Free Energy of Subunit Interactions. Hemerythrin*

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ABSTRACT: The association–dissociation equilibrium of hemerythrin has been studied by equilibrium ultracentrifugation. Dilution of the protein leads to extensive dissociation of the octamer at concentrations below about 0.04%. The equilibrium concentration distribution indicates that only monomers are present in equilibrium with octamers; and the association constant for $8Hr = Hr_8$ is 3.4×10^{36} M^{-7} for the azide complex of hemerythrin at 5°. This corresponds to a standard free-energy change of 6 kcal/mole for formation of 1 mole of monomer from octamer. An examination of free-energy changes associated with small perturbations in structure of hemerythrin, or other proteins, indicates that these could have pronounced effects on the conformation of the macromolecule.

An overwhelming majority of proteins, in their native states, are constituted of subunits (for a recent list, see Klotz, 1967). In any specific case once the subunit nature and stoichiometry have been established it becomes of interest to examine the interactions between these constituents of the oligomeric macromolecule. The energetics of these interactions can be established readily if the oligomer and its dissociated structural units are in equilibrium with each other.

Hemerythrin, the oxygen-carrying protein of a number of marine organisms, is composed of eight subunits (Klotz and Keresztes-Nagy, 1963). Hybridization of hemerythrin with a chemically labeled derivative has shown that the octamer dissociates reversibly (Keresztes-Nagy *et al.*, 1965) and sedimentation velocity studies (Klapper *et al.*, 1966) have indicated that this equilibrium is between octameric and monomeric species. This system is thus ready for a quantitative examination by sedimentation equilibrium methods.

Theory

General treatments of association equilibria have been carried out recently by several investigators (Adams and Williams, 1964; Jeffrey and Coates, 1966; Adams, 1967; Albright and Williams, 1968; Adams and Lewis, 1968). Since sedimentation velocity studies of hemerythrin indicated only monomer and octamer as present in appreciable quantities, we have expressed the equilibrium equations for this special, simple situation. We have also assumed that the solutions are ideal in behavior. Experimental observations can then be analyzed in terms of the resultant equations to determine whether the actual behavior is adequately described within this framework.

For the monomer–octamer equilibrium we may write

$$8Hr = Hr_8;\ K_8 = (Hr_8)/(Hr)^8 \tag{1}$$

The following definitions are also convenient: C_1 = moles/l. of monomer species; C_8 = moles/l. of octamer species; C_t = moles/l. of all dissolved hemerythrin in monomeric units. From these definitions it follows that

$$C_t = C_1 + 8C_8 = C_1 + 8K_8C_1^8 \tag{2}$$

Furthermore, in view of the definition of weight-average molecular weight,

$$M_w = \frac{\Sigma C_i M_i^2}{\Sigma C_i M_i} \tag{3}$$

we find for the monomer–octamer equilibrium system

$$M_w = \frac{M_1}{C_t}(C_1 + 64K_8C_1^8) \tag{4}$$

To evaluate the equilibrium constant, K_8, the usual approach proceeds to determine C_1 as a function of C_t. For an ideal solution one may use the equation of Steiner (1952)

$$\ln (C_1/C_t) = \int_0^{C_t}\left(\frac{M_1}{M_w} - 1\right)\frac{dC_t}{C_t} \tag{5}$$

In practice a numerical integration is made of the right-hand side of eq 5.

Once M_w is known as a function of C_1, and of C_t, K_8 can be evaluated. One simple procedure is to rearrange eq 4 to

$$\frac{M_w}{M_1}\frac{C_t}{C_1} = 1 + 64K_8C_1^7 \tag{6}$$

A graph of the left-hand side of eq 6 *vs.* C_1^7 should be linear, the slope of the line being proportional to K_8.

The fit of the experimental data to K_8 may also be visualized

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more directly by reversing the computations and generating a smooth curve of M_w *vs.* C_t from eq 2 and 4.

Materials and Methods

Oxyhemerythrin was prepared from the coelomic fluid of the marine worm *Golfingia gouldii* by procedures previously described (Klotz *et al.*, 1957). The protein was oxidized to the Fe(III) form, methemerythrin, by the procedure described by Keresztes-Nagy and Klotz (1965) except that the exhaustive dialysis was against Tris-cacodylate buffer (pH 7). Dialysis was continued until the spectrum of the sample was that of metaquohemerythrin. The buffer was prepared by adjusting the pH of a 0.1 M Tris solution to pH 7 with solid cacodylic acid. Hemerythrin samples for sedimentation equilibrium were converted into the azide form by dialysis of the metaquo form against a sample of the above buffer which was also 0.05 M in sodium azide.

The sulfhydryl group of hemerythrin is susceptible to oxidation. If some oxidation occurs, the resulting "crippled" monomer is unable to participate in the association–dissociation equilibrium of the native protein and becomes a contaminant in the system. To remove any traces of crippled monomer, a concentrated solution (20–30 mg/ml) of metaquohemerythrin was passed through a Sephadex G-75 column (48 × 1.5 cm). The resulting separation of crippled monomer from native octamer was found to be excellent. To prevent subsequent oxidation of hemerythrin SH groups, 10^{-3} M mercaptoethanol was incorporated into the Tris-cacodylate buffer.

Sodium azide (Fisher Scientific Co., purified grade) had traces of insoluble material as well as a definite yellow color in a 5 M solution. For this reason, it was purified as follows. Chelex-100 (Bio-Rad Laboratories, Inc.) chelating resin (100–200 mesh) was washed as described by the manufacturer and the fines were removed. A solution (5 M) of sodium azide was filtered into a beaker containing the resin and stirred for 24 hr. The resin was removed by filtration and replaced with fresh resin for another 24-hr period. The resin was again removed by filtration and the sodium azide was twice recrystallized by the addition of ethanol. The final crystals were dried *in vacuo* at 70° for 24 hr. A solution (5 M) of the purified crystals was clear and had no significant absorbance between 600 and 350 nm.

Protein solutions of the required concentration were made by diluting the stock solution with dialysate. Protein concentrations were determined both spectrophotometrically (Keresztes-Nagy and Klotz, 1965) and with a Brice–Phoenix differential refractometer. The standard filter on the refractometer was replaced with the 546.1-nm (Baird-Atomic interference) filter used in the sedimentation equilibrium experiments. The initial concentration, in fringes, j_0, was then calculated from eq 7

$$j_0 = \frac{l}{\lambda} \Delta n \tag{7}$$

where l = path length of the center piece (30 mm), λ is the wavelength (546.1 nm), and Δn is the difference in refractive index between protein solution and dialysate.

All sedimentation equilibrium experiments were performed in a cell with a 30-mm, six-channel centerpiece[1] similar to the commercial 12-mm centerpiece supplied by the Spinco Division, Beckman Instrument Co. Uncertainties in initial concentration caused by wall absorption were minimized as described by Adams and Lewis (1968). Also their protocol was followed for filling the cell.

The experiments reported here were performed at 5° or at 25° in a Spinco Model E analytical ultracentrifuge, equipped with an An-E rotor and with adjustable Rayleigh optics. The optics were aligned according to the directions of Gropper (1964) except that the camera lens was always focussed on the two-thirds plane of the cell.

Both low-speed and high-speed equilibrium experiments were necessary to span the desired concentration range. The low-speed experiments (8,225–10,589 rpm) followed the recommendations of LeBar and Baldwin (1962) and Richards *et al.* (1968). Low-speed runs were subjected to the overspeed conditions described by Hexner *et al.* (1961). The high-speed experiments at 27,690 rpm were performed, as described by Yphantis (1964), on the same samples as the low-speed experiments simply by changing rotor speed. Each experiment was preceded and followed by a base-line determination at each of the speeds of the experiment; for this purpose all six channels of the cell were filled with distilled water.

Data were collected photographically on Kodak III-G spectrographic plates. Fringe displacements were measured with a Gaertner microcomparator which was equipped with a high-intensity substage illuminator and a 60× objective lens.

The previously determined (Klotz and Keresztes-Nagy, 1963) partial specific volume, 0.735 cc/g, was used in all calculations. The density of the buffer was determined pycnometrically to be 1.015 g/cc. For the reasons discussed by Adams and Lewis (1968) no layering oil was used.

Results

Molecular weights were calculated from the experimental data using the relations in eq 8,

$$M_{w,\,app} = M_{w,r} = A \frac{d \ln c}{dr^2} \tag{8}$$

$$A = \frac{2RT}{(1 - \bar{v}\rho)\omega^2}$$

where c is the concentration of protein at the distance, r, from the center of rotation, R is the gas law constant, T the absolute temperature, $\bar{v}$ the partial specific volume of solute, ρ the density of the solution, and ω the angular velocity of the rotor. The identity of the apparent weight-average molecular weight, $M_{w,\,app}$, and the actual weight-average molecular weight, $M_{w,r}$, follows from the assumption of ideal behavior in solutions (Adams and Williams, 1964). This assumption seems well justified in solutions of ionic strength 0.10 (or greater) and protein concentrations below 10 mg/ml.

The necessary calculations were carried out on a Contro

[1] The authors wish to thank Professor D. A. Yphantis for his gift of a 30-mm six-channel centerpiece and for a copy of the computer program which was used in conjunction with the high-speed experiments.

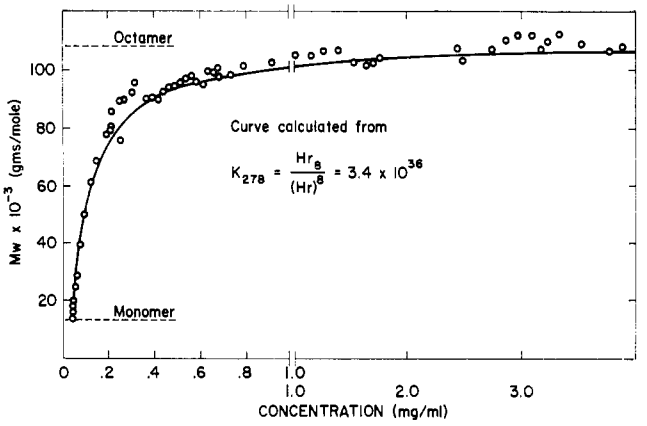

FIGURE 1: Weight-average molecular weight of azidehemerythrin as a function of protein concentration, at pH 7 and 5°. The curve has been calculated assuming only monomers and octamers are in equilibrium, with $K_8 = 3.4 \times 10^{36}$.

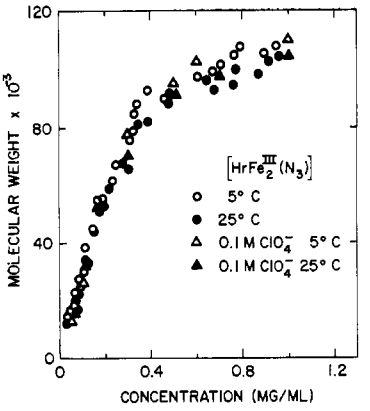

FIGURE 2: Effect of temperature and of ClO_4^- ion on dissociation of azidehemerythrin at pH 7: (○) 5°, (●) 25°, (△) in presence of ClO_4^- at 5°, and (▲) in presence o. ClO_4^- at 25°.

Data Corp. 6400 digital computer. The program for the low-speed sedimentation equilibrium experiments was written in this laboratory while that for the high-speed sedimentation equilibrium experiments was written by D. E. Roark and D. A. Yphantis.[1]

The dependence of M_w on concentration of metazidehemerythrin at pH 7 and 5° is illustrated in Figure 1. A concentration range of over 100-fold was covered. It is evident that the octamer is the overwhelmingly dominant species at high concentrations of hemerythrin (above ~0.05%). When the concentration drops below 0.5 mg/ml, the monomer appears in increasing proportion and the fraction of octamer drops precipitously.

The information shown in Figure 1 was used to provide the data for the integrations required in eq 5 to evaluate C_1. The results of such serial integrations are summarized in Table I.

Combination of the information on the variation of M_w with C_t and of C_1 with C_t provided the data needed for eq 6. A graph of M_wC_t/M_1C_1 *vs.* C_1[7] was linear, as is required if only monomeric and octameric species are present in significant concentrations. From the slope of this linear graph an equilibrium constant, K_8, of 3×10^{36} M^{-7} was evaluated.

A better direct fit of K_8 to the data in Figure 1 was obtained by successive approximations using eq 2 and 4. A value of K_8 near 3×10^{36} was chosen, C_1 at a given C_t was calculated from eq 2, and then M_w computed for this value of C_t, from eq 4. This procedure was reiterated for the entire range of C_t and the calculated curve for M_w compared with observations. The entire procedure was then repeated for various values of K_8. A best fit of computed curve of M_w *vs.* C_t was obtained for a K_8 of 3.4×10^{36} M^{-7}. Variation by a factor of two from this value of K_8 gave higher and lower calculated curves for M_w *vs.* C_t which encompassed 90% of all experimental observations below 0.3 mg/ml. It should be noted that a variation of 100% in K_8 corresponds to one of 2% in C_1, since it is C_1^8 which is linked to K_8, as can be seen in eq 1.

The effect of temperature on the dissociation behavior of metazidehemerythrin was also examined. Since the dissociation curve may vary slightly from sample to sample of protein, due to the presence of small amounts of crippled monomer, the temperature dependence was determined on a single sample by the following procedure.

Metazidehemerythrin (pH 7 and 5°) was placed in the ultracentrifuge and sedimented at 8766 rpm until equilibrium was reached. Photographs of the interference pattern were then taken. At this speed the rotor and cell were warmed to 25°, heat being supplied by the RTIC unit of the ultracentrifuge, and sedimentation was continued until a new equilibrium was established. Again photographs were taken. The rotor was then accelerated to 27,680 rpm at 25° and the system was again allowed to equilibrate, after which a third set of interference photographs were taken. Finally the rotor was cooled to 5° while rotating at 27,680 rpm. When equilibrium was attained, a fourth set of photographs was taken. From these data, M_w as a function of concentration was calculated at each temperature for both the low- and high-speed situations. As is evident in Figure 2 there is very little effect of temperature over this range on the association–dissociation behavior of hemerythrin.[2]

It has been shown recently (Darnall *et al.*, Garbett and Klotz, 1968) that perchlorate ion is bound at a site other than the

TABLE 1: Equilibrium Concentrations of Monomer and Variation of Equilibrium Constant with Hemerythrin Concentration.

C_t (mg/ml)	C_1/C_t	C_1 (mg/ml)	$K_8 \times 10^{-36}$ (M^{-7})
0.055	0.884	0.0486	2.2
0.360	0.206	0.0736	3.5
0.870	0.0970	0.0844	3.2
1.920	0.0473	0.0936	3.4

[2] The curve at 5° in Figure 2 is shifted slightly toward higher concentrations than its counterpart in Figure 1. We believe this is due to a slightly higher content of "crippled monomer" in the sample of hemerythrin used for the temperature experiments. It is for this reason that a single sample of protein was used to examine the temperature coefficient of dissociation.

iron locus of hemerythrin. In the presence of perchlorate there is a large decrease in the rate of formation of metazide-hemerythrin from metaquohemerythrin, as well as a marked decrease in the reactivity of the SH group with mercurials. It seemed possible that these phenomena might be a reflection of the effect of perchlorate on the dissociation of the oligomer. Sedimentation equilibrium experiments were run therefore both at 5° and at 25° in the presence of 0.1 M ClO_4^- ion. The same temperature cycle was used as described for experiments in the absence of perchlorate. As the data in Figure 2 show, perchlorate ion does not shift the monomer–octamer equilibrium. Likewise, since experiments were carried out at two temperatures, we see, once again, that the temperature coefficient of the equilibrium is very small.

The iron at the active site of hemerythrin can bind a variety of ligands besides azide, both in the octameric and monomeric state. In the presence of SCN^-, the dissociation curve for hemerythrin was not significantly different from that of a corresponding control in the presence of azide. Two other liganded forms of the protein are the pH-dependent metaquo and methydroxy derivatives. At pH 7 in the absence of other ligands the iron atom binds water; at pH 8.5 this H_2O molecule is converted into, or displaced by, an OH^- ion. As can be seen from Figure 3, the dissociation of metaquohemerythrin is not significantly different from that of an azide control. On the other hand the association constant for the monomer–oligomer equilibrium of methydroxyhemerythrin is definitely slightly larger (at pH 8.5) than that of aquohemerythrin (at pH 7).

It has recently been found (D. W. Darnall, and I. M. Klotz, 1969; unpublished observations) that a met-tiron-hemerythrin can also be prepared (in which the tiron ligand is the large molecule, 1,2-dihydroxybenzene-3,5-disulfonic acid). The tiron ligand cannot be coordinated with the iron when the protein is in the octameric form, but it is bound to the iron when hemerythrin is in the monomeric state, and a nondialyzable complex with an iron to tiron ratio of 1:1 is formed. This complex is still stable if the monomer is then reconverted to the octameric species to give $[HrFe_2(III)(tiron)_2]_8$. The presence of these two large ligands at the iron locus might be expected to have a pronounced effect on the association–dissociation process. As Figure 3 shows, tiron–hemerythrin does indeed dissociate more readily[3] than the other complexes examined, although the shift is not as dramatic as might have been expected. Experiments with tiron were done in triplicate and at three different initial concentrations and all three curves overlap within a few per cent. This indicates that any heterogenity of the sample was minimal.

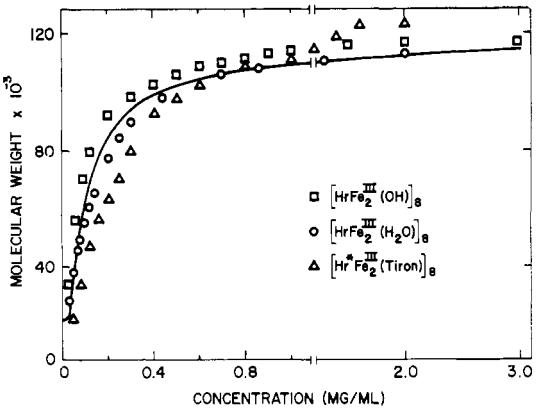

FIGURE 3: Dissociation of hemerythrins with different ligands on Fe: (□) methydroxyhemerythrin, (○) metaquohemerythrin, and (△) met-tiron-hemerythrin.

Discussion

One of the most noteworthy features of this study is that a *single* equilibrium constant, K_8, fits the dissociation behavior very well. This fit is particularly well shown in Table I, which illustrates the constancy of K_8 over a 40-fold range in concentration of hemerythrin.

Since the single equilibrium constant K_8 does describe the dissociating hemerythrin system, it is apparent that this equilibrium involves overwhelmingly only the monomer and octamer species (eq 1). In other words these equilibrium centrifugation studies do not reveal the presence of any significant quantities of other oligomeric intermediates, such as dimer or tetramer. Hemerythrin thus behaves differently from, for example, hemoglobin (Vinograd and Hutchinson, 1960; Guidotti and Craig, 1963; Guidotti *et al.*, 1963; Rosemeyer and Huehns, 1967; Neer *et al.*, 1968; Schachman and Edelstein, 1966) in which dimers play an important role, or from lactic dehydrogenase (Millar *et al.*, 1969) in which dimers and tetramers are intermediates between monomer and octamer.

Association of monomers in hemerythrin thus is a highly cooperative process if only final octamers are detectable. It seems relevant to ask how strong this cooperativity need be to reduce intermediate oligomeric species to negligible concentration. We have approached this question in the following way.

Without explicitly taking statistical factors into account, and assuming that the degree of cooperativity is the same for each associating step, we may define a cooperativity parameter, α, by eq 9, where k_i is the equilibrium constant for the

$$\alpha = \frac{k_{i+1}}{k_i} \tag{9}$$

addition of a monomeric A molecule to oligomer A_{i-1} containing $(i - 1)$ monomeric subunits

$$A + A_{i-1} = A_i \quad k_i = \frac{(A_i)}{(A)(A_{i-1})} \tag{10}$$

If one writes explicitly the individual steps in the successive associations represented by eq 10, then one can show readily that

$$\frac{(A_i)}{(A)^i} = \prod_2^i k_j \tag{11}$$

[3] It should be noted that the molecular weight of tiron-hemerythrin is larger than for azidehemerythrin since two tiron residues per monomer contribute an additional 500 to the molecular weight of the monomer and 4000 to that of the octamer. If normalized to the azide curve, the tiron M_w–C_t curve would be somewhat more displaced than is apparent in Figure 3.

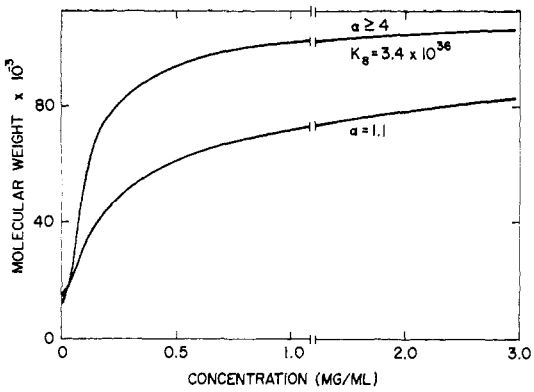

FIGURE 4: Computed curves of M_w, molecular weight, *vs.* concentration of hemerythrin for different values of cooperativity parameter α.

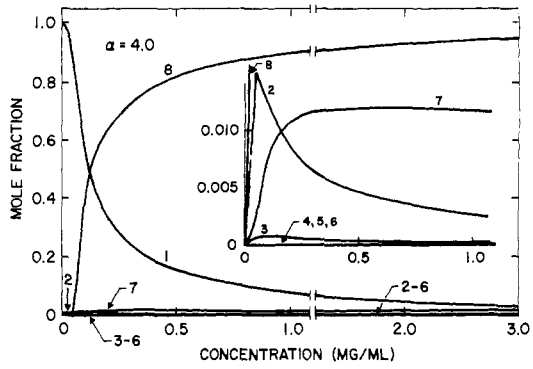

FIGURE 5: Computed curves of mole fraction (in monomer units) of oligomeric species i *vs.* concentration of hemerythrin for an $\alpha = 4$. The integer labeling each curve gives i, the number of subunits in the respective oligomer (1 = monomer, 2 = dimer, etc). The insert shows the same data for $2 \leq i \leq 7$ on an expanded scale to illustrate how low are the concentrations of these species.

Making use of α from eq 9 we may write

$$\begin{aligned} k_3 &= \alpha k_2 \\ k_4 &= \alpha k_3 = \alpha^2 k_2 \\ &\cdots \\ k_i &= \alpha k_{i-1} = \alpha^{i-2} k_2 \end{aligned} \tag{12}$$

Combining the relations of eq 12 with eq 11 we find

$$\begin{aligned} A_i &= (\prod k_j)(A)^i = k_2{}^{i-1}(\alpha \cdot \alpha^2 \ldots \alpha^{i-2})A^i \\ &= k_2{}^{i-1}\alpha^\sigma A^i \end{aligned} \tag{13}$$

where

$$\sigma = [1 + 2 + \ldots (i-2)] = \frac{(i-1)(i-2)}{2} \tag{14}$$

The experimentally fitted single association constant for a monomer–i-mer equilibrium would be written as

$$K_i = \frac{(A_i)}{(A)^i} \tag{15}$$

From eq 13 and 15 it follows that

$$k_2{}^{i-1}\alpha^\sigma = K_i \tag{16}$$

Taking an experimental determination of K_i we can treat α as a parameter, calculate k_2, and then each k_i. With this information, we can then compute a curve of M_w *vs.* concentration of protein, as well as curves for the mole fraction of each oligomeric species.

We have applied this method of analysis to the hemerythrin system, using $K_8 = 3.4 \times 10^{36}$ M^{-7}. Figure 4 shows that the calculated M_w curves quickly converge for $\alpha \geq 4$, giving a limiting curve that fits well with the experimental one (Figure 1). For $\alpha = 4$, computed curves for the mole fraction (in monomeric units) of monomer and octamer (Figure 5) agree well with corresponding curves calculated from the experimental observations on the assumption that only a monomer–octamer equilibrium exists (no intermediate species) with $K_8 = 3.4 \times 10^{36}$ M^{-7} (Figure 6). The insert in Figure 5 also shows how very trivial the equilibrium concentrations of dimer, trimer, etc., would be for a system with a cooperativity factor $\alpha = 4$.

Such a value for α corresponds to a very small free-energy *increment* for the $(i + 1)$th association as contrasted to the ith

$$\frac{k_{i+1}}{k_i} = \alpha \tag{17}$$

$$\Delta(\Delta G^\circ) = RT \ln \frac{k_{i+1}}{k_i} = RT \ln 4 = 0.8 \text{ kcal}$$

Thus it would require only a very minor increase in interface contact area between incoming monomer and each successive oligomeric species to permit such a minor increment in cooperativity free energy; and yet such a minor increase in contact interface could be reflected as a steeply rising association curve.

The very crucial role that a minor change in structure may play in the association–dissociation energetics can also be seen in another way. For comparison of proteins with different numbers of subunits it should be convenient to distribute the free energy of association of the highest oligomer equally among the monomers and to define a free energy of formation of monomer, $\Delta G^\circ{}_m$, as follows

$$n\text{A} = \text{A}_n \qquad \frac{\Delta G^\circ}{n} = -\Delta G_m{}^\circ = -\frac{1}{n} RT \ln K_n \tag{18}$$

$\Delta G_m{}^\circ$ may be viewed as the difference in (free) energy levels between the monomeric and oligomeric states of a subunit. For hemerythrin, for example,

$$\Delta G_m{}^\circ = \frac{1}{8} RT \ln K_8 = 5.8 \text{ kcal/mole of monomer} \tag{19}$$

We may now examine the effect of small changes in $\Delta G_m{}^\circ$

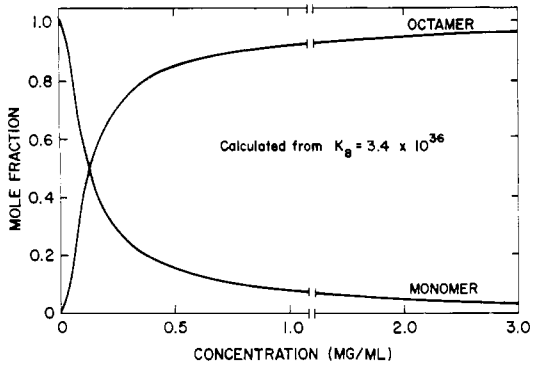

FIGURE 6: Curves of mole fraction of monomer and of octamer calculated from experimental data for M_w on the assumption that no intermediate species exist in significant concentration. The experimental K_8 of 3.4×10^{36} was used for this calculation.

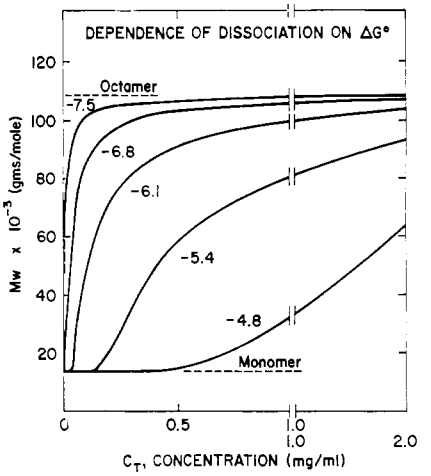

FIGURE 7: Curves of M_w *vs.* concentration of hemerythrin computed for different values of $-\Delta G_m°$, the difference in free-energy levels between monomeric and octameric state of the subunit.

on the association–dissociation equilibrium. Some typical calculations for hemerythrin are summarized in Figure 7. A change of only 2 kcal in $\Delta G_m°$ (from 6.8 to 4.8 in Figure 7) would convert this quaternary structure into one that would appear completely monomeric in the range below 0.5 mg/ml, and overwhelmingly dissociated (instead of, as actually, almost completely in the octameric state) up to very high concentrations of protein. Even a change of 0.8 kcal would markedly affect the M_w *vs.* C_t curve (Figure 7).

Although obvious, it should be mentioned explicitly that $\Delta G_m°$, being a difference, can be changed by variation of $G°$ for either the initial or final state. Changes in $\Delta G_m°$ may arise either from a small perturbation in the interactions of side chains of neighboring monomers in the interface area within the oligomer or from a modification of interactions of such chains in the dissociated monomer with the surrounding solvent.

Energetic quantities in themselves cannot establish the molecular origin of the interactions, or changes in interactions. On the other hand, it is useful to keep in mind that minor changes in structure or orientation of small groups are accompanied by substantial changes in free energy. For example, replacement of an Asn by an Asp residue, with the presumed ionization of the side-chain carboxyl of the latter, would be accompanied by a substantial free-energy change, which to a first approximation should be given by that of a typical COOH ionization

$$COOH = COO^- + H^+ \quad \Delta G° = 6 \text{ kcal} \qquad (20)$$

A somewhat smaller but appreciable $\Delta G°$ would be associated with removal of a COOH from apolar surroundings to an aqueous environment. In a different but related direction we might note that immobilization of an amide residue (eq 21)

$$CHRCONH_{flexible} = CHRCONH_{immobile} \quad \Delta S° = -5\text{eu} \qquad (21)$$

should be accompanied by an entropy loss of 3–7 eu (Schellman, 1958) which in itself corresponds to a $\Delta G°$ of 1.5 kcal. As a glance at Figure 7 shows, changes in $\Delta G_m°$ as small as 1–2 kcal are accompanied by marked perturbations of the oligomeric state of a protein.

Only for hemoglobin have subunit contacts been established in precise terms (Perutz *et al.*, 1968). When mutant hemoglobins, containing single amino acid residue replacements, are examined (Perutz and Lehmann, 1968) it is indeed found that single substitutions in the $\alpha\beta$ subunit interfaces may produce marked effects on the dissociation of tetramer. Hemoglobin Kansas, for example, in which an Asn → Thr substitution occurs, is much more easily dissociated than hemoglobin A (both proteins being compared in the oxy form). The change in dissociation constant for tetramers to dimers corresponds to a $\Delta G°$ of 2–3 kcal. In hemoglobin Philly a Tyr is replaced by a Phe in the subunit interface region and in this case there is increased dissociation into monomers. In a related sense, but in interactions involving further aggregation rather than dissociation, the replacement of Glu → Val in going from hemoglobin A to hemoglobin S is accompanied by striking changes in the solubility of the deoxygenated protein.

Single residue replacements in the primary structure are *intrinsic* changes which may lead to modifications in quaternary structure. In principle equal changes in $\Delta G_m°$ could also be produced by *extrinsic* perturbants, such as added ligands which are bound at low concentrations, or by solutes which change the themodynamic potential of the aqueous solvent (Klotz, 1966). Profound effects on the quaternary state of a protein may thus result from very limited, minor changes in the molecular nature or environment of as little as one side chain.

References

Adams, E. T., Jr. (1967), *Biochemistry 6*, 1864.

Adams, E. T., Jr., and Lewis, M. S. (1968), *Biochemistry 7*, 1044.

Adams, E. T., Jr., and Williams, J. W. (1964), *J. Am. Chem. Soc. 86*, 3454.

Albright, D. A., and Williams, J. W. (1968), *Biochemistry 7*, 67.

Darnall, D. W., Garbett, K., and Klotz, I. M. (1968), *Biochem. Biophys. Res. Commun. 32*, 264.

Gropper, L. (1964), *Anal. Biochem. 7*, 401.

Guidotti, G., and Craig, L. C. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 54.
Guidotti, G., Konigsberg, W., and Craig, L. C. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 774.
Hexner, P. E., Radford, L. E., and Beams, J. W. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1848.
Jeffrey, P. D., and Coates, J. H. (1966), *Biochemistry 5*, 489.
Keresztes-Nagy, S., and Klotz, I. M. (1965), *Biochemistry 4*, 919.
Keresztes-Nagy, S., Lazer, L., Klapper, M. H., and Klotz, I. M. (1965), *Science 150*, 357.
Klapper, M. H., Barlow, G. H., and Klotz, I. M. (1966), *Biochem. Biophys. Res. Commun. 25*, 116.
Klotz, I. M. (1966), *Arch. Biochem. Biophys. 116*, 92.
Klotz, I. M. (1967), *Science 155*, 697.
Klotz, I. M., and Keresztes-Nagy, S. (1963), *Biochemistry 2*, 455.
Klotz, I. M., Klotz, T. A., and Fiess, H. A. (1957), *Arch. Biochem. Biophys. 68*, 284.
LeBar, F. E., and Baldwin, R. L. (1962), *J. Phys. Chem. 66*, 1952.
Millar, D. B., Frattali, V., and Willick, G. E. (1969), *Biochemistry 8*, 2416.
Neer, E. J., Konigsberg, W., and Guidotti, G. (1968), *J. Biol. Chem. 243*, 1971.
Perutz, M. F., and Lehmann, H. (1968), *Nature 219*, 902.
Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G. (1968), *Nature 219*, 131.
Richards, E. G., Teller, D. C., and Schachman, H. K. (1968), *Biochemistry 7*, 1054.
Rosemeyer, M. A., and Huehns, E. R. (1967), *J. Mol. Biol. 25*, 253.
Schachman, H. K., and Edelstein, S. (1966), *Biochemistry 5*, 2681.
Schellman, J. (1958), *Compt. Rend. Trav. Lab. Carlsberg 30*, 363.
Steiner, R. F. (1952), *Arch. Biochem. Biophys. 39*, 333.
Vinograd, S., and Hutchinson, W. O. (1960), *Nature 187*, 216.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Macromolecule–Small Molecule Interactions. Strong Binding and Cooperativity in a Model Synthetic Polymer*

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ABSTRACT: Exceptionally strong binding of organic anions is exhibited by polyethylenimine derivatives with apolar side chains. Conformationally compact, water-soluble polymers have been prepared with pendant butyryl, hexanoyl, or lauroyl aliphatic groups, or with carbobenzoxytyrosine or carbobenzoxytryptophan aromatic residues. All of these complex much more extensively with methyl orange than do proteins such as serum albumin or β-lactoglobulin.

The dependence of binding on concentration of small anion as well as the spectra of the complexes show that strong cooperative interactions appear with increased uptake of small molecule. These polymers offer attractive macromolecules for insertion of catalytic sites in addition to binding sites.

The binding of small molecules and ions by serum albumin has been of interest for many years (Klotz *et al.*, 1946; Klotz, 1949) because these complexes provide an insight into general biomacromolecular interactions with substrates and modifiers. Stoichiometric and energetic quantities characteristic of these interactions were obtained readily, particularly by equilibrium–dialysis techniques. However, an understanding of the detailed molecular nature of binding has been more elusive, although it has been clear that a combination of apolar and ionic interactions contribute to the strength of the complexes (Klotz, 1946).

If one truly understands a biochemical interaction it should be possible to reproduce it *de novo* with materials of nonbiological origin. Thus one might expect water-soluble synthetic polymers containing suitable apolar and ionic side chains to exhibit strong affinities for small molecules. Many such polymers have been examined (for references see Klotz and Sloniewsky, 1968). In our previous experience, however, no linear-chain type of water-soluble polymer was found to bind small molecules with an avidity comparable to that of serum albumin.

Such polymers, however, have highly swollen, extended conformations in aqueous solution, as is evident from their high intrinsic viscosities. In contrast serum albumin with an intrinsic viscosity near 4 ml/g must be relatively compact. It seemed possible, therefore, that if one could create a polymer with a high *local* concentration of apolar and ionic

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