_8 for oxyhemerythrin obtained at pH 7.0 in this study are higher than values previously reported for the azide, thiocyanate, and aquo complexes of methemerythrin. These differences are consistent with the observation that ligands such as azide and thiocyanate promote dissociation (Klapper and Klotz, 1968; Rao and Keresztes-Nagy, 1972). These differences may also reflect in part the formation of oxidized or "crippled" monomer on prolonged centrifugation (Langerman and Klotz, 1969), a problem not encountered in these present studies owing to the relative rapidity of gel filtration. Finally, analyses of the trailing boundaries of the elution profiles, obtained under conditions of rapid equilibrium at pH 7.0, provide direct confirmation that the apparent order of the association–dissociation reaction is 8 ($n = 7.1 + 0.8$). This work thus clearly demonstrates the utility of frontal gel chromatography in the study of protein association–dissociation equilibria. The technique is rapid, simple, and sensitive to changes in pH, ionic strength, temperature, and other parameters. This technique not only permits easy detection of relatively subtle changes in the association–dissociation behavior of a protein but also permits accurate measurements at very low protein concentrations.

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Gel Filtration Studies of Oxyhemerythrin. II. Effect of Temperature and Ionic Strength on the Association–Dissociation Equilibria†

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ABSTRACT: The effects of temperature and ionic strength on the association of oxyhemerythrin have been studied. $\Delta H°$ and $\Delta S°$ for association at pH 7.0 are −2.6 kcal and +16.5 eu per mol of monomer. These values suggest that solvent adjacent to the surface of the protein undergoes rearrangement on association. Increasing ionic strength is observed to promote dissociation while decreasing the rate of attainment of equilibrium between monomers and octamers. Qualitatively similar results are observed on lowering the pH from 7.0 to 4.8, thereby linking the effects of increasing ionic strength to those of protonation of specific amino acid residues at the subunit contacts of hemerythrin. The apparent enthalpy of ionization of the amino acid residue controlling dissociation at acidic pH was found to be −1.9 to +2.1 kcal/mol. These values are consistent with a carboxyl group.

In the previous paper in this series we have demonstrated that the dissociation of oxyhemerythrin is markedly pH dependent, increasing below pH 6.0 and above pH 8.5 (Tan et al., 1975). One prototropic residue per subunit appears to be responsible for the enhanced dissociation at acidic pH values. On the basis of its apparent pK_a and results of previous chemical modification studies, it was suggested that this could be either a carboxyl or imidazole group (Tan et al., 1975; Fan and York, 1969; Klippenstein, 1972). At alkaline pH values possibly two different ionizable groups are responsible for enhanced dissociation. On the basis of the pH dependency of the extent of dissociation it was suggested that ionic interactions and/or hydrogen bonds contribute to the stability of octameric hemerythrin.

In order to clarify the role of prototropic amino acid side chains in subunit assembly, to expand the available thermodynamic data describing the subunit interactions, and to explore the contribution of electrostatic forces to the stability of octamer, we have studied the effects of pH, temperature, and ionic strength on the dissociation of oxyhemerythrin. In this present paper we will show that the dissociation of oxyhemerythrin is both temperature and ionic strength dependent. The data will support the suggestion that ionic interactions and/or hydrogen bonds between acidic and basic amino acid residues represent a component of the binding force at the subunit contacts of hemerythrin. In addition, the heat of ionization of the amino acid side chain controlling dissociation at acid pH has been measured and this value is compatible with a carboxyl group.

Experimental Section

Materials. Marker proteins used in the calibration of Sephadex columns have been described previously (Tan et al., 1975). Blue Dextran and Sephadex G-75 were obtained from Pharmacia Fine Chemicals, Inc. The marine worm, *Golfingia gouldii*, was supplied alive by the Marine Biological Laboratories, Woods Hole, Mass. All buffers were prepared from deionized, distilled water and analytical grade chemicals. Buffer solutions were filtered through a 0.22-μ Millipore membrane and partially degassed prior to use.

Oxyhemerythrin was isolated from the coelemic fluid of the sipunculid worm, *Golfingia gouldii*, by the procedure of Klotz et al. (1957). The crystalline protein so obtained was stored at 3°. Stock solutions of oxyhemerythrin were prepared fresh each week and the protein concentration was determined by absorbancy measurements at 280 nm (Keresztes-Nagy and Klotz, 1965).

Frontal Elution Chromatography. Frontal elution was performed as described previously (Tan et al., 1975). The concentration of protein applied to the column was adjusted so that the experimental values of the weight average sieve coefficient ($\bar{\sigma}_w$) fell within the linear region of the column calibration curve. The void volume (V_0) and the internal volume (V_i) were determined from the elution volume of

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