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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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Blue Dextran and potassium chromate as described by Henn and Ackers (1969). The molecular sieve coefficients (σ) of the calibrating standards were determined from their elution volumes (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 Data. The weight average partition coefficients ($\bar{\sigma}_w$) for oxyhemerythrin at various protein concentrations were determined from the elution profiles as described previously (Tan et al., 1975). These $\bar{\sigma}_w$ values were then used to determine α, the weight fraction monomer, and $\bar{M}_w$, the weight average molecular weight (Tan et al., 1975).

Results

Effect of Temperature and pH on the Dissociation of Oxyhemerythrin. Calculation of the weight average molecular weight ($\bar{M}_w$) and the apparent average molecular weight ($\bar{M}_{app}$) for an interacting protein system from its elution profile has been described previously (Tan et al., 1975). Figure 1 shows the variation in $\bar{M}_{app}$ of oxyhemerythrin with pH at 5, 25, and 30° and at a protein concentration of 30 μg/ml. The pH-$\bar{M}_{app}$ profiles are flat-topped, bell-shaped curves suggesting the involvement of acidic and basic amino acid residues in the association reaction. It can be seen that at each pH the extent of dissociation increased with increasing temperature.

All the qualitative aspects of the association–dissociation reaction which had previously been observed at 25° (Tan et al., 1975) remained essentially the same at 5 and 30°. Thus below pH 6.4 elution profiles were characteristic of a two-component system in which complete resolution of the two components were attained. Sieve coefficients (σ) were determined from the centroid elution volumes of the fast and slow moving components and were found to correspond to octamer and monomer, respectively. Above pH 6.4 elution profiles were characteristic of a rapid equilibrium and the various forms of hemerythrin were not resolved on passage through the column. Between pH 6.6 and 7.4 elution profiles obtained at 5 and 30° displayed a unimodal leading edge and a bimodal trailing boundary characteristic of a rapid polymerization (Winzor and Scheraga, 1963; Ackers and Thompson, 1965; Zimmerman et al., 1971). The order of the reaction (n) was determined from the position of the minimum ($\bar{V}_{min}$) in the bimodal trailing boundary essentially as described previously (Tan et al., 1975). The value obtained for n was close to 8. Between pH 8.0 and 8.8 elution profiles at 5 and 30° were characteristic of a rapid dimerization reaction (Winzor and Scheraga, 1963; Zimmerman and Ackers, 1971). On the basis of earlier results at 25° it was assumed that these profiles also reflected the occurrence of intermediate aggregation states (Tan et al., 1975). Finally above pH 9.0 elution profiles obtained at 5 and 30° were characteristic of a rapid polymerization. The calculated value of n was consistent with a dimer–octamer equilibrium in the range of protein concentrations studied. This is apparent on inspection of Figure 1 where it can be seen that the limiting value of $\bar{M}_{app}$ approached at 30° and high pH corresponds to the molecular weight of dimer.

Calculation of Association Constants. Association constants were calculated for the slow association–dissociation reaction below pH 6.4 directly from the measured concentrations of the fast and slow moving components in the elution profiles and on the basis of n equal to 8. These are collected in Table I along with the calculated values of $\Delta G°$. Association constants were calculated for the rapid polymerization reactions between pH 6.6 and 7.4 and above pH 9.0 according to eq 1 (Ackers and Thompson, 1965):

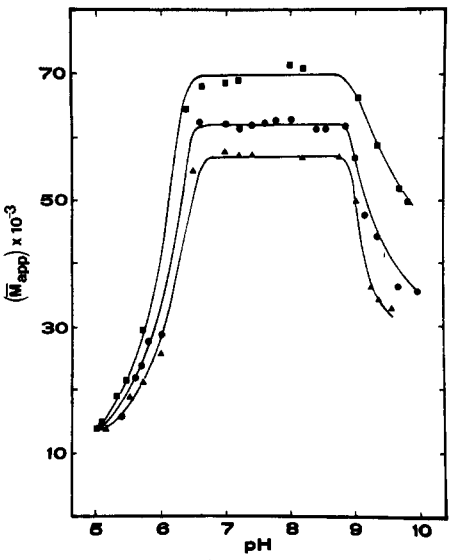

FIGURE 1: pH dependency of $\bar{M}_{app}$ for a 30-μg/ml solution of oxyhemerythrin in Tris-cacodylate buffer, I = 0.01 M. (■) 5°; (●) 25°; (▲) 30°.

$$K_{eq} = \frac{(1 - \alpha_1)M_m^{n-1}}{n\alpha_1^n C_0^{n-1}} \quad (1)$$

In this equation C_0 is the concentration of protein in grams/liter, M_m is the molecular weight of monomer, and α_1 is the weight fraction monomer and is determined according to eq 2 (Ackers and Thompson, 1965):

$$\alpha_1 = \frac{(\sigma_p - \bar{\sigma}_w)}{(\sigma_p - \sigma_m)} \quad (2)$$

In the pH region between 6.8 and 7.4 σ_m and σ_p were taken to be σ_1 and σ_8 (Tan et al., 1975) and n was thus 8. Above pH 9.0 σ_m and σ_p were assumed to correspond to dimer and octamer and n was thus 4 (Tan et al., 1975). The calculated values for the association constants along with the corresponding values of $\Delta G°$ are presented in Table I.

Figure 2 shows the pH dependency of the logarithm of the association constants obtained between pH 4.8 and 7.4 and at 30°. The circles represent the experimental data and the solid line was calculated with the aid of the empirical equation:

$$K_{obsd} = \frac{K_0 + K_H([H]/K_a)^n}{1 + ([H]/K_a)^n} \quad (3)$$

In this equation K_{obsd} is the experimentally determined association 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 8; and K_a is the acid dissociation constant of the amino acid residue in monomeric hemerythrin which controls the association–dissociation reaction. 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 6.36, 5.12, 1.05×10^{44} M^{-7}, and 1.26×10^{35} M^{-7}, respec-

Table I: pH Dependency of the Association Constant for Oxyhemerythrin at 5 and 30° in Tris-Cacodylate Buffer, I = 0.01 M.[a]

pH	Protein Concn (mg/l.)	K_{eq} (M^{-7})	$-\Delta G°$ (kcal/mol of Monomer)
	Part 1, Temp = 5°		
4.80	100	3.88×10^{36}	5.82
4.90	100	4.72×10^{36}	5.83
5.00	100	5.87×10^{36}	5.85
5.10	30	5.45×10^{37}	6.00
5.31	30	6.60×10^{38}	6.18
5.46	30	1.98×10^{39}	6.25
5.70	30	3.59×10^{40}	6.45
6.36	30	2.16×10^{45}	7.61
6.64	30	1.35×10^{46}	7.34
7.00	30	1.56×10^{46}	7.35
7.20	30	1.80×10^{46}	7.36
pH	Protein Concn (mg/l.)	K_{eq} (M^{-3})	$-\Delta G°$ (kcal/mol of Dimer)
9.04	30	7.40×10^{19}	6.32
9.35	30	1.22×10^{19}	6.07
9.69	30	3.68×10^{18}	5.91
9.83	30	2.4×10^{18}	5.85
	Part 2, Temp = 30°		
4.90	180	1.30×10^{35}	6.09
5.00	180	1.10×10^{35}	6.08
5.19	180	2.70×10^{35}	6.14
5.50	30	5.01×10^{38}	6.71
5.70	30	1.89×10^{39}	6.81
6.00	30	9.96×10^{39}	6.93
6.41	30	5.80×10^{43}	7.59
7.00	30	1.24×10^{44}	7.64
7.20	30	9.02×10^{43}	7.56
7.40	30	1.06×10^{44}	7.63
pH	Protein Concn (mg/l.)	K_{eq} (M^{-3})	$-\Delta G°$ (kcal/mol of Dimer)
9.04	30	2.58×10^{18}	6.38
9.28	30	1.78×10^{17}	5.98
9.40	30	1.16×10^{17}	5.92
9.60	30	8.64×10^{16}	5.87

[a] Data at 25° have been presented previously (Tan et al., 1975).

tively. The corresponding values for these constants at 5° were 6.50, 4.98, 2.82×10^{46}, and 3.98×10^{36} and n was also found to be 8.

Calculation of Thermodynamic Parameters. The apparent heat of ionization (ΔH_i) of the amino acid residue participating in the subunit contacts of hemerythrin was estimated from the temperature dependency of its pK_a according to the relationship:

$$\Delta H_i = 2.303R \frac{d(pK_a)}{d(1/T)} \quad (4)$$

Accordingly, $2.303R(pK_a)$ was plotted as a function of $1/T$. This is shown in Figure 3. Upper curve represents the temperature dependency of the pK_a of the amino acid residue in monomeric hemerythrin controlling association and lower curve represents the temperature dependency of the pK_a of the same amino acid residue in octameric hemerythrin. The slopes yielded values for ΔH_i of +2.1 and −1.9 kcal/mol, respectively.

The enthalpy change for the association of oxyhemerythrin between pH 4.8 and 7.4 was calculated according to the equation:

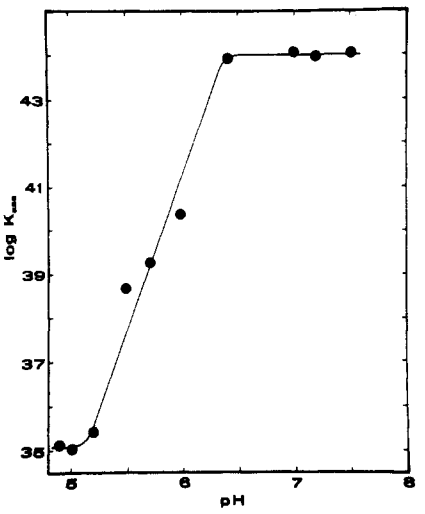

FIGURE 2: pH dependency of the association constant for oxyhemerythrin at 30° in Tris-cacodylate buffer, I = 0.01 M. In this pH range only monomers and octamers are present in equilibrium. The circles are the experimental points and the solid line was calculated (see text for details).

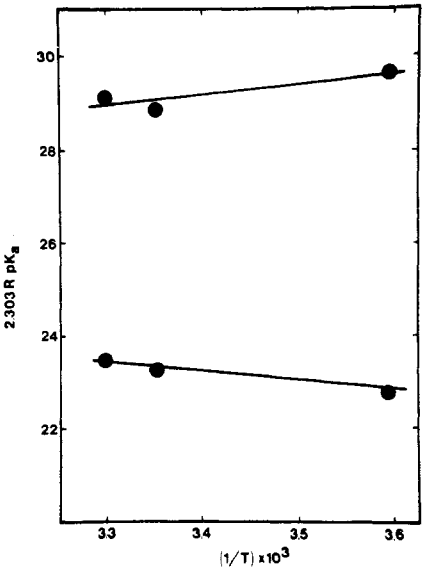

FIGURE 3: The heat of ionization of the amino acid residue controlling association at acidic pH. The buffer is Tris-cacodylate, I = 0.01 M. Upper line, the pK_a of the amino acid residue in monomeric hemerythrin; lower line, the pK_a of the amino acid residue in octameric hemerythrin.

$$\log \frac{K_{(T2)}}{K_{(T1)}} = -\frac{\Delta H°}{2.303R}\left[\frac{1}{T_2} - \frac{1}{T_1}\right] \quad (5)$$

using values for the equilibrium constant obtained between 5 and 30° and on the assumption that $\Delta H°$ did not change appreciably in this temperature range. Figure 4 shows a plot of $\Delta H°$ vs. pH. At neutral pH, $\Delta H°$ for the association reaction was small and negative, being about −2.7 kcal/mol of monomer. This value agrees reasonably well with results obtained previously by gel filtration (Rao and Keresztes-Nagy, 1972) and by microcalorimetry (Langerman and Sturtevant, 1971). At more acidic pH, $\Delta H°$ for the association reaction was about −4.1 kcal/mol of monomer. The midpoint of the plot gives an apparent pK_a of 5.2 for the amino acid residue influencing the association reaction.

The association–dissociation reaction of oxyhemerythrin was studied in greater detail in pH 7.0. Association con-

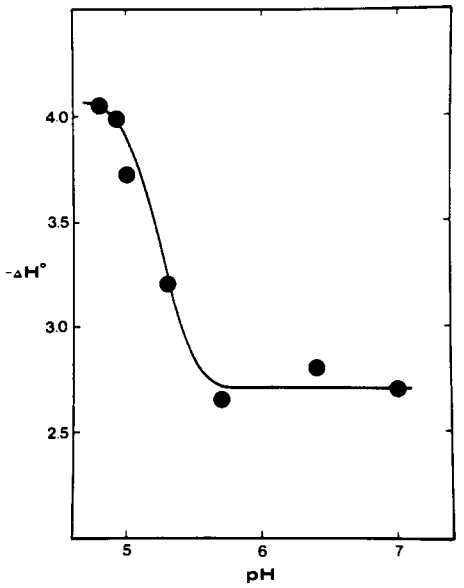

FIGURE 4: The effect of pH on the enthalpy of association of oxyhemerythrin. The buffer is Tris-cacodylate, $I = 0.01$ M.

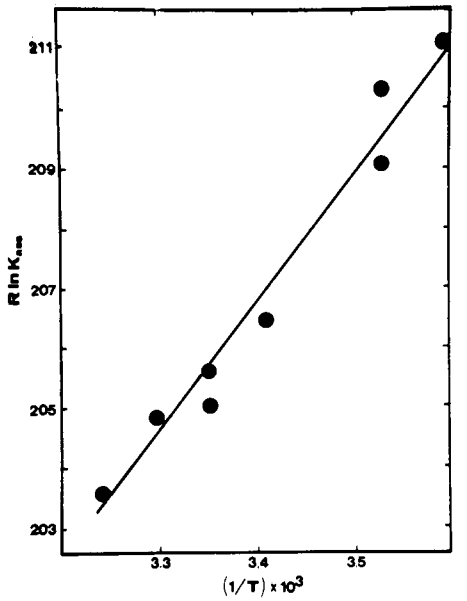

FIGURE 5: The enthalpy of association of oxyhemerythrin at pH 7.0. The buffer is Tris-cacodylate, $I = 0.01$ M.

stants were obtained at 5, 10, 20, 25, and 30° and there were plotted according to the equation:

$$\frac{d(R \ln K)}{d(1/T)} = -\Delta H^\circ \qquad (6)$$

The results of such a plot are shown in Figure 5. From the slope, ΔH° at pH 7.0 was calculated to be -2.6 ± 1 kcal/mol of monomer in excellent agreement with the value of -2.7 kcal/mol obtained previously at just two temperatures. The entropy change for the association reaction at pH 7.0 was calculated according to the equation:

$$\Delta S^\circ = \frac{(\Delta H^\circ - \Delta G^\circ)}{T} \qquad (7)$$

The value for ΔS° at 25° was $+16.5 \pm 5$ eu/mol of monomer. Equation 7 was also used to estimate the entropy change at pH 4.8. The value obtained was about $+6.5 \pm 6$ eu/mol of monomer.

Effect of Ionic Strength on the Dissociation of Oxyhemerythrin. The study of the effect of ionic strength on the association–dissociation reaction of oxyhemerythrin was carried out by varying the concentration of Tris-cacodylate buffer and sodium sulfate in the reaction medium. These salts were chosen because their respective anions do not form coordination complexes with the iron of hemerythrin. These salts were, therefore, assumed to bind either not at all or only very poorly to hemerythrin.

In 0.01 M Tris-cacodylate buffer at pH 7.0, the dissociation of oxyhemerythrin was a rapid reaction as evidenced by the presence of a single sharp leading edge and an unresolved bimodal trailing edge (Tan et al., 1975). Such elution profiles were observed at all temperatures studied between 5 and 30°. An example is shown in Figure 6. As the ionic strength was increased above 0.1, the rate of equilibration between interacting species of hemerythrin decreased resulting in their progressive resolution on transport through the column. This became more pronounced at lower temperatures and, at 10° and ionic strengths above 0.25, elution profiles displayed two well-separated leading and trailing boundaries indicating complete resolution of species (Figure 6). From their centroid elution volumes and sieve coefficients, the interacting forms of hemerythrin were

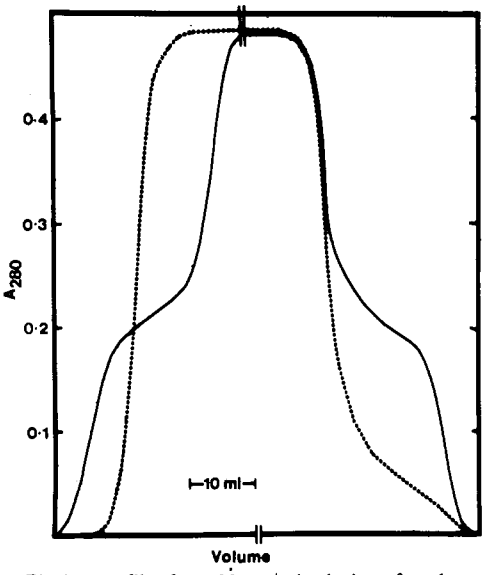

FIGURE 6: Elution profiles for a 30-μg/ml solution of oxyhemerythrin. The buffer is Tris-cacodylate (pH 7.0). (···) 25°, $I = 0.01$ M; (—) 10°, $I = 0.25$ M.

identified as monomer and octamer. Sodium sulfate was found to be more effective than Tris-cacodylate in decreasing the rate of equilibration between monomer and octamer. Accordingly, complete resolution of these species was attained at 10° and ionic strengths above 0.15.

The effect of ionic strength on the dissociation of hemerythrin is shown in Figure 7a. In this figure equilibrium constants are expressed as dissociation constants. It is apparent that increasing ionic strength resulted in increased dissociation of oxyhemerythrin. A comparison of the two curves reveals, however, that Tris-cacodylate and sodium sulfate are not equally effective. The effectiveness of sodium sulfate in promoting dissociation was found to be pH dependent (Figure 7b). The extent of dissociation was several fold greater at pH 6.0 than at pH 7.0.

Discussion

Previous studies on the effect of pH on the dissociation of

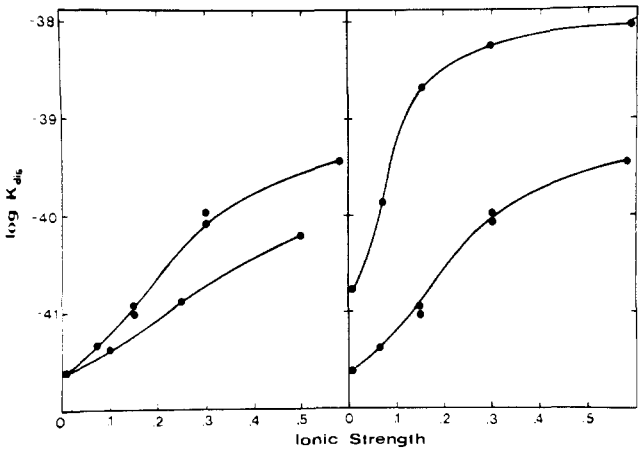

FIGURE 7: (a) The effects of ionic strength on the dissociation of oxyhemerythrin at pH 7.0. The buffer is Tris-cacodylate. Lower curve, with increasing concentration of Tris-cacodylate; upper curve, with increasing concentration of sodium sulfate. (b) The effects of pH and ionic strength on the dissociation of oxyhemerythrin. The buffer is Tris-cacodylate containing increasing amounts of sodium sulfate at the indicated ionic strengths. Lower curve, pH 7.0; upper curve, pH 6.0.

oxyhemerythrin at 25° suggested the presence of acidic and basic amino acid residues at or near the subunit contacts. Titration of these prototropic groups altered both the nature and extent of dissociation (Tan et al., 1975). In this present study it was shown that the qualitative characteristics of the dissociation reaction previously observed at 25° were preserved at 5 and 30° (Figure 1). From the variation in K_{eq} and $\Delta H°$ between pH 4.8 and 7.4 it was shown that the acidic amino acid residue controlling dissociation had an apparent pK_a between 5.0 and 6.0. In order to distinguish between a carboxyl group and an imidazole group, the effect of temperature on its apparent pK_a was studied (Figure 3). The enthalpy of ionization was found to be +2.1 and −1.9 kcal/mol for this amino acid residue in monomeric and octameric hemerythrin, respectively. Average values of $\Delta H°$ reported for some carboxyl groups range from about −1 to +2 kcal/mol. This is in contrast to values of +7 to +8 kcal/mol for the imidazole side chain of histidine (Edsall and Wyman, 1958). On this basis it is reasonable to conclude that the amino acid side chain controlling dissociation at acid pH is a carboxyl group. This conclusion is in agreement with the chemical modification studies of Klippenstein (1972).

The presence of a carboxyl group at the subunit contacts of hemerythrin, the requirement that this group must be unprotonated for maximum stability of the octamer, and the observation that dissociation is enhanced above pH 8.5 (Figure 1) suggests that a bond between a carboxylate and a basic amino acid residue contributes to the binding forces at the subunit contacts. This may be an ionic and/or hydrogen bond.

Values for the various thermodynamic parameters describing the association reaction of oxyhemerythrin indicate that the driving force for subunit association is largely entropic; $\Delta S°$ is +16.5 ± 5 eu/mol of monomer while $\Delta H°$ is small being about −2.6 ± 1 kcal/mol of monomer. These values are entirely representative of results obtained for other protein association reactions (Berson and Yalow, 1959; Singer and Campbell, 1955a,b). The major conclusion usually inferred from such results is that the transfer of water from the subunit interfaces into the bulk solvent is the thermodynamically significant event accompanying subunit association. A second conclusion which might be made is that extensive changes in the tertiary structure of the subunits probably do not accompany subunit association.

As a first approximation it may be assumed that the decrease in the stability of octamer observed on lowering the pH from 7.0 to 4.8 reflects solely the loss of an interaction between a carboxyl group and a second amino acid side chain. The difference between the thermodynamic parameters for association at pH 7.0 and at pH 4.8 should then represent the carboxylate interaction at pH 7.0. The difference in $\Delta G°$ of about −1.6 kcal/mol of monomer is in the range expected of an ion pair and/or hydrogen bond. However, the increment in $\Delta H°$ between pH 4.8 and 7.0 is +1.5 kcal/mol of monomer and the increment in $\Delta S°$ is approximately +10 eu. These values are surprising since it is generally suggested that ion pair formation and hydrogen bond formation accompanied by the transfer of the participants from water into a less polar environment results in a negative enthalpy change and a small positive or even negative entropy change (Kauzmann, 1959; Scheraga, 1963). This disagreement between the expected and observed values for $\Delta H°$ and $\Delta S°$ may reflect the fact that the reactions upon which the expected values are based are inappropriate models for hemerythrin. Thus the positive values reported here for the change in $\Delta H°$ and $\Delta S°$ between pH 7.0 and pH 4.8 are similar to values obtained for the formation of an ion pair between an aspartate carboxylate and an N-terminal amino group in the active conformation of α-chymotrypsin (Fersht, 1972) and for the binding of trichloroacetate and iodate to the cationic active site of acetoacetic decarboxylase (Fridovich, 1963). Near zero enthalpy and positive entropy changes are also observed for the binding of both large and small anions to albumin (Scatchard et al., 1950; Karush and Sonenberg, 1949).

While the thermodynamic data may be equivocal, the observation that increasing ionic strength promotes dissociation clearly suggests the presence of polar residues at the subunit contacts of hemerythrin. It is striking that increasing ionic strength like decreasing pH not only promotes dissociation but also decreases the rate of equilibration between monomers and octamers such that they are resolved on chromatography. Sodium sulfate is more effective than Tris-cacodylate in promoting dissociation and in decreasing the rate of equilibration between interacting species. These results clearly link the effects of increasing ionic strength to the effects of protonating specific carboxyl groups at the subunit contacts of hemerythrin, thereby providing additional support for the role of ionic and/or hydrogen bonds in stabilizing octameric hemerythrin. It is interesting to note that for hemoglobin interchain hydrogen bonding between aspartate residues and asparagine residues contributes significantly to the stability of the tetramer (Bonaventura and Riggs, 1968; Perutz and Lehman, 1968). Accordingly, hemoglobin also undergoes dissociation on lowering the pH from 7.0 to 5.0 (Rossi Fanelli et al., 1964) and on addition of salts (Rossi Fanelli et al., 1961; Benesch and Benesch, 1962).

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Pyridoxal 5′-Phosphate and Analogs as Probes of Coenzyme–Protein Interaction in *Bacillus alvei* Tryptophanase†

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ABSTRACT: Tryptophanase from *Bacillus alvei* was resolved from its coenzyme, pyridoxal phosphate, by treatment with cysteine followed by column chromatography. Spectrophotometric titration of apoenzyme with pyridoxal-P showed 1 mol of pyridoxal-P bound per 52,000 g of enzyme. Kinetic analysis of coenzyme binding showed hyperbolic activation curves with a K_m of 1.6 μM. Pyridoxal-P was used as a natural active site probe in spectrophotometric studies to distinguish differences in the active center of holotryptophanase and reconstituted enzyme that were not apparent by other techniques. The pK_a for holotryptophanase is 7.9 while the pK_a for reconstituted apoenzyme is 8.4. Apotryptophanase binds 2-nor, 2′-methyl, 2′-hydroxy, 6-methyl, and *N*-oxide pyridoxal-P to form analog enzymes distinguishable on the basis of absorption spectra and relative activity in catalyzing both the α,β-elimination and β-replacement reactions of tryptophanase. Apoenzyme also binds pyridoxal but pyridoxal analog enzyme is not active.

Tryptophanase from *Bacillus alvei* was previously reported (Hoch and DeMoss, 1966) to bind 1 mol of pyridoxal-P[1] per 125,000 g of enzyme when apotryptophanase was prepared by extensive dialysis against Tris–EDTA buffer. Techniques for successfully resolving pyridoxal-P enzymes to form apoprotein vary considerably (Wada and Snell, 1962; Newton et al., 1965; Shaltiel et al., 1966; Matsuzawa and Segal, 1968; Dowhan and Snell, 1970; etc.). Many apoprotein forms of pyridoxal-P enzymes which by kinetic and spectrophotometric criteria are resolved of coenzyme show a characteristic ability to bind 1 mol of pyridoxal-P per 50,000–60,000 g of enzyme (Wilson, 1963; Wilson and Meister, 1966; Novogrodsky and Meister, 1964; Tate and Meister, 1969; Kakimoto et al., 1969; Cowell, 1972) instead of the value of 1 mol per 125,000 g seen for *B. alvei* tryptophanase.

In this investigation *B. alvei* holotryptophanase was resolved of pyridoxal-P by treatment with cysteine followed by column chromatography. Our aim was to characterize

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[1] Abbreviations used are: pyridoxal-P, pyridoxal 5′-phosphate; Q_{max}, maximal fluorescence quenching; bicine, *N,N′*-bis(2-hydroxyethyl)glycine.