Allosteric Interpretation of the Oxygen-Binding Reaction of Human Hemoglobin Tetramers†

Enrico Di Cera, Charles H. Robert, and Stanley J. Gill*
Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215
Received November 13, 1986; Revised Manuscript Received February 23, 1987

ABSTRACT: An allosteric model is presented that provides a simple explanation for the low population of triply ligated species, relative to the other species, in the oxygenation of human hemoglobin tetramers as found in high-concentration studies [Gill, S. J., Di Cera, E., Doyle, M. L., Bishop, G. A., & Robert, C. H. (1987) *Biochemistry* (preceding paper in this issue)]. The model is a quantitative interpretation of the Perutz mechanism [Perutz, M. F. (1970) *Nature* (*London*) *228*, 726–739] and is based on a number of structural and thermodynamic findings so far reported in the analysis of hemoglobin properties. Human hemoglobin is assumed to exist in two quaternary states: the T or low-affinity state and the R or high-affinity state. An extreme chain heterogeneity in the T state is postulated so that oxygen binds only to the α chains. Nearest-neighbor interactions between the α chains may lead to cooperativity within the T state. The R state is noncooperative, and both the α and β chains have equal oxygen affinity.

In the preceding paper (Gill et al., 1987) we have analyzed precise differential oxygen-binding data, obtained by means of a thin-layer apparatus (Dolman & Gill, 1978), according to the Adair equation (Adair, 1925). The results show a negligible value for the overall Adair constant β_3, directly related to the population of the triply ligated species. This observation comes from a purely phenomenological interpretation of the oxygen-binding reaction of human hemoglobin tetramers. Although low populations of intermediate species have been indicated previously (Imai, 1982; Chu et al., 1984), the strikingly asymmetric distributions seen in the preceding study demand consideration of a possible underlying mechanism. In this paper, we propose a possible explanation of the phenomenon.

In 1970 Perutz suggested a sterochemical mechanism of cooperative effects in hemoglobin (Perutz, 1970) based on X-ray crystallographic studies and expressing many features of the allosteric MWC[1] model (Monod et al., 1965). According to this mechanism the cooperative effects arise from the equilibrium between two alternative quaternary states, the deoxy "T" or low-affinity state and the oxy "R" or high-affinity state. In the absence of ligand the T structure is hypothesized to be more stable, due to the presence of several salt bridges which constrain the $\alpha^1\beta^2$ and $\alpha^2\beta^1$ interfaces. As oxygenation proceeds, the salt bridges are progressively loosened within the T state and a transition to the high-affinity R state is then observed. The low affinity of the T structure is suggested to arise from a "tension" at the heme, which opposes the transition from the high-spin unligated state of the iron to the low-spin state when ligand is bound (Perutz, 1972). Therefore ligand uptake implies a change in the tertiary structure as predicted by the "induced-fit" theory of Koshland (1958). In addition, steric factors hinder oxygen binding to the iron atom of the β chain in the T state. Perutz pointed out that the position of the E helix relative to the heme plane is such that Val67β sterically blocks the oxygen-binding site. In going to the R state the E helix shifts by about 1 Å, making room for oxygen. In contrast, the α chain has room for oxygen in both quaternary states. In Perutz's mechanism the consequence of these differences at the heme pockets of the α and β chains is that oxygen first binds to the α chains in the T state and then, after the quaternary transition has occurred, to the β chains in the R state.

Perutz's mechanism provided new insight into the cooperative properties of hemoglobin and has inspired many structural and thermodynamic studies aimed at investigating the main aspects of the proposed scheme. The existence of only two quaternary states, the T and the R structures, is indicated by the dovetailed nature of the $\alpha^1\beta^2$ and $\alpha^2\beta^1$ interfaces (Fermi, 1975; Perutz, 1976; Baldwin & Chothia, 1979; Shaanan, 1983; Fermi et al., 1984), which allows no other stable positions. The idea of tension at the heme in the T structure has been supported by the finding of a change in the symmetry of the proximal histidine position on ligand binding (Gelin & Karplus, 1977), which is associated with a movement of the iron atom toward the heme plane (Baldwin & Chothia, 1979). The existence of tertiary structural changes on ligand binding has been clearly substantiated by the discovery of an "allosteric core" (Gelin et al., 1983), which undergoes definite structural changes on oxygen binding. The strong, preferential binding to the α chain in the T state with respect to the β chain was initially questioned by Fermi (1975) with his discovery of a water molecule attached to the distal histidine in the α-heme pocket, which was thought to provide steric hindrance comparable to Val67 in the β chain. The controversy was, however, largely resolved by Takano (1977), who found a similar blockage in deoxymyoglobin. Since myoglobin is known to have high oxygen affinity, the water molecule attached to the distal histidine is unlikely to provide any steric hindrance. The role of Val67 in blocking the β-heme pocket has been recently confirmed by high-resolution X-ray analysis of the deoxy structure (Fermi et al., 1984). Recent support also comes from crystallographic studies (Brzozowski et al., 1984) of doubly oxygenated crystals, presumably obtained under triple-point

† Aspects of this paper were presented at a meeting in Paris on Heme Proteins (July 2–4, 1986) in honor of Jeffries Wyman's 85th birthday. This work was supported by National Institutes of Health Grant HL22325.

[1] Abbreviations: Hb, hemoglobin; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; MWC, Monod–Wyman–Changeux; KNF, Koshland–Nemethy–Filmer.

0006-2960/87/0426-4003$01.50/0

conditions (Gill & Richey, 1984), which show a T state hemoglobin with oxygen bound to the α chains but not to the β chains.

The absence of cooperativity within either quaternary state, as hypothesized by the simple MWC model (Monod et al., 1965), so that all cooperativity originates from the quaternary transition, is another element assumed in the original Perutz mechanism. No evidence that contradicts this assumption for binding in the R state has been so far suggested. However, indirect evidence suggests intrinsic cooperativity of the T state, as demonstrated by proton NMR studies on Hb M Milwaukee (Fung et al., 1977) and on normal adult Hb (Viggiano & Ho, 1979). On the basis of these results Perutz modified his original mechanism in the spirit of the KNF model (Koshland et al., 1966), postulating a sequential rise in oxygen affinity in the T state generated by sequential loosening of its constraining hydrogen bonds (Perutz, 1976, 1978, 1979). The R state would still be noncooperative since unconstrained (Perutz, 1976).

In the present study we have drawn the main features of Perutz's mechanism into a quantitative thermodynamic framework that provides an interpretation of the low relative population of triply ligated species in the oxygen-binding reaction of human hemoglobin. The model takes into account fundamental postulates of both the MWC and KNF allosteric models and is inspired by the generalized view of allosteric control in biological macromolecules as formulated in the concept of "nesting" (Wyman, 1984). It allows a detailed description of the experimental data, as demanded by the high resolution achieved in the study presented in the preceding paper.

THE MODEL

The allosteric model presented here is based on the following tenets: (i) Hemoglobin exists in two alternative quaternary structures, the T or low-affinity state and the R or high-affinity state. (ii) In the T state only the α chains can bind oxygen in a significant amount, the affinity of the β chains being negligible. (iii) The T state can show cooperative behavior arising from interaction between the α subunits. (iv) The R state is noncooperative, and the α and β chains have the same affinity.

All these statements have a precise physical significance based on both structural and thermodynamic findings on human hemoglobin. The first is based on the crystallographic evidence so far collected (Perutz, 1970; Fermi, 1975; Baldwin & Chothia, 1979). The second statement is strictly based on the steric hindrance for Val67 at the heme pocket in the β chain. Negligible affinity for the β chain in the T state (Perutz, 1970) has been recently implicated by the crystallographic data of Brzozowski et al. (1984). The third tenet is substantiated by the proton NMR studies of Ho and colleagues (Fung et al., 1977; Viggiano & Ho, 1979), who suggest cooperativity within the T state. The last is included for simplicity and appears appropriate in the absence of any experimental evidence supporting cooperativity within the R state. The affinity in either state can of course be changed by allosteric effectors.

The binding polynomial P, or binding partition function, for hemoglobin according to this model can be written as

$$P = \frac{L}{L+1}\{1 + 2k_{T\alpha}x + \gamma k_{T\alpha}{}^2x^2\} + \frac{1}{L+1}\{1 + k_Rx\}^4 \quad (1)$$

where x is the ligand activity, L is the familiar allosteric constant ([TO]/[RO]), $k_{T\alpha}$ and k_R are respectively the association constants for oxygen binding to the α chain in the T state and to either chain in the R state, and γ is an interaction constant that defines positive ($\gamma > 1$) or negative ($\gamma < 1$) interactions within the T state. When $\gamma = 1$, the α chains become independent in the T state. The inclusion of direct subunit–subunit interactions (Koshland et al., 1966) within the framework of a quaternary equilibrium (Monod et al., 1965) constitutes the "cooperon" idea (Di Cera, 1985), an interpretation of the phenomenological concept of nesting (Wyman, 1984; Robert et al., 1987), which arises when cooperative effects are hypothesized to result from contributions at different structural levels. In this model the α chains in the T state, since they interact, form a cooperon (an "α_2-cooperon"), which contributes cooperativity at the first structural level. The quaternary equilibrium between the T and R states involves all the subunits and contributes cooperativity at a second structural level.

The mechanism that leads to the low population of triply ligated species rests on the negligible affinity of the β chains in the T state combined with a large value of the allosteric constant L. For sufficiently high values of L the first two oxygen-binding events are seen essentially to occur in the T state. For a third molecule of oxygen to be bound, the transition to the R state, where the β chains are exposed with high affinity, must occur (regardless of the value of L). This aspect of the α_2-cooperon model makes it different from the MWC model and other modified versions of the MWC model (Szabo & Karplus, 1972; Ogata & McConnell, 1972; Herzfeld & Stanley, 1974; Minton & Imai, 1974), where an increase of L progressively shifts the quaternary transition toward higher stages of ligation.

Writing the Adair equation (Adair, 1925) as

$$P = 1 + \beta_1x + \beta_2x^2 + \beta_3x^3 + \beta_4x^4 \quad (2)$$

and combining eq 1 and 2, one can express β_3 and β_4 in terms of the α_2-cooperon model parameters as

$$\beta_3 = 4\kappa_R{}^3/(L+1) \quad (3a)$$

$$\beta_4 = \kappa_R{}^4/(L+1) \quad (3b)$$

Since β_4 is always well-defined (Gill et al., 1987), eliminating κ_R in both expressions above yields

$$\beta_3 = 4\beta_4{}^{3/4}/(L+1)^{1/4} \quad (4)$$

By progressively increasing the constant L, we can recover a value for β_3 as low as we please. However, in order for β_4 to be well-defined, an increase of L must lead to a resulting increase of κ_R according to eq 3b. Therefore, since β_3 is undetermined because negligibly small, a correlation between L and κ_R is expected in the data fitting and is in fact found.

RESULTS

The data discussed in this study are the same used for the phenomenological Adair analysis outlined in the preceding paper (Gill et al., 1987). A brief summary of the experimental conditions is given in Table I. The results of fitting the proposed model to the data are shown at the top in Table II, with best-fit values and one standard deviation confidence intervals as determined by F-testing (Magar, 1972). The standard error of a point for these fits is shown in the first column at the bottom of Table II.

A rigorous test of a model's validity is the comparison of the "goodness of fit" (Bevington, 1969) obtained from fitting the model to the data with that obtained from the fit of the Adair equation. The statistical method for excluding a given model with respect to the phenomenological Adair model is based on a comparison of standard errors according to the

Table I: Description of Experimental Conditions for Data Sets Discussed in This Paper

	buffer	EDTA	NaCl	pH	T (°C)	[heme] (mM)
A	0.1 phosphate			7.5	25	12
B	50 mM HEPES	40 μM	0.1 M	7.4	25	8
C	50 mM HEPES	40 μM	0.1 M	7.4	25	4
D	50 mM HEPES	40 μM	0.1 M	7.4	25	2
E	0.1 M HEPES	40 μM	5 mM	7.4	25	8
F	50 mM HBG[a]	40 μM	0.1 M	9.1	25	4
G	50 mM HBG[a]	40 μM	0.1 M	6.9	25	4
H	0.1 M Tris-HCl	1 mM	0.1 M	7.4	21.5	4
I	0.1 M Tris-HCl	1 mM	0.1 M	7.4	21.5	2
J	50 mM HEPES	40 μM	0.1 M	7.5	25	4
J*[b]	50 mM HEPES	40 μM	0.1 M	7.5	25	4

[a] HBG = 50 mM each of HEPES, bicine, and glycine. [b] * = reversibility test: J, decreasing saturation; J*, increasing saturation (Gill et al., 1987).

Table II

Model Parameters and Corresponding Confidence Intervals within One Standard Deviation (67%) Obtained by Fitting the Data Sets Reported in Table I

	$\kappa_{T\alpha}$ (Torr^{-1})	L ($\times 10^{-8}$)	κ_R (Torr^{-1})	γ
A	0.020	11.0	20.9	14.2
	0.015, 0.023	0.16, ∞	8.1, ∞	8.3, 25.4
B	0.042	2.5	21.3	9.6
	0.031, 0.052	0.042, ∞	7.5, ∞	2.9, 16.5
C	0.052	3.2	22.2	5.0
	0.042, 0.080	0.008, ∞	4.8, ∞	1.4, 8.6
D	0.028	15.3	21.2	15.3
	0.023, 0.044	0.008, ∞	5.1, ∞	3.8, 27.0
E	0.087	0.37	20.3	4.3
	0.066, 0.110	0.032, ∞	11.2, ∞	2.8, 7.6
F	0.329	0.032	23.0	1.8
	0.225, 0.436	0.004, ∞	12.8, ∞	0.9, 5.5
G	0.028	19.0	20.5	3.7
	0.022, 0.033	0.13, ∞	5.9, ∞	1.8, 6.0
H	0.084	1.1	20.2	0.8
	0.056, 0.111	0.03, ∞	7.3, ∞	0.4, 4.5
I	0.074	1.2	20.3	1.6
	0.056, 0.100	0.03, ∞	7.5, ∞	0.7, 4.4
J	0.053	1.8	21.4	5.5
	0.037, 0.084	0.003, ∞	4.2, ∞	1.1, 9.8
J*	0.052	1.2	19.9	8.9
	0.038, 0.079	0.017, ∞	6.4, ∞	1.6, 15.0

Standard Error of a Point ($\times 10000$) for Fits of the Data Sets Reported in Table I according to Different Models[a]

	α_2-cooperon[a]	MWC[a]	α_2-cooperon[a,b]	Adair
A	3.2 (54)	4.1 (86)	4.8 (96)	3.1
B	3.5 (63)	5.7 (99)	6.9 (99)	3.2
C	3.8 (57)	4.5 (86)	4.4 (76)	3.6
D	1.3 (41)	2.6 (99)	2.2 (99)	1.2
E	17.2 (41)	23.6 (94)	30.7 (99)	15.9
F	2.9 (63)	3.4 (86)	3.2 (81)	2.6
G	1.5 (59)	2.1 (94)	2.0 (92)	1.4
H	2.8 (54)	3.2 (74)	2.9 (59)	2.7
I	1.5 (59)	1.9 (89)	1.6 (69)	1.4
J	4.3 (55)	4.9 (76)	4.9 (76)	4.1
J*	2.1 (57)	3.3 (99)	3.2 (97)	2.0

[a] The percent confidence in excluding a given model with respect to the Adair equation is shown in parentheses. [b] With γ fixed to 1.

F-test (Magar, 1972). As shown in Table II, application of the α_2-cooperon model to the analysis of the binding capacity data reported in the preceding paper (Gill et al., 1987) gives standard errors of the fits not significantly worse than those obtained by application of the Adair equation; in fact, the model can be excluded with a maximum confidence of only 63%. On the other hand, application of the MWC model gives a poor fit in general and can be excluded with up to 99% confidence. Modified versions of the MWC model that account for a possible heterogeneity of the chains in the T state (Ogata & McConnell, 1972; Herzfeld & Stanley, 1974) give almost the same result as the simple MWC model, even when the affinity of the β chains is dropped to zero in the data-fitting process. In this case, these models become identical with the α_2-cooperon model for $\gamma = 1$, and the confidence in excluding such a model is high, as seen in the third column at the bottom of Table II. This result demonstrates the necessity of a cooperativity between the α chains along with an extreme heterogeneity of the different chains in the T state.

Application of the α_2-cooperon model predicts a value for κ_R of about 21 Torr^{-1}. However, due to the strong correlation between κ_R and L as previously noted, we found any other value of $\kappa_R > 21$ Torr^{-1} to be consistent with fitting the data within the confidence interval of one standard deviation, provided L is allowed to increase as well. As far as the cooperativity within the α_2 dimer in the T state is concerned, the values of γ show positively cooperative oxygen binding in almost all cases. In Figure 1 we report the model prediction for the various species fractions in hemoglobin oxygenation for four relevant cases sketched in Table I. As expected, the population of the triply ligated species is much smaller than any other.

Discussion

Comparison of experimental data and theoretical models is a critical step in the thermodynamic study of ligand binding and control processes of biological macromolecules. Hemoglobin has played a major role in the interplay between experimental investigation and mechanistic interpretation of protein action. The low population of triply ligated species in the oxygen-binding reaction of hemoglobin tetramers (Gill et al., 1987) can be quantitatively explained in terms of Perutz's mechanism (Perutz, 1970, 1979), whose features have been substantiated in later crystallographic studies (Fermi, 1975; Gelin & Karplus, 1977; Baldwin & Chothia, 1979; Gelin et al., 1983; Shaanan, 1983; Brzozowski et al., 1984; Fermi et al., 1984). We have found that an extreme heterogeneity between the α and β chains in the T state, with $\kappa_{T\beta} = 0$, is necessary to account for a low value of β_3. However, this condition is not sufficient. A high affinity of the R state is necessary as well as a large value of the allosteric constant L. An accurate fit of the experimental data discussed here requires the inclusion of cooperativity within the T state. This feature agrees with the proton NMR studies (Viggiano & Ho, 1979) and more recent findings on the cooperative free energy levels of oxidized hemoglobin in its reaction with cyanide ion (Smith & Ackers, 1985; Gill et al., 1986). The model proposed here allows a high accuracy in fitting the data, while other models, like the MWC model (Monod et al., 1965), resulted in poor fits. This result is not new, since the consistency of the simple MWC model as applied to human hemoglobin function has long been questioned (Perutz, 1970, 1976; Szabo & Karplus, 1972; Ogata & McConnell, 1972; Herzfeld & Stanley, 1974; Minton & Imai, 1974; Viggiano & Ho, 1979; Smith & Ackers, 1985; Gill et al., 1986).

Whenever the analysis of a physical phenomenon is cast in terms of a mechanistic model, rather than in terms of a purely phenomenological interpretation, it necessarily suffers from approximations. The extreme chain heterogeneity suggested for the T state with respect to oxygen binding is a case in point. This feature might be seen to be at variance with kinetic measurements of oxygen binding to the deoxyHb (Sawicki & Gibson, 1977), where a two-component decay process was attributed to oxygen binding to the α and β chains. The assignment by Sawicki and Gibson of the two components observed in their studies cannot be regarded as unique as soon as cooperativity within the T state is hypothesized. A model

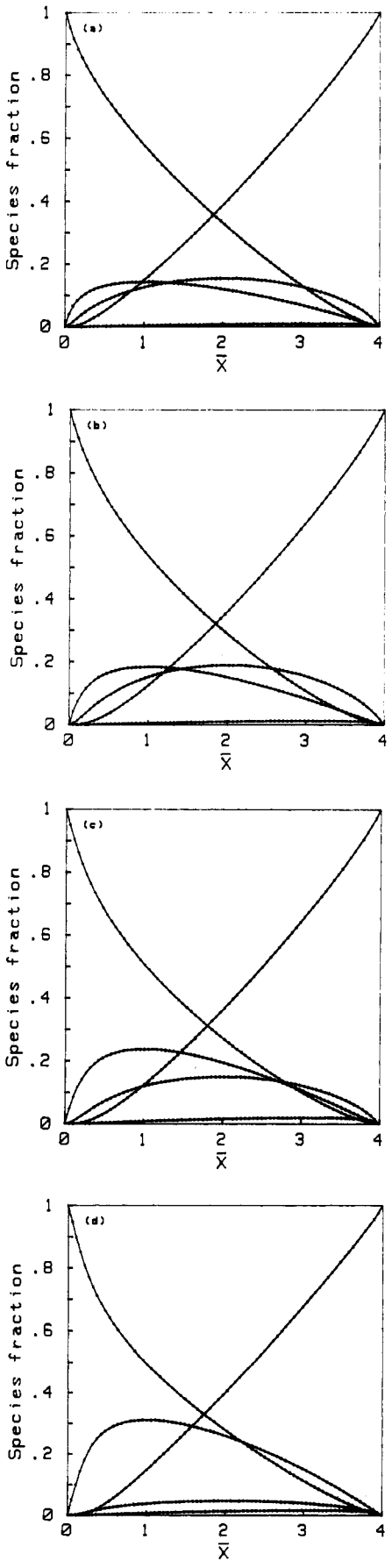

FIGURE 1: Fractional populations of the five ligated species of human hemoglobin for cases A (a), B (b), E (c), and H (d), as reported in Table I, vs. the average number $\bar{X}$ of oxygens bound. Theoretical curves were drawn by using values of the model parameters given in Table II.

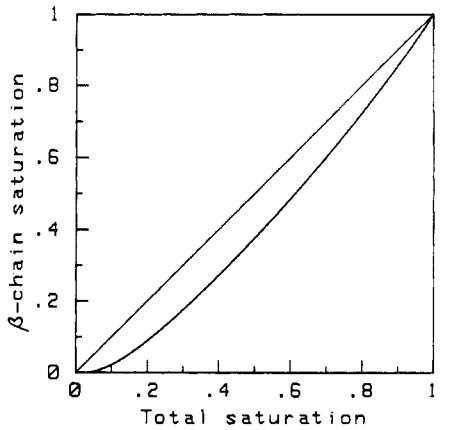

FIGURE 2: Oxygen saturation of the β chain as a function of the total oxygen saturation for case E as reported in Table I. The lag of the β chain ligation is nowhere greater than 0.12 fractional saturation.

such as the one presented here would predict multicomponent decay in a kinetic investigation of the T-state molecule, even with a zero affinity for the β chains, and thus qualitatively represents an alternative interpretation of the kinetic data.

A plot of the oxygen saturation of the β chains vs. the total oxygen saturation using the present model is shown in Figure 2. As can be seen from this plot for case E in Table I, the lag of the β chain ligation is not as dramatic as might initially be supposed from the assumption of extreme heterogeneity in the T state and is of the magnitude found experimentally through fluorine NMR measurements (Huestis & Raftery, 1975). However, the fluorine NMR results may be inconsistent with a more recent proton NMR study (Viggiano & Ho, 1979). In any event, the assumption of marked heterogeneity provides a simple explanation in molecular terms of the unmeasurable contribution of the triply ligated species in the oxygen-binding reaction of human hemoglobin.

The α_2-cooperon model can be shown to be related to the I_2H_4-cooperon model (Gill et al., 1986), which provides a mechanistic explanation for the three cooperative free energy levels discovered in a phenomenological study of hemoglobin oxidation intermediates (Smith & Ackers, 1985). The binding polynomial for this model is

$$P = \frac{L}{L+1}\{1 + 2(\kappa_{T\alpha} + \kappa_{T\beta})y + [\gamma(\kappa_{T\alpha}^2 + \kappa_{T\beta}^2) + 4\delta\kappa_{T\alpha}\kappa_{T\beta}]y^2 + 2\delta^2\gamma\kappa_{T\alpha}\kappa_{T\beta}(\kappa_{T\alpha} + \kappa_{T\beta})y^3 + \delta^4\gamma^2\kappa_{T\alpha}^2\kappa_{T\beta}^2y^4\} + \frac{1}{L+1}\{1 + \kappa_R y\}^4 \quad (5)$$

where y is, in this case, the inverse of electron activity a_e (Gill et al., 1986) defined as $a_e = \exp(-FE/RT)$ (where F is the Faraday constant, E is the oxidation potential, R is the gas constant, and T is the absolute temperature), δ is the interaction constant for the four heterologous (H_4) $\alpha\beta$ pairs, γ is the interaction constant for the two isologous (I_2) $\alpha\alpha$ and $\beta\beta$ pairs, and $\kappa_{T\beta}$ is the affinity of the β chain in the T state. The other parameters are as in eq 1. Interestingly, when $\kappa_{T\beta}$ is dropped to zero, eq 5 collapses into a form equivalent to eq 1. In contrast to the oxygen-binding properties, the redox properties of the α and β chains seem to be similar in the T state.

It should be mentioned that the model proposed here has no prediction regarding the affinity of the β chains for other ligands. The induced-fit concept (Koshland, 1958) implies differing effects for different ligands. On the other hand, as far as carbon monoxide is concerned, a $\{\alpha(Fe(II)-CO)\beta(Mn(II)\}_2$ crystal shows a T-like structure (Arnone et al., 1986). It has been reported in a preliminary way that crystals

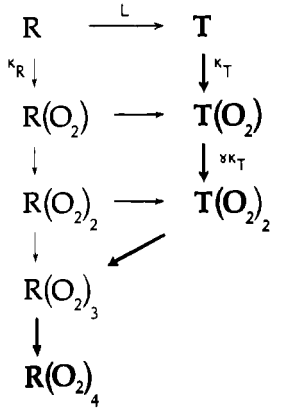

FIGURE 3: Schematic representation of oxygen ligation of human hemoglobin in terms of the α_2-cooperon model; species represented in bold type are the major intermediates to be found. The oxygenation pathway is indicated by the bold arrows.

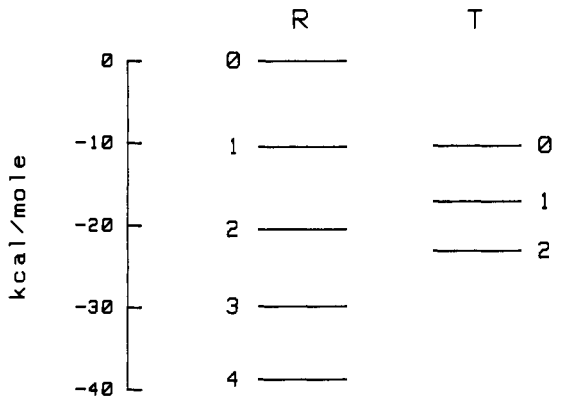

FIGURE 4: Standard free energy levels for the model as applied to case E with parameters reported in Table II.

of $\{\alpha(Mn(II))\beta(Fe(II)-CO)\}_2$ exist in a T form (Blough & Hoffman, 1984). However, further investigation has shown that the crystals were presumably unligated since they broke upon additional saturation with CO (A. Arnone, personal communication). More recently, NMR studies of monoliganded Fe–Co hybrid hemoglobin in its reaction with CO have indicated that the binding to the β chain induces more profound change in the quaternary structure of hemoglobin than the binding to the α chain and that the tetramer ligated at the α chain has more T-state character than the tetramer ligated at the β chain (Inubushi et al., 1986). These results are in agreement with the prediction from oxygen studies of the present model that ligation of the β chains can only occur in the molecule after the quaternary transition to the R state.

In Figure 3 we schematically represent the model. The bold type in this figure shows the major species, i.e., any species whose population reaches a maximum greater than 10% of the total. The path of oxygenation is indicated by the bold arrows. The standard free energy level diagram (Figure 4) demonstrates the greatly different oxygen-binding properties of the two quaternary states of human hemoglobin and shows the thermodynamic basis of the oxygenation pathway.

The low population of triply ligated species implies an extremely high asymmetry in the binding curve, as can be easily verified by a glance at Figure 1, and has an apparent physiological relevance, since hemoglobin can retain a high degree of cooperativity even at high saturations. In Figure 5 we show the degree of cooperativity as a function of the logarithm of oxygen partial pressure for case A reported in Table I. The expected asymptotic value of unity at high degree of saturation lies far beyond physiological availability (40–140 Torr of

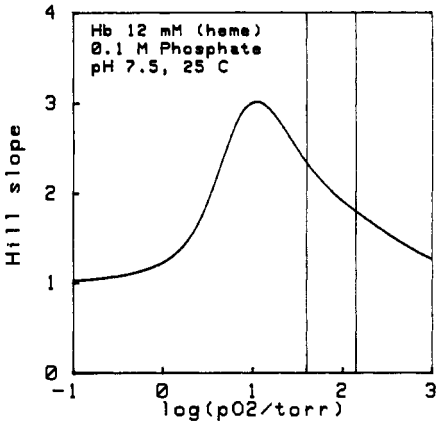

FIGURE 5: Degree of cooperativity (Hill slope) for human hemoglobin for case A in Table I, as allowed by the model parameters reported in Table II. The physiological range of oxygen partial pressure (40–140 Torr) is shown by vertical lines.

oxygen partial pressure). Weber (1982) considered the strong asymmetry of the oxygen-binding curve and suggested as an explanation a marked difference in the interaction between the α chains with respect to that between β chains. He noted that hemoglobin could be seen to work by loading and unloading the β chains. The model presented here demonstrates the validity of Weber's conclusion, but in a totally different framework. Taking the place of direct subunit–subunit interactions of different order (Weber, 1982) are two quaternary states in equilibrium and a strong heterogeneity of the chains with respect to oxygen binding in a cooperative T state. This justifies the tetrameric $\alpha_2\beta_2$ structure on functional grounds.

As we have seen, this model constitutes a synthesis of many structural and thermodynamic features of hemoglobin. However, other possible interpretations, depending upon one's point of view, cannot be excluded. We have been strongly influenced by the general allosteric concept and its support from detailed structural information. As new insights are developed these ideas will necessarily require extension. In spite of the simplicity of the model presented here it perhaps provides a base for interpretation of the products of such exploration.

ACKNOWLEDGMENTS

We gratefully acknowledge Michael L. Doyle and Gary A. Bishop for helpful suggestions and criticisms. We thank Max Perutz for drawing our attention to recent work on iron–manganese hybrids from Brian Hoffman's laboratory, and we appreciate having unpublished information from Arthur Arnone on the behavior of $\{\alpha(Mn(II))\beta(Fe(II))\}_2$ crystals. Finally, we thank reviewer 1 for pointing out to us the results of Inubushi et al. (1986).

REFERENCES

Adair, G. S. (1925) *J. Biol. Chem. 63*, 529–535.

Arnone, A., Rogers, P., Blough, N. V., McGourty, J. L., & Hoffman, B. N. (1986) *J. Mol. Biol. 188*, 693–706.

Baldwin, J., & Chothia, C. (1979) *J. Mol. Biol. 129*, 175–220.

Bevington, P. R. (1969) in *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.

Blough, N. V., & Hoffman, B. M. (1984) *Biochemistry 23*, 2875–2882.

Brzozowski, A., Derewenda, Z., Dodson, E., Dodson, G., Grabowski, M., Liddington, R., Skarzynski, T., & Vallely, D. (1984) *Nature (London) 307*, 74–76.

Chu, A. H., Turner, B. W., & Ackers, G. K. (1984) *Biochemistry 23*, 604–617.

Di Cera, E. (1985) Dissertation, Universitâ Cattolica del Sacro Cuore, Rome, Italy.
Dolman, D., & Gill, S. J. (1978) *Anal. Biochem. 87*, 127-134.
Fermi, G. (1975) *J. Mol. Biol. 97*, 237-256.
Fermi, G., Perutz, M. F., Shaanan, B., & Fourme, R. (1984) *J. Mol. Biol. 175*, 159-174.
Fung, L. M., Minton, A. P., Lindstrom, T. R., Pisciotta, A. V., & Ho, C. (1977) *Biochemistry 16*, 1452-1462.
Gelin, B. R., & Karplus, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 801-805.
Gelin, B. R., Lee, A. W., & Karplus, M. (1983) *J. Mol. Biol. 171*, 489-559.
Gill, S. J., & Richey, B. (1984) *Nature (London) 310*, 160-161.
Gill, S. J., Robert, C. H., Coletta, M., Di Cera, E., & Brunori, M. (1986) *Biophys. J. 50*, 753-757.
Gill, S. J., Di Cera, E., Doyle, M. L., Bishop, G. A., & Robert, C. H. (1987) *Biochemistry* (preceding paper in this issue).
Herzfeld, J., & Stanley, H. E. (1974) *J. Mol. Biol. 82*, 231-265.
Huestis, W. H., & Raftery, M. A. (1975) *Biochemistry 14*, 1886-1892.
Imai, K. (1982) in *Allosteric Effects in Haemoglobin*, Cambridge University Press, Cambridge, UK.
Inubushi, T., D'Ambrosio, C., Ikeda-Saito, M., & Yonetani, T. (1986) *J. Am. Chem. Soc. 108*, 3799-3803.
Koshland, D. E. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 98-104.
Koshland, D. E., Nemethy, G., & Filmer, D. (1966) *Biochemistry 5*, 365-385.
Magar, M. E. (1972) in *Data Analysis in Biochemistry and Biophysics*, Academic, New York.
Minton, A. P., & Imai, K. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1418-1421.
Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88-118.
Ogata, R. T., & McConnell, H. M. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 335-339.
Perutz, M. F. (1970) *Nature (London) 228*, 726-739.
Perutz, M. F. (1972) *Nature (London) 237*, 495-499.
Perutz, M. F. (1976) *Br. Med. Bull. 32*, 195-208.
Perutz, M. F. (1978) *Sci. Am. 239(6)*, 92-125.
Perutz, M. F. (1979) *Annu. Rev. Biochem. 48*, 327-386.
Robert, C, H., Decker, H., Richey, B., Gill, S. J., & Wyman, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1891-1895.
Sawicki, C. A., & Gibson, Q. H. (1977) *J. Biol. Chem. 252*, 5783-5788.
Shaanan, B. (1983) *J. Mol. Biol. 171*, 31-59.
Smith, F. R., & Ackers, G. K. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5347-5351.
Szabo, A., & Karplus, M. (1972) *J. Mol. Biol. 72*, 163-197.
Takano, T. (1977) *J. Mol. Biol. 110*, 569-584.
Viggiano, G., & Ho, C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3673-3677.
Weber, G. (1982) *Nature (London) 300*, 603-607.
Wyman, J. (1984) *Q. Rev. Biophys. 17*, 453-488.

Viscosity Dependence of Ethidium–DNA Intercalation Kinetics

Robert B. Macgregor, Jr., Robert M. Clegg,* and Thomas M. Jovin

Abteilung Molekulare Biologie, Max-Planck-Institut für biophysikalische Chemie, D-3400 Göttingen, Federal Republic of Germany

Received September 2, 1986; Revised Manuscript Received December 15, 1986

ABSTRACT: The kinetics of ethidium intercalation into double-stranded poly[d(G-C)] were investigated by use of repetitive pressure-jump chemical relaxation at 20 °C in low ionic strength (0.1 M NaCl) aqueous buffers containing either glycerol or methanol. The viscosity of the various solvents differed by more than an order of magnitude while other physical properties (e.g., dielectric constant) remained approximately constant. The single-reciprocal kinetic relaxation time (τ^{-1}) increases linearly with DNA concentration. The observed association rate constant is lower in all organic–aqueous mixtures than in water and is inversely proportional to the viscosity. These results provide evidence for an additional step in the intercalation mechanism which is identified as an obligatory DNA conformational change preceding ethidium intercalation. From the data presented, the equilibrium constant of this local conformational change is $\sim 10^{-3}$, i.e., greatly favoring the structure incapable of intercalation. The corresponding kinetics were not directly determined; however, in order to be consistent with all of the data the forward and/or reverse rate constants of the conformational change must be larger than the rate of the intercalation reaction. Thus, it is proposed that the rate of the conformational change back to the nonintercalating B-DNA structure is greater than ~ 500 s^{-1}, implying a rate of opening greater than ~ 0.5 s^{-1}, in agreement with other hydrogen exchange and NMR data. The observed overall rate constant for the dissociation of ethidium is inversely proportional to the solvent density, possibly reflecting a dependence on the solvent free volume. The overall volume change of intercalation is less negative in the organic–aqueous solvent mixtures than in water.

The physical and chemical processes leading to intercalation of a dye between successive DNA base pairs are expected to have a complex dependence on the immediate environment of the macromolecule. If the potential intercalator is charged, it will interact electrostatically with the polyanionic DNA and condensed counterions, producing at least a partial displacement of the latter. X-ray crystal structures of intercalation complexes suggest that the static conformation in the imme-

0006-2960/87/0426-4008$01.50/0