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Analysis of Cooperativity in Hemoglobin. Valency Hybrids, Oxidation, and Methemoglobin Replacement Reactions†

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ABSTRACT: An allosteric model proposed previously for structure–function relations in hemoglobin is applied to the analysis of low- and high-spin valency hybrids. By assuming that the low-spin oxidized chains have the tertiary structure of oxygenated chains while the high-spin oxidized chains have a tertiary structure intermediate between that of deoxygenated and oxygenated chains, the model parameters associated with the different valency hybrids can be obtained, and their equilibrium properties can be estimated. The hybrid results are used also to provide an interpretation of methemoglobin and its ligand replacement reactions and of the oxidation–reduction equilibrium of normal hemoglobin. For the various systems studied, it is found that the effects of pH and 2,3-diphosphoglycerate are in agreement with the model.

To understand the mechanism of cooperative ligand binding by the hemoglobin tetramer, it is not sufficient to know the structure and properties of the completely deoxygenated (Hb) and fully oxygenated ($Hb(O_2)_4$) species. Information about the intermediates ($Hb(O_2)$, $Hb(O_2)_2$, $Hb(O_2)_3$) that occur in the course of the oxygenation reaction is required. Such knowledge is difficult to obtain in a highly cooperative system like hemoglobin because the equilibrium concentra-

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tion of partially oxygenated species is small relative to the limiting forms. It has not been possible to crystallize the intermediates for X-ray analysis and they are difficult to observe and identify in solution even by sensitive techniques like nuclear magnetic resonance spectroscopy and spin labeling. One approach is provided by kinetic measurements, since higher concentrations of intermediates can occur under nonequilibrium conditions. Until now, however, most of the available data have come from equilibrium studies of "hybrid" hemoglobins, which are treated as models for the partially oxygenated intermediates (Antonini and Brunori, 1971).

A variety of hybrids have been studied. All of these are presumed to be primarily tetrameric systems in which the two α chains or the two β chains are modified in some way. The known hybrids include the *valency hybrids* in which either the α or β chains have been oxidized (Antonini and Brunori, 1971), the *metal hybrids,* in which the ferrous ion of either the α or β chains has been replaced by cobaltous ion (Hoffmann and Peterling, 1970; Yonetani et al., 1973; Yamanoto et al., 1974), the *heme hybrids* in which one type of chain contains a heme group involving protoporphyrin IX and the other type a heme group involving a modified porphyrin (Yonetani et al., 1973; Yamanoto et al., 1974), and the *de-metal* hybrids in which the heme group of either the α or the β chains has been replaced by protoporphyrin IX without iron (Treffry and Ainsworth, 1974). Analysis of the hybrid hemoglobin data is concerned with understanding their equilibrium properties and with evaluating their potential for providing information concerning the nature of partially oxygenated intermediates of normal hemoglobin. In what follows, we focus on the valency hybrids, which have been studied in greatest detail; a separate paper will discuss the metal hybrids.

The valency hybrids can be characterized by the spin state of the ferric iron in the oxidized chains. In the low-spin hybrids (such as cyanomet and azomet) the iron is expected to have its equilibrium position in the plane of the porphyrin ring (disregarding any interaction with the protein), while in the high-spin hybrids (such as fluoromet and aquomet) the iron should lie about 0.3 Å out of the porphyrin plane in accord with X-ray results for model compounds (Hoard, 1971; Hoard and Scheidt, 1973). This suggests that the unoxygenated low-spin valency hybrids can be regarded as models for the normal hemoglobin intermediates $Hb(O_2)_2$ in which either two α or two β chains have been oxygenated; the high-spin valency hybrids, by contrast, do not correspond directly to their oxygenated analogs but rather have some intermediate form. It is shown in this paper how these ideas can serve to determine the structural parameters in a simple model for the equilibrium properties of the high- and low-spin valency hybrids. The model proposed for the valency hybrids is found to be applicable also to the properties and replacement reactions of methemoglobin and to the oxidation–reduction equilibrium of normal hemoglobin. In developing and applying the model, we have been concerned with the qualitative relationships that can be obtained by its use and have not stressed quantitative details, except where they can aid our understanding of the interactions involved.

Section 1 provides the theoretical background required to understand the subsequent arguments. A thermodynamic description of ligand binding is specialized to dimer behavior and it is shown how a model of the allosteric type for relating structure and function in hemoglobins can be applied to the hybrids. The properties of the low-spin valency hybrids are considered in section 2 and of the high-spin valency hybrids in section 3. It is demonstrated that low- and high-spin hybrids can be understood by using the same model with structurally motivated changes in its parameters. In section 4, it is shown that the arguments developed for the valency hybrids are consistent with the properties of methemoglobin and provide a simple explanation of the cooperativity found in certain ligand replacement reactions. A corresponding treatment of the oxidation–reduction equilibrium of normal hemoglobin is given in section 5. The concluding discussion is presented in section 6.

(1) Thermodynamic Description and the Allosteric Model

The equilibrium of a macromolecule M with N binding sites for a ligand X at concentration (activity) λ can be described by a generating function (Szabo and Karplus, 1972) defined as:

$$\Xi_N(\lambda) = \sum_{s=0}^{N} \lambda^s A_s \tag{1}$$

where A_s is the macroscopic equilibrium constant for binding of s ligands:

$$M + sX \rightleftharpoons MX_s \tag{2}$$

The utility of the generating function, Ξ_N, lies in the fact that each term $\lambda^s A_s$, $s = 0, 1, \ldots N$, is proportional to the probability that s ligands are bound. Thus, the fractional saturation, $\langle y \rangle$, of the macromolecule with ligand is given by:

$$\langle y \rangle = \frac{\langle s \rangle}{N} = \frac{\sum_{s=0}^{N} s\lambda^s A_s}{\sum_{s=0}^{N} \lambda^s A_s} = \frac{\lambda}{N}\frac{\partial}{\partial \lambda}[\ln \Xi_N(\lambda)] \tag{3}$$

From eq 3, it is clear that the ligand binding curve is uniquely determined by the N parameters $A_1, A_2, \ldots A_N$ ($A_0 = 1$). It is their detailed interpretation which provides the link between structure and function that corresponds to an understanding of the binding properties of the macromolecule (Szabo and Karplus, 1972; A. Szabo and M. Karplus, manuscript to be published).

For the present application to hybrid hemoglobins, we consider eq 1–3 specialized to the dimer case ($N = 2$). By a "dimer" we mean any species, such as the valency hybrids, which has two equivalent ligand binding sites even it it is composed of more than two subunits. For the dimer, eq 1 reduces to

$$\Xi_2(\lambda) = A_0 + A_1\lambda + A_2\lambda^2 \tag{4}$$

In eq 4, the consecutive terms are proportional, respectively, to the probability that none, one, and two ligands are bound. If K_i is the Adair equilibrium constant for the reaction $MX_{i-1} + X \rightleftarrows MX_i$, then $A_0 = 1$, $A_1 = K_1$, and $A_2 = K_1K_2$. The fractional saturation $\langle y \rangle$ obtained from eq 3 and 4 is:

$$\langle y \rangle = \frac{1}{2}\lambda\frac{\partial}{\partial\lambda}[\ln \Xi(\lambda)] = \frac{1}{2}\frac{A_1\lambda + 2A_2\lambda^2}{A_0 + A_1\lambda + A_2\lambda^2} \tag{5}$$

If the system is "homogeneous" (i.e., it is free from impurities, M is not dissociating, and there are no interactions between different M), eq 4 and 5 provide an exact and complete phenomenological description. From eq 5, applied to oxygenation with λ equal to the partial pressure (p) of O_2, it follows that the partial pressure at half saturation, $p_{1/2}$, and the Hill coefficient, n, at $p_{1/2}$ are given by:

$$p_{1/2} = \sqrt{A_0/A_2} = 1/\sqrt{K_1K_2} \quad (6a)$$

$$n = \frac{4}{2 + \sqrt{A_1^2/A_2A_0}} = \frac{4}{2 + \sqrt{K_1/K_2}} \quad (6b)$$

Solving eq 6a and 6b for K_1 and K_2, we find

$$K_1 = \frac{2(2 - n)}{n} \frac{1}{p_{1/2}} \quad (7a)$$

$$K_2 = \frac{n}{2(2 - n)} \frac{1}{p_{1/2}} \quad (7b)$$

Thus, the Adair constants and hence the entire binding curve for a macromolecule with two binding sites are uniquely specified by n and $p_{1/2}$. A corresponding result is not applicable, in general, to more complicated cases, such as the hemoglobin tetramer.

An important consequence of eq 5, first noted by Wyman (1967), is that the two-site binding curve (i.e., $\langle y \rangle$ *vs.* log λ) must be symmetric about $\lambda_{1/2}$. The significance of this statement based solely on thermodynamic reasoning is that if the measured binding curve shows asymmetry, the sample studied must be "heterogeneous"; i.e., it does not obey eq 4 and 5, and therefore cannot correspond simply to ligand binding by a dimer. (For analysis of a dimer including dissociation, see Appendix A.) Thus, caution must be used in interpreting the n values from such measurements; we shall return to this point in the following sections.

Generalized Allosteric Model. To go beyond a thermodynamic description of the hybrid hemoglobins, it is necessary to introduce assumptions which make it possible to use available structural information in a determination of their functional properties. For this purpose, we utilize a detailed model proposed previously for structure-function relations of hemoglobin and extend the qualitative discussion of the valency hybrids given in the original paper (Szabo and Karplus, 1972). In its simplest formulation the model can be written in the generalized Monod-Wyman-Changeux form for an allosteric system (Szabo and Karplus, 1972; A. Szabo and M. Karplus, manuscript to be published; Ogata and McConnell, 1972a,b). For the tetramer with inequivalent chains, the generating function appropriate to the model is:

$$\Xi_4(\lambda) = L(1 + c_\alpha K_\alpha \lambda)^2(1 + c_\beta K_\beta \lambda)^2 + (1 + K_\alpha \lambda)^2(1 + K_\beta \lambda)^2 \quad (8)$$

where L is the equilibrium constant (in the absence of ligand) between the oxyquaternary structure (referred to as R) and the deoxyquaternary structure (T), K_α and K_β are the chain ligand binding constants in the R state, and c_α and c_β represent the reduction of the binding constants in the T state.

To be able to specialize eq 8 to the hybrid hemoglobins, it is appropriate to summarize the nature of the detailed model and to show how the assumptions involved provide a structural basis for the phenomenological parameters K_α, K_β, c_α, c_β, and L. It is assumed that there are two quaternary structures for the tetramer (T and R) and within each quaternary structure each subunit chain has two tertiary structures (unliganded and liganded). Within a given quaternary structure, subunits bind ligand independently since it is assumed that the tertiary structure of one chain is not altered directly by the state of ligation of another (A. Szabo and M. Karplus, manuscript to be published). Furthermore, certain interactions (primarily inter- and intrachain salt bridges) are assumed to be affected by tertiary and quaternary structural changes (Perutz, 1970). The interactions can be of three types (A. Szabo and M. Karplus, manuscript to be published): they are altered only when the quaternary structure goes from T to R (quaternary linked), they are altered only in going from the unliganded to the liganded tertiary structure (tertiary linked), and they are altered both when the quaternary structure changes from T to R and when the tertiary structure changes from unliganded to liganded in the T state (tertiary and quaternary linked). For structural reasons (Perutz, 1970), we do not include the possibility that the interactions are modified significantly only on ligation when the tetramer is in the R state. In eq 8, the parameter L contains contributions from interactions coupled to the quaternary structural change alone or to both quaternary and tertiary structural changes. The parameters c_α and c_β are composed of interactions coupled to both tertiary and quaternary structural changes. They represent the effect of constraints which lead to the reduction of the affinity for ligand in the T state. This reduction can be due to the presence of salt bridges which must be broken if a subunit in the T state (but not in R) is to bind ligand and/or to other stereochemical factors (e.g., if the neighborhood of the heme in the T state is such that ligand binding requires certain groups to move out of the way) (Perutz, 1970). An interaction (salt bridge) linked only to tertiary structural change (ligation of a subunit) would alter K_α or K_β. It would, therefore, affect primarily the affinity (i.e., the $p_{1/2}$ value), though it could have an indirect effect on cooperativity by introducing greater inequivalence in the apparent chain binding constants.

From the above discussion, the parameter L for hemoglobin is expected to be independent of the type of ligand, while both K_α, K_β and c_α, c_β are expected to be different for different ligands. In particular, if the tertiary structural change on ligation is not the same for two ligands, they are expected to have different values of c (c_α and c_β); such is the case for oxygenation vs. oxidation (see below).

We now apply the allosteric model to a hemoglobin valency hybrid in which the β chains have been oxidized ($\alpha_2\beta_2^+$); the argument holds for a hybrid in which the α chains are oxidized if the labels α and β are interchanged. The appropriate dimer generating function is:

$$\Xi_{2,\beta^+}(\lambda) = L_h^\alpha(1 + c_\alpha K_\alpha \lambda)^2 + (1 + K_\alpha \lambda)^2 \quad (9)$$

where L_h^α is the L constant for the hybrid and K_α and c_α are the parameters for the unoxidized α chains. Since L_h^α is the equilibrium constant between the two quaternary structures, the fraction of hybrids in the T state in the absence of ligand is:

$$f_T^\alpha = \frac{L_h^\alpha}{L_h^\alpha + 1} \quad (10)$$

Comparison of eq 9 with eq 4, 6a, and 6b shows that

$$p_{1/2} = \frac{1}{K_\alpha}\left(\frac{L_h^\alpha + 1}{L_h^\alpha c_\alpha^2 + 1}\right)^{1/2} \simeq \frac{(L_h^\alpha + 1)^{1/2}}{K_\alpha} \quad (11a)$$

$$n = \frac{2}{1 + \frac{L_h^\alpha c_\alpha + 1}{[(L_h^\alpha + 1)(L_h^\alpha c_\alpha^2 + 1)]^{1/2}}} \simeq \frac{2}{1 + \frac{L_h^\alpha c_\alpha + 1}{(L_h^\alpha + 1)^{1/2}}} \quad (11b)$$

where the approximate equalities hold for $(L_h^\alpha c_\alpha^2 \ll 1)$. We can eliminate L_h^α from eq 11a and 11b and obtain:

$$n = \frac{2\kappa_{1/2}(1 + c_\alpha)}{(1 + \kappa_{1/2})(1 + c_\alpha\kappa_{1/2})} \quad (12a)$$

where $\kappa_{1/2} = K_\alpha p_{1/2}$. For a weakly cooperative system ($\kappa_{1/2} \simeq 1$, $n \simeq 1$) we expand eq 12a for n about $(\kappa_{1/2} - 1)$ and find:

$$n \simeq 1 + \frac{(1 - c_\alpha)}{2(1 + c_\alpha)}(\kappa_{1/2} - 1) \simeq 1 + \log \kappa_{1/2} \quad (12b)$$

for $n \leq 1.3$, $c_\alpha \leq 0.05$. Equation 12b shows that for the dimer in the region of low n, the change in n at constant K_α is directly proportional to the change in $\log p_{1/2}$.

To illustrate the above equations for the two-site case, we plot in Figures 1a and 1b, respectively, n and $\log \kappa_{1/2}$ against $\log L_h$ for $c = 0.01$ and $c = 0.05$. In Figure 2 we plot n vs. $\log \kappa_{1/2}$ for the same values of c. From Figure 1 we see that for small c ($c \leq 0.01$), no mechanism which changes L_h alone can raise $\log \kappa_{1/2}$ from zero by more than 0.5 without raising n above 1.5. Figure 2 shows that for low values of n ($n \leq 1.3$) the change in n is nearly equal to the change in $\kappa_{1/2}$ for $c \leq 0.05$, in agreement with eq 12b. It is only in the region of higher n that the change in affinity does not affect the n value significantly; i.e., the value of n is "buffered." The significance of these results will appear in the subsequent sections when we consider the correlation of the properties of low- and high-spin valency hybrids.

(2) Low-Spin Valency Hybrids

The cyanomet hybrids [$\alpha_2\beta_2^+(CN^-)$ and $\alpha_2^+(CN^-)\beta_2$] have been extensively studied; other low-spin valency hybrids (e.g., with N_3^- and OH^- as ligands) have also been examined but fewer data are available for them. To correlate their properties with one another and with those of normal hemoglobin, we must determine how L_h and c_α, c_β are dependent on the structure of the hybrids. The simplest assumption is that for a low-spin hybrid (e.g., cyanomet) the ferric chains have the same tertiary structure as an oxygenated chain, while the ferrous chains are unaffected. Thus, the structure of the low-spin hybrids $\alpha_2^+(ls)\beta_2$ and $\alpha_2\beta_2^+(ls)$ is assumed to be identical with the partially oxygenated intermediates $\alpha_2(O_2)\beta_2$ and $\alpha_2\beta_2(O)_2$, respectively. From a comparison of eq 8 and 9, this implies that:

$$\begin{aligned} &\alpha_2\beta_2^+(ls): && L_h^\alpha = Lc_\beta^2 \\ &\alpha_2^+(ls)\beta_2: && L_h^\beta = Lc_\alpha^2 \end{aligned} \quad (13)$$

where L, c_α, and c_β refer to normal hemoglobin. Such a relation was used explicitly by Ogata and McConnell (1972a,b) and implicitly by Szabo and Karplus (1972) in their consideration of the cyanomet hybrids. It follows directly from the assumption that the formation of the low-spin complex has the iron in the plane of the porphyrin and that the salt bridges or other interactions coupled to both tertiary and quaternary structures are altered in the same way as in the oxygenated form. It is certainly possible that even though in these hybrids the iron is low spin and lies in the plane of the porphyrin ring, the tertiary structure of the oxidized chains is not identical with the oxygenated chains; e.g., the iron–proximal-histidine distance might well be different in an oxidized vs. an oxygenated chain. If that were true, eq 13 would be satisfied only approximately (see below). A generating function having the *form* given by eq 9 would still be applicable with the constant L_h different from that given by eq 13.

One of the successes of an allosteric model of the type described here is that it can qualitatively account for a large body of experimental data concerning the low-spin valency hybrids by assuming that eq 13 is approximately valid with $L_h^\alpha < L_h^\beta$, so that the unliganded $\alpha_2\beta_2^+(ls)$ hybrid lies closer to the oxyquaternary structure (R state) than does $\alpha_2^+(ls)\beta_2$ (Ogata and McConnell, 1972a,b). In terms of eq 13, this requires that the interactions coupled to both the tertiary structure of the β chains and the quaternary structure are stronger than the corresponding interactions involving the α chains ($c_\beta < c_\alpha$). The following is a list of observations which are consistent with this interpretation: (1) the Hill coefficient, n, is greater for $\alpha_2^+(ls)\beta_2$ than for $\alpha_2\beta_2^+(ls)$ (Brunori et al., 1970; Banerjee et al., 1973b); (2) the hyperfine shifted proton magnetic resonance spectrum of the subunit containing the oxidized iron changes upon the oxygenation of $\alpha_2^+(ls)\beta_2$ but remains invariant for the hybrid $\alpha_2\beta_2^+(ls)$ at pH 7 in phosphate buffer (Ogawa and Shulman, 1972); (3) a larger change in the electron spin resonance (ESR) spectrum of a spin label attached to the reactive SH group of the β chains is observed on the oxygenation of $\alpha_2^+(ls)\beta_2$ than of $\alpha_2\beta_2^+(ls)$ (Ogawa et al., 1968); (4) the SH groups at 93β are more reactive in unliganded $\alpha_2\beta_2^+(ls)$ than in $\alpha_2^+(ls)\beta_2$ (Maeda and Ohnishi, 1971); (5) the 2,3-diphosphoglycerate binding constant of $\alpha_2^+(ls)\beta_2$ is larger than that for $\alpha_2\beta_2^+(ls)$ (Ogata and McConnell, 1972a,b; Bauer et al., 1973). The recent observations of Henry and Banerjee (1973) on the ESR spectrum of NO in the $\alpha_2(NO)\beta_2$ and $\alpha_2\beta_2(NO)$ hybrids are also in agreement with the above conclusions on the relative strengths of the α and β chain interactions. They find that the ESR spectrum of $\alpha_2(NO)\beta_2$ changes when the β chains are oxygenated while that of $\alpha_2\beta_2(NO)$ is invariant upon oxygenation of the α chains.

When one attempts to make the above correlations quantitative (Szabo and Karplus, 1972; Ogata and McConnell, 1972a,b), certain difficulties arise. The most serious involves the interpretation of the fact that the cyanomet hybrids appear to have a normal Bohr effect ($\Delta \log p_{1/2} \simeq 1.0$ between pH 7 and 9) (Banerjee et al., 1973b) while the n values are close to unity ($\alpha_2^+(CN^-)\beta_2$, $n \simeq 1.3$; $\alpha_2\beta_2^+(CN^-)$, $n = 1.0$) and independent of pH. These results, if correct, require that all the salt bridges responsible for the alkaline Bohr effect are broken only when the tertiary structure of subunit changes from unliganded to liganded and are independent of quaternary structure. As described in section 1, such salt bridges would effect only K_α and K_β and would, therefore, not alter the cooperativity of the hybrids. In the model previously proposed for hemoglobin structure–function relations (Szabo and Karplus, 1972), most of the salt bridges were coupled to quaternary, as well as to tertiary structure. One of the salt bridges involved in the Bohr effect (i.e., the intrachain salt bridge between the imidazole group of histidine 146β and the carboxyl group 94β) was assumed to depend only on tertiary structural changes. A corresponding coupling to tertiary structure alone of certain salt bridges involved in the Bohr effect contribution of the α chains is also possible, particularly because there is still a part of the Bohr effect that is not completely accounted for (Kilmartin and Rossi-Bernardi, 1973). However, indirect evidence (i.e., that modified hemoglobins have a significant Bohr effect if "frozen" in the deoxyquaternary structure and none if "frozen" in the oxy structure) suggests that the dominant contribution to the Bohr effect comes from salt bridges linked to both quaternary and tertiary structural changes (Szabo and Karplus, 1972; A. Szabo and M. Karplus, manuscript to be pub-

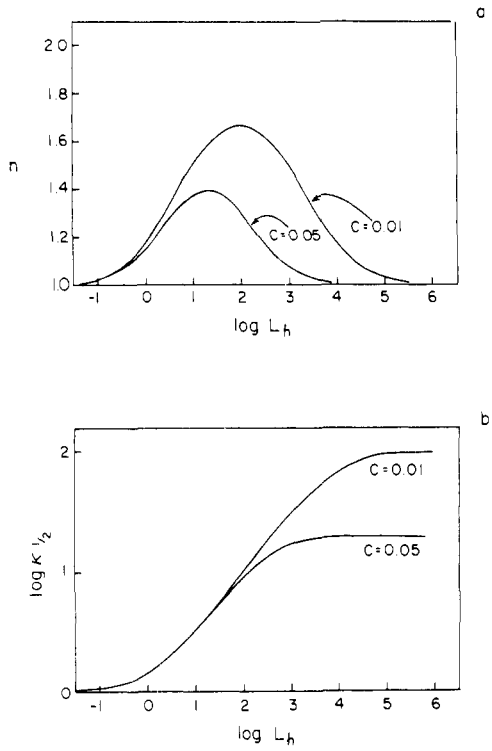

FIGURE 1: Allosteric model for hemoglobin hybrids of (a) Hill coefficient n and (b) scaled affinity log $\kappa_{1/2}$ as a function of log L_h for c = 0.01 and 0.05.

lished; J. Baldwin, manuscript to be published). For the valency hybrids, this implies that a normal Bohr effect requires quaternary transition on oxygenation; that is, the unliganded hybrids must have a value of f_T significantly different from zero (eq 10). Associated with the required quaternary transition, the n values must be on the order of 1.5 at neutral pH. To show this, we first estimate L_h for the valency hybrids; we drop α and β subscripts for simplicity. Using eq 11a we can write:

$$L_h \simeq (p_{1/2}K)^2 - 1 \qquad (14)$$

where K is the oxygen binding constant of a subunit in the oxyquaternary structure (see eq 9). Free-chain measurements of K yield values of ~3 $mmHg^{-1}$ at pH 7 (Bunn and Guidotti, 1972; J. V. Kilmartin, private communication), while the detailed model for hemoglobin (Szabo and Karplus, 1972) suggests $K \simeq 3$–5 $mmHg^{-1}$. With K values in this range and the experimental $p_{1/2}$ values of Banerjee et al. (1973b) and Brunori et al. (1970) for cyanomet ($p_{1/2} \simeq 2.5$) and azomet ($p_{1/2} \simeq 1.1$) hybrids at neutral pH, we obtain from eq 14 that $(L_h)_{cyanomet} \gtrsim 60$ and $(L_h)_{azomet} \gtrsim 10$. Normal hemoglobin at pH 7 has $L \simeq 10^5$ (Szabo and Karplus, 1972), so that from eq 13 the estimated c values are $c(CN^-) \simeq 0.024$ and $c(N_3^-) \simeq 0.01$, which are somewhat larger than the corresponding c value for hemoglobin ($c \sim 0.005$), perhaps reflecting the fact that the tertiary structure of low-spin met chains is not identical with that of oxygenated chains. For these values of L_h and $c \sim 0.01$, Figure 1a shows that the expected n values are greater than 1.5. Furthermore, for these hybrid systems at pH >7, log $\kappa_{1/2}$ is in a range such that eq 12 and Figure 2 should be applicable. This implies that as pH increases, the change in n should be proportional to that in log $\kappa_{1/2}$. At high pH (pH 9), Banerjee et al. (1973b) find $p_{1/2} \simeq 0.3$, which requires $n \simeq 1.0$ according to Figure 2 and a deoxygenated hybrid

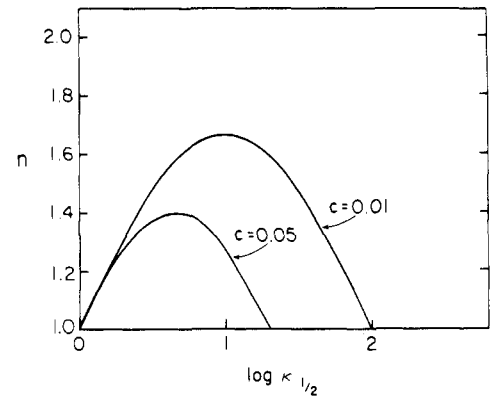

FIGURE 2: Allosteric model for hemoglobin hybrids; Hill coefficient n vs. the scaled affinity log $\kappa_{1/2}$ for c = 0.01 and 0.05.

mainly in the oxyquaternary structure. Although $n \simeq 1.0$ at pH 9, its values does not increase significantly at lower pH.[1]

The dilemma posed by the above analysis may be linked to the fact that the experimental binding curves are asymmetric whereas *independent of detailed models* (see section 1) the curves must be symmetric if the system is homogeneous. This indicates that the preparations must contain some impurities or the hybrids are dissociating. We assume that the n values are much more sensitive to heterogeneities than the affinities. In other words, the observed changes in $p_{1/2}$ (factors of 5 to 10) are taken to be meaningful while the essential invariance of n is considered as an artefact. It is proposed that experiments on pure hybrids (if such were possible) would yield greater n values in agreement with the predictions of the model. An analogous situation has occurred in the measurement of n values for the cyanomet hybrids in the presence and absence of 2,3-diphosphoglycerate. Haber and Koshland (1971) reported that 2,3-diphosphoglycerate affects the affinity of $\alpha_2^+(CN^-)\beta_2$ without changing the n value. However, more recent experiments by Maeda et al. (1972) show that for $\alpha_2^+(CN^-)\beta_2$ at pH 7.4 in 0.1 M NaCl, the addition of 2×10^{-3} M diphosphoglycerate to the stripped hybrid raises n from 1.17 to 1.52, while producing a change in log $p_{1/2}$ of 0.22. As can be seen from Figure 2, this result is in agreement with the predictions of the allosteric model if it is assumed that the primary effect of diphosphoglycerate is to increase L_h (Szabo and Karplus, 1972; Ogata and McConnell, 1972a,b; Herzfeld and Stanley, 1974).

Minton (1974) has recently published an attempt to fit certain data for the cyanomet hybrids by an allosteric model of the type considered here. Assuming that eq 13 is exact, he had difficulty obtaining quantitative agreement with the hybrid data. This is not surprising since, as we have suggested above, the constant $c(CN^-)$ appears to be different from the c value for hemoglobin. Moreover, the hybrid measurements of Maeda et al. (1972) used by Minton are asymmetric and so are inherently inconsistent with ligand binding by a dimer (see section 1).

(3) High-Spin Valency Hybrids

The tertiary structure of the oxidized chains in high-spin valency hybrids (e.g., the aquomet and the fluoromet hy-

[1] R. Banerjee (private communication) has found on a reexamination of the hybrid data used in Banerjee et al. (1973b) that it suggests an increase in n with decreasing pH, though the scatter of the results ($1.0 \leq n \leq 1.5$) is too great to make a definitive statement.

brids) is expected to be different from that for the low-spin hybrids. As described in the introductory statement, the iron of the oxidized chains in the former appears to have its minimum energy position out of the porphyrin plane by about 0.3 Å, while in the low-spin ferric chains and oxygenated hemoglobin, the iron radius is such that its equilibrium position is in the porphyrin plane. Hence, it is reasonable to assume that the tertiary structure of high-spin oxidized chains in the deoxyquaternary structure is intermediate between the unliganded and liganded forms (Szabo and Karplus, 1972). It was suggested further (Karplus, 1972) that the oxidized high-spin chains of hemoglobin molecules in the deoxyquaternary structure would have tertiary–quaternary coupled salt bridges that are destabilized but not broken; in the oxyquaternary structure, which is assumed to be essentially the same as that of oxygenated hemoglobin, the corresponding salt bridges would be broken. These predictions have been confirmed by the recent X-ray study of aquomethemoglobin in acrylamide gel containing crystals (Anderson, 1973). It is, of course, possible that even for an oxygenated subunit in the deoxyquaternary structure, the salt bridges are only strained and not broken; such a reinterpretation of the model (Szabo and Karplus, 1972; A. Szabo and M. Karplus, manuscript to be published) would require only that the strain on oxygenation be larger than that on oxidation. Use of the above results in the model developed in section 1 requires that the value of L_h for the high-spin hybrids be larger than that in the low-spin hybrids. Formally, we can write (the primed values refer to high spin):

$$\begin{aligned} &\alpha_2\beta_2^{+}(\text{hs}): && L_{h'}^{\alpha} = L(c_\beta')^2 \\ &\alpha_2^{+}(\text{hs})\beta_2: && L_{h'}^{\beta} = L(c_\alpha')^2 \end{aligned} \tag{15}$$

where $c_\beta' > c_\beta$ and $c_\alpha' > c_\alpha$. Consequently, for either the α or the β chains oxidized in the hybrid:

$$L_h(\text{hs}) > L_h(\text{ls}) \tag{16}$$

with L_h(ls) close to the effective L value for $Hb(O_2)_2$. This means that the deoxyquaternary structure (T) makes a larger contribution to the equilibrium in the deoxygenated high-spin hybrids than to the low-spin hybrids (i.e., $f_T^{hs} > f_T^{ls}$) and that, as can be seen from Figure 1b:

$$(p_{1/2})_{hs} > (p_{1/2})_{ls} \tag{17}$$

The experimental results of Banerjee et al. (1973b) are in agreement with this analysis. They find at pH 7 the $p_{1/2}$ value of the fluoromet hybrids to be 5.5 mmHg, as compared with 2.5 and 1.1 mmHg for the low-spin cyanomet and azomet hybrids under comparable conditions (see above). Using eq 14, we estimate that $L_{h'}^{\alpha} \simeq L_{h'}^{\beta} > 330$ for the fluoromet hybrids. Ignoring, for simplicity, differences between the α and β chains, we can use the estimates of L_h in eq 13 and 15 to obtain:

$$\frac{c(F^-)}{c(CN^-)} \simeq 2.4; \quad \frac{c(F^-)}{c(N_3^-)} \simeq 5.7 \tag{18}$$

Clearly, these ratios are approximate. However, we shall show how to use them in the following section to analyze certain reactions of methemoglobin with high- and low-spin ligands.

A number of additional correlations between the properties of high- and low-spin hybrids are consistent with the above interpretation. The value of $p_{1/2}$ of $\alpha_2\beta_2^{+}(H_2O)$ is similar to the $p_{1/2}$ of $\alpha_2\beta_2^{+}(F^-)$, while the $p_{1/2}$ of $\alpha_2\beta_2^{+}(OH^-)$ is similar to the $p_{1/2}$ of $\alpha_2\beta_2^{+}(CN^-)$. This is in accord with the fact that OH^- is a "stronger" ligand than H_2O in the sense that the presence of OH^- as the sixth ligand leads to low-spin iron while H_2O results in a high-spin iron. It is of interest, and may be significant, that this behavior of OH^- and H_2O is the inverse of that found in the usual spectrochemical series for octahedral complexes (Day and Selbin, 1962). In a comparison involving NO intermediates, Henry and Banerjee (1973) found that the NO electron paramagnetic resonance spectra of $\alpha_2(NO)\beta_2$, $\alpha_2(NO)\beta_2^{+}(F^-)$, and $\alpha_2(NO)\beta_2^{+}(H_2O)$ were very similar, but very different from the spectra of $\alpha_2(NO)\beta_2(O_2)$ and $\alpha_2(NO)\beta_2^{+}(CN^-)$, which were again similar. One other prediction concerns the relative n values of the high- and low-spin complexes. As pointed out in section 2, Figure 2 should be applicable to these systems with $c \simeq 0.01$. This implies that n should be proportional to log $\kappa_{1/2}$ in the observed range, so that $(n)_{hs} > (n)_{ls}$. In fact, the results of Banerjee et al. (1973b) are not in agreement with this prediction, in that they find low n values for both high- and low-spin hybrids (e.g., $n = 1.1$ for $\alpha_2\beta_2^{+}(N_3^-)$ and $n = 1.2$ for $\alpha_2\beta_2^{+}(F^-)$, respectively). However, since the binding curves are markedly asymmetric, it is reasonable to suggest that the measured n values are too low (see sections 1 and 2).

(4) The Ligand Replacement Reactions of Methemoglobin

The analysis of the high-spin and low-spin hemoglobin valency hybrids in the previous sections provides the information necessary for an understanding of the ligand replacement reactions of methemoglobin of the type

$$Hb^{+}(Y)_4 + 4X \rightleftharpoons Hb^{+}(X)_4 + 4Y \tag{19}$$

where X and Y can be F^-, CN^-, N_3^-, and H_2O or OH^- depending on the pH. We assume that the allosteric model of section 1 is applicable and that the c parameters determined for the hybrids are appropriate for the methemoglobin with the corresponding ligand. If L is the allosteric constant for normal (unoxidized) hemoglobin, the allosteric constant L_m for methemoglobin with a particular ligand is Lc^4 where the c parameter is the one appropriate for that ligand. Thus, the fraction, g_T, of the methemoglobin that exists in the deoxyquaternary (T) structure is given by (see eq 10):

$$g_T = \frac{L_m}{L_m + 1} = \frac{Lc^4}{Lc^4 + 1} \tag{20}$$

In the oxygenation reaction, $L \simeq 10^5$ at pH 7, $c < 0.01$, and $Lc^4 \ll 1$, so that for the completely oxygenated tetramer, $Hb(O_2)_4$, essentially all of the molecules are in the oxyquaternary structure. In methemoglobin bound to ligands that yield a high-spin iron, we have seen in section 3 that the value of c can be significantly larger ($c \simeq 0.06$). Thus, it is possible that $Lc^4 \geq 1$ and that by eq 20, the species $Hb^{+}(Y)_4$ still has an important contribution from the deoxy (T) structure. From the analysis of the valency hybrids (sections 2 and 3) we have:

$$\begin{aligned} &\text{low pH:} && c(F^-) \simeq c(H_2O) > c(CN^-) \simeq c(N_3^-) \\ &\text{high pH:} && c(F^-) > c(OH^-) \simeq c(CN^-) \simeq c(N_3^-) \end{aligned} \tag{21a}$$

Since the L value which corresponds to the deoxy-/oxyquaternary equilibrium of hemoglobin in the absence of ligands is by its definition the same for all species at a given pH, eq 20 gives:

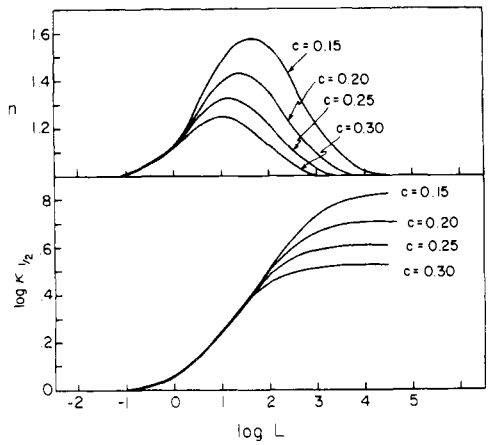

FIGURE 3: Allosteric model for ligand replacement reactions; Hill coefficient n and scaled affinity log $\kappa_{1/2}$ vs. log L for c = 0.15, 0.20, 0.25, and 0.30.

$$\text{low pH:} \quad (g_T)_{F^-} \simeq (g_T)_{H_2O} > (g_T)_{CN^-} \simeq (g_T)_{N_3^-} \tag{21b}$$

$$\text{high pH:} \quad (g_T)_{F^-} > (g_T)_{OH^-} \simeq (g_T)_{CN^-} \simeq (g_T)_{N_3^-}$$

In the replacement reaction shown in eq 19, if $Hb^+(Y)_4$ exists primarily in the T state (i.e., $(g_T)_Y > 0.5$) while $Hb^+(X)_4$ exists primarily in the R state (i.e., $(g_T)_X < 0.5$), there would be a quaternary structural change in the reaction and cooperative behavior would be expected. It is clearly possible in replacement reactions to also have cooperativity involving a quaternary structural change from R to T.

To make the above discussion more quantitative, we construct the generating function for the replacement reaction. If we define K_{XY} to be the equilibrium constant for the replacement of Y by X on a subunit in methemoglobin in the oxyquaternary (R) structure, the generating function for the binding of X by $Hb^+(Y)_4$ is:

$$\Xi[X] = Lc_Y^4(1 + \frac{c_X}{c_Y}K_{XY}[X])^4 + (1 + K_{XY}[X])^4 \tag{22}$$

where the concentration of X is relative to that of Y. If the total concentration of Y is much greater than the hemoglobin concentration, the free concentration of Y will remain constant during the course of the replacement reaction; such is the case when Y is H_2O and OH^- in a buffered solution. Equation 22 is of the same form as the generating function for oxygen binding to hemoglobin in the Monod-Wyman-Changeux model (Monod et al., 1968). To allow for the inequivalence between the α and the β chains, a similar argument can be used to obtain an appropriately modified form of eq 8. Such a generalization, although it would allow us to describe n values less than unity, is not made here because it would complicate the development without adding qualitative insight. From eq 22 it follows that the replacement reaction is expected to be cooperative when (a) $Lc_Y^4 > 1$ and $c_Y > c_X$, so that $Lc_X^4 < 1$; (b) $Lc_Y^4 < 1$ and $c_Y < c_X$, so that $Lc_X^4 > 1$. In case (a) the quaternary structure changes from deoxy to oxy in the replacement reaction and in case (b) it changes from oxy to deoxy. It is important to note that in eq 22 we expect $c_{eff} = c_X/c_Y$ to be much larger than the value appropriate for oxygenation ($c \lesssim 0.01$). To illustrate the nature of the cooperative behavior for such large values of c, we plot in Figure 3 n and log $\kappa_{1/2}$ vs. L ($L = L_m = Lc_Y^4$) for c_{eff} = 0.15, 0.2, 0.25, and 0.3. We note the maximum n value is less than 1.6 and that the range of L values over which the system is cooperative is small relative to that for hemoglobin oxygenation.

We now apply the theory outlined above to the reaction to methemoglobin at low and high pH with F^- and N_3^- for which data have been obtained recently by Banerjee et al. (1973a). In contrast to the valency hybrids, where experimental difficulties are likely to invalidate determinations of n (see sections 2 and 3), the methemoglobins are relatively more stable and better behaved so that n values for the replacement reactions (even for n near unity) should be meaningful. At low pH (pH 6), methemoglobin exists in the aquomet form. Since $c(H_2O) \simeq c(F^-)$, we expect the reaction in which fluoride replaces H_2O to be noncooperative. This would mean $n \simeq 1$ for equivalent chains or a value slightly less than unity for inequivalent chains. The experimental value of n at pH 6 is 0.92 (Banerjee et al., 1973a). For the reaction of aquomethemoglobin with azide, the value of $c_{eff} = [c(N_3^-)/c(H_2O)] \simeq 0.18$ so that cooperativity ($n > 1.2$) is possible for log L_m between ~0.5 and 2.8 (see Figure 3). Experimentally (Banerjee et al., 1973a), it is found that $n = 1.4$ for this reaction, indicating that under the experimental conditions aquomethemoglobin is more in the T state while azomethemoglobin is primarily in the R state (case (a) mentioned above). For the L value corresponding to hemoglobin at pH 7 ($L \simeq 2 \times 10^5$) and $c(H_2O) \simeq 0.06$ from above and the previous section, we find $L_m \simeq 2.6$, which yields $n \simeq 1.3$ from Figure 3 in satisfactory agreement with experiment. At high pH (9) the situation is different since methemoglobin exists in the hydroxymet form. For this pH, the reaction with azide is predicted to be noncooperative since $c(N_3^-) \simeq c(OH^-)$. However, the reaction with F^- should be slightly cooperative ($c(F^-)/c(OH^-) \simeq 2.3$) (case (b)). This is in accord with the experimental observations of Banerjee et al. (1973a), who find that n equals 1.02 for the azide reaction and 1.15 for the fluoride reaction at high pH.

From Figure 3 we note that, because c_{eff} is near unity, the cooperativity in the replacement reactions is very sensitive to the value of L_m and, therefore, to that of L. One way of altering L is to vary the concentration of phosphates. It would be of interest to examine the changes in cooperativity of the different replacement reactions under completely stripped conditions (i.e., in the absence of phosphate buffer) as well as at high concentrations of 2,3-diphosphoglycerate. In both limits for certain cases, the n values might be significantly reduced relative to those found by Banerjee et al. (1973a). This sensitivity to external conditions might well account for the fact that some workers (Epstein and Stryer, 1968) have not observed cooperativity in the replacement reactions.

(5) Oxidation-Reduction Equilibrium of Hemoglobin

In this section, we employ the allosteric model of section 1 to relate the oxygenation and oxidation of hemoglobin. The essential element of the argument is that the parameter L is unique for a given hemoglobin under a set of conditions, while both c and K depend on the reaction taking place. As already described in sections 3 and 4, the high-spin oxidized subunits of aquomethemoglobin have an intermediate tertiary structure in the deoxyquaternary state, so that the parameter c is larger than that for oxygenation. This means that the quaternary structure of aquomethemoglobin is less shifted toward the oxy form than is fully oxygenated hemoglobin. These ideas are sufficient for a qualitative understanding of the redox properties of hemoglobin. The interpretation given here is in essential agreement with

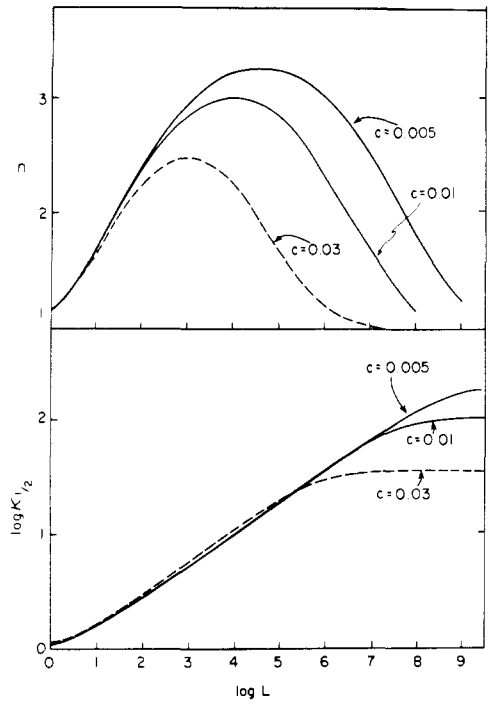

FIGURE 4: Allosteric model values of Hill coefficient n and scaled affinity log $\kappa_{1/2}$ vs. log L for c = 0.005, 0.01, and 0.03; the solid curves correspond to hemoglobin oxygenation and the dotted curve to oxidation.

the discussion of Perutz et al. (1974), which is in part based on the present analysis.

In Figure 4 we present the variation of n and log $\kappa_{1/2}$ with log L for c = 0.005, 0.01, and 0.03. Similar curves have been given by Rubin and Changeux (1966) and Edelstein (1971). For the oxidation-reduction process log $\kappa_{1/2}$ is proportional to $E_{1/2} - E_{1/2}{}^0$ where $E_{1/2}$ is the midpoint oxidation-reduction potential (Antonini and Brunori, 1971) and $E_{1/2}{}^0$ is the reference value corresponding to an independent subunit in the oxyquaternary structure. In Figure 4, the solid curves with c values of 0.005 and 0.01 are appropriate for oxygenation, while the dashed curve (c = 0.03) is taken as representative of oxidation; the actual value of c for both reactions depends on pH (see below). Since the same value of L applied to both processes, we can use the results obtained earlier in the analysis of oxygenation (Szabo and Karplus, 1972). For pH 7, L was found to be $\sim 2 \times 10^5$. This yields $n \simeq 3$ for oxygenation, while for oxidation n is calculated to be ~1.7 in agreement with experiment (Antonini and Brunori, 1971). To determine how n changes with pH, we need to know the pH dependence of the model parameters. The dominant effect (see section 1) comes from Bohr-effect-involved salt bridges that are coupled to both quaternary and tertiary structures. For oxygenation, this implies that L decreases and c increases; there is a possible additional effect on K from the salt bridges coupled to tertiary structure alone (Szabo and Karplus, 1972; A. Szabo and M. Karplus, manuscript to be published). For oxygenation at pH 9, the estimated value of L is $\sim 5 \times 10^2$ and c increases to 0.01–0.02 so that $n \simeq 2.5$ (Szabo and Karplus, 1972); that is, n is only weakly dependent on pH in the alkaline range. For the oxidation-reduction reaction, the situation is very different. Although L decreases, the value of c changes less than in oxygenation since there are two competing factors which affect c. As the pH is increased, the salt bridges are weakened as they are in oxygenation so that c tends to increase. However, at high pH, the aquomet form goes over to one with OH^- as the ligand. In sections 3 and 4, we saw that $c(OH^-) < c(H_2O)$. The relative magnitude of the two effects is difficult to estimate and for the sake of simplicity we assume that c for oxidation is independent of pH. Consequently, we see from the dotted curve in Figure 4 that as L decreases from 1×10^5 to 5×10^2, we expect the cooperativity for the oxidation reaction to increase significantly. This is in agreement with experiment (Brunori et al., 1969).

If the L value is such that for a given c the corresponding n is near its maximum, it can be shown that the release of Bohr protons is proportional to the oxygenation or oxidation reaction (Szabo and Karplus, 1972; Ogata and McConnell, 1972a,b). In the case of oxygenation, the range of L values between pH 7 and 9 is such that the criterion is well satisfied and the release of Bohr protons is proportional to the uptake of oxygen (Szabo and Karplus, 1972; Wyman, 1948). However, for oxidation, it is clear from Figure 4 that only at high pH would such a proportionality be expected and that at low pH the release of protons would lag behind to a significant degree. This conclusion is in agreement with the experiments of Brunori et al. (1969). In the presence of high concentrations of inositol hexaphosphate (IHP) at low pH, L increases to a limiting value on the order of 10^8. From Figure 4, we see that n for oxygenation is expected to decrease (Tyuma et al., 1973), as should the n for oxidation; the latter is expected to approach unity in agreement with experiment (Kilmartin, 1973). From Figure 4, we see also that both $p_{1/2}$ and $E_{1/2}$ increase with IHP due to the increase in L.

An interesting set of experiments was done by Brunori et al. (1967). They determined the effect of blocking the SH groups of Cys-β-93 with iodoacetamide. In terms of the allosteric model, the dominant result of this alteration is that L is decreased and c increased from its normal value. In the case of oxygenation, the effect would be a slight decrease in n and in the value of $p_{1/2}$. The effect on the oxidation reaction is expected to be greater, since a decrease of L coupled with a small change in c moves the system into the range where n is near its maximum (Figure 4). Consequently, for this modified system n for oxidation should be fairly insensitive to pH and the release of Bohr protons should be proportional to oxidation, in agreement with the experiment (Brunori et al., 1967).

Corresponding arguments apply to other modified or mutant hemoglobins in that Figure 4 allows us to unify the qualitative predictions of the allosteric model for oxygenation and oxidation. In species with high L values ($L \simeq 10^8$), oxygenation and oxidation should give very different n values whereas for species with low L ($L \simeq 10^2$), the n values for the two processes are expected to be similar. Moreover, for species with very low L values, organic phosphates should increase n for both oxidation and oxygenation.

There are a number of additional features of the hemoglobin tetramer which must be included in a more quantitative description of the oxidation process. For the oxidation Bohr effect, a detailed consideration of the contribution made by the replacement of the water molecule by hydroxide ion is required. Also, it appears that the α and β chains are more functionally inequivalent in oxidation than they are in oxygenation (MacQuarrie and Gibson, 1971; Banerjee and Cassoly, 1969). MacQuarrie and Gibson (1971) have shown, however, that contrary to the predictions of

Banerjee and Cassoly (1969) based on the properties of free α and β chains, the chain heterogeneity does not change with pH between 6.1 and 8.7. Nevertheless, a more complete description of the oxidation reaction of hemoglobin and its mutants must be based on an allosteric model with inequivalent α and β chains (Szabo and Karplus, 1972; Ogata and McConnell, 1972a,b). Only such a model could correctly describe n values which are less than one, a situation which can occur in mutants frozen in one of the quaternary structures.

(6) Conclusions

It has been shown that an allosteric model for the structure–function relations in normal hemoglobin can be used to interpret the behavior of high-spin and low-spin valency hybrids, the methemoglobin ligand replacement reactions, and the hemoglobin redox reaction. The results obtained provide evidence for the hypotheses that the hemoglobin tetramer can be regarded as having two quaternary structures (oxy and deoxy), in both of which the subunits can have a range of tertiary structures depending on the nature of the ligand and that, in a given quaternary structure, changes in the tertiary structure of one subunit do not directly induce significant alterations in the tertiary structure of the other subunits. Of most importance for understanding the systems considered here is the difference in tertiary structure between subunits with high-spin (out-of-plane) and low-spin (in-plane) ferric iron; the latter can be assumed to correspond approximately to the oxygenated subunit while the former are expected to have an intermediate tertiary structure. This interpretation permits one to estimate the changes, as a function of ligand, in the constraining interactions coupled to quaternary and/or tertiary structure and, thereby, to determine the model parameters for each type of system. The variation in the Hill coefficient, n, and the affinity, $p_{1/2}$, for the reactions can be calculated from these parameters. The trends obtained for the affinity are in agreement with experiment but the predicted changes in the n values have not been observed. Using a model independent argument, it is pointed out that the available n values may be inaccurate because heterogeneous preparations were studied.

Acknowledgments

We wish to thank B. Pullman for inviting us to the Institut de Biologie Physico-Chimique, where we had many useful discussions on the subject of the valency hybrids with R. Banerjee and R. Cassoly. One of us (A.S.) wishes to thank M. F. Perutz for his hospitality during a stay at the Medical Research Council Laboratory of Molecular Biology, where he benefited from numerous discussions with M. F. Perutz, J. Baldwin, and J. V. Kilmartin.

Appendix

Dissociation of Dimers. For a macromolecular dimer, each with one binding site, the binding curve is not symmetric, in general, if significant dissociation occurs. To formulate the expression for the binding curve in this case, we consider the equilibra:

$$M_2 \overset{K_D}{\rightleftharpoons} 2M$$
$$M + X \overset{K}{\rightleftharpoons} MX$$
$$M_2 + X \overset{K_1}{\rightleftharpoons} M_2X$$
$$M_2X + X \overset{K_2}{\rightleftharpoons} M_2X_2$$

The binding curve is:

$$\langle y \rangle = \frac{K_1\lambda + K_1K_2\lambda^2 + f(\lambda)K(X)}{2(1 + K_1\lambda + K_1K_2\lambda) + f(\lambda)(1 + K\lambda)}$$

where $f(\lambda)$ is the ratio $[M]/[M_2]$ at ligand concentration λ. Introducing $[M]_T$, the total concentration of species M:

$$[M]_T = [M] + [MX] + 2[M_2] + 2[M_2X] + 2[M_2X_2]$$

we find:

$$f(\lambda) = \frac{K_D(1 + K\lambda) + \sqrt{K_D^2(1 + K\lambda)^2 + 8K_D[M]_T(1 + K_1\lambda + K_1K_2\lambda^2)}}{2[M]_T}$$

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Amino Acid Sequence Studies on Plasmin-Derived Fragments of Human Fibrinogen: Amino-Terminal Sequences of Intermediate and Terminal Fragments†

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ABSTRACT: The progressive changes in amino-terminal sequence brought about by the digestion of human fibrinogen by plasmin have been studied. In addition, the limit products (fragments D and E) have been isolated and characterized in the same way. These studies have confirmed the generally accepted scheme of fibrinogen being changed into a large molecular weight fragment X, which in turn is converted into an intermediate fragment Y and a limit fragment D, followed by the breakdown of fragment Y into an additional fragment D and another core fragment E. Our data allow the precise identification of several of the junctions being attacked, including one in a region of the γ chain whose sequence has not previously been reported. The cleavages are not singular in any case, however, and, as suggested by others, intermediate species exist which correspond to "early D," "late D," etc. In addition to localizing the exact bonds split by plasmin, we have been able to sequentially position the core fragments relative to each other, since the γ-chain amino terminus of fragment D has been found to be contiguous to the known carboxy-terminal sequence of fragment E.

During the course of the last 15 years many different groups have studied the pattern of plasmin degradation of human fibrinogen (Nussenzweig *et al.*, 1961; Marder *et al.*, 1969; Gaffney and Dobos, 1971; Furlan and Beck, 1972; Pizzo *et al.*, 1972; Mills, 1972; Kowalska-Loth *et al.*, 1973; Mosesson *et al.*, 1973; *inter alia*). In recent years most of the data have been acquired by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In general, the results have been consistent with a scheme whereby fibrinogen is first transformed into a somewhat smaller molecule, fragment X, which is then split asymmetrically into a fragment D and a larger fragment Y (Marder, 1970). Fragment Y is subsequently broken into a second fragment D and a limit fragment E. The pattern is consistent with a dimeric fibrinogen molecule in which the fragment E represents a central domain and the two fragments D are disposed symmetrically about a central axis. A few investigators have challenged this interpretation, especially with regard to the numbers of fragments D and E which result, Mosesson *et al.* (1973) contending that there is only one fragment D per molecule, and Plow and Edgington (1974) claiming that there are really two fragments E. The situation is confused in part by the fact that plasmin degradation is not an entirely specific process, and there are "early" and "late" species of each of the major fragments, the relative amounts of which depend on the exact conditions of digestion.

In this article we report a detailed study of the plasmin digestion course for human fibrinogen, combining the use of sodium dodecyl sulfate gel electrophoresis for monitoring the fragmentation progress with amino-terminal sequence studies for identifying cleavage points. Our data generally confirm previous notions of an asymmetric cleavage scheme which ultimately yields two fragments D and one fragment E. They add to previous reports in that we have identified precise cleavage points in several instances, including some fragmentation sites in regions whose amino acid sequences have not previously been reported.

Experimental Section

Materials

Human fibrinogen was prepared from blood bank plasma by a cold ethanol fractionation procedure (Doolittle *et al.*, 1967). U..S. Red Cross human plasmin in 50% glycerol (10 C.T.A. U/ml) was kindly provided by Dr. Alan Johnson, New York University Medical Center. Soybean trypsin inhibitor (SBTI)[1] was purchased from Worthington Bio-

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[1] Abbreviations used are: SBTI, soybean trypsin inhibitor; TATG, thioacetylthioglycolic acid; PAS, periodic acid–Schiff; Gdn· HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate.