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Cooperative Interactions and Determination of Protein Association–Dissociation Equilibria. Hemerythrin*

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ABSTRACT: Cooperative interactions between different sites in a protein, such as hemerythrin, composed of several subunits, can be accounted for if (1) the oligomer is in equilibrium with monomer; (2) the monomer has a greater affinity for a particular ligand than does the oligomer; and (3) the monomer has a different reactivity for a given reagent than does oligomer. Further evidence for the validity of the second assumption in regard to hemerythrin has now been obtained in studies of the binding of ligands to the iron site. The apparent affinity of the protein for thiocyanate ion increases with decreasing protein concentration, as would be expected if the monomer binds more strongly. In a complementary manner, the variation of ligand binding with protein concentration may be used to evaluate the equilibrium constant K_P for $A_n = nA$. Equations and suitable extrapolations have been developed for this ligand binding analysis of dissociation, and dissociation constants and related parameters have been evaluated for hemerythrin.

Hemerythrin, a nonheme iron-containing protein of sipunculids, is composed of eight subunits (Keresztes-Nagy and Klotz, 1963a). This protein reacts with iron-coordinating ligands (Keresztes-Nagy and Klotz, 1965) and with sulfhydryl reagents (Keresztes-Nagy and Klotz, 1963b). These reactions are easily and independently detectable. When an external ligand is bound by the iron the visible spectrum of the protein is changed; in contrast, reaction with sulfhydryl reagents results in dissociation of the octameric protein to monomers. On the other hand, these two reactions are not fully independent of each other. When the protein iron is not coordinated with external ligands the SH groups react only slowly with organic mercurials or *N*-ethylmaleimide; in the presence of iron-coordinating anions, such as azide or thiocyanate, the SH groups react readily (Keresztes-Nagy and Klotz, 1965).

The dependence of the SH group reactivity on the environment about the iron is an example of cooperative interactions. A reaction at one site of the protein affects the reactivity of a second site. A molecular interpretation of this phenomenon in hemerythrin has been proposed (Keresztes-Nagy and Klotz, 1965). The model described is based on three assumptions. (1) The octameric protein is in equilibrium with a small amount of monomer; (2) the monomer has a greater affinity for iron-coordinating anions than does the octamer; and (3) the SH group in the monomer is more reactive than the SH group in the octamer. This model may be represented schematically as

$$A_8 \rightleftharpoons 8A$$

$$A + B \longrightarrow A\text{–}B$$

where A_8 is the octamer, A the monomer, and B the sulfhydryl reagent. The assumptions of this model are sufficient to explain the cooperative interaction; the attachment of an anion such as thiocyanate or chloride to iron of the protein shifts the protein dissociation

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equilibrium toward the monomer, and thus enhances the apparent reactivity of the SH groups. This model is similar to one proposed by Monod *et al.* (1965) to explain the phenomenon of allosteric interactions, with the difference that the conformational change postulated for hemerythrin is the dissociation of the oligomeric protein.

Some information has already been obtained to show that the first two assumptions of our model are valid. Since native and lightly succinylated hemerythrins hybridize at neutral pH values (Keresztes-Nagy *et al.*, 1965) the octamer is in equilibrium with a smaller subunit. That this subunit is the monomer has been shown directly (Klapper *et al.*, 1966). The sedimentation of hemerythrin in a gravitational field has been determined with an analytical ultracentrifuge equipped with a split-beam, photoelectric-scanner optical system (Schachman *et al.*, 1962; Lamers *et al.*, 1963). Dilution of hemerythrin below 0.4% concentration results in dissociation of the octameric protein into monomeric subunits with no apparent formation of intermediate-sized species. Thus the validity of the first assumption has been established.

The ultracentrifugation experiments also provided evidence for the correctness of the second assumption. At low concentrations, thiocyanate was found to promote the dissociation of hemerythrin into its monomeric subunits. Thus, the dissociation of the protein is linked to the binding of the anion. Detailed analysis of such linked functions has been provided previously (Wyman, 1948, 1964). The important point to be stressed here is that the effect of the anion, thiocyanate, on the dissociation equilibrium of hemerythrin is a reflection of the greater affinity that the monomer has for the ligand than does the octamer. That the monomer would have the greater affinity was foreshadowed in earlier binding measurements made with subunits obtained by reaction of the protein's sulfhydryl groups with *N*-ethylmaleimide (Keresztes-Nagy and Klotz, 1965).

In this paper we shall present further, independent evidence to show that the unmodified monomer has the larger affinity for the ligand thiocyanate. Since the degree of hemerythrin aggregation depends on its concentration (Klapper *et al.*, 1966), the measurement of ligand binding at various protein concentrations can be used to determine the affinity of the monomer for thiocyanate relative to that of the octamer. Since the protein disaggregates on dilution, the apparent binding constant for ligand should increase with decreasing protein concentration only if the monomer binds ligand more strongly than does the octamer. We have investigated the binding of thiocyanate by hemerythrin over a wide range of protein concentration, and have found that the apparent constant does indeed increase as the protein is diluted.

Because the binding properties of hemerythrin depend on the state of protein aggregation, the *apparent* ligand binding constants reflect the protein disaggregation equilibrium constant, as well as the affinities of the various protein forms for ligand. Hence if one could determine the *true* binding affinities of each of the separate protein species present in solution, then, in principle, the amounts of each of the protein species in solution could also be determined. The procedure by which ligand binding may be used to study protein association–dissociation equilibria will, therefore, be outlined at the outset.

Ligand-Binding Method of Studying Dissociation Equilibria

Let us represent an oligomer (A_n)–monomer (A) equilibrium of a multichain protein by eq 1.

$$A_n = nA \tag{1}$$

Then we may write the following relationship for the equilibrium constant, K_P, for the dissociation.

$$K_P = \frac{(A)^n}{(A_n)} \tag{2}$$

If the following conditions are applicable, K_P (and a variety of other constants) can be readily determined. (1) The equilibrium must be between A_n and A with no significant amounts of intermediate aggregates.[1] (2) The protein must bind small ligand molecules (L) reversibly. (3) The affinity of monomer for the ligand must differ from that of oligomer for the same small molecule.

Under these circumstances we can represent the equilibria of relevance by eq 3.

$$\begin{array}{ccc} A_n & \overset{K_P}{=\!=} & nA \\ +L & & +L \\ {\scriptstyle k_1}\big| & & \big\|{\scriptstyle K_1} \\ A_nL & & AL + (n-1)A \\ \vdots & & \vdots \\ A_nL_n & \overset{K_{PL}}{=\!=} & n(AL) \end{array} \tag{3}$$

The equilibrium constant K_P has already been defined (eq 2). The other equilibrium quotients are specified by the following relationships.

$$K_1 = \frac{(AL)}{(A)(L)} \tag{4}$$

$$\left.\begin{aligned} k_1 &= \frac{(A_nL)}{(A_n)(L)} \\ k_2 &= \frac{(A_nL_2)}{(A_nL)(L)} \\ &\vdots \\ k_n &= \frac{(A_nL_n)}{(A_nL_{n-1})(L)} \end{aligned}\right\} \tag{5}$$

[1] If significant amounts of intermediate species are present the protein dissociation constants may still be obtained, but the analysis becomes more complicated (see the Appendix).

$$K_{PL} = \frac{(AL)^n}{(A_nL_n)} \tag{6}$$

To relate these equilibrium constants to experimental quantities it is convenient to define an experimental parameter K_{app} as

$$K_{app} = \frac{\alpha}{(1-\alpha)}\frac{1}{(L)} \tag{7}$$

where α is the fraction of total available sites for ligand, on all forms of the protein, to which L is actually bound. A molecular species specification for α is

$$\alpha = \frac{(AL) + (A_nL) + 2(A_nL_2) + \cdots + n(A_nL_n)}{(A_T)} \tag{8}$$

where (A_T) is the total concentration of ligand binding sites and hence of protein expressed in moles per liter (of monomer units). Since $1 - \alpha$ is the fraction of sites which are open, *i.e.*, do not have a bound L, we may represent it by

$$1 - \alpha = \frac{(A) + n(A_n) + (n-1)(A_nL) + \cdots + (A_nL_{n-1})}{(A_T)} \tag{9}$$

From an examination of eq 7 it is evident that K_{app} can be evaluated experimentally if α, the fraction of sites occupied with ligand, and (L), the free-ligand concentration, can be determined. In practice either or both of these quantities may be easily obtained if the protein spectrum changes when L is bound, or if the absorption spectrum of the ligand is altered when it is attached to the protein. Other methods such as equilibrium dialysis (Klotz, 1953) or ultracentrifugation (Schachman, 1963) may also be used.

Substitution of eq 8 and 9 into eq 7 yields

$$K_{app} = \frac{1}{(L)}\frac{(AL) + \sum_{i=0}^{n} i(A_nL_i)}{(A) + \sum_{i=0}^{n}(n-i)(A_nL_i)} \tag{10}$$

When the appropriate relations from eq 4 to 6 are substituted into eq 10, we obtain

$$K_{app} = \frac{K_1(A) + (A_n)[k_1 + 2k_1k_2(L) + \cdots + n(k_1\cdots k_n)(L)^{n-1}]}{(A) + A_n[n + (n-1)k_1(L) + \cdots + (k_1\cdots k_{n-1})(L)^{n-1}]} \tag{11}$$

[2]

From eq 11 it is evident that at the limiting condition of zero free ligand, K_{app} is still a finite number

$$\lim_{(L)\to 0} K_{app} = \frac{K_1(A) + k_1(A_n)}{(A) + n(A_n)} = K_{app}^* \tag{12}$$

From this it follows by rearrangement that

$$\frac{(A)}{(A_n)} = \frac{nK_{app}^* - k_1}{K_1 - K_{app}^*} \tag{13}$$

Conservation of mass requires that, in the absence of ligand

$$(A_T) = (A) + n(A_n) \tag{14}$$

Thus after K_{app}^*, k_1, and K_1 are known, we can use eq 13 and 14 to calculate (A) and (A_n) and hence from eq 2 we can compute the protein–subunit dissociation constant K_P.

K_{app}^* is obtained experimentally by extrapolation, according to eq 12, of experimental data for K_{app} obtained from eq 7. By straightforward algebraic manipulation of eq 11, one can show also that

$$\lim_{(A_T)\to 0} K_{app} = K_1 \tag{15}$$

Finally by rearrangement of eq 12 one obtains

$$K_{app}^* = \frac{K_1\dfrac{(A)}{(A_n)} + k_1}{\dfrac{(A)}{(A_n)} + n} \tag{16}$$

from which it follows that

$$\lim_{(A_T)\to\infty} K_{app}^* = \frac{k_1}{n} \tag{17}$$

Thus all the parameters required in eq 13 can be obtained from experimental observations.

In the above derivation we assumed that all the monomer subunits are identical. If this is not the case then the equilibrium should be represented as

$$A_aB_bC_c\cdots = aA + bB + cC + \cdots \tag{18}$$

where A, B, and C represent distinct subunits, a, b, and c being the respective numbers of each type. In place of eq 12 we would now obtain

$$\lim_{(L)\to 0} K_{app} = \frac{k_1(A_aB_bC_c\cdots) + [K_A(A) + K_B(B) + K_C(C) + \cdots]}{n(A_aB_bC_c\cdots) + [(A) + (B) + (C) + \cdots]} = K_{app}^* \tag{19}$$

[2] If the binding of ligand L to any one site on the octamer is independent of the binding at any other site on this oligomer, then the constants k_1 are related to each other in a statistical manner and eq 11 can be markedly simplified (see the Appendix).

where

$$n = a + b + c + \cdots \tag{20}$$

and K_A, K_B, and K_C are the ligand-binding constants for each type of monomer. As eq 18 indicates, whenever an oligomer $A_aB_bC_c$ dissociates, the different types of subunit are produced in definitely related amounts, in proportion to the coefficients a, b, c... Hence we may write

$$\frac{(A)}{a} = \frac{(B)}{b} = \frac{(C)}{c} = (\xi) \tag{21}$$

where (ξ) represents a normalized concentration. Insertion of the relations of eq 21 into eq 19 leads to

$$K_{app}^* = \frac{k_1(A_aB_bC_c) + [K_A a(\xi) + K_B b(\xi) + K_C c(\xi) + \cdots]}{n(A_aB_bC_c) + [a(\xi) + b(\xi) + c(\xi) + \cdots]} \tag{22}$$

This can now be reduced in straightforward algebraic steps to

$$\frac{n(\xi)}{(A_aB_bC_c\cdots)} = \frac{nK_{app}^* - k_1}{\frac{aK_A + bK_B + cK_C + \cdots}{n} - K_{app}^*} \tag{23}$$

which is formally analogous to eq 13. The essential difference between these two equations lies in the first term in the denominator; in eq 23 we have an average ligand binding constant for the different subunits in place of K_1, the single ligand binding constant when all subunits are identical. In an analogous fashion we now obtain

$$\lim_{(A_T)\to 0} K_{app} = \frac{aK_A + bK_B + cK_C + \cdots}{n} \tag{24}$$

instead of K_1 (see eq 15). On the other hand, we find that even for nonidentical subunits eq 17 is still valid.

In summary, then, even if all the monomer subunits are not identical the ligand binding equations can be applied in the same way as those for identical subunits. The extrapolations of the experimental parameter K_{app} prescribed by eq 12, 15, and 17, or of eq 19, 24, and 17, yield the equilibrium constants needed to describe the equilibria described in eq 3.

On the other hand, if the dissociation $A_n = nA$ does not proceed directly but occurs in sequential steps through a series of intermediates A_{n-i}, then the experimental problem becomes more complicated. An analysis of such a sequential equilibrium is given in the Appendix.

In this communication we shall show that thiocyanate binding to hemerythrin is linked to protein dissociation. Using the analysis described above we have roughly estimated the dissociation constant for the octamer–monomer equilibrium.

Experimental Methods

Oxyhemerythrin was prepared, by a previously described procedure (Klotz *et al.*, 1957), from the coelomic fluid of the marine worm *Golfingia gouldii* (also known as *Phascolosoma gouldii*). Protein concentration was determined from the optical density at 500 mμ using 1100 (liters per centimeter per mole of iron) as the extinction coefficient (Keresztes-Nagy and Klotz, 1965).

The protein was oxidized to methemerythrin in the following manner. Potassium ferricyanide, in a 1.5–2 molar excess over protein iron, was added to approximately 1 g of protein in 40 ml of 2.0 M sodium chloride. The oxidation was performed at room temperature for 2 hr, and then at 4° overnight. Methemerythrin was separated from the oxidizing agent by passage through a column (1.0 × 15 cm) of Dowex 1-X8 (200–400 mesh) which had been previously cleaned by the method of Hartree (1964), equilibrated with 1.0 M perchloric acid, and then washed with water. The protein passes through the column, while the ferri- and ferrocyanides are absorbed at the top.

Chloride was removed by extensive dialysis of the methemerythrin against 0.1 M sodium perchlorate. The protein was stored in 0.1 M sodium perchlorate at 4°. Under these conditions the protein was stable for several months. If the protein was frozen, some denaturation occurred. Before use the protein was dialyzed against the buffer of choice. Methemerythrin concentration was determined from the optical density at 355 mμ using 3220 (liters per centimeter per mole of iron) as the extinction coefficient (Keresztes-Nagy and Klotz, 1965).

The binding of thiocyanate to methemerythrin was monitored by measuring the optical density at 452 mμ with a Cary 14R recording spectrophotometer. Binding curves were obtained by adding aliquots of thiocyanate to a solution of the protein. After each addition the system was allowed to come to equilibrium as evidenced by no further change in optical density at 452 mμ. Potassium thiocyanate in 0.100 M Tris (cacodylate) buffer (pH 7.00 ± 0.02) was added to a solution of methemerythrin in the same buffer and at 10.0 ± 0.2°. To cover as wide a range of protein concentration as possible, cells with quartz windows and lengths varying from 0.50 to 10.00 cm were used.

The fraction of protein binding thiocyanate after each addition of the anion was calculated by dividing the incremental optical density change by the total change that accompanied saturation. The bound thiocyanate was obtained by multiplying the fraction of bound protein by the total protein (monomer) concentration. The free thiocyanate concentration was calculated as the difference between the total thiocyanate and bound thiocyanate.

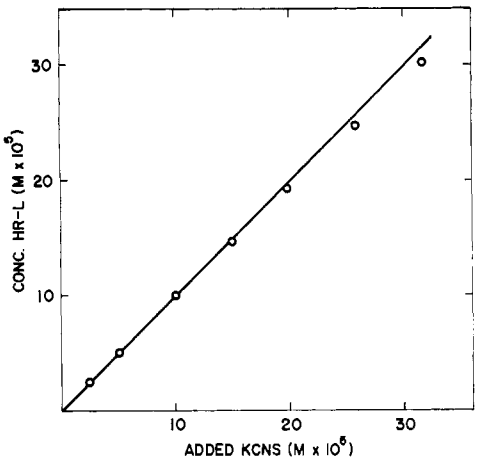

FIGURE 1: Titration of methemerythrin with KSCN to establish stoichiometry of iron–ligand combination.

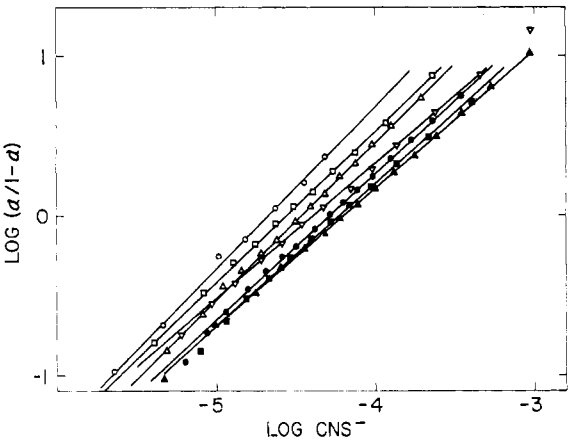

FIGURE 2: Dependence of thiocyanate binding on concentration of methemerythrin. Protein concentration in moles per liter of subunit: (○) 0.221×10^{-5}, (□) 0.876×10^{-5}, (△) 1.86×10^{-5}, (▽) 3.59×10^{-5}, (●) 7.29×10^{-5}, (■) 13.4×10^{-5}, and (▲) 23.3×10^{-5}.

Results

Stoichiometry of Thiocyanate Binding. Because the binding of thiocyanate to hemerythrin results in a large alteration of the protein's spectrum, the fraction of protein to which ligand is bound, or α, is readily obtained. From α, and from the total amounts of ligand and protein in the system, one can calculate the free ligand concentration provided that the stoichiometry of binding is known. The stoichiometry of the hemerythrin–thiocyanate interactions was determined by a direct titration of a concentrated hemerythrin solution with thiocyanate. The direct titration is a plausible method since the binding constant has been previously estimated to be of the order of 10^4 (Keresztes-Nagy and Klotz, 1965).

A 1.05×10^{-3} M solution of methemerythrin (calculated in terms of monomer units) in 0.100 M Tris (cacodylate) (pH 7.00 ± 0.02) at 10.0 ± 0.2° was titrated with potassium thiocyanate dissolved in the same buffer. The results, shown in Figure 1, were plotted as the concentration of ligand-containing protein *vs.* the total ligand concentration. The stoichiometry of the reaction was obtained from the slope of the initial linear portion of the curve. The value of this slope was 0.99 indicating that one thiocyanate molecule is bound to one monomer unit. The same stoichiometry was found previously for the binding of azide and of oxygen to hemerythrin (Keresztes-Nagy and Klotz, 1965).

The Dependence of Thiocyanate Binding on the Protein Concentration. The binding of thiocyanate to methemerythrin over a range of protein concentrations is presented in Figure 2. Each curve represents the results obtained at one protein concentration. The binding affinity of the protein for the ligand increases as the protein is diluted, as is evident from the shift of the binding curves to the left in Figure 2. Apparent binding constants, some of which are listed in Table I, were calculated from the 50% saturation point at each protein concentration. A 100-fold variation in the protein concentration is accompanied by a threefold change in the apparent binding constant. The slopes of the Hill plots (not shown here) are also listed in Table I. At all protein concentrations the value of the Hill n coefficient is close to one; thus curves of bound thiocyanate *vs.* free thiocyanate would all be nearly hyperbolic. At the present time we do not know whether the deviations from 1.0 are experimentally significant.

Discussion

A dissociation equilibrium such as $A_n = nA$ can be studied quantitatively if in principle one can evaluate the equilibrium concentrations of the oligomer and monomer. In many systems, one of the participants, usually A, is present in such low concentrations that its detection is difficult. In such circumstances one usually resorts to some perturbation method. The simplest perturbation is dilution, for in terms of LeChatelier's principle it is obvious that the dissociation equilibrium would be shifted toward subunits. In practice dilute protein solutions are difficult to examine, although

TABLE I: Equilibrium Coefficients for Hemerythrin and Hill Coefficients as a Function of Protein Concentration.

Protein Concn (M × 10^6) (in moles of monomer unit)	K_{app} at $\alpha = 0.5$ (M × 10^{-4})	Hill Coefficient	$K_P \times 10^{88}$
2.21	4.69	1.0	0.03
8.76	3.60	0.95	80
18.6	2.90	0.99	20
72.9	1.92	0.91	0.3

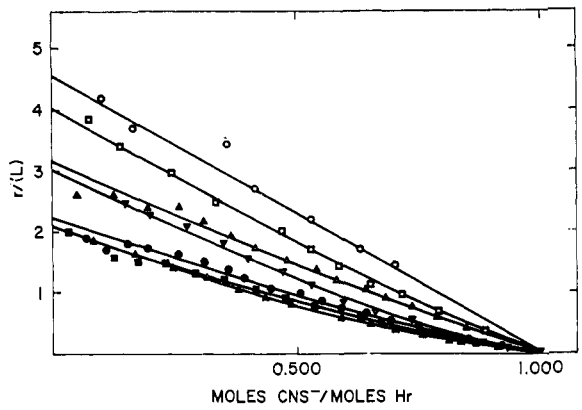

FIGURE 3: Extrapolation of binding data to zero ligand concentration at each of several protein concentrations. Protein concentrations in moles per liter of subunit: (○) 0.221×10^{-5}, (□) 0.876×10^{-5}, (△) 1.86×10^{-5}, (▽) 3.59×10^{-5}, (●) 7.29×10^{-5}, (■) 13.4×10^{-5}, and (▲) 23.3×10^{-5}.

recent scanner attachments (Schachman *et al.*, 1962; Schachman and Edelstein, 1966) promise to overcome these limitations. An alternative perturbation method involves the addition of high concentrations of a third component, such as urea or salt, to the protein solution. However, introduction of the third component is accompanied by difficulties in analysis of the experimental observations. On the other hand, if the dissociation equilibrium is linked to the binding of ligands, then the addition of these ligands at low concentrations will shift the equilibrium without a significant change in the nature of the bulk solvent. This cooperative interaction may then be used as a method to study quantitatively the dissociation of the macromolecule into its subunits.

The applicability of the ligand binding method has been tested in a preliminary manner using the data summarized in Figure 2. To obtain k_1, K_{app} should be plotted against the reciprocal of the protein concentration so that an extrapolation to infinite protein concentration (*i.e.*, $(\text{protein})^{-1} \rightarrow 0$) can be made readily. For this extrapolation one must use values of K_{app} at low α's. At low α's, however, the precision of the data declines. The procedure we have finally adopted is to carry out a double extrapolation. (1) Values of $r/(L)$, where r is the moles of ligand bound per mole of hemerythrin (expressed in terms of subunit), were extrapolated to zero ligand concentration (Klotz, 1953) for each concentration of protein (Figure 3); and (2) the intercepts of these extrapolations were then extrapolated (Figure 4) to infinite protein concentration (*i.e.*, $(\text{protein})^{-1} \rightarrow 0$). The value for $k_1/8$ so obtained is 2.09×10^4.

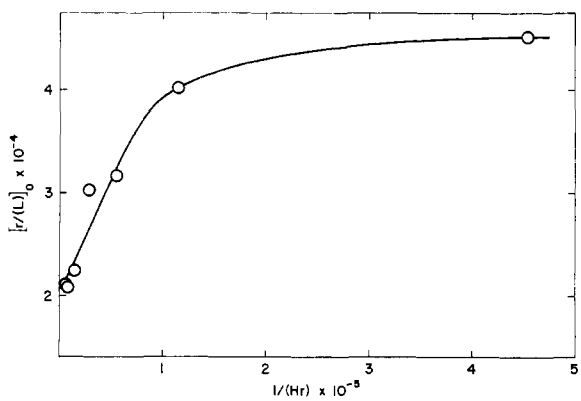

FIGURE 4: Extrapolation of intercepts of Figure 3 to infinite protein concentration.

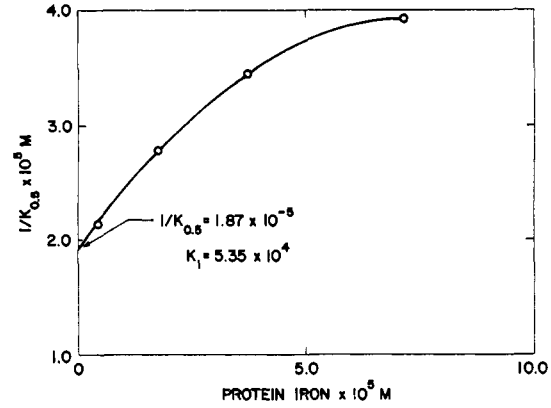

FIGURE 5: Extrapolation of $1/K_{app}$ to zero protein concentration.

For K_1, K_{app} must be extrapolated to zero protein concentration. For graphical convenience $1/K_{app}$ was actually used as the ordinate so that K_1 is the reciprocal of the intercept on this axis (Figure 5). In the hemerythrin–thiocyanate system, K_1 is 5.35×10^4. In the monomer, only one site is available for ligand. Hence, there can be no electrostatic repulsive interactions between successively bound ligands. A double extrapolation, as for k_1, is unnecessary, therefore, and the most precise experimental data, at $\alpha = 0.5$, can be used directly for the single extrapolation.

K_{app}* was obtained at four different protein concentrations by plotting K_{app} *vs.* (L) and extrapolating to (L) = 0. In these extrapolations we have no recourse but to depend heavily on measurements made at low α, where the precision of the experiments becomes poor. From the intercepts obtained at each protein concentration, $(A)/(A_8)$ was calculated by means of eq 13, and then K_P (eq 2) was computed. The results are summarized in Table I. A value of approximately 10^{-38} (M^7) is, thus, obtained for the dissociation constant of octameric hemerythrin.

It must be recognized that K_P contains the monomer concentration raised to the eighth power. Errors in the evaluation of (A) are thus greatly magnified in K_P. For example, a very optimistic estimate of the uncertainties in K_1, k_1, and K^* is 5%. At a protein concentration of near 1×10^{-4} M, these uncertainties would

introduce a possible error in $(A)/(A_8)$ of a factor of 2. In turn this would change K_P by 250-fold. At a very low (or very high) protein concentration, as in any type of stoichiometric titration, the error in the constituent parameters would be even greater and hence the variation in K_P could be even larger. Thus we see that the apparent large variation of K_P in Table I reflects primarily the high exponential power in the factor $(A)^8$.

Although the binding data are treated on the assumption of an octamer–monomer equilibrium with no significant intermediates, we cannot at present rule out other dissociation modes. If octamer does dissociate directly to the monomer, the protein dissociation constants as calculated above should be the same at all protein concentrations. The data from which K's were calculated are not accurate enough to check for self-consistency. The experimental evidence to date, however, supports an octamer–monomer equilibrium. Furthermore in studies of dissociation of the protein by reaction with organic mercurial (Keresztes-Nagy and Klotz, 1963b) or by dilution of the protein (Klapper *et al.*, 1966), there was no indication of any protein species other than the octamer and monomer.

Conclusion

Hemerythrin is not the only protein in which the affinity for ligands is dependent on protein concentration. Roughton *et al.* (1955) have noted that both the shape and position of the oxygenation curves of hemoglobin change with dilution of the protein. Similar phenomena have been reported for the binding of guanosine triphosphate to glutamic dehydrogenase (Colman and Frieden, 1966) and of aspartate to glutamic–aspartic transaminase (Polyanovski and Ivanov, 1964). In all of these systems it should be possible to obtain protein dissociation constants from the ligand binding data. The thermodynamic basis for these relationships has been recognized for some time (Wyman, 1948, 1964; Briehl, 1963). Application to disaggregating or aggregating proteins has been very limited, however. The equations and procedures described in this paper should facilitate such application. Thus, another method becomes available for the quantitative assessment of the thermodynamic parameters that govern dissociation of an oligomer into its subunits.

Appendix

Sequential Equilibrium. For all oligomers of three or more subunits dissociation may occur sequentially; *i.e.*, the dissociation of one subunit at a time. Rather than solve the general case, we shall deal with the trimer–dimer–monomer model. The solution of this problem will give us the information we require to discuss the general case.

Assume the equilibrium given in the equation

$$A_3 \overset{K_{P,1}}{\rightleftharpoons} A_2 + A_1 \overset{K_{P,2}}{\rightleftharpoons} 3A \qquad (25)$$

We then make the following definitions.

$$K_{P,1} = (A_2)(A)/(A_3) \qquad (26)$$

$$K_{P,2} = (A)^2/(A_2) \qquad (27)$$

$$K_1 = (AL)/(A)(L) \qquad (28)$$

$$l_1 = (A_2L)/(A_2)(L) \qquad (29)$$

$$l_2 = (A_2L_2)/(A_2L)(L) \qquad (30)$$

$$k_1 = (A_3L)/(A_3)(L) \qquad (31)$$

$$k_2 = (A_3L_2)/(A_3L)(L) \qquad (32)$$

$$k_3 = (A_3L_3)/(A_3L_2)(L) \qquad (33)$$

Then in place of eq 10 we write

$$K_{app} = \frac{1}{(L)} \times \frac{(AL) + (A_2L) + 2(A_2L_2) + (A_3L) + 2(A_3L_2) + 3(A_3L_3)}{(A) + (A_2L) + 2(A_2) + (A_3L_2) + 2(A_3L) + 3(A_3)} \qquad (34)$$

Substituting eq 28–33 into eq 34 we obtain

$$K_{app} = \frac{[K_1(A) + l_1(A_2) + 2l_1l_2(A_2)(L) + k_1(A_3) + 2k_1k_2(A_3)(L) + 3k_1k_2k_3(A_3)(L)^2]}{[(A) + l_1(A_2)(L) + 2(A_2) + k_1k_2(A_3)(L)^2 + 2k_1(A_3)(L) + 3(A_3)]} \qquad (35)$$

It follows from this that

$$\lim_{(L)\to 0} K_{app} = \frac{K_1(A) + l_1(A_2) + k_1(A_3)}{(A) + 2(A_2) + 3(A_3)} = K_{app}^* \qquad (36)$$

When no ligand is present

$$(A_T) = (A) + 2(A_2) + 3(A_3) \qquad (37)$$

The solution of the sequential trimer equilibrium is thus analogous to that of the oligomer–monomer equilibrium. There are, however, two additional unknowns; therefore, to solve eq 36 and 37 explicity two additional operations are required. These two operations may be obtained with the analytical ultracentrifuge using any two of the procedures proposed by Adams and Filmer (1966) or Steiner (1953a,b). Alternatively, the two additional operations may be provided by additional ligand binding studies.

A binding analysis could be performed using a second ligand, L′. In this case eq 36 becomes

$$\lim_{(L')\to 0} K_{app} = \frac{K_1'(A) + l_1'(A_2) + k_1'(A_3)}{(A) + 2(A_2) + 3(A_3)} \qquad (38)$$

where K_1', l_1', and k_1' are the monomer, dimer, and trimer binding constants[3] for the ligand L'.

An analysis of the dependence of K_{app}* (eq 36) on the protein concentration can also be made. Substitution of eq 37 and 36 followed by differentiation leads to

$$K_{app}^* + \frac{(A_T)dK_{app}^*}{d(A_T)} = \frac{K_1 d(A)}{d(A_T)} + \frac{l_1 d(A_2)}{d(A_T)} + \frac{k_1 d(A_3)}{d(A_T)} \quad (39)$$

Differentiating eq 26, 27, and 37 and applying the proper algebraic manipulations we obtain

$$\frac{d(A_2)}{d(A_T)} = 2(A)^{-1}(A_2)\frac{d(A)}{d(A_T)} \quad (40)$$

$$\frac{d(A_3)}{d(A_T)} = 3(A)^{-1}(A_3)\frac{d(A)}{d(A_T)} \quad (41)$$

$$\frac{d(A)}{d(A_T)} = \frac{(A)}{(A) + 2^2(A_2) + 3^2(A_3)} \quad (42)$$

Then we may substitute eq 40–42 into eq 39 to obtain

$$K_{app}^* + \frac{(A_T)dK_{app}^*}{d(A_T)} = \frac{K_1(A) + 2l_1(A_2) + 3k_1(A_3)}{(A) + 2^2(A_2) + 3^2(A_3)} \quad (43)$$

Thus, the slope of the curve obtained from a plot of K_{app}* *vs.* (A_T) and titration with a second ligand would provide the two additional operations required.

Without deriving the general solution we can see that for each additional intermediate in the sequential equilibrium two additional unknowns are introduced. Therefore, for each intermediate two new operations are required. The oligomer–monomer equilibrium can be solved explicitly with four operations, the trimer sequential equilibrium with six operations, and the tetramer sequential equilibrium with eight operations. Clearly, there is a limit as to the number of operations which will be experimentally accessible. Furthermore the accuracy of any calculated parameter decreases as the number of operations increases.

Statistical Relations in Ligand Binding to Oligomer. Let us assume that the binding of L at any one site of the oligomer is independent of the binding of any other site. Then we may write [see, *e.g.*, Klotz, 1953]

$$k_i = \frac{n - i + 1}{i}k \qquad i = 1, 2, 3 \ldots n \quad (44)$$

where n is the total number of sites, k the intrinsic binding constant, and k_i the individual binding constants.

To solve this problem for the oligomer–monomer equilibrium we rearrange eq 11 to the following

$$\frac{(A)}{(A_n)} = \frac{K_{app}\sum_{i=1}^{n}(n - i + 1)(L)^{i-1}\Pi k_{i-1} - \sum_{i=1}^{n} i(L)^{i-1}\Pi k_i}{K_1 - K_{app}} \quad (45)$$

where

$$\sum_{i=1}^{n}(n - i + 1)(L)^{i-1}\Pi k_{i-1} = n + (n-1)k_1(L) + \cdots + (k_1 \cdots k_{n-1})(L)^{n-1}$$

$$\sum_{i=1}^{n} i(L)^{i-1}\Pi k_i = k_1 + 2k_1k_2(L) + \cdots + n(k_1 \cdots k_n)(L)^{n-1}$$

Substituting the relations generated from eq 44 into eq 45 we obtain

$$\frac{(A)}{(A_n)} = \frac{n(K_{app} - k)}{K_1 - K_{app}}\left[1 + (n-1)k(L) + \frac{(n-1)(n-2)k^2(L)^2}{1\cdot 2} + \cdots + k^{n-1}(L)^{n-1}\right] \quad (46)$$

When $k(L) \ll 1$, eq 46 can be reduced to

$$\frac{(A)}{(A_n)} = \frac{n(K_{app} - k)}{K_1 - K_{app}}[1 + k(L)]^{n-1} \quad (47)$$

or alternatively

$$\frac{(A)}{(A_n)} = \frac{nK_{app} - k_1}{K_1 - K_{app}}[1 + k_1(L)/n]^{n-1} \quad (48)$$

Since K_{app}, K_1, and k_1 have all been previously determined, ligand binding curves can be generated using eq 48. From the correspondence between the experimental and generated binding curves one would be able to test the hypothesis of independent binding sites.

[3] If

$$\frac{K_1}{K_1'} = \frac{l_1}{l_1'} = \frac{k_1}{k_1'}$$

determination of either K_1' or k_1', using the same procedures by which K_1 and k_1 were obtained, will yield the value of l_1/l_1'.

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