Let

$$A = \frac{Q_1}{1 + \omega^2\tau_1^2}$$
$$B = \frac{Q_2}{1 + \omega^2\tau_2^2}$$
$$C = \frac{Q_3}{1 + \omega^2\tau_3^2} \qquad (A.15)$$
$$f_2 = 0.39 - f_3$$

substitution into eq A.11 and rearrangement yields

$$f_3 = \frac{0.61A(\bar{\tau} - \tau_1) + 0.39(\bar{\tau} - \tau_2)B}{C(\tau_3 - \bar{\tau}) + B(\bar{\tau} - \tau_2)} \qquad (A.16)$$

The degree of saturation of oxalate binding sites, S, is then

$$S = f_3/0.39 \qquad (A.17)$$

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Cooperativity of Binding of Anilinonaphthalenesulfonate to Serum Albumin Induced by a Second Ligand†

David A. Kolb‡ and Gregorio Weber*

ABSTRACT: When a ligand X is multiply bound to energetically identical, noninteracting sites of a protein, cooperative binding of this ligand can be induced by the presence of a second ligand Y. This effect should appear whenever multiple interactions exist between the bound X and Y ligands, and vanish when the concentration of Y is made sufficiently large to ensure Y saturation at all concentrations of X. These predictions have been verified for the binding of 8-anilino-1-naphthalenesulfonate to serum albumin, when Y, the effector ion, is 3,5-dihydroxybenzoate. In the presence of 2 mM dihydroxybenzoate, the Hill coefficient for anilinonaphthalenesulfonate binding rose steadily from 1 to 1.5 as the number of molecules of ligand bound increased from 1 to 3.3 per albumin molecule. The theory of interactions between isolated ligands, applied in the previous paper (D. A. Kolb and G. Weber (1975), *Biochemistry,* preceding paper in this issue), is extended to cases of multiple interactions, and applied here to show that the experimental results are tolerably well reproduced for a model in which four anilinonaphthalenesulfonate molecules are homogeneously coupled to four molecules of dihydroxybenzoate by free energies of 3.0 and 3.5 thermal units.

Cooperative ligand binding by proteins is classically exemplified by the equilibria of several ligands with hemoglobin (Wyman, 1964) and has more recently been observed in other oligomeric proteins (Changeux et al., 1968; Er-el et al., 1972). In many more cases the dependence of catalytic reaction velocity upon substrate concentration has been considered to reflect cooperative substrate binding (Kirschner, 1968). Monod et al. (1965) explained these cooperative effects as arising out of the requirement for symmetry conservation that they postulated as operating in oligomeric protein aggregates. On the other hand, Weber (1972) has shown that cooperative binding of a multiply bound ligand X arises inevitably as a result of positive or negative *multiple* interactions between two or more molecules of X on one

† From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received May 14, 1975.* This paper is taken from the Ph.D. dissertation of D.A.K., University of Illinois, 1975. This work was supported by Grant GM 11223 from the National Institutes of Health.

‡ Present address: Spex Industries, Metuchen, N.J. 08854.

side and one or more molecules of another ligand Y on the other. There is nothing in this simple free energy theory that requires the effects to be restricted to oligomeric molecules. Consequently, we have looked for, and found, a case in which cooperative binding of a ligand (8-anilino-1-naphthalenesulfonate, ANS)[1] by a single chain protein (bovine serum albumin) is induced by addition of a second ligand (3,5-dihydroxybenzoate, 3,5-DHB). These two ligands, ANS and DHB, interact negatively when both are bound to the molecule of bovine serum albumin as a polyvalent complex.

Origin of These Experiments

In extending the study of the binding of ANS and bovine serum albumin of Daniel and Weber (1966), Pasby (1969) observed by the use of equilibrium dialysis that at least 15 ANS molecules could be bound per protein molecule at neutral pH, but that only the four or five molecules bound at the lower ANS concentration contributed appreciably to the fluorescence efficiency of the complexes. It is known that when water is added to an ethanol or propanol solution of ANS it acts as a collisional quencher with a Stern–Volmer constant of 5 M^{-1}. It seems natural then to conclude that two types of anion-binding sites exist in bovine serum albumin. One kind of site (inner sites) is sufficiently hidden from water to prevent its interaction with ANS during the fluorescence lifetime (16 nsec). In the other class of sites (outer sites) interaction of the excited ligand with water is strong enough to quench the fluorescence. The observations of Pasby (Figure 1) indicate that the inner sites have a much higher affinity for ANS than the outer sites, a preference that might be expected because the partition of ANS between water and butanol is highly favorable to the latter medium, where ANS displays a fluorescence efficiency and spectrum comparable to those that it shows when occupying the strong binding sites in albumin. Daniel and Weber (1966) and Anderson and Weber (1969) showed that electronic energy transfer takes place among the ANS molecules bound to the inner sites, and Pasby (1969) showed that transfer also occurs from inner-bound to outer-bound ANS molecules. The outer site fluorescence, although small, is not negligible, and compensates almost exactly for the decreased fluorescence efficiency that would result from transfer of the excitation to completely nonfluorescent outer sites. The net result of this compensation is that fluorescence titration curves of ANS reflect only the occupancy of the strongly fluorescent sites. There is reason to suppose that anions more hydrophylic than ANS, such as the polyhydroxybenzoates, would be preferentially bound to the outer sites, and that in occupying these sites they would exert an antagonistic effect, probably by electrostatic repulsion, to the binding of ANS to the inner sites. If the concentration of such an antagonist ion is adjusted to be of the order of a few times its dissociation constant, it would progressively dissociate as ANS is bound to the inner sites, facilitating further ANS binding in seemingly cooperative fashion. At still higher concentrations the hydrophylic anion would not dissociate appreciably from the outer sites upon ANS binding and the cooperative effect should disappear.

[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; 2,6-DHB, 2,6-dihydroxybenzoic acid; 2,4-DHB, 2,4-dihydroxybenzoic acid; 3,5-DHB, 3,5-dihydroxybenzoic acid; 4-HB, *p*-hydroxybenzoic acid.

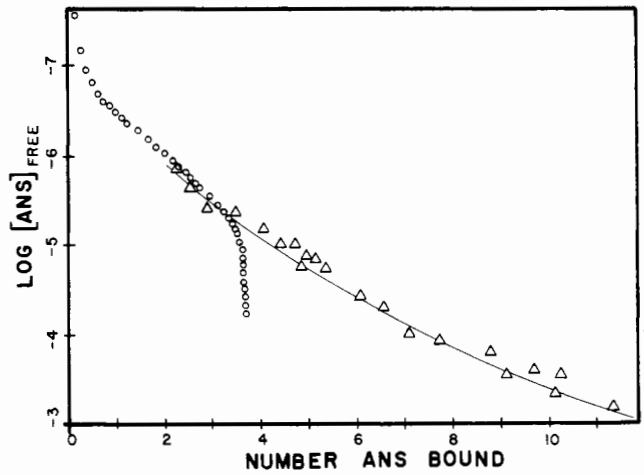

FIGURE 1: Comparison of titration of bovine serum albumin with ANS by fluorescence (○) and equilibrium dialysis (△) methods. Equilibrium dialysis data are from Pasby (1969).

Theory of Interactions in Polyvalent Complexes

If N molecules of ligand X and M molecules of ligand Y are bound per protein molecule there are $NM + N + M$ liganded species of the form PX_JY_K. Each of these forms has

$$\binom{N}{J} \cdot \binom{M}{K} = I$$

site isomers in which X and Y are differently distributed among the binding sites. In each polyvalent complex it is possible to define a mean free energy coupling among the X and Y ligands by a simple extension of concepts already examined (Weber, 1972, 1975). Let $\Delta F°(X_J)$ denote the standard free energy of binding of J molecules of X to the protein in the absence of Y, and similarly $\Delta F°(Y_K)$ denote the standard free energy of binding of K molecules of Y in the absence of X. Let $\Delta F°(X_J/Y_K)$ denote the standard free energy change in the reaction $JX + PY_K \rightarrow PX_JY_K$ and $\Delta F°(Y_K/X_J)$ the standard free energy change in the reaction $KY + PX_J \rightarrow PX_JY_K$. Free energy conservation (e.g., Weber, 1975) gives the relation

$$\Delta F°(X_JY_K) = \Delta F°(X_J) + \Delta F°(Y_K/X_J) = \Delta F°(Y_K) + \Delta F°(X_J/Y_K) \quad (1)$$

where $\Delta F°(X_JY_K)$ is the standard free energy change in the reaction:

$$P + JX + KY \rightarrow PX_JY_K$$

From eq 1

$$\Delta F°(X_J/Y_K) - \Delta F°(X_J) = \Delta F°(Y_K/X_J) - \Delta F°(Y_K) = \overline{\delta F_{JK}} \quad (2)$$

defining the average free energy coupling $\overline{\delta F_{JK}}$ between X and Y in the PX_JY_K complex. $\overline{\delta F_{JK}}$ is formed from the contributions of the I site isomers of the PX_JY_K complex according to the equation:

$$\overline{\delta F_{JK}} = \left[\sum_{i=1}^{I} \delta F_{JK}(i) \exp(-\delta F_{JK}(i)/RT)\right] \Big/ \sum_{i=1}^{I} \exp(-\delta F_{JK}(i)/RT) \quad (3)$$

Equations 1 and 2 may be combined to give

$$\Delta F°(X_JY_K) = \Delta F°(X_J) + \Delta F°(Y_K) + \overline{\delta F_{JK}} \quad (4)$$

which expresses the standard free energy of formation of the PX_JY_K complexes as the sum of the separate free energies of formation of PX_J and PY_K and the average free energy of interaction of the ligands in the polyvalent complex.

The average number of molecules of X and Y bound by the protein at equilibrium are respectively:

$$\bar{n}_X = \sum_{J=0}^{N}\sum_{K=0}^{M} J[PX_JY_K]/D \quad (5)$$

$$\bar{n}_Y = \sum_{J=0}^{N}\sum_{K=0}^{M} K[PX_JY_K]/D \quad (6)$$

$$D = \sum_{J=0}^{N}\sum_{K=0}^{M} [PX_JY_K] \quad (7)$$

If the successive dissociation constants of X in the absence of Y are $K_1, K_2, \ldots K_N$ and those of Y in the absence of X are $L_1, L_2, \ldots L_M$ we can set

$$a_J = (K_1K_2 \ldots K_J)^{-1} = \exp(-\Delta F^\circ(X_J)/RT) \quad (8)$$

$$b_K = (L_1L_2 \ldots L_K)^{-1} = \exp(-\Delta F^\circ(Y_K)/RT) \quad (9)$$

Combining the last two equations with (4) gives

$$[PX_JY_K] = [P]\binom{N}{J}[X]^J a_J \binom{M}{K}[Y]^K b_K \times \exp(-\overline{\delta F_{JK}}/RT) \quad (10)$$

where $[PX_JY_K]$, [P], [X], and [Y] designate the thermodynamic equilibrium concentrations of the complexes, the unliganded protein, and the two ligands, respectively. Using the abbreviations

$$f(X, J) = \binom{N}{J}[X]^J$$
$$f(Y, K) = \binom{M}{K}[Y]^K \quad (11)$$

and introducing eq 10 into (5) we obtain

$$\bar{n}_X = \frac{\sum_{J=0}^{N}(Jf(X, J)a_J \sum_{K=0}^{M} f(Y, K)b_K \exp(-\overline{\delta F_{JK}}/RT))}{\sum_{J=0}^{N}(f(X, J)a_J \sum_{K=0}^{M} f(Y, K)b_K \exp(-\overline{\delta F_{JK}}/RT))} \quad (12)$$

We can further define a mean free energy coupling between X and Y in the PX_J complexes, weighted according to the values of K by the equation

$$\bar{m}_J = \frac{\sum_{K=0}^{M} f(Y, K)b_K \exp(-\overline{\delta F_{JK}}/RT)}{\sum_{K=0}^{M} f(Y, K)b_K} \quad (13)$$

Using this definition, eq 12 may be put into the characteristic Adair form

$$\bar{n}_X = \frac{\sum_{J=0}^{N} Jf(X, J)a_J'}{\sum_{J=0}^{N} f(X, J)a_J'} \quad (14)$$
$$a_J' = a_J\bar{m}_J$$

The N apparent dissociation constants for X of the Adair eq 14, $(K_1', K_2' \ldots)$, are determined by the quantities

$$a_J' = (K_1'K_2' \ldots K_J')^{-1} = (K_1K_2 \ldots K_J)^{-1}\bar{m}_J \quad (15)$$

As shown by eq 13 these are not true constants but functions of [Y]. However, if the total concentration of Y is kept constant at a value $[Y_0]$ and if $[Y_0]/M \gg [P_0]$ where $[P_0]$ is the molar concentration of protein in the solution, the changes in [Y] attending the dissociation or association of Y from or to the protein will be negligible. We can then set $[Y] = [Y_0]$, making the K' quantities constant *for each fixed value of* $[Y_0]$. The computation of the dissociation constants for X, over a whole range of $[Y_0]$ values can give a detailed picture of the influence of Y upon the binding characteristics of X. When $[Y_0]$ and $M[P_0]$ are comparable, the additional condition $[Y] = [Y_0] - \bar{n}_Y[P_0]$ must be introduced in the computations. Such a situation, which occurs, for example, when hemoglobin in the presence of a comparable molarity of diphosphoglycerate is titrated with oxygen, has been analyzed before (Weber, 1972).

Materials and Methods

Crystallized bovine serum albumin was purchased from Armour. Concentrated solutions (2%) in 0.1 M pH 7 potassium phosphate buffer were passed over a Sephadex G-150 column to eliminate aggregates and other impurities. The pooled column fractions containing bovine serum albumin were stored at 4°C, and, over a period of 4 months, showed no deterioration as measured by acrylamide disc gel electrophoresis. Concentrations of bovine serum albumin solutions were determined from absorption measurements using an absorption coefficient at 278 nm of $4.44 \times 10^4\ M^{-1}\ cm^{-1}$. Bovine serum albumin solutions for all binding studies were in 0.1 M pH 7 potassium phosphate buffer.

ANS was prepared as the magnesium salt according to the method of Weber and Young (1964). The potassium salt of ANS was prepared by making a solution of the magnesium salt basic with potassium hydroxide in an excess of potassium chloride, then recrystallizing by addition of ethanol. ANS concentrations were determined from absorption measurements based on an absorption coefficient of $6.3 \times 10^3\ M^{-1}\ cm^{-1}$ at 350 nm. This value, based on microanalysis of different batches of ANS, is in good agreement with the value recently reported by Ferguson and Cahnmann (1974). The earlier value of 4.9×10^3 used by this lab (Weber and Young, 1964) was based on the magnesium content of the ANS crystals. Analysis of different batches of ANS shows that the magnesium content is consistently high compared to the carbon content, probably due to the coprecipitation of other magnesium salts. Quinine bisulfate was prepared as in the previous paper (Kolb and Weber, 1975).

2,6-Dihydroxybenzoic acid (2,6-DHB), 2,4-dihydroxybenzoic acid (2,4-DHB), 3,5-dihydroxybenzoic acid (3,5-DHB), and *p*-hydroxybenzoic acid (4-HB) were purchased from Aldrich Chemical Company. All were further purified by recrystallization from water. Other reagents used were of reagent grade and were used without further purification.

Results

The binding of ANS to bovine serum albumin was measured by the fluorescence titration method of the previous paper. ANS was found to bind to four equivalent sites that could be measured by the increased fluorescence of ANS when bound to the protein. Comparison of these binding data with those determined by Pasby (1969) by equilibrium dialysis (Figure 1) demonstrated that the binding measured by the fluorescence method corresponded to the four tightest sites measured by equilibrium dialysis. Although more than 15 different sites could bind ANS with differing affinities, the fluorescence titration method detected only the first four sites.

In solutions of $1.0 \times 10^{-6}\ M$ bovine serum albumin containing no reducing agent, titration with ANS, both by the

method of Daniel and Weber (1966) and by the method described in the previous paper, proved very erratic, detecting about two binding sites for ANS on bovine serum albumin. The number of sites detected proved to be inversely proportional to the amount of stirring the protein solution underwent, even if the stirring was done manually with a small Teflon paddle. Small amounts of 2-mercaptoethanol (10^{-3} M) inhibited the loss of binding power without changing the other binding characteristics. Blocking the free sulfhydryl on bovine serum albumin with iodoacetamide by the method of Nikkel and Foster (1971) achieved the same results as the addition of 2-mercaptoethanol. The blocking technique reduced the free sulfhydryl detectable by Ellman's analysis (Ellman, 1959) from 0.58 mol/mol of bovine serum albumin to less than 0.05 mol/mol of bovine serum albumin.

Bovine serum albumin as received from Armour contained as much as 18% of aggregates as detected by acrylamide disc gel electrophoresis. Other workers have also found similar amounts (Stewart, 1973; Hagenmaier and Foster, 1971) so that this anomaly was not unexpected. Binding studies on protein solutions containing significant amounts of aggregates were erratic, both in stoichiometry of binding and strength of binding. "Monomer" bovine serum albumin isolated on a Sephadex G-150 column had a consistent stoichiometric ratio of four ANS sites per bovine serum albumin molecule and consistent binding affinities. Binding studies on "monomer" bovine serum albumin only are reported.

Residual amounts of free fatty acids have been shown to be present in bovine serum albumin preparations (Chen, 1967; Sogami and Foster, 1968). Since fatty acids compete with ANS for binding sites (Santos and Spector, 1972), it was desirable to check that the 0.7 mol of free fatty acid per mol of bovine serum albumin detected by the method of Chen (1967) did not interfere with ANS binding studies. Comparison of the binding of ANS by bovine serum albumin with the residual free fatty acid present and with the free fatty acid removed by the method of Sogami and Foster (1968) showed no detectable differences, implying that the residual free fatty acid does not interfere with ANS binding to the four sites detected fluorometrically. The alternative explanation that the fatty acid was bound much more weakly than ANS is untenable since the free fatty acid remains on the protein after dialysis. Also, fatty acid binding has been shown to be of comparable strength to that of ANS (Fletcher et al., 1970, among others).

By fluorescence titration ANS was found to bind to four different sites per bovine serum albumin molecule with a dissociation constant of 8.9×10^{-7} M. The Bjerrum plot of the results (Figure 2) had a span of 1.9 log units of ANS concentration between 10 and 90% ANS saturation, indicating that the binding was to independent and equivalent sites. A plot of the Hill coefficient as a function of the number of ANS bound confirmed that the ANS binding was to identical independent sites. All the points on this plot fell on or within experimental error of a Hill coefficient of one (Figure 4).

The hydroxybenzoic acids differed mainly in the strength of their interactions with ANS bound to bovine serum albumin. 2,5-DHB inhibited ANS binding the most strongly; a 1.2×10^{-3} M concentration was sufficient to raise the ANS dissociation constant to 2.5×10^{-5} M with a Hill coefficient at half ANS saturation of 1.15 (Figure 2). The disadvantage of 2,6-DHB was that it apparently competed

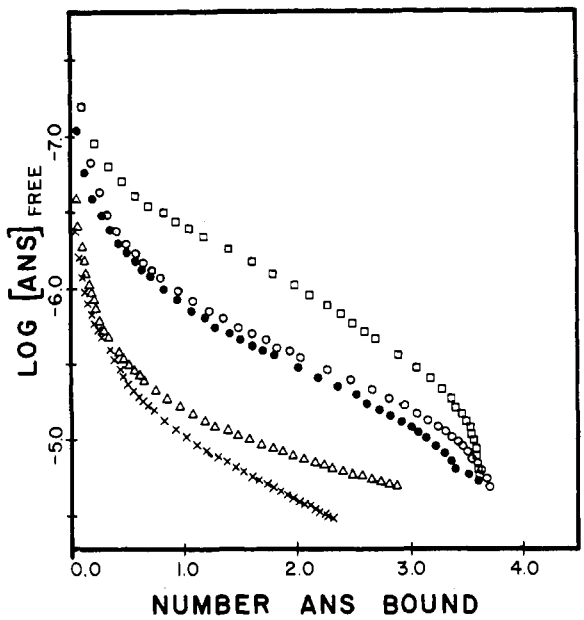

FIGURE 2: Comparison of the binding of ANS by bovine serum albumin in the presence of different effectors. (□) No effector present; (○) 2.0×10^{-3} M 3,5-DHB; (●) 1.0×10^{-3} M 4-HB; (△) 5.0×10^{-3} M 2,4-DHB; (X) 1.2×10^{-3} M 2,6-DHB. Titrations are of 1.0×10^{-6} M bovine serum albumin in the presence of 10^{-3} M 2-mercaptoethanol.

strongly for the ANS binding sites, with the result that the ANS dissociation constant was so large as to make the measurement of a complete ANS titration impossible by fluorescence means. 3,5-DHB inhibited ANS binding much less strongly, a 2.0×10^{-3} M concentration raising the ANS dissociation constant to 2.8×10^{-6} M with a Hill coefficient at half ANS saturation of 1.15. This weaker inhibition allowed higher concentrations of the effector 3,5-DHB to be used advantageously. The higher effector concentrations meant that the concentration of unbound effector remained constant during a titration. The weaker interaction with ANS binding made it possible to increase the effector (3,5-DHB) concentration enough to look for the disappearance of cooperative interactions, while still being able to complete a major portion of the titration. 2,4-DHB and 4-HB were also tested. 2,4-DHB interacted with ANS in a manner similar to that of 2,6-DHB, a 5×10^{-3} M concentration being sufficient to raise the ANS dissociation constant to 1.3×10^{-5} M 4-HB had properties very similar to those of 3,5-DHB, a 1.0×10^{-3} M concentration raising the ANS dissociation constant to 3.2×10^{-6} M with a Hill coefficient at half ANS saturation 1.2.

As is shown in Figure 3, 3,5-DHB interacted with ANS binding in more than a simple competitive manner. At 3,5-DHB concentrations of 2×10^{-4} M and below there was little or no change in the ANS dissociation constant from the 8.9×10^{-7} M found in the absence of 3,5-DHB and no change in the degree of cooperativity as shown by the span of the Bjerrum plot. At a 3,5-DHB concentration of 2.0×10^{-3} M the ANS dissociation constant was raised to 2.8×10^{-6} M, and at the same time, the span of the Bjerrum plot was reduced by about 0.3 log unit. This cooperative effect was also seen in the plot of the Hill coefficient as a function of ANS saturation (Figure 4). The maximum Hill coefficient attained was 1.5 at 3 ANS molecules bound per bovine serum albumin molecule (on the average). At a higher 3,5-DHB concentration of 2.0×10^{-2} M, the span of the Bjerrum plot increased to near normal and the ANS disso-

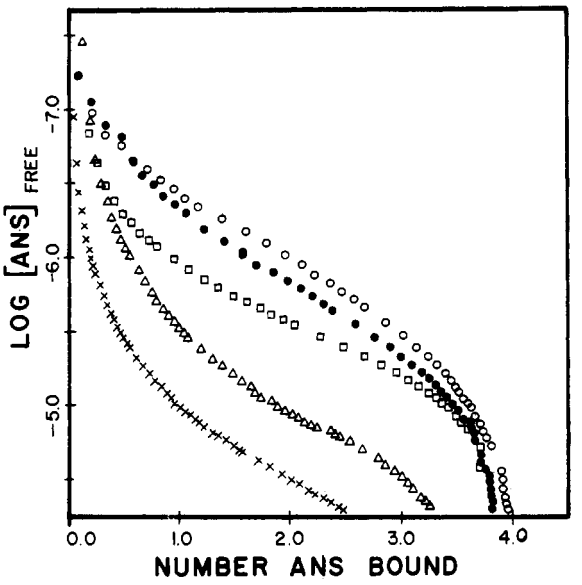

FIGURE 3: Effect of 3,5-DHB on the binding of ANS by bovine serum albumin. (○) No effector; (●) 2.0×10^{-4} *M* 3,5-DHB; (□) 2.0×10^{-3} *M* 3,5-DHB; (△) 2.0×10^{-2} *M* 3,5-DHB; (X) 6.0×10^{-2} *M* 3,5-DHB. Titrations are of 1.0×10^{-6} *M* bovine serum albumin in the presence of 10^{-3} *M* 2-mercaptoethanol.

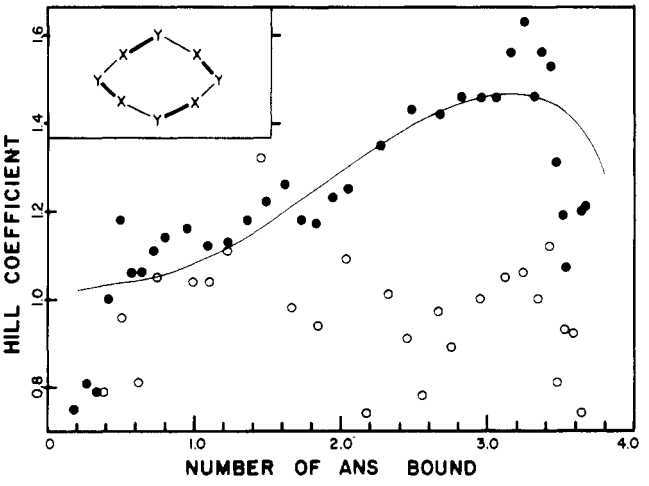

FIGURE 4: Hill coefficient for ANS binding by bovine serum albumin as a function of the number of ANS bound. The theoretical curve is for the model shown in inset where each effector Y interacts with two ligands X with interaction energies of 3.0 and 3.5*RT* units and [Y]/*K*(Y) = 4. The experimental points are computed from titrations of 1.0×10^{-6} *M* bovine serum albumin in the presence of 10^{-3} *M* 2-mercaptoethanol: (○) no effector; (●) 2.0×10^{-3} *M* 3,5-DHB. The values of the Hill coefficient at each point were calculated as the ratio of finite differences $\Delta \log (4/\bar{n} - 1)/\Delta \log$ [ANS] for neighboring points in the titration curves. The greater dispersion of the results in the absence of effector results mainly from the lower values of free ANS in this case, which increases the uncertainty in the measured quantities.

ciation constant was raised to 1.1×10^{-5} *M*. Increasing the 3,5-DHB concentration still further to 6.0×10^{-2} *M* raised the ANS dissociation constant still further to 3.5×10^{-5} *M*, but the shape of the Bjerrum plot did not change further. This lack of limit to the shift in the ANS dissociation constant implied that the 3,5-DHB was not only interacting with ANS as an effector from other sites, but was also competing directly for the same binding sites. For this reason, an interaction energy between ANS and 3,5-DHB could not be directly determined.

To eliminate the possibility that the divalent magnesium

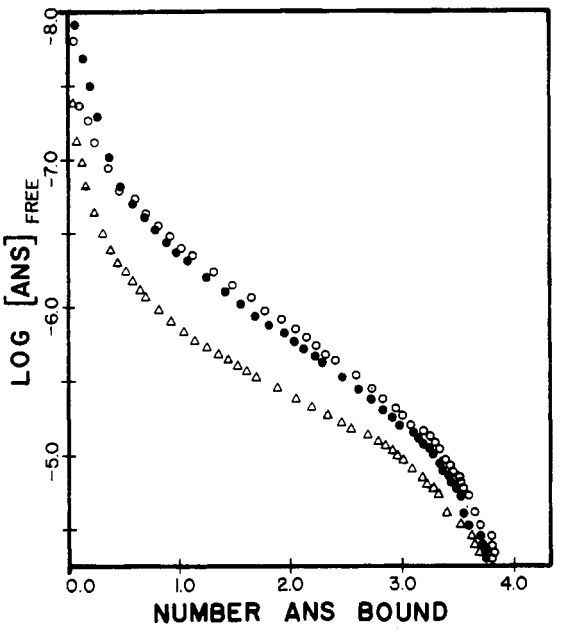

FIGURE 5: Effect of Mg^{2+} ion on the binding of ANS by bovine serum albumin. (●) Mg^{2+} salt of ANS—no effector; (○) K^+ salt of ANS—no effector; (△) K^+ salt of ANS and 2.0×10^{-3} *M* 3,5-DHB. Titrations are of 1.0×10^{-6} *M* bovine serum albumin in the presence of 10^{-3} *M* 2-mercaptoethanol.

cation was acting as a complexing agent and affecting the interaction between ANS and 3,5-DHB, the experiments were repeated using the potassium salt of ANS instead of the magnesium salt. The results of all experiments using the potassium salt of ANS were the same as those using the magnesium salt (Figure 5).

The method of titration used assumes that the quantum yield of ANS bound to bovine serum albumin is the same in the presence or absence of the different effectors used. The validity of this assumption was demonstrated by the fact that the measured fluorescence lifetime of ANS bound to bovine serum albumin is the same at saturation of the ANS binding sites with ANS in the presence and absence of 3,5-DHB. In addition, a stoichiometric titration of bovine serum albumin with ANS is not affected by the presence of 3,5-DHB.

Discussion

The interactions of ANS and 3,5-DHB bound to bovine serum albumin do not lend themselves to the straightforward analysis that was possible when the interacting ligands were oxalate and NADH bound to lactate dehydrogenase (Kolb and Weber, 1975). NADH and oxalate are sufficiently different, so that the changes in the free energy of binding of one ligand in the presence of the other may be assumed to arise from energy coupling in the ternary complexes with negligible or no contribution from direct competition for each other's binding site. This assumption is obviously not tenable in the case of binding of ANS and 3,5-DHB by bovine serum albumin, where some affinity of the latter ligand for what we called the inner sites, albeit smaller than the affinity for the outer sites, is to be expected. Then, if the concentration of 3,5-DHB is increased indefinitely the competition for the inner sites will result in a continuous decrease in the binding affinity of ANS, rather than the attainment of a clear limiting value as was the case for the NADH-oxalate pair. Consequently, a value of *K*(ANS/

3,5-DHB) cannot be directly determined and ΔF_{XY} obtained from the ratio of this value to K(ANS). An indirect method must be adopted, namely the comparison of the free energy change and Hill coefficients experimentally obtained with those of some simple interactive models computed according to the theory sketched before.

At a millimolar concentration of 3,5-DHB many molecules of this anion are bound, probably in excess of 10. Therefore a large variety of coupling schemes between these molecules and the four molecules of ANS bound to the inner sites may be conceived. We arbitrarily assumed that only four of the dihydroxybenzoate molecules were energetically coupled to ANS. The coupling scheme adopted is shown in the inset of Figure 4. The continuous curve in this figure gives the variation of the Hill coefficient with number of ANS molecules bound per serum albumin molecule when the two coupling free energies postulated are assumed to have values of +3.0 and +3.5RT units, and the dissociation constant of 3,5-DHB is assumed to have a unique value of 5×10^{-4} M. Apart from fitting well the values of the Hill coefficients this model predicts an increment in the value of log ANS at the midpoint of the titration curve of 0.7 unit not too distant from the observed value of 0.5. Evidently there are too many adjustable parameters to give these computations a value other than the demonstration of the suitability of the qualitative explanation of the effects.

Conclusions

The appearance of cooperative binding effects in a single-chain protein, induced by the binding of a second ligand, negates the proposition that these effects are to be found exclusively in oligomeric proteins, or that structural symmetry is necessarily involved in their production. The possibility must be kept in mind that the cooperative binding effects, either directly observed by measurements of chemical equilibria, or inferred from observations on steady-state catalysis, arise from interactions of two kinds of ligands bound to the protein and do not have the relation to overall protein conformation that is often postulated. In a recent study Shaklai et al. (1975) have demonstrated that the cooperative oxygen binding by *Levantina hierosolima* arises from oxygen-calcium ion interactions and is not directly related to the changes in protein aggregation in the system. (Klarman et al., 1975).

As we have discussed elsewhere (Weber, 1972, 1975) the globular proteins on account of their multiple, weak internal interactions and their low-dielectric constant core are well suited to the establishment of ligand interactions. Many cases are known of polyvalent, multiple ligand binding so that the conditions for the appearance of effects like those studied here must be present in many, perhaps in most of them. In attempting to demonstrate these effects two important requisites must be kept in mind. Firstly, a number of titrations must be carried out maintaining the concentration of all the effector ligands constant, while only one particular ligand concentration, the titrating liand, is varied. The effector concentration must then be varied over a considerable range to define the optimum for the observation of cooperativity of binding of the titrating ligand, and to prove that it vanishes for concentrations much lower or much higher than the optimum. Secondly, a satisfactory calculation of the Hill coefficient along the titration curve, such as is presented in Figure 4, demands a considerable density of experimental points (Weber and Anderson, 1965) of sufficient precision. Because these conditions are often difficult to fulfill, the literature of ligand binding shows few examples of the kind that we have described here. However, since ligand interactions are involved in some of the most important protein functions, the efforts to describe them as completely as possible appears to be not just valuable but altogether indispensable to our understanding of those functions.

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