Ligand Binding and Internal Equilibria in Proteins†

Gregorio Weber*

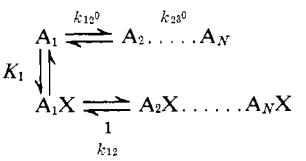
ABSTRACT: An examination is carried out of the relations between the binding of small ligands to proteins (micro-associations), the tautomerizations of the peptide chain (conformational changes) and the second- and higher order interactions among the chains (macroassociations). This task is facilitated by the introduction of "apparent" and "conditional" standard free energies. By consideration of the simultaneous binding of two ligands which stabilize different conformations of the peptide chain it is demonstrated that a protein cannot adopt an absolute conformation dependent upon the binding of one ligand in particular. It is also shown that multiple binding of one ligand in the presence of a second ligand can give rise to cooperative effects whether the two types of ligands oppose or enhance each other's binding. The coupling between the free energies of micro- and macro-association is introduced by considering a dimer made up of two identical protomers. Coupling between the two association processes may occur whether the ligand binding has simple, cooperative, or anticooperative character. The relations between changes in macroassociation and in quaternary structure are then examined and it is made clear that the latter are only a particular case of the former which obtains when the protomer concentrations are greater than all relevant macrodissociation constants of the system. The coupling of micro- and macroassociations is generalized to multimers of any order by considering it to be the sum of pairwise protomer interactions. Explicit calculations have been made for various models of tetramers of the type $\alpha_2\beta_2$. Some of the models are formally very close to those of direct heme–heme interaction proposed by Pauling in 1935 but admit a very different physical interpretation. The interactions possible between macroassociations and the binding of two types of ligands are then discussed. Computations have been carried out for a system that mimics the hemoglobin–oxygen–diphosphoglycerate equilibrium. The view that diphosphoglycerate is always released upon complete oxygenation is shown to be contrary to the principles of chemical equilibrium. The multiplicity of interactions and the variety of effects that follow from them show that multimer proteins are unlikely to be limited to a very small number of allowed conformations, as is currently believed. This latter view is contrasted with the one emerging from our analysis which considers the protein as subject to the same stochastic limitations that are known to apply to simpler molecules. The flexibility of behavior that this implies is believed necessary for the explanation of many important physiological effects.

In considering the possible classes of chemical equilibria in proteins in solution, we are naturally led to distinguish the following possibilities: (a) the binding equilibria between the protein and added ligands, usually small molecules; (b) the internal, first-order equilibria between parts of the same peptide chain; (c) the second- and higher order equilibria among the several covalent chains that constitute the multichain proteins, or among the macromolecules in single-chain proteins. Evidently no one of these equilibria can be treated in isolation from the others. In the interactions of proteins and small ligands the changes in the free energy of the system upon ligand binding correspond not only to the process under point a but they must, to a negligible or appreciable extent, depending upon the case, involve contributions from processes under the three headings.

The interdependence of all these processes may be described in principle by the use of the theory of linked functions due to Wyman (1948, 1964, 1965). The theory possesses the necessary generality but does not lend itself to an intuitive approach, so necessary in the practical applications. We have therefore taken an alternative, and in our opinion more direct route, by the introduction of apparent free energies and more important of conditional free energies. We have used no other principle besides that of free-energy conservation. This principle is the key to any consideration of dependent equilibria as is made clear in the more recent of Wyman's publications. The simple way in which we have used it, if not the most rigorous, seems to us the one which makes most transparent the relations among the processes dependent on ligand binding.

Binding by an Isolated Peptide Chain

In the simplest case, that of a unique, isolated peptide chain binding a single mole of ligand, the interchain equilibria under c are excluded and the processes a and b above are the only operative ones. If the chain can exist in a number of conformations $A_1 \ldots A_N$ the possible equilibria take the form



The observations upon the equilibrium of the chain with the small ligand X in solution yield only an apparent dissociation constant K_{app} defined[1] without regard to the possible protein

† From the Department of Biochemistry, School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801. *Received October 11, 1971.*

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[1] For the definition of dissociation constants K, binding constants R, and corresponding free energies of dissociation and association, respectively, see Appendix.

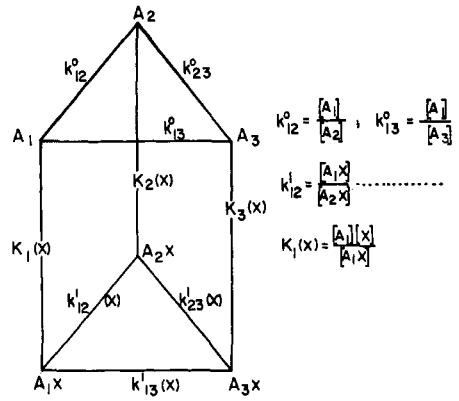


FIGURE 1: A_1, A_2, and A_3: chain conformations. k^0_{12} and k^0_{13}: first-order equilibrium constants of unliganded conformations. $k^1_{12}(X)$ and $k^1_{13}(X)$: first-order equilibrium constants of X-liganded conformations. $K_1(X)$, $K_2(X)$, and $K_3(X)$: second-order equilibrium constants of X.

forms present before or after ligand binding. K_{app} is therefore defined by the eq 1.1.

$$K_{app}(X) = \frac{([A_1] + [A_2] + \ldots [A_n])[X]}{[A_1X] + \ldots\ldots + [A_NX]} \qquad (1.1)$$

K_{app} may always be written as a function of the first-order equilibrium constants that govern the equilibria among the several isomeric liganded and unliganded forms of the chain (Weber, 1965). For simplicity we may assume that we are dealing with three chain conformers only (A_1, A_2, and A_3) and that in the absence of ligand the equilibrium greatly favors A_1 to the point that we can consider A_1 to be *the* unliganded conformation in solution. We shall determine the effects that result from combination with the ligands X and Y which, respectively, stabilize the forms A_2 and A_3. We assume that X and Y bind at different places thus excluding from the beginning the trivial case of competition of X and Y for the same binding site. The transformations among the species assumed imply the equilibria and the equilibrium constants shown in Figure 1.

$$k_{12}{}^0 = \frac{[A_1]}{[A_2]} \qquad k_{13}{}^0 = \frac{[A_1]}{[A_3]}$$

$$k_{12}{}^1(X) = \frac{[A_1X]}{[A_2X]} \qquad k_{13}{}^1(X) = \frac{[A_1X]}{[A_3X]} \qquad (1.2)$$

$$K_1(X) = \frac{[A_1][X]}{[A_1X]}$$

A similar scheme corresponds to the binding of Y. We can easily deduce from (1.2) that for the case in which X alone is present in solution

$$K_{app}(X) = K_1(X)\frac{1 + (k_{12}{}^0)^{-1} + (k_{13}{}^0)^{-1}}{1 + (k_{12}{}^1)^{-1} + (k_{13}{}^1)^{-1}} \qquad (1.3)$$

From our hypothesis of the prevalence of A_1 in the absence of ligand $(k_{12}{}^0)^{-1}$, $(k_{13}{}^0)^{-1} \ll 1$. Also if ligand X stabilizes preferentially the form A_2X, we must have 1, $(k_{13}{}^1)^{-1} \ll (k_{12}{}^1)^{-1}$. With these assumptions (1.3) becomes

$$K_{app}(X) \simeq K_1(X)\cdot k_{12}{}^1(X) = K_2(X)\cdot k_{12}{}^0 \qquad (1.4)$$

$$\Delta F_{app}(X) \simeq \Delta F_1(X) + \Delta F_{12}{}^1(X) = \Delta F_2(X) + \Delta F_{12}{}^0$$

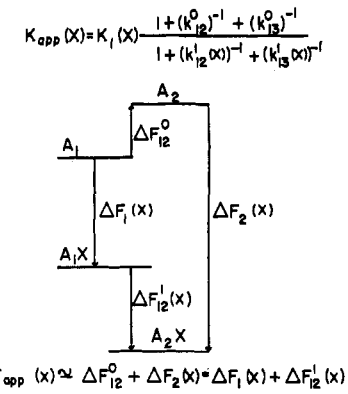


FIGURE 2: Significance of ΔF_{app}, the apparent free energy of binding.

The apparent free energy of binding as shown in Figure 2 is determined in part by the displacement of the internal equilibrium among the chain tautomers. In the same way, if ligand Y stabilizes the form A_3

$$\Delta F_{app}(Y) \simeq \Delta F_1(Y) + \Delta F_{13}{}^1(Y) = \Delta F_3(Y) + \Delta F_{13}{}^0 \qquad (1.5)$$

We can understand without difficulty the extent of the stabilization of a given conformation by each separate ligand, X or Y. The question that presents itself is: "What is the conformation assumed when both X and Y are in solution in concentrations comparable to or greater than their respective apparent dissociation constants?"

The overall energetics of the system is described by three apparent free energies, $\Delta F_{app}(X)$, $\Delta F_{app}(Y)$, and $\Delta F_{app}(XY)$, defined as the free-energy changes in the binding of 1 mole of X, 1 mole of Y, or both together by 1 mole of protein, respectively. As shown in Figure 3 there are two further conditional free energies of binding, $\Delta F_{app}(X/Y)$ and $\Delta F_{app}(Y/X)$, corresponding to the binding of X when Y is already bound and the binding of Y when X is already bound, respectively. Free-energy conservation results in the relations

$$\Delta F_{app}(X) + \Delta F_{app}(Y/X) = \Delta F_{app}(Y) + \Delta F_{app}(X/Y) \qquad (1.6)$$

$$-\Delta F_{app}(X) + \Delta F_{app}(X/Y) = -\Delta F_{app}(Y) + \Delta F_{app}(Y/X) = \Delta F_{XY} \qquad (1.7)$$

$$\Delta F_{app}(XY) = \Delta F_{app}(X) + \Delta F_{app}(Y) + \Delta F_{XY} \qquad (1.8)$$

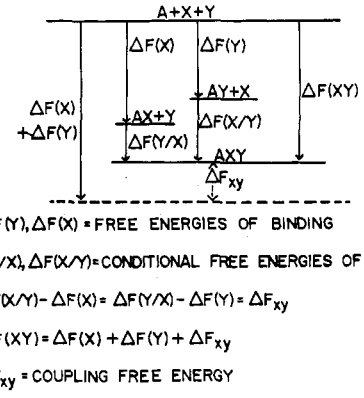


FIGURE 3: Conditional free energies of binding $\Delta F(X/Y)$ and $\Delta F(Y/X)$ and coupling free energy ΔF_{XY}.

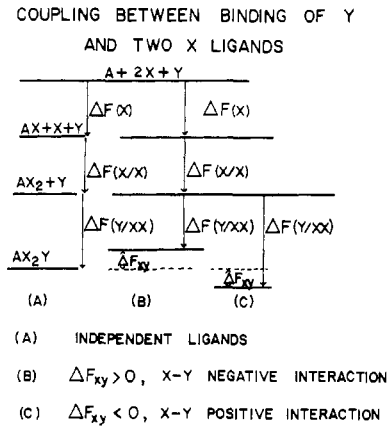


FIGURE 4: Coupling between X and Y, when 2 moles of X and 1 of Y are bound.

ΔF_{XY} represents the unique difference between the free energies of binding of one ligand with the unliganded chain and with the chain saturated with the other ligand. ΔF_{XY} is thus a coupling free energy between the two ligands. We can distinguish three cases depending upon its value. (1) $\Delta F_{XY} = 0$.[2] There is no appreciable interaction between X and Y. If the conformations A_2 and A_3 are distinguishable by some physical criterion the binding of X and Y must modify substantially independent regions of the chain. (2) $\Delta F_{XY} < 0$. In this case the ligands promote each other's binding and they must necessarily stabilize the same conformation, or to put it in more general terms A_2 and A_3 must be much closer to each other than to A_1. (3) $\Delta F_{XY} > 0$. This case in which the ligands hinder each other's binding is the more interesting of the three. If the concentration of free X is large in comparison to $K(X/Y) = K(X)\cdot\exp(\Delta F_{XY}/RT)$ the site for X will be virtually saturated. Similarly if the concentration of free Y is large compared to $K(Y/X) = K(Y)\cdot\exp(\Delta F_{XY}/RT)$ the site for Y will also be saturated. When both X and Y are present at these concentrations, the conformation of the chain will not correspond to A_2 or A_3 but some intermediate form which partakes from the character of both although differing from either by the free-energy content ΔF_{XY} derived from the binding of *both* ligands. We have therefore demonstrated that if a protein combines with two ligands that oppose each other's binding–in the nontrivial sense of not occupying the same binding site–the conformation is one determined by both acting together. When we recall the large number of ligands from protons and other ions onward present in the biological environment or added for experimental purposes we may feel sure that some of them must have a negative influence upon the binding of others and therefore that we *cannot ascribe to the protein an absolute conformation brought about by the binding of one ligand species in particular without regard for the presence of other ligands.*

Moreover whatever conformational changes are to be expected when a given ligand is added will critically depend upon the concentration of the components of the system. If in the example just analyzed we suppose the concentration of Y to be intermediate between $K(Y)$ and $K(Y/X)$ addition of an excess of X will result in the release of Y and the characteristic appearance of the A_2, or X-stabilized conformation. On the other hand, if Y is in concentration appreciably larger than $K(Y/X)$, addition of X will not produce the release of Y, nor the appearance of the A_2 conformation, but one which differs from it by a free energy close to or equal to ΔF_{XY}.

From the conditions of equilibrium the saturation of the protein chain for X or Y is easily determined. The dissociation constants corresponding to the energies $\Delta F(X)$, $\Delta F(Y)$, $\Delta F(X/Y)$, and $\Delta F(Y/X)$ are given by the equations

$$\begin{aligned} K(X) &= \exp(\Delta F(X)/RT) \\ K(Y) &= \exp(\Delta F(Y)/RT) \\ K(X/Y) &= K(X)\cdot\exp(\Delta F_{XY}/RT) = \beta\cdot K(X) \\ K(Y/X) &= \beta\cdot K(Y) \end{aligned} \tag{1.9}$$

The saturations[3] are

$$S_X = ([AX] + [AXY])/([A] + [AX] + [AY] + [AXY])$$
$$S_Y = \ldots.$$

$$\begin{aligned} S_X &= [X]/\left[[X] + \beta K(X)\frac{[Y] + K(Y)}{[Y] + \beta K(Y)}\right] \\ S_Y &= [Y]/\left[[Y] + \beta K(Y)\frac{[X] + K(X)}{[X] + \beta K(X)}\right] \end{aligned} \tag{1.10}$$

As shown by eq 1.10 if the free concentration of one of the ligands is kept constant and the other is varied, a normal titration curve is obtained with an apparent dissociation constant depending upon the concentration of the other ligand. If $\Delta F_{XY} < 0$ (or $\beta < 1$) the ligands enhance each other's binding and the apparent dissociation constant is smaller than the constant observed when one ligand alone is present. If $\Delta F_{XY} > 0$ ($\beta > 1$) the opposite is observed. If both ligands vary simultaneously, an apparent cooperative or anticooperative effect is obtained depending upon β being smaller or larger than one.

An example of the relation between ligands that we have just described may be given as follows. Suppose the peptide chain in question to be a molecule of polyglutamate. The conformational change brought about by the binding of protons is the random coil–helix transition. A ligand L may be expected to bind well to the coil but for steric reasons fail to bind, or bind much more weakly to the helix. If the concentrations of both protons and L are large enough the system will adopt an intermediate conformation, neither fully that of a helix or a coil but most probably consisting of segments of helix interrupted at those points where L is bound. There is no reason to believe that peptide chains in proteins behave in any different fashion, as regards the conformations that are imposed by the binding of ligands.

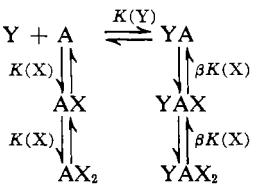
Multiple Ligand Interactions. The interactions between two types of ligands bound to the same protein chain need not be confined to one of each class, and particularly interesting effects occur when one molecule of ligand Y can affect two or more X sites at the same time. The physical nature of the interactions whether exerted through modification of the conformation or to simple electrostatic effects is unimportant, a single thermodynamic description being sufficient for all cases.

[2] $\Delta F_{XY} < 0$ (>0) means that ΔF_{XY} has the same (opposite) sign to $\Delta F(X)$ and $\Delta F(Y)$.

[3] It will be observed that S_X and S_Y obey the reciprocity relation characteristic of linkage (Wyman, 1964), namely $[X](dS_Y/d[X]) = [Y](dS_X/d[Y])$.

Consider for simplicity that the molecule A is capable of binding two moles of ligand X and one of Y at spatially different sites so that the species AX_2Y may be formed at finite concentrations within experimental reach. The possible interactions among the ligands X and Y are shown by means of the free-energy level scheme of Figure 4. A identifies the conditions of independent ligand binding in which the total free energy of formation of the complex AX_2Y is the sum of the free energies of formation of the separate complexes AX_2 and AY. B represents the case in which X and Y oppose each other binding ($\Delta F_{XY} > 0$), and C is the case in which X and Y stabilize each other's complex ($\Delta F_{XY} < 0$). As depicted in the figure Y affects equally the binding of both X ligands so that the free energy of coupling of each X with Y equals $\Delta F_{XY}/2$. This restriction is not generally necessary.

The equilibria and the dissociation constants involved are



(1.11)

Setting $[Y]/K(Y) = \epsilon$, the average number of moles of X bound by A equals

$$\bar{n}_x = 2\frac{\frac{[X]}{K(X)}\left[\frac{1+\epsilon\beta^{-1}}{1+\epsilon}\right] + \frac{[X]^2}{K(X)^2}\left[\frac{1+\epsilon\beta^{-2}}{1+\epsilon}\right]}{1 + 2\frac{[X]}{K(X)}\left[\frac{1+\epsilon\beta^{-1}}{1+\epsilon}\right] + \frac{[X]^2}{K(X)^2}\left[\frac{1+\epsilon\beta^{-2}}{1+\epsilon}\right]} \quad (1.12)$$

The binding is seen to correspond to a system with *two* dissociation constants for X, $K_1(X)$, and $K_2(X)$

$$K_1(X) = K(X)\frac{1+\epsilon}{1+\epsilon\beta^{-1}} \qquad K_2(X) = K(X)\frac{1+\epsilon\beta^{-1}}{1+\epsilon\beta^{-2}} \quad (1.13)$$

$$\frac{K_1(X) - K_2(X)}{K(X)} = \frac{\epsilon(1-\beta^{-1})^2}{(1+\epsilon\beta^{-1})(1+\epsilon\beta^{-2})} \quad (1.14)$$

Since both numerator and denominator of eq 1.14 are always positive it follows that $K_1(X) \gtrless K_2(X)$, the equality applying only when $\beta = 1$, that is, in the absence of X–Y interactions. Thus whether these X–Y interactions are positive or negative they *always* give rise to cooperative effects. If $\beta < 1$, that is when X and Y stabilize each other's binding, cooperativity is only observed when $\epsilon < 1$, that is when Y is present but the AY complex is mostly dissociated. Contrariwise if $\beta > 1$, that is when Y and X oppose each other's binding, cooperativity is only observed when $\epsilon > 1$, that is when the AY complex is formed. In both cases if the concentration of Y is very much larger than $\beta K(Y)$, no cooperative effects are observed but the unique dissociation constant for X appears then increased or decreased by the presence of Y. In other words for the appearance of cooperative effects the concentration of Y must be such that the AY complex changes its degree of saturation in the presence of X.

Effect of Interchain Interactions

In introducing the effects of protomer interactions enumerated as c above it becomes necessary to distinguish clearly between two classes of second-order processes. Those that involve a protomer and a small molecule will be called here *microassociations*, while those involving protomers alone will be called *macroassociations*. We start the description of the relations between micro- and macroassociations with the simplest case: two identical protomers that can associate giving rise to a dimer–monomer equilibrium. The dimer A_2 combines successively with two moles of X giving A_2X and A_2X_2. The quantities $^2\Delta F_0$, $^2\Delta F_1$, $^2\Delta F_2$ are the free energies of association of the species A_2, A_2X, and A_2X_2, respectively, from the protomers A, AX. ΔF_{21}, ΔF_{22} are the standard free energies of the reactions $A_2 + X \rightarrow A_2X$, and $A_2X + X \rightarrow A_2X_2$. ΔF_{11} is the free energy of the isolated protomer–ligand association ($A + X \rightarrow AX$). The coupling between the processes of macro- and microassociation is shown in Figure 5 by means of a free-energy level scheme. It is easily deduced from the figure that

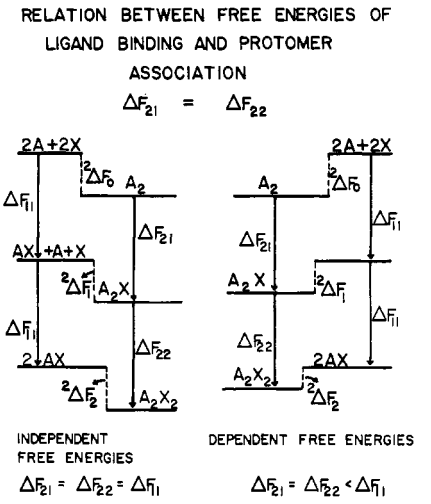


FIGURE 5: Relations between the free energies of ligand binding and the association of the protomers. Case of equal ligand binding free-energies ($\Delta F_1 = \Delta F_2$). At left case of independence of macroassociation from ligand binding. At right case in which the free energies of macro- and microassociation are dependent upon each other.

$$\Delta F_{21} = \Delta F_{11} + {}^2\Delta F_1 - {}^2\Delta F_0 \quad (2.1)$$

$$\Delta F_{22} = \Delta F_{11} + {}^2\Delta F_2 - {}^2\Delta F_1 \quad (2.2)$$

If the intrinsic free energy of binding of the two protomers is the same, as assumed above, ΔF_{21} and ΔF_{22} can only differ when the free energies of macroassociation fail to obey the equality

$$ {}^2\Delta F_1 - {}^2\Delta F_0 = {}^2\Delta F_2 - {}^2\Delta F_1 \quad (2.3)$$

$$ {}^2\Delta F_1 = {}^1/_2({}^2\Delta F_0 + {}^2\Delta F_2) \quad (2.4)$$

which implies that $^2\Delta F_1$ is the mean of $^2\Delta F_0$ and $^2\Delta F_2$, the free energies of association of the unliganded and fully liganded dimers, respectively. Equation 2.4 may be satisfied in two cases which represent two very different physical situations

$$ {}^2\Delta F_0 = {}^2\Delta F_1 = {}^2\Delta F_2 \quad (2.5)$$

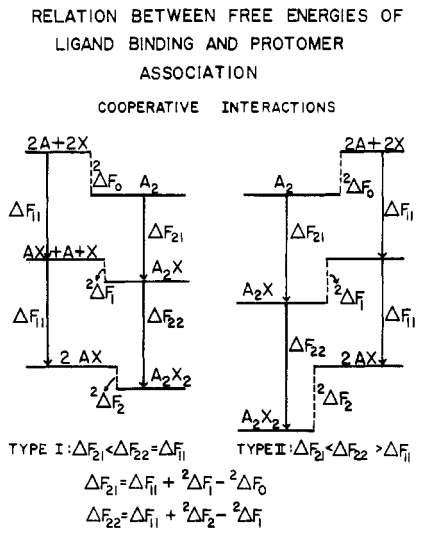


FIGURE 6: Relation between the free energies of ligand binding and association of the protomers. Cases of cooperative ligand binding. At left the case in which ligand binding promotes dissociation of the dimer; at right case in which it promotes association of the protomers.

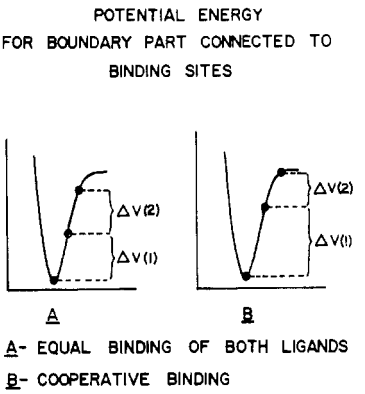


FIGURE 7: Showing the possible changes in potential energy at the boundary between protomers following ligand binding. $\Delta V(1)$ and $\Delta V(2)$: changes in potential energy associated with the binding of the first and second ligand. (A) Case of $\Delta V(1) = \Delta V(2)$; (B) case in which $\Delta V(1) > \Delta V(2)$.

in which case ligand binding and protein association are independent phenomena, and

$$^2\Delta F_0 \neq {}^2\Delta F_1 \neq {}^2\Delta F_2 \qquad (2.6)$$

In this latter case macro- and microassociations are dependent but eq 2.4 holds because the first and second ligands bound make equal contributions to the free energy of protomer association. The important point follows that the equality $\Delta F_{21} = \Delta F_{22}$ does not permit one to conclude that micro- and macroassociations are independent.

Cooperative and Anticooperative Processes. Evidently if $\Delta F_{21} \neq \Delta F_{22}$ eq 2.4 is not obeyed. Cooperative processes, that is those in which $\Delta F_{21} < \Delta F_{22}$, require that

$$^2\Delta F_2 - {}^2\Delta F_1 > {}^2\Delta F_1 - {}^2\Delta F_0 \qquad (2.7)$$

As shown by Figure 6 cooperative behavior can arise both when the greater part in the change of macroassociation free energy takes place upon the binding of the first or second ligand. These two cases are however easily distinguishable in practice.

If ligand binding promotes dissociation and $^2\Delta F_0 > {}^2\Delta F_1 \sim {}^2\Delta F_2$ we must necessarily have $\Delta F_{21} < \Delta F_{11}$, a decreased affinity of the dimer for the first ligand bound as compared to the isolated protomer. On the other hand, if ligand binding promotes association and $^2\Delta F_0 \sim {}^2\Delta F_1 < {}^2\Delta F_2$ it follows that $\Delta F_{21} \sim \Delta F_{11} < F_{22}$ and the affinity of the dimer for the first ligand is equivalent to that of the isolated protomer while the affinity for the second ligand is greater than them. Anticooperative cases follow, *mutatis mutandis*, corresponding rules.

We have already seen that changes in the free energy of protomer association and ligand binding are possible when $\Delta F_{21} = \Delta F_{22}$. Therefore cases of cooperative and anticooperative binding are simply those cases of dependence of macro- and microassociations in which there is an unequal sequential contribution of ligand binding to the free energy of protomer association.

The Physical Basis of the Interaction between Macro- and Microassociations. When a ligand is bound by a protein interactions arise in which groups in the ligand and the protein participate and certain points along the peptide-chain-forming part of the binding site become subjected to forces not present before. Because of the continuity of the covalently bonded peptide chain and the compact character of its folding the effects of these forces may be transmitted and felt at many points in the structure. The places where the strains thus set are largest and where the local bonds are weakest are those where structural rearrangements are most likely to take place. In multimer proteins an additional region of this kind is provided by the boundary between subunits. The bonds between the residues or atoms at both sides of the boundary are generally much weaker than those that maintain the tertiary structure within each protomer, as witnessed by the fact that dissociation and hybridization appear to take place far more readily than unfolding and denaturation. From these considerations it follows that we must expect changes in the free energy of protomer association with ligand binding. Figure 7 gives an oversimplified physical representation of the kind of difference that may be responsible for cooperativity when ligand binding results in boundary displacements. It shows the usual potential energy curve as a function of distance between a pair of short-range interacting structures. The points on the curves correspond, from bottom to top, to the energies for zero, one, and two ligands bound by the dimer. In A the effects of binding the first and second ligands are almost or exactly equal. The free energy of macroassociation will change with binding but no cooperativity will be observed. In B the binding of the first ligand will produce most of the total boundary change and cooperative binding will ensue. It is clear that the necessary condition for cooperativity shown in B is by no means sufficient. For example, a pair of protomers α and β with two contacts having the property B may cancel each other's effects. The contribution of ligand binding to the free energy of macroassociation may be expected to be only a few times the thermal energy, to judge from the observations on hemoglobin (Rossi-Fanelli *et al.*, 1964), and a free-energy change of this small magnitude need only to involve changes in a few atomic contacts. We know from the structural studies of Perutz and coworkers (Perutz *et al.*, 1968; Bolton and Perutz, 1970) that the interprotomer contacts in hemoglobin involve

some hundred atomic contacts for each pair so that the possibility of detecting and pinpointing the relevant contacts by present-day methods of study of proteins in solution seem most unlikely. Even more so the changes set up at the binding site of the remaining unliganded protomers when one of them is bound by a ligand may be dismissed as virtual unobservables. Therefore the negative results of the nuclear magnetic resonance observations of half-liganded hemoglobin reported by Shulman *et al.* (1969) are fully to be expected. The same may be said of the observations to the effect that deoxygenation of crystals of lamprey or sheep hemoglobin does not result in disorder of the lattice (Chance and Rumen, 1967) or in conspicuous optical retardation changes (B. Chance and Inouye, personal communication) although oxygen is bound cooperatively by the crystals as well as by the solutions.

Relations of Macroassociation and Quaternary Structure. Descriptive theories of the origin of cooperative or anticooperative binding in multichain proteins have postulated as dependent equilibria the microassociation and a process of macrotautomerization involving the mutual stabilization of certain conformations of the protomers. *All* such processes of tautomerization are included within the scheme of eq 2.1 and 2.2 as particular cases that appear *when the protomer concentration is greater than the largest of the three macrodissociation constants of the dimer.* In this case the unliganded and liganded dimers must differ in some structural detail of the interacting protomers to account for the different ease of dissociation. In principle changes in macroequilibria, *at protomer concentrations large compared to the macrodissociation constants,* may be described by specifying either the free energies of association of the possible aggregates of liganded and unliganded protomers (macroassociation constants) or a series of tautomerization constants describing the new conformations in the protomer aggregates following the successive stages of ligand binding. The number of tautomeric protomer arrangements necessary to describe all the theoretically possible cases must be *at least* as large as the number of liganded states being then three for a dimer and five for a tetramer. Thus the assumption of Monod *et al.* (1965) that a protein multimer may exist in only two mutually exclusive conformations limits the description to cases without changes in conformation, which results in independent ligand binding and cases of tautomerization which are *always* accompanied by cooperative ligand binding. Anticooperative cases, and cases of apparently independent ligand binding with changes in conformation (eq 2.4 and 2.6), *cannot* be described by the simple scheme of two unique conformations. A more general scheme that assumes multiple tautomerizations and makes it possible to described the two last types is due to Koshland *et al.* (1966). Koshland *et al.* (1966) define first-order constants K_{AB}, K_{AA}, and K_{BB}—of which the last two are composite values—dependent upon three free energies of tautomerization: $\Delta F(A \rightarrow B)$ belonging to an isolated protomer, $\Delta F(AA \rightarrow BB)$ and $\Delta F(AA \rightarrow AB)$ belonging to a protomer pair. The principal difference between the formulations of Monod *et al.* (1965) and Koshland *et al.* (1966) and the view presented here is that in the former two changes in ligand binding are always associated with overall structural changes, while in our view the changes in ligand binding are the result of free-energy conservation, have no simple one to one relation with overall structural changes and can be modified by a variety of local circumstances.

It must be pointed out that the thermodynamic description of the system by means of tautomerizations although formally equivalent in many cases to the description by means of the free energies of macroassociation is in reality far more restricted in scope and less useful than the latter. To this effect consider the following. (a) The characterization of tautomerizations in molecules involving as large a number of potential degrees of freedom as proteins is confined to some structural detail that happens to be observable. Tautomerizations involving one protomer alone cannot be separated in any simple way from those involving two neighboring protomers, a distinction which is crucial for the recognition of changes in intersubunit interactions as distinct from others. (b) It appears unlikely than any method can furnish us with a precise determination of the tautomerization constants necessary to characterize completely the system. In contradistinction the free energies of association of the protomers are well-defined and measurable thermodynamic quantities. Their determination is possible in those cases in which the macrodissociation constants are greater than 10^{-7} M and it may be expected that refinements of present day methods may decrease this value by at least three to five orders of magnitude in the future. (c) Since the tautomerization constants are not directly accessible they turn out in practice to be simply numbers which are chosen to fit the experimental curves giving the dependence of ligand binding upon free-ligand concentration. Resolution of these curves is certainly not very accurate, and possibly not unique[4] if the number of ligands bound is more than two per multimer. (d) The tautomerization constants are first-order quantities while the free energies of association of the macroaggregates are second order in protomer concentration for dimers and include still higher orders if the number of protomers exceeds two per macromolecule. They are capable of describing the behavior of the system at all protein concentrations. The tautomerization constants apply only when the protomer concentrations exceed appreciably all macrodissociation constants of the system and become meaningless at concentrations below these.

A quantitative relation between the free energies of macroassociation and the tautomerization free energy is easily established. Writing eq 2.1 and 2.2 in the form

$$\Delta F_{21} - \Delta F_{11} = {}^2\Delta F_1 - {}^2\Delta F_0 \qquad (2.8)$$

$$\Delta F_{22} - \Delta F_{11} = {}^2\Delta F_2 - {}^2\Delta F_1 \qquad (2.9)$$

The left-hand side may be recognized as the free energy of binding measured by taking as zero level the free energy of binding of the isolated protomer. On the right-hand side we have the difference between the free energies of aggregation before and after addition of ligand, which must therefore equal the free energy of tautomerization. As written above the equations assume the complete aggregation of the protomers into dimers, and are therefore valid in practice provided the protein concentration is appreciably higher than all the macrodissociation constants involved. At lower protein concentration the free energies of macroassociation are sufficient, together with the value of ΔF_{11} for a complete descrip-

[4] Experimental titration curves may be fitted reasonably well by more than one set of dissociation constants. Compare, for example, the values of Roughton and Lyster (1965) with those of Gibson (1970) for the same experimental set. Qualitative conclusions from such values are often ambiguous. For example, it is currently assumed (*e.g.*, Cornish-Bowden and Koshland, 1970) that equal binding constants imply noninteracting subunits, contrary to eq 2.1 and 2.6.

tion of the system. The stipulation of the free energy of tautomerization, which is the difference between two values of free energies of macroassociation, would be insufficient for the purpose.

Generalization to All Multimers. The simple dimer treatment sketched above may be generalized to aggregates of any order which are treated as the sum of pairwise protomer interactions.

To perform the necessary calculations for a molecule made up of N protomers it becomes necessary to specify: (1) the free energies of binding of the N isolated protomers; (2) the interacting pairs of protomers (there can by up to $N(N-1)/2$ interacting pairs although the number to be considered will be usually smaller if some pairs have no common boundaries); (3) for each interacting pair ij four free energies of association: $\Delta F(i(0)j(0))$, $\Delta F(i(1)j(0))$, $\Delta F(i(0)j(1))$, $\Delta F(i(1)j(1))$. The first corresponds to the unliganded dimer, the second and third to the uniliganded dimer, and the fourth to the biliganded dimer.

With these specifications it is possible to calculate the free-energy difference between any two aggregates of any order up to N, at all possible states of ligation. More particularly we are interested to compute, for the purpose of comparison to experiment: (a) the N-average free energies of ligand binding $\Delta F_{NJ}(1 < J < N)$ of the N-mer; (b) the free energies of dissociation of the N-mer into all possible half-molecules made up of $N/2$ protomers, in the unliganded and fully liganded forms.

To compute the N intrinsic average free energies of binding ΔF_{NJ} we first determine the free-energy F_J of a particle with J ligands. This is made up of two contributions: one from microassociations and the other from macroassociations. The microassociation free energies are those of J isolated liganded protomers. The free energies of macroassociation required to yield a particle of N protomers, J of which are liganded, are specified under 2 and 3 above. It must be noticed that the J ligands may be distributed in $\binom{N}{J}$ ways among the N protomers. Therefore, the free energies F_J are average values given by the relation

$$\bar{F}_J = \frac{\sum_i^{\binom{N}{J}} F_J(i)\cdot\exp(F_J(i)/RT)}{\sum_i^{\binom{N}{J}} \exp(F_J(i)/RT)} \qquad (2.10)$$

where the index i refers to one of the $\binom{N}{J}$ possible distributions. Finally the average intrinsic free energy of binding for the Jth ligand is given by

$$\Delta\bar{F}_{NJ} = \bar{F}_J - \bar{F}_{J-1} \qquad (2.11)$$

The last equation displays a relation contained also in eq 2.1. The intrinsic free energies of binding are the differences between the total free energies of the unliganded and liganded aggregates, respectively. Any quantity—representing actually a contribution to protomer affinity independent of ligand binding—may be added to both $\bar{F}_J$ and $\bar{F}_{J-1}$ without affecting the result. It follows that from the experimentally determined free energies of binding it is possible only to determine differences in free energies of aggregation but not their absolute values. Of the macroassociation constants the most likely one to be experimentally determined is the dissociation of the N-mer into halves. If there are several possible ways for such dissociation to occur, the changes with ligand binding for each of them may be predicted from the set of assumptions 1–3 above, but it would not be possible to decide which of them actually obtains in practice, precisely because of the undeterminate additive constant to both terms in the right-hand side of eq 2.11.

Explicit Calculations for a Tetramer. Of the many protein systems that exhibit multiple binding only hemoglobin and serum albumin have been sufficiently studied to warrant an attempt at quantitative analysis of the binding. Serum albumin being a single-chain molecule presents no interest from the point of view of the relations between micro- and macroassociations. Consequently we have made calculations only for a tetramer of the hemoglobin type. In our computations we have tried to use a minimum number of assumptions although keeping as close as possible to what is definitely known about hemoglobin. In the specifications discussed under heading 1 above we considered two alternatives. (1a) All protomers have equal intrinsic affinities for the ligand; (1b) the ligand affinity of one kind of chain differs from the other kind by a small amount. This is known to be the case for the isolated α and β chains of hemoglobin (Brunori *et al.*, 1968). Under the second heading, we have restricted the possible interactions to those between the α and β chains and neglected those between α and α or β and β. From the point of view of the energetic description of the system the assumption of two contacts for each subunit rather than the maximum of three should not prove to be a significant restriction.

As regards the type of protomer interaction it is known that in hemoglobin, since the affinity for the ligand at low degrees of saturation is much less than that of the isolated protomer, the interaction between protomers must act as a binding constraint and addition of the first ligand bound must produce the largest if not the total effect (eq 2.7 and Figure 61). Consequently we have adopted the simplest assumption. Addition of ligand to either of the two protomers of the pair produces the total effect. Therefore $\Delta F(i(0)j(1)) = \Delta F(i(1)j(0)) = \Delta F(i(1)j(1)) = C$; $\Delta F(j(0)i(0)) = C + \delta F$. There is in consequence a single value of $\delta F(ij)$ for the free-energy change brought about by the binding of ligand for each protomer pair. We shall refer to this quantity as the coupling energy between binding site and boundary. Each protomer is assumed to have contacts at two boundaries so that there are in all four active boundaries. To each of this a different value of $\delta F(ij)$ could apply but on account of the twofold symmetry of the system the maximum number of distinct values of $\delta F(ij)$ is reduced to two. The various kinds of models considered differ only in the absolute values δF_1 and δF_2 assigned to the energy couplings between binding site and boundary.

The models examined are shown in Figure 8, where the arrows show the boundaries which represent a constraint for binding at a given site. As shown in Table I models of type I give rise always to anticooperative binding if the ligand affinities of the protomer are equal, whether δF is a negative or a positive quantity. This result may be contrasted with the answer that could be obtained in these cases if instead of basing the changes in ligand binding upon free-energy conservation as done here, we had postulated a simple tautomerization upon binding. No restraint being placed upon the tautomerization constants assumed we could construct asymmetric models of type I endowed with cooperative properties. Models of type II yield apparent independent binding if the affinities of the isolated protomers are supposed equal, and anticooperative binding if these affinities are different. These conclusions as regards models of types I and II are almost intuitive and

TABLE I: Relative Binding Constants, Mean Affinity Changes, and Hill Coefficients for Tetramers $\alpha_2\beta_2$.[a]

δF	Δ Log [X]	R_2/R_1	R_3/R_1	R_4/R_1	$H_{0.5}$	H_2	$H_{3.5}$
			Type I				
1.0	0.30	0.88	0.72	0.63	0.96	0.88	0.96
2.0	0.99	0.77	0.28	0.22	0.90	0.62	0.90
3.0	1.57	0.80	0.083	0.066	0.89	0.37	0.89
			Type II				
2.0	0.0	1.0	1.0	1.0	1.0	1.0	1.0
2.0[b]	0.10	0.88	0.72	0.63	0.96	0.88	0.96
			Type III				
1.0	0.22	1.78	1.53	2.72	1.15	1.19	1.15
2.0	0.44	4.83	1.53	7.84	1.33	1.26	1.34
3.0	0.65	15.3	1.31	20.1	1.48	1.17	1.48
4.0	0.87	47.4	1.15	54.6	1.60	1.10	1.58
			Type IV				
1.5/1.5	0.65	3.85	5.21	20.1	1.58	1.98	1.58
2.0/2.0	0.87	6.51	8.39	54.6	1.91	2.39	1.91
2.5/2.5	1.09	11.0	13.4	148.0	2.32	2.79	2.32
3.0/3.0	1.30	18.7	21.6	403.0	2.74	3.14	2.74
			Type V				
2.0/1.0	0.65	4.83	4.26	20.9	1.55	1.89	1.56
2.0/3.5	1.19	23.3	10.5	245.0	2.33	2.77	2.34
3.0/1.0	0.83	14.1	3.87	35.6	1.66	1.77	1.55
3.0/2.5	1.19	15.3	16.01	245.0	2.51	2.95	2.50
Values of Human Stripped Hemoglobin and Hemoglobin–Diphosphoglycerate Complex According to Tyuma *et al.* (1971a)							
Stripped		3.7	9.5	55		2.53	
Hb–DPG		4.6	2.5	544		3.00	

[a] The types are those of Figure 8. Δ log [X] is the depression of the midpoint of the titration curve. log [X] = 0, for $\delta F_1 = \delta F_2 = 0$ and equal protomer affinities. R_2/R_1, R_3/R_1, R_4/R_1 = values of intrinsic binding constants relative to the first. $H_{0.5}$, H_2, $H_{3.5}$ values of the Hill coefficients when the number bound has the value of the subscripts. The values of δF are in RT units. [b] Assumes that the free energy of binding of two isolated protomers is greater than that of the other two by an amount equal to RT.

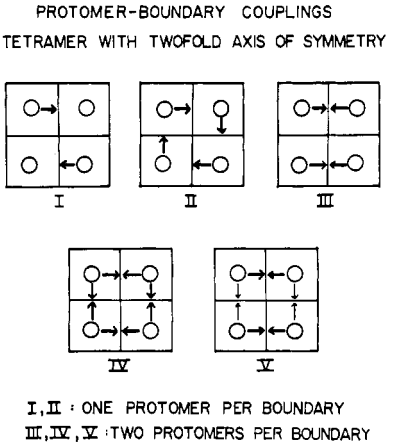


FIGURE 8: Energy coupling between binding sites and boundaries for a tetramer with twofold symmetry. The arrows connect the boundaries where the constraint to binding resides with the corresponding binding sites. The roman numerals refer to to the various types of coupling for which computations were made.

permits us to formulate the rule that *for the appearance of cooperativity the common boundary must represent a constraint for both binding sites.* In models of type III two independent dimers are assumed, and because of the twofold symmetry a unique value of δF is operative. No interactions between the two dimers are assumed, yet the ratios R_1/R_3 and R_4/R_2 are evidently different from eq 1.1 as shown in Table I. The cause of this may be understood by the schemes of Figure 9. The affinities of the four sites are low (L) in the unliganded tetramer. The first ligand binds always to an L site. The second, however, has the choice of two L and one high-affinity or H site. The second free energy of binding will therefore be appreciably smaller than that of an H site unless the free-energy H is incomparably larger than L (eq 2.10 and 2.11). The third ligand bound has the choice of a large number of molecules with two L sites or a smaller number with two H sites. Its free energy of binding will be therefore larger than that of an L site. The last ligand is always bound to an H site. Therefore, the fact that $R_1 < R_3$; $R_2 < R_4$ is the result of entropic, or statistical causes.

Whatever coupling free energy δF is chosen the value of the Hill coefficient at midsaturation in a model of type III can never be greater than two. Models of type III cannot possibly represent hemoglobin where the Hill coefficient has usually a value of 2.7 and may reach occasionally up to 3.1 (Rossi-Fanelli *et al.*, 1961).

Models of type IV and V are "cross-linked" models in the sense that binding at each site affects always the two boundaries of the subunit. Models IV, the simplest, are those in which $\delta F_1 = \delta F_2$. In these models $R_1 < R_2 \sim R_3 < R_4$. The origin of these differences is schematized in Figure 10 for the case in which all isolated protomers have similar ligand affinities. Much has been made of the larger value of R_4 in comparison to the other constants as an indication of interactions involv-

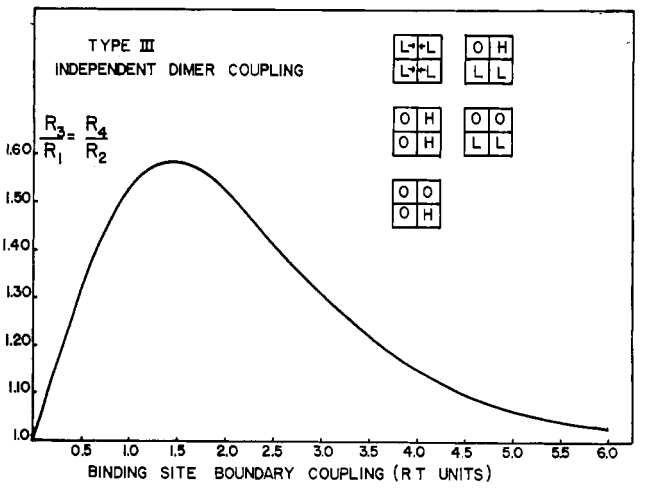


FIGURE 9: Showing the lack of degeneracy in cases of type III (independent dimers) when ΔF is not large in comparison to RT. The schemes on the upper right show the affinities of the sites (H = high, L = low) as progressive binding takes place and explains how the degeneracy is lifted.

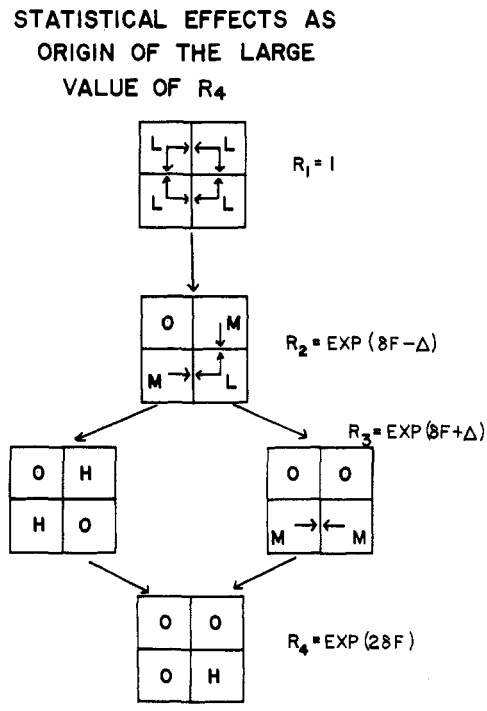


FIGURE 10: Showing how the fourth binding constant R_4 becomes larger than the others as a result of a purely statistical effect depending upon the progressive disappearance of the binding constraints. A model of type IV with a unique value for δF was assumed for simplicity. O indicates an occupied binding site. L, M, and H indicate the relative ligand affinities of the unoccupied binding sites (low, medium, and high, respectively). A binding site adjacent to two unbound protomers is L, one adjacent to one bound and one unbound protomer is M, and one adjacent to two bound protomers is H. Δ represents a free energy—small in comparison with δF—dependent only upon the statistical distribution of the ligands and the value of δF.

ing the whole tetramer and indirectly of a unique conformational change upon binding. As shown in Figure 10 the large relative value of R_4 is simply explained as a statistical consequence of the progressive disappearance of binding constraints as the sites become occupied.[5]

Both models IV and V can give rise to Hill coefficients at midsaturation of the magnitude observed in hemoglobin. One must conclude that a cross-linked model is indispensable in reaching those values and that hemoglobin must be a cross-linked tetramer in which binding at one site affects simultaneously two boundaries. This conclusion is essentially that reached by Guidotti (1967) from his experiments on the effects of the tetramer–dimer equilibrium upon the cooperativity in hemoglobin.

The results of the calculations for models I–V are summarized in Table I. which lists the ratios of the instrinsic binding constants R_2/R_1, R_3/R_1, and R_4/R_1 together with the values of the Hill coefficients that are observed when 0.5, 2, and 3.5 moles are bound, respectively. It will be noticed that a ratio of R_4/R_1 close to 200 and a Hill coefficient of 2.8 at midsaturation are reached in models IV and V when the free energies of the binding site–boundary coupling reach a value of approximately $5RT$ or 3 (kg cal)/protomer. Model V is the only

[5] The direct interaction between heme groups originally assumed by Pauling (1935) lead him to a description of oxygen binding by hemoglobin which if formally similar to, although physically very different from, that of our models III and IV.

TABLE II: Changes in Affinity and in Hill Coefficients at Midsaturation after Complete Dissociation of the Tetramer into Dimers.[a]

δF	Constraint Lost	% Change in Δ Log [X]	$H(4)$	$H(2)$
		Type IV		
2.0/2.0	2	50	2.39	1.46
2.5/2.5	2.5	50	2.79	1.55
3.0/3.0	3	50	3.14	1.63
		Type V		
2.0/3.5	2	36	2.76	1.63
	3.5	64		1.46
3.0/2.5	3	55	2.95	1.63
	2.5	45		1.55

[a] The change in affinity is given as per cent change in Δ log [X] (see Table I).

type which can reproduce a feature often reported for the actual determined binding constants. According to Gibson (1970), Roughton and Lyster (1965), and Tyuma *et al.* (1971a), the relation among the constants most often observed is $R_3 < R_2 < R_4$. This feature appears as a natural consequence of the assumption that $\delta F_1 \neq \delta F_2$, which in turn follows from consideration of the very different nature of the contacts between α_1 and β_1, on one hand, and α_1 and β_2, on the other, shown by the X-ray structure (Perutz *et al.*, 1968), or by the fact that one mode of dissociation of the tetramer is predominant over the alternative one. Models of type IV cannot give rise to the desired relation among the constants whether the isolated protomers are assumed to have equal or somewhat different affinities.

In Table II we have gathered a couple of examples of the changes in overall affinity and cooperativity that result from dissociation of the tetramer into dimers. Only the dimerization obtained by dilution would be acceptable for a valid experimental comparison. It is quite clear that no firm conclusions could be reached if splitting into dimers is achieved by addition of another ligand or change in pH since there is no reason to believe that the relations of macro- and microassociations in the parent tetramer would be the same as those obtaining in the absence of ligand or at a different pH.[6]

The Interactions between Macro- and Microassociations for the Case of Ligands of Several Types. A further stage of complexity is introduced when several types of ligands can modify each other's binding either through a change in the macroassociation constants or in a direct fashion. The only case of this type well investigated up to the present is that of the dependence of the oxygen–hemoglobin equilibrium upon the presence of diphosphoglycerate (Benesch and Benesch, 1967; Chanutin and Curnish, 1967). However, the literature on enzymes contains many examples which indicate that such interdependence must be very common, even unavoidable in multimer proteins. The situation in which a ligand Y can affect the binding of 2 or more moles of X is particularly

[6] Changes in the binding of buffer ions and inherent instability of the isolated protomers or dimers may prevent also a valid comparison of the tetramer to the products of dissociation by dilution.

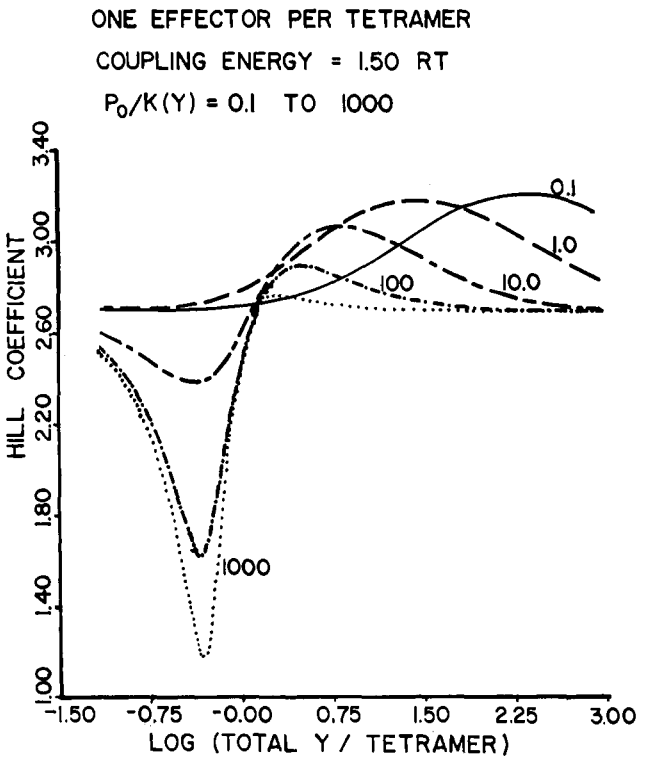


FIGURE 11: Effect of a further compled equilibrium with 1 mole of Y upon the binding of X by a tetramer with binding constraints of type IV and $\Delta F = 2.375RT$. $[P_0]$, total protein concentration. $[Y_0]$, total Y concentration. $K(Y)$: dissociation constant of the protein–Y complex. The figure shows the change in Hill coefficient for X binding at $\bar{n}_X = 2$ as a function of log ($[Y_0]/[P_0]$), for $\Delta F_{XY} = 1.5RT$ and different values of $[P_0]/K(Y)$. For details of computation, see Appendix.

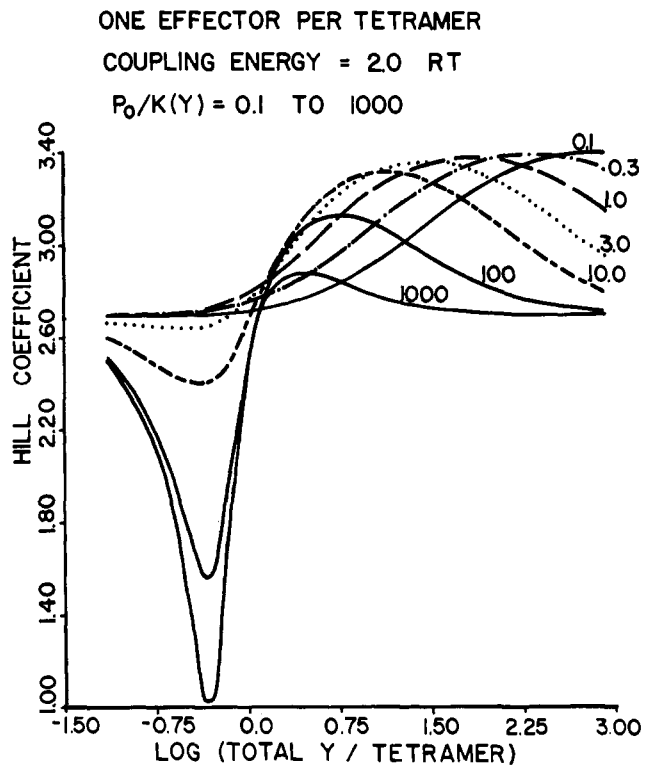


FIGURE 12: Same as Figure 11 with $\Delta F_{XY} = 2.0RT$.

likely to be realized in a multimer by the binding of Y at the boundary of two or more protomers. The effects observed depend critically upon the value of ΔF_{XY}, the number of Y molecules involved, the equivalence or otherwise of the interactions between the X and Y ligands and the concentrations of protein and Y present.

As an example we have made computations for a tetramer binding 4 moles of X and 1 or 2 moles of Y. It will thus be recognized that the computations attempt to describe the effects of diphosphoglycerate upon the O_2–hemoglobin equilibrium (X = O_2; Y = DPG). As a model for stripped hemoglobin we have taken an evenly cross-linked tetramer (type IV) with $\delta F = 2.375RT$. This value gives a Hill coefficient of 2.69 at midsaturation. Experimental values of 2.5–2.7 (Gibson, 1970; Tomita and Riggs, 1971; Tyuma *et al.*, 1971a,b) are quoted for stripped hemoglobin. The number of XY interactions was kept equal to four, so that in cases in which a single molecule of Y is bound this interacts equally with the X ligands at the four protomers, or if two molecules of Y are bound each of them interacts with the molecules of X bound at only two of the four protomers. As typical values of ΔF_{XY} we have taken $1.5–2.5RT$ units because the maximum increases in oxygen pressure at midsaturation due to addition of DPG are close to one logarithmic unit. The computations were carried out as described in the Appendix. The computed values include the changes in X affinity at midsaturation ($\Delta \log [X]_{1/2}$), the Hill coefficient observed at $\bar{n}_X = 2$ and the extent of release of Y from the complex. As a measure of the latter we define a "relative Y saturation change" as: (Y saturation at $\bar{n}_X = 0.4$ − Y saturation at $\bar{n}_X = 3.6$)/0.8. Figures 11–16 show the dependence of these quantities upon variable $[Y_0]/[P_0]$, where Y_0 and P_0 are the *total* concentrations of Y and protein in the solution. As parameters we have taken $[P_0]/K(Y)$, where $K(Y)$ is the dissociation constant of the Y–protein complex, and ΔF_{XY} the coupling free energy. The results may be summarized as follows.

Decrease in X Affinity. This changes monotonically with Y_0 and approaches a limit of $0.434\Delta F_{XY}$ logarithmic units when the saturation of Y does not change with X saturation (upper tracings of Figures 14–16).

Changes in Hill Coefficients at Midsaturation. Depending upon the number of moles of Y that may be bound, $[P_0]/K(Y)$ and $[Y_0]/[P_0]$ the Hill coefficient at midsaturation may increase, decrease, or remain unchanged. Experimentally these three possibilities have been observed (Tyuma *et al.* 1971a,b; Tomita and Riggs 1971). If only 1 mole of Y is bound per tetramer, Figures 11 and 12 show that the Hill coefficient rises or undergoes no change when $[Y_0]/[P_0]$ equals 1 or more, but that it decreases or undergoes no change if $[Y_0]/[P_0]$ is less than one. The former case corresponds to the experimental conditions of Tyuma *et al.* (1971a), the second to those of Tomita and Riggs (1971). Table III shows, by the comparison of some computed values to those of Tyuma *et al.* (1971a), that with very simple assumptions the equilibrium theory given in the Appendix can account for the direction and approximate value of the changes that follow the addition of DPG. In the cases studied by Tomita and Riggs only decreases in Hill coefficients are reported at all values of $[Y_0]/[P_0]$. Figures 11–13 show that this behavior is observed when 2 moles of Y are bound per tetramer, or when 1 mole is bound with very high affinity. We can accordingly predict that mouse hemoglobin binds 1 mole of DPG/tetramer with an affinity some 100 times greater than that of human hemoglobin or that it binds 2 moles of DPG per tetramer with affinity similar to human hemoglobin. The smaller decreases in Hill coefficients ob-

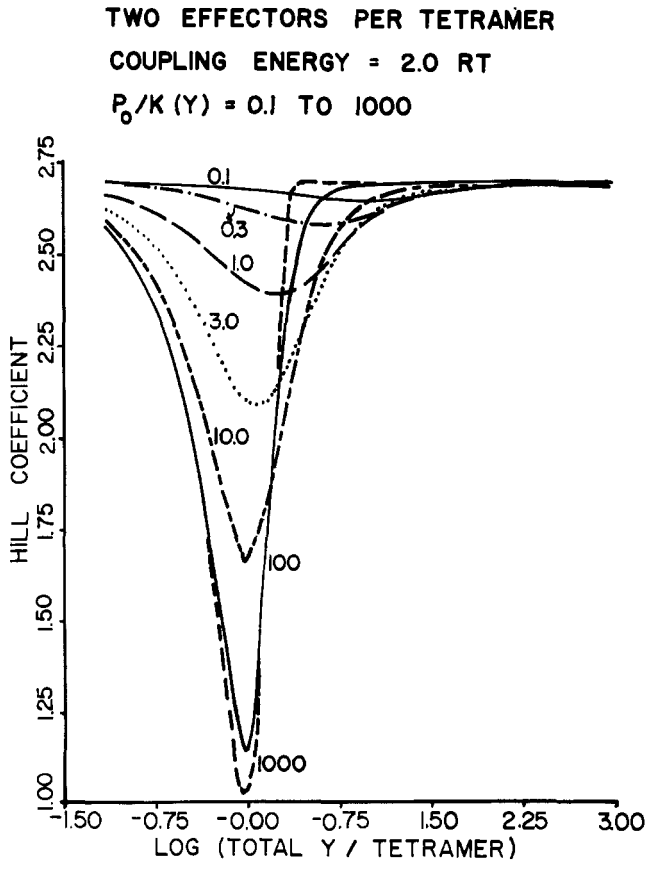


FIGURE 13: Same plot as in Figure 11, but for equilibrium with 2 moles of Y binding with a single dissociation constant K(Y). $\Delta F_{XY} = 2.0RT$. For details of computation, see Appendix.

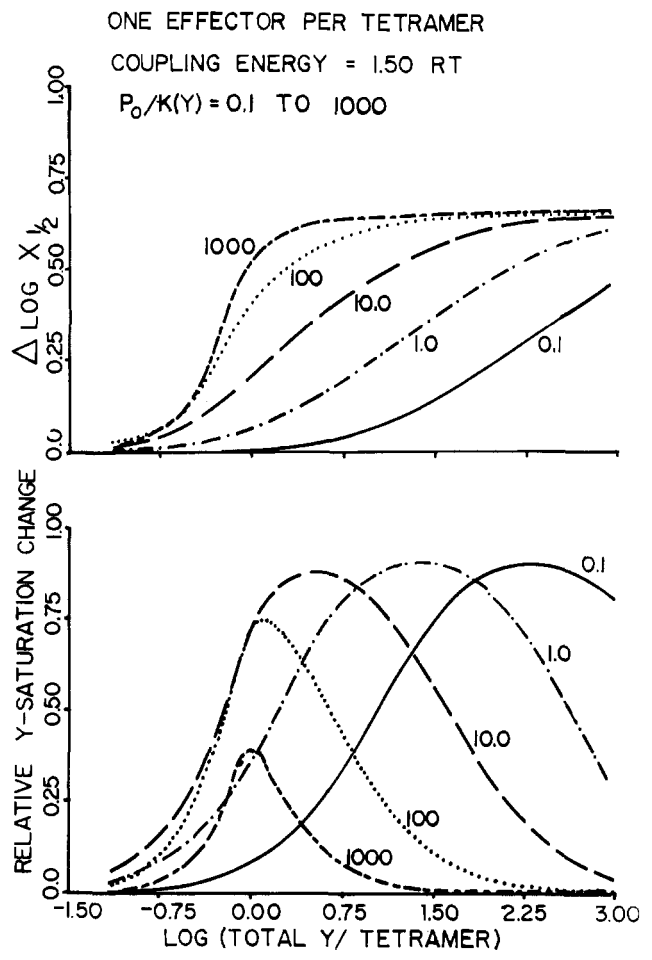


FIGURE 14: Effect of a further coupled equilibrium with 1 mole of Y. The upper tracings show the change in free X concentration at midsaturation from that corresponding to $[Y_0] = 0$. The lower tracings give the "relative Y saturation" defined as: (Y saturation at $\bar{n}_X = 0.4$ − Y saturation at $\bar{n}_X = 3.6$)/0.8. The abscissas are the same (log $[Y_0]/[P_0]$) for upper and lower tracings. Otherwise same conventions as in Figure 11.

served upon increase of buffer ionic strength and the eventual invariance in 0.02 M phosphate (Figure 1 of Tomita and Riggs' paper) are what we should qualitatively predict to follow when K(Y) is increased by competition with less effective anions.

Changes in Y Saturation. Figures 14–16 (lower tracings), as well as eq 1.12 show that when the Hill coefficient is unchanged by the addition of Y, Y saturation must be complete and invariant with X saturation. Thus, a mechanism in which the release of DPG upon complete oxygenation is obligatory for the attainment of a unique structure characteristic of oxyhemoglobin (Perutz, 1970) is ruled out by the principles of chemical equilibrium.[7]

The remarkably different effects in the action of diphosphoglycerate depending upon the concentration of the reagents and the presence of competing ions is an evident reminder that the subtleties of control and regulation of biological activity through ligand binding are to be sought in the detailed consideration of all the interacting equilibria rather than in some inherently unique property of the protein structure.

TABLE III

	Computed Values ($\Delta F_{XY} = 2.5$, $[P_0]/K(Y) = 10$)	Exptl Values
$[Y_0]/[P_0]$	145	133
Δ log $[X]_{1/2}$	+0.80	+0.89
Change in Hill coefficient	+0.68	+0.55

Conclusions

The most important difference between complex formation by small molecules and ligand binding by proteins is that in the latter case the equilibria involved are not restricted to those degrees of freedom involved in the molecular contacts. Although participation of other equilibria in the case of small molecules is well recognized, for example, the variation in the pK of groups not directly involved in binding, they constitute the exception rather than the rule. In ligand binding by pro-

[7] Tyuma *et al.* (1971b) have recently reported that when DPG is replaced by inositol hexaphosphate (IHP) the increase in Hill coefficient over that of stripped hemoglobin is no longer observed and the ratio R_4/R_1 doubles instead of increasing by a factor of ten as in the DPG case. In consequence they conclude "that the mechanism of action of IHP must be quite different from that of DPG." However, as Figures 11 and 12 show quite clearly the observed changes are exactly what would be expected from the much greater affinity of IHP. In particular the smaller change of R_4/R_1 reflects the fact that the point has been reached at which IHP saturation does not change much with oxygen saturation.

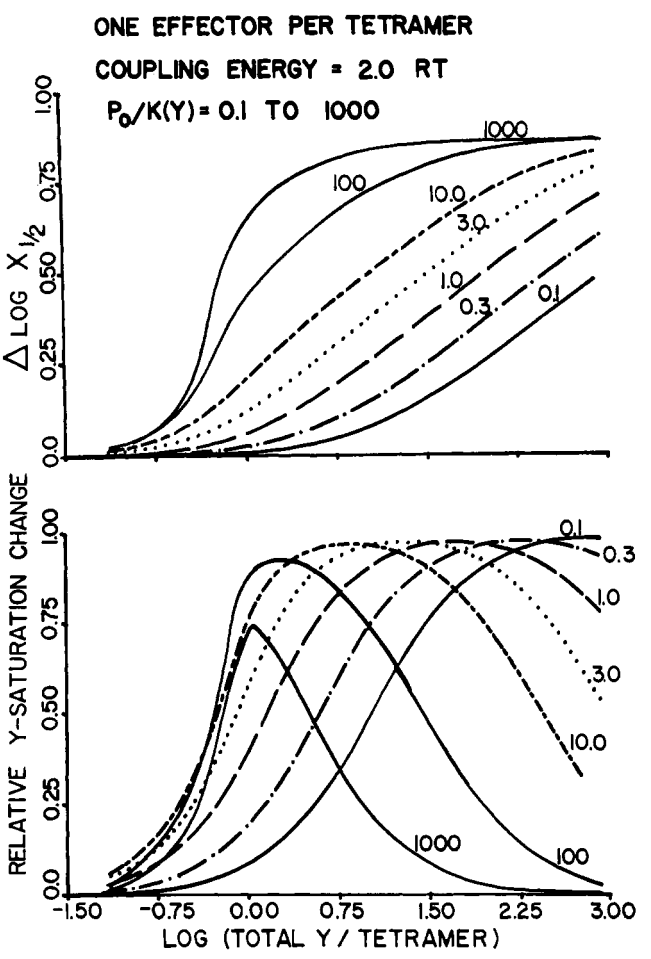


FIGURE 15: Same as Figure 14 but with $\Delta F_{XY} = 2.0RT$.

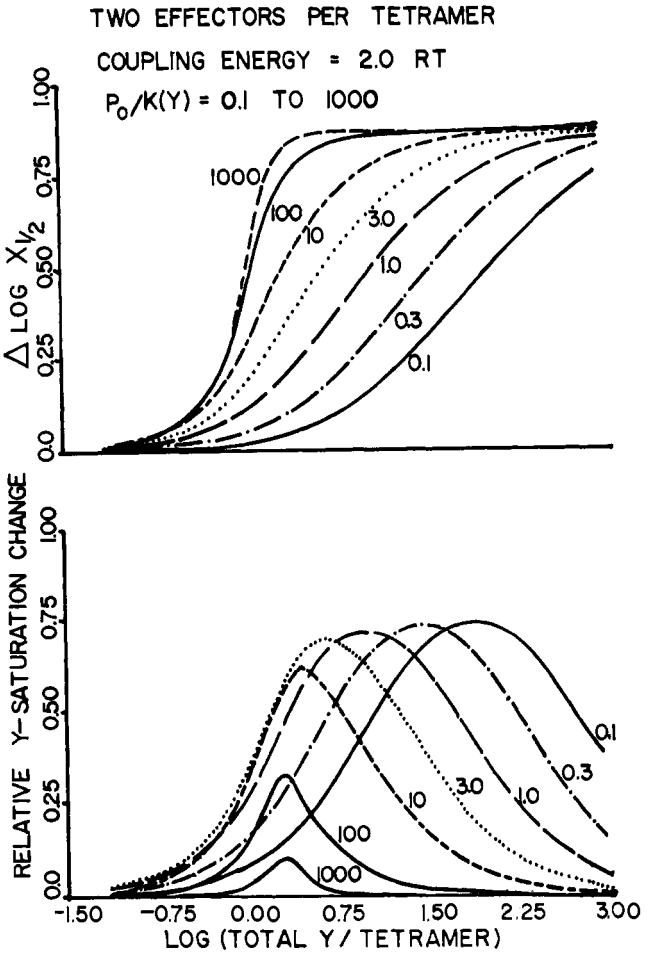


FIGURE 16: Coupled equilibria with 2 moles of Y having the same value of $K(Y)$. Otherwise as Figure 14 with $\Delta F_{XY} = 2.0RT$. For details of computation, see Appendix.

teins it is safe to say that the opposite is the case and that the problems of ligand binding may be considered solved—as far as thermodynamic description goes—only when the various equilibria connected with binding are demonstrated and the total free energy of binding is apportioned among them. These equilibria include tautomerizations of the protein chain, association with other protomers, and interactions among bound ligands. The only principle that may be applied with confidence to all of them is that of free-energy conservation. In this respect binding systems obey something quite akin to the principle of D'Alembert, valid in the mechanical domain. D'Alembert's principle (*e.g.*, Mach, 1912) states that part of the applied forces may be inoperative at the point of application if they participate also in a system of balanced constraints. The conservation of mechanical energy applies to these cases just as the conservation of free energy applies to binding. The "further systems of balanced constraints" are in the latter case the equilibria among different parts of the chain, or among protomers. The inoperative forces have their counterpart in the diminished free energy of binding of the first ligand bound in cooperative systems. This missing free energy is employed in displacing a preestablished equilibrium at some point other than the binding site itself. From the variety of possibilities involved it appears that the first and most important task in understanding multiple binding systems should be the enumeration of all the potential ligands present and the determination of the energetic couplings among them. Since proteins are polyelectrolytes which can hardly be handled except in the presence of considerable molar excess of ions it becomes necessary to determine whether specific buffer ion binding and interaction takes place. Unfortunately, the methods for determining such specific ion interactions are far from adequate. The need for investigation of this aspect is clearly shown by the discovery of the important effects that phosphate ions and organic phosphate esters have upon the oxygen–hemoglobin equilibrium. This particular example indicates the necessity of considering at once *all* the intervening equilibria if any one of them is to be correctly interpreted. Accordingly complete confidence may not be placed at this time in the supposition that the binding constants of stripped hemoglobin are the result of an equilibrium in which oxygen and hemoglobin are alone involved.[8]

Uniqueness or Multiplicity of Protein Conformations? The analysis that we have carried out, stressing as it does the multiplicity of the equilibria involved, naturally suggests that the attempt to describe proteins as existing in a very restricted number of equilibrium conformations is most unlikely to be correct.

The observations performed upon proteins in solution, in particular those nondestructive experiments that rely upon spectroscopic techniques, can reveal only average values of some property. The averaging process extends both over the population of molecules at any given time and over a char-

[8] Saroff (1970) has argued that contrary to the commonly held view (Wyman, 1948; Antonini *et al.*, 1963) proton dissociation and cooperativity are dependent phenomena.

acteristic time that depends both upon the method used and the conditions of observation, but is always finite. In most cases this characteristic time is sufficient for extensive molecular motions and rearrangements to take place. A demonstration of the "uniqueness" of protein conformation in solution by present-day methods is therefore out of the question. Every measurement in solution must be considered that of an average value of the property that is measured. Its variation with pH, temperature, or ligand additions is a variation in average value the manner of formation of which is unknown. The discussion of these changes in terms of unique structural models does not appear profitable because of these limitations. Postulation of a small number of *states* to facilitate the description of the system is permissible provided it is realized that: (1) these states are themselves average states of unknown composition; (2) there are many examples of undoubted continuous distributions in which postulation of discontinuous transitions between a small number of supposedly unique states is sufficient for an accurate description of the system. The seeming inconsistency involved here is due to the insufficiency of the methods of observation to extract any other information beyond the average population of the supposed unique states.

In discussions concerning the changes in properties of proteins in solution, particularly in those cases showing the mutual influence of ligands bound to them, these fundamental premises are all too often forgotten and unique, "exclusive" conformations are postulated although no experiment is known to the author which either requires such uniqueness of conformation or may be used to infer it. It seems in fact that we can explain the properties of proteins in solution without introducing any other principles than those that obtain in the case of much smaller molecules. In extending these to the proteins we must remember some special circumstances that operate in their case. (1) Proteins are constituted of a number of semiindependent domains. Stretches of α helix or β structure are obvious examples but many regions must exist in which the stabilizing influences do not extend beyond the nearest neighbors. (2) In each semiindependent domain structural changes must take place depending upon a local activation energy, probably not large but sufficient to maintain the domain in a characteristic conformation most of the time. (3) Pending a demonstration to the contrary there is little reason to believe that there is much, if any, dependence of one domain upon another beyond that imposed by the physical continuity of the covalent chain. A consequence of this would be that although to each domain a characteristic structure may be assigned, if the number of semiindependent domains is at all large the probability of finding all of them at once in their average conformation becomes very low indeed. Thus there would not even be an average conformation which the whole of the protein retains most of the time. The situation does not differ in principle from that in a crystal. There is an overall long-range order that is preserved but without requiring that the short-range order be also preserved at all times and everywhere. Lattice vacancies are continuously being created and filled; molecular groups undergo rotations and translations without the crystal losing its individuality or changing its average properties. A protein molecule may be expected to behave in many respects like a low-melting point crystal.[9]

[9] Indeed in all natural systems, chemical and biological, the long-range order depends only statistically upon the conservation of the short-range order, a situation that does not apply at all to man-made machines.

The point of view presented here as alternative to the existence of a unique conformation could be considered to add little more than a corrective to an extreme point of view. There is however an important difference. Proponents of the existence of unique conformations related by discontinuous changes are led, by the very nature of their assumptions, to consider the protein as endowed with the mechanical properties that we reserve for macroscopic objects. In particular the accessibility of certain groups, catalytic properties, and reactivity to other macromolecules, that is the most important functional properties, are made to depend upon all-or-none, quasi-mechanical transitions from one state to another, rather than in a chemical equilibrium of which the nature and concentration of all the reactants in the medium are the final determinants. We have discussed this aspect in relation to the equilibrium of oxygen and diphosphoglycerate with hemoglobin. It seems worth adding that if all the effects are seen as the consequence of ordinary chemical equilibria, the protein appears as one more reagent exhibiting the stochastic characteristics that we attribute to any molecular population.

The opposite view would represent the proteins as having escaped the stochastic limitations and behaving in a completely deterministic, almost mechanical fashion. This impression is strongly conveyed by the interpretation of the functionalism of hemoglobin that Perutz (1970) has placed upon his remarkable investigations of the crystal structure of oxy- and deoxyhemoglobins. It is also implicit in the many references in the literature to "switch" mechanisms and "flip-flop" mechanisms applied to proteins the properties of which vary in the presence of ligands, as if these systems had more in common with the bistable electronic devices from which the names are borrowed than with reagents participating in a complex chemical equilibrium. The main reason for stressing our point of view is that complex chemical equilibria contain a wealth of possibilities for the explanation of many physiologically important processes and that these possibilities of explanation are lost from the start, or at least greatly obscured if the system is credited with the inflexible mechanical properties of a macroscopic object.

Acknowledgment

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Appendix

Statistical and Intrinsic Binding Constants. Throughout this paper in dealing with second-order processes I have used sometimes binding constants, denoted by R, and sometimes dissociation constants, denoted by $K = R^{-1}$. To R and K correspond, respectively, free energies of association and dissociation of the complexes which are equal in absolute value and differ only in the sign. All the free energies of second-order processes may be assumed to have the same sign, and therefore all the equations in which they appear relate to the absolute value of the free energies, which may be interpreted as free energies of association or dissociation by preceding them with the appropriate sign. The free-energy changes in first-order processes are defined in each case by the ratio which represents the first-order equilibrium constant k.

The binding or dissociation constants in cases of multiple

binding ($R_1 \ldots R_N$; $K_1 \ldots K_N$) are given in the literature sometimes as *statistical* constants, in which case the equilibrium is formulated as

$$A_N X_J + X = A_N X_{J+1} \tag{A.1}$$

$$R_S = [A_N X_{J+1}]/([A_N X_J]\cdot[X]) \tag{A.2}$$

and at other times as *intrinsic* constants formulated as

$$(N - J)A_N X_J + X = (J + 1)A_N X_{J+1} \tag{A.3}$$

$$R_I = (J + 1)[A_N X_{J+1}]/((N - J)[A_N X_J][X]) \tag{A.4}$$

$$R_I = ((J + 1)/(N - J))R_S \tag{A.5}$$

$$\Delta F_I = \Delta F_S - RT \ln ((J + 1)/(N - J)) \tag{A.6}$$

Evidently the statistical free energy of binding lumps together the true or intrinsic free energy and an entropy contribution arising from the multiplicity of sites. In a tetramer with four identical independent binding sites the four intrinsic constants are equal while the statistical constants are in the ratios 4:3/2:2/3:1/4, respectively. The calculations of (2.10) and (2.11) yield intrinsic free energies of binding. To calculate $\bar{n}$, the number of moles of ligand bound, use is made of the intrinsic binding constants in the form

$$\bar{n} = \frac{\sum_0^N \binom{N}{J} J R_1 \ldots R_J [X]^J}{\sum_0^N \binom{N}{J} R_1 \ldots R_J [X]^J} \tag{A.7}$$

In the experimental studies of Roughton and Lyster (1965) and Gibson (1970) the binding constants quoted are statistical values, while Tyuma *et al.* (1971) quote intrinsic constants.

Conditional Free Energies of Binding. In general the conditional free energy of binding $\Delta F(i/jk\ldots)$ is defined as the standard free-energy change in the reaction

$$Ajk\ldots + i \longrightarrow Aijk\ldots \tag{A.8}$$

The difference between this free energy and the unconditional free energy (or simply the free energy) $\Delta F(i)$ of the reaction

$$A + i \longrightarrow Ai \tag{A.9}$$

is always a coupling free energy $\Delta F_{i/jk\ldots}$. The "linkage map" (Wyman, 1965) determining which of these ligands affect each other's binding may be obtained by varying the concentration of one ligand at a time and measuring the change in saturation of the others. It must be recalled that two ligands may appear uncoupled when they are the only ones present but appear coupled in the presence of a third ligand. Therefore, it is not possible to describe in general the couplings operating in the system by measurements in which two ligands alone are present and the rest are absent. Nevertheless, one may expect that in many cases the interactions operating among the ligands may be expressed as the sum of the interactions of pairs of ligands taken in isolation from the others (independent pairwise interactions).

We wish to demonstrate that this hypothesis may be experimentally tested. For the case of there ligands X, Y, and Z, we have

$$\Delta F(XYZ) = \Delta F(XY) + F\Delta(Z/XY) = \Delta F(YZ) + \Delta F(X/YZ) = \ldots \tag{A.10}$$

and using eq 1.8

$$\Delta F(XYZ) = \Delta F(X) + \Delta F(Y) + \Delta F_{XY} + \Delta F(Z/XY) = \ldots \tag{A.11}$$

$$3\Delta F(XYZ) = 2(\Delta F(X) + \Delta F(Y) + \Delta F(Z)) + \Delta F(X/YZ) + \Delta F(Z/XY) + \Delta F_{XY} + \Delta F_{YZ} + \Delta F_{ZX} \tag{A.12}$$

$$\Delta F(XYZ) = \Delta F(X) + \Delta F(Y) + \Delta F(Z) + \Delta F_{XYZ} \tag{A.13}$$

where ΔF_{XYZ} is the sum of all the interactions of X, Y, and Z in the complex containing the three ligands. Evidently if

$$\Delta F_{XYZ} = \Delta F_{XY} + \Delta F_{YZ} + \Delta F_{ZX} \tag{A.14}$$

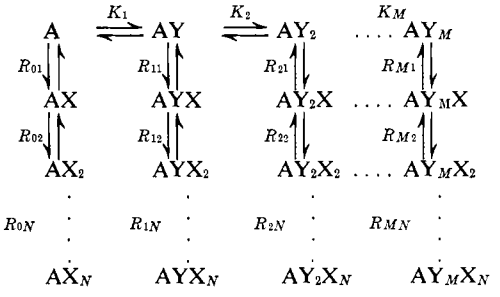
we must have

$$\Delta F(X/YZ) + \Delta F(Y/ZX) + \Delta F(Z/XY) - \Delta F(X) - \Delta F(Y) - \Delta F(Z) = 2(\Delta F_{XY} + \Delta F_{YZ} + \Delta F_{ZX}) \tag{A.15}$$

Since all the quantities in the last equation are subject to experimental determination the hypothesis that the couplings among X, Y, and Z are pairwise independent may be put to the test.

Computation of Binding, in the Case of Multiple Interactions among Two Types of Ligands. If an N-mer binds N moles of X and M moles of Y there will be in general $2MN + M + N$ distinct ligand addition steps involving an equal number of binding constants. Of these only $(N + 1)(M + 1) - 1$ are independent. As a convenient set of independent constants we shall consider those determining the $M + 1$ series of N additions of X to the species $AY_J (0 < J < M)$ and the M additions of Y to the unliganded species A. The binding steps corresponding to these constants are those shown in Scheme I.

SCHEME I



Let the average binding constants for the N successive steps of binding of X by the species AY_J be denoted by $R_{J1}, \ldots R_{JN}$. We shall set the product $R_{1K}R_{2K}\ldots R_{JK}$ equal to C_{JK} and $C_{0K} = 1$. Binding functions S_J and W_J may then be defined by the equations

$$S_J \equiv \sum_{K=0}^{N} \binom{N}{K} [X]^K C_{JK} \tag{A.16}$$

$$W_J \equiv \sum_{K=0}^{N} K \binom{N}{K} [X]^K C_{JK} \tag{A.17}$$

With these definitions

$$[P_0] = \sum_{J=0}^{M} [AY_J]S_J \quad (A.18)$$

$$[Y_0] - [Y] = \sum_{J=0}^{M} J[AY_J]S_J \quad (A.19)$$

where $[P_0]$ is the total protein concentration and $[Y_0]$ the total Y concentration in the solution. Setting $\epsilon = [Y]/[Y_0]$, we have for $J \geq 0$

$$[AY_J] = \binom{M}{J}[A]\epsilon^J[Y_0{}^J]B_J \quad (A.20)$$

where B_J equals the product $(K_1 K_2 \ldots K_J)^{-1}$, and $B_0 = 1$. From this and the previous two equations

$$\sum_{J=0}^{N}\binom{M}{J}S_J[Y_0{}^J]B_J\epsilon^J(\epsilon + (J[P_0]/[Y_0]) - 1) = 0 \quad (A.21)$$

The variable ϵ is introduced to stress the dependence of the system upon the parameters $[Y_0]/K_1 \ldots [Y_0]/K_M$, the ratios of the total Y ligand concentration to the dissociation constants of the AY_M complex. Evidently if the binding constants $R_{01} \ldots R_{MN}$ for X and the dissociation constants $K_1 \ldots M_M$ of the AY_M complex are specified together with [X], the free X concentration, the equilibrium concentration of free Y is obtained by solving eq A.21 of degree $M + 1$ in ϵ. The average number of moles of X bound per mole of protein is

$$\bar{n}_X = \frac{\sum_{J=0}^{M}\binom{M}{J}W_J\epsilon^J[Y_0{}^J]B_J}{\sum_{J=0}^{M}\binom{M}{J}S_J\epsilon^J[Y_0{}^J]B_J} \quad (A.22)$$

and the average number of moles of Y bound per protein is

$$\bar{n}_Y = ([Y_0]/[P_0])(1 - \epsilon) \quad (A.23)$$

The above equations have been used in the computation of the values of log [X], Y saturation and Hill coefficients for X binding at various values of $\bar{n}_X$, when, respectively, 1 or 2 moles of Y may be bound per tetramer. Thus we have in eq A.16–A.22 $N = 4$; $M = 1$ or 2. The values $R_{01} \ldots R_{04}$ of the binding constants of X in the absence of Y have been assumed to be those of a tetramer with binding constraints of type IV and $\delta F = 2.375\ RT$ units. The Hill coefficient of this system at $\bar{n}_X = 2$ equals 2.69. The values of R_{11} up to R_{M4} are determined by the free-energy couplings assumed to exist between X and Y. We have also assumed that the dissociation constants of the AY_M complexes are identical when 2 moles of Y are bound per tetramer.

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