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Kinetics of hemoglobin and transition state theory

(cooperativity/diffusion-controlled reactions/linear free energy relationships)

ATTILA SZABO

Department of Chemistry, Indiana University, Bloomington, Indiana 47401

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ABSTRACT **Experimental evidence indicates that the way cooperativity in hemoglobin is manifested kinetically depends on the nature of the ligand. It is shown that the qualitative differences in the kinetics of NO, O_2, CO, and bulky isocyanides can be understood in structural terms in a framework based on transition state theory.**

The mechanism of cooperative ligand binding by hemoglobin cannot be completely elucidated without considering its kinetic aspects. In contrast to equilibrium properties of hemoglobin (1–8), the relationship between its kinetic behavior and its structure is poorly understood. I shall show that transition state theory or absolute reaction rate theory (4), which has long been used to correlate structure and reactivity in physical organic chemistry, provides a framework in which the essential features of the kinetics of ligand binding by hemoglobin and the free chains can be understood qualitatively in structural terms. First, the experimental information on the kinetics of the free chains and hemoglobin reacting with a variety of ligands will be summarized and it will be shown that the way cooperativity manifests itself kinetically is crucially dependent on the nature of the ligand. Then, a phenomenological interpretation of the qualitative trends displayed by the kinetic data will be proposed. In order to go beyond this and begin to understand the data in structural terms, I consider the basic ideas of transition state theory, focusing on the relationship between the structures of reactants and products and that of the transition state or activated complex that is the transient species with the largest free energy along the reaction path. After the results obtained with the isolated chains have been used to define ideas on the structure of the transition state and its dependence on the nature of the reactants, I turn to the analysis of hemoglobin kinetics.

It should be stressed that the approach developed here is clearly oversimplified and is but a first step toward the goal of understanding the kinetics of hemoglobin. The dynamics of ligand binding by an allosteric protein are extremely complex. Even for the reaction of myoglobin with small ligands such as O_2 and CO, Austin *et al.* (5) must invoke several barriers along the reaction path to explain their data. To make progress, it is assumed that hemoglobin controls the kinetics largely by modulating the height of the barrier closest to the heme. In other words, the focus is on a single transition state in which the metal–ligand bond is partially formed and the steric constraints imposed by the heme pocket are included in an effective way.

Experimental information

The essential features of the kinetics of isolated chains and those of hemoglobin are summarized in Tables 1 and 2, respectively. The precise values of the various rate constants may be somewhat uncertain but we are interested primarily in their qualitative trends. Table 1 contains the "on" (k') and "off" (k) constants as well as the equilibrium constants (K) for the reactions of NO, O_2, CO, and various isocyanides of increasing bulkiness with the isolated chains. The entries are averages of the rates for α and β chains. α,β chain heterogeneity is important for the bulky isocyanides, particularly for *n*-butylisocyanide (BuNC) (10) for which $k'_\alpha = 7 \times 10^4\ M^{-1}\ sec^{-1}$, $k'_\beta = 4 \times 10^5\ M^{-1}\ sec^{-1}$, $k_\alpha = 0.4\ sec^{-1}$, and $k_\beta = 1.4\ sec^{-1}$. The following results in Table 1 are important. (*i*) Both NO and O_2 react with the chains extremely quickly ($\sim 5 \times 10^7\ M^{-1}\ sec^{-1}$). (*ii*) The large difference between the affinities for O_2 and NO is almost exclusively due to differences between the "off" constants. (*iii*) Although CO binds more strongly than O_2, it reacts at $1/10$ the speed. (*iv*) The isocyanides react much more slowly than the smaller ligands. (*v*) Increasing the bulkiness of isocyanides reduces their binding constants; kinetically, this is reflected primarily in the reduction of their "on" constants.

These results will serve as the basis for correlating the kinetic data in Table 2 with the structure of hemoglobin. Table 2 illustrates how cooperativity is manifested kinetically for a series of ligands by supplying the ratio of the intrinsic (i.e., per subunit) "on" (primed) and "off" (unprimed) rate constants for the first and fourth binding steps. Information on chain heterogeneity is included for BuNC, which reacts faster with the β chains than with the α chains (16): $k^{\beta'}{}_1/k^{\alpha'}{}_1 \cong 20$–$100$ and $k^{\beta'}{}_4/k^{\alpha'}{}_4 \cong 10$. In all cases, k'_4 and k_4 are close to the corresponding values for isolated chains.

The following are the important results emerging from Table 2. (*i*) For NO, the "on" constants are independent of ligation, and cooperativity arises almost exclusively from the decrease in the "off" constant. (*ii*) For O_2, there is a slight increase in the rate of the "on" reaction, but cooperativity is again largely manifested by a decrease in the "off" constant. (*iii*) For CO, there is both a significant increase in the "on" constant and a decrease in the "off" constants. In fact, the former appears to be dominant. (*iv*) For BuNC, cooperativity is reflected equally by an increase in the "on" and a decrease in the "off" constants for the quickly reacting β chains. On the other hand, for the slowly reacting α chains, cooperativity is due almost exclusively to an increase in the "on" constant.

Phenomenological interpretation

Table 2 shows that the kinetics of cooperative ligand binding depend crucially on the nature of the ligand. For ligands that react very rapidly with the free chains (e.g., O_2 and NO), cooperativity results primarily from a decrease in the "off" rates. On the other hand, for more slowly reacting ligands, the protein increases its affinity for the last ligand by both decreasing the "off" and increasing the "on" constants. In the case of the re-

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Abbreviation: BuNC, *n*-butylisocyanide.

Table 1. Kinetics of isolated chains

Ligand (ref.)*	Constants "On" $k' \times 10^{-5}$, M^{-1} sec^{-1}	"Off" k, sec^{-1}	$K \times 10^{-5}$, M^{-1}
NO (6)	250	2.5×10^{-5}	10^7
O_2 (7)	500	20	25
CO (8)	40	0.01	4000
EtNC (9)	3	0.2	10
BuNC (10)	2.3	0.9	2.5
iPrNC (9)	0.5	0.15	3
tBuNC (9)	0.05	0.4	0.1

* EtNC, BuNC, IprNC, and tBuNC are ethyl, *n*-butyl, isopropyl, and tertiary butyl isocyanides, respectively. The literature references are in parentheses.

action with BuNC, for the slowly reacting α chains, cooperativity is due almost exclusively to an increase in the "on" rates.

To establish just how fast ligands such as O_2 and NO react, we must estimate the limit set by the rate of diffusion, assuming that, once a ligand gets near the iron, it is bound. Suppose there is small circular hole of radius R in the protein and a ligand of radius R_o reacts once it has diffused through it. The diffusion-controlled rate for this model is (17)

$$k'_{DC} = 4D(R - R_o) \qquad [1]$$

in which D is the sum of the diffusion constants of the ligand and the protein. To estimate this rate, we take D equal to the diffusion constant of O_2 in water ($D = 2 \times 10^{-5}$ cm^2 sec^{-1}) and use $R_o = 1.6$ Å as the effective radius of O_2 and $R = 2.6$ Å as the radius of the hole in the protein. This gives $k'_{DC} = 5 \times 10^8$ M^{-1} sec^{-1}, which is only an order of magnitude larger than the observed rate of oxygen binding to the free chains. This rate must be an overestimate because not all molecules that enter the pocket will react and, in fact, the hole leading into the heme pocket is too small (1) to allow even a small ligand to enter, so that thermal fluctuations in the size of the hole are crucial. Therefore Eq. 1 gives the rate constant for a model in which the reaction always occurs once the ligand strikes a surface area of radius R centered on the hole leading into the heme pocket.

According to this analysis the rates of reactions of O_2 and NO approach the diffusion-controlled limit. In the limiting case of the reaction of hemoglobin with the first ligand being entirely diffusion controlled, the protein can increase the binding constant of the last ligand only by decreasing its "off" rate. This applies to NO and O_2 for which the increase in binding constants is predominantly manifested by changes in the "off" constants (Table 2). If the reaction with the first ligand is considerably slower than the limit set by diffusion, the protein can increase its affinity by both decreasing the "off" and increasing the "on" rates. This happens for more slowly reacting ligands.

Table 2. Kinetics of hemoglobin*

Ligand	k'_4/k'_1	k_1/k_4
NO	1 (11)	~100 (6)
O_2	4 (12)–8 (13)	150 (13)
CO	40 (14)	10 (15)
BuNC (16)		
α	17–90	1
β	6	6

* The literature references are in parentheses.

To understand the reason we shall now consider the reaction path.

Transition state theory and Hammond's postulate

Transition state theory in its simplest form states that for the reaction

$$A + B \underset{k}{\overset{k'}{\rightleftharpoons}} AB$$

the rate constants k' and k are given by

$$k' = \frac{\kappa T}{h} \exp[-(G^{\ddagger} - G_R)/RT] \qquad [2a]$$

$$k = \frac{\kappa T}{h} \exp[-(G^{\ddagger} - G_P)/RT] \qquad [2b]$$

in which κ and h are Boltzmann's and Planck's constants, respectively, G_R and G_P are the Gibbs free energies of the reactants and products, and $G^{\ddagger}$ is the free energy of the transition state or activated complex. A standard state of 1 M has been assumed. $G^{\ddagger} - G_R$ is the free energy of activation for the "on" reaction. $G^{\ddagger} - G_P$ is the free energy of activation for the "off" reaction. The equilibrium constant K is

$$K = k'/k = \exp[-(G_P - G_R)/RT]. \qquad [3]$$

$G^{\ddagger}$ is the maximum free energy of the composite system A and B as the two reactants approach each other from infinity. At first the free energy of the system increases; then, as A and B get closer and bond formation begins, this increase slows until the point is reached such that further bond formation decreases the free energy of the system. The partially bonded species A···B at this point is called the activated complex or transition state.

The kinetics of a reaction depend on the properties of the reactants and products and also on the properties of the transition state. Although the theory (4) shows how $G^{\ddagger}$ can be calculated, this is difficult even for moderately complex organic reactions. To make progress, one has to focus on trying to predict qualitatively how the rates change when the structures of the reactants or products are altered. Intuitively it would appear that, the more thermodynamically favored a reaction is, the faster it should proceed. Unfortunately, this is just not the case in general. However, if the transition state for a reaction has structural features intermediate between those of the reactants and products, factors that alter the stability of the reactants or products will have a corresponding effect on the energy of the transition state (18–22). In addition, if the two similar reactions are compared, the transition state for the more exothermic reaction is expected to occur earlier along the reaction path—i.e., it will be more reactant-like. This is a corollary of Hammond's postulate which states that, if there is an unstable intermediate on the reaction pathway, the transition state for the reaction will resemble this intermediate (20). Consequently, for reactions with reactant-like transition states, the change in the free energy of the transition state ($\Delta G^{\ddagger}$) closely parallels changes in free energy of the reactants (ΔG_R) but is largely unaffected by changes in the free energy of the products. The reverse is true for product-like transition states. These are special cases of the more quantitative statement (19)

$$\Delta G^{\ddagger} = \alpha \Delta G_P + (1 - \alpha)\Delta G_R. \qquad [4]$$

in which the ΔGs are respective free energy changes due to structural modifications in the reactants and products and the parameter $\alpha (0 \leq \alpha \leq 1)$ is a measure of the position of the transition state along the reaction path (i.e., $\alpha = 0$ means that it is completely reactant-like and $\alpha = 1$, that it is completely

product-like). Any real transition state lies somewhere between these extremes. Only if the reaction is entirely diffusion controlled is the transition state completely reactant-like.

Eq. 4 forms the basis for the linear free energy relationships often used in organic chemistry (22). To see this, we rewrite Eq. 4 as

$$\Delta(G^{\ddagger} - G_R) = \alpha\Delta(G_P - G_R) \quad [5]$$

which, by differentiation of Eqs. 2a and 3, can be shown to be identical to

$$\frac{\Delta k'}{k'} = \frac{\alpha\Delta K}{K}. \quad [6]$$

Integrating this relationship gives

$$\ln\frac{k'_1}{k'_2} = \alpha \log\frac{K_1}{K_2} \quad [7]$$

which relates the "on" rate constants (k') for two reactions, 1 and 2, to the equilibrium constants (K). It immediately follows from Eq. 7 that the "off" constants for the reactions satisfy

$$\ln\frac{k_1}{k_2} = (\alpha - 1)\ln\frac{K_1}{K_2}. \quad [8]$$

These equations show that for a completely reactant-like transition state ($\alpha = 0$) an increase in the equilibrium constant, K, is reflected exclusively in a decrease in the "off" rate while the "on" rates are unaffected. On the other hand, when the transition state is completely product-like ($\alpha = 1$) an increase in the equilibrium constant is reflected in an increase in the "on" rate while the "off" rates do not change. To apply these ideas to the reaction of hemoglobin with different ligands we shall now consider the nature of the transition state and how it depends on the ligands.

Kinetics of isolated chains

The binding of a ligand by a single-chain heme protein can be pictured as follows. The ligand diffuses through the solution via an opening into the protein. Once inside, it moves toward the iron, bouncing off various side chains and distorting the protein, to an extent that depends on the nature of the ligand, before it gets close enough to begin bonding with the iron. As this bond becomes stronger, the iron gradually moves into the plane of the porphyrin ring, thus causing additional conformational changes. The thermodynamic affinity of the protein for the ligand is determined by how much of the intrinsic energy of the ligand–iron bond is expended to change the conformation of the protein. Note the distinction between two types of ligand-induced conformational changes. The first, which can be called the "direct," is simply steric distortion of the heme pocket and the second, which can be called "indirect," is triggered by the motion of the iron. In hemoglobin, nature could have chosen either or both the direct and the indirect approach to control the ligand affinity of the heme. The Perutz stereochemical mechanism (1, 2) of hemoglobin includes both types of control but places emphasis on the importance of the indirect one. A major objective of this paper is to determine whether hemoglobin kinetics can be qualitatively understood in terms of indirect control alone—i.e., without invoking changes in the accessibility of the heme in the course of ligation,

To discuss kinetics we must determine where along the reaction path the transition state lies. That is, we must know to what extent the iron–ligand bond has formed and how far the iron has moved at the point where the free energy is maximal. This depends on the nature of the ligands. First, consider the kinetics of the free chains.

Table 1 shows that both NO and O_2 are bound very strongly. The enthalpy of reaction for O_2 is about -14 kcal (-6.7×10^4 J) (23); no data exist for NO but it is probably even more exothermic. Moreover, these ligands react with a rate approaching the diffusion-controlled limit, which suggests reactant-like transition states. This is also consistent with the large difference between their binding constants being due mainly to changes in the "off" rates. CO is bound much more strongly than O_2 but reacts at about $1/10$ the speed, implying that the transition state lies further along the reaction path (i.e., less reactant-like) than it does for NO and O_2. Why does CO have a larger activation energy for binding than the other small ligands? The explanation may lie in the differences between their electronic structures and the nature of their bonds with the iron, but it is tempting to attribute the relatively slow "on" rate of CO to distortion of the heme pocket resulting from its preference to bind to the iron in a linear fashion. The recently reported structures of sperm whale carboxymyoglobin (24) and carboxyhemoglobin (25) show that the CO oxygen is displaced from the heme axis; the CO carbon is not visible but Heidner *et al.* (25) advanced good reasons why it should also lie off the heme axis. The displacements were shown to be due to steric hindrance by the N_ϵ of His-E7 and the C_{γ_2} of Val-E11; the same is probably true in myoglobin. Unlike O_2 and NO, which bind to model iron porphyrin complexes in a bent configuration (26, 27), CO binds in a linear fashion. Collman *et al.* (28) have attributed the decreased affinity of heme proteins for CO, compared with unencumbered model compounds, to steric hindrance.

I suggest that this steric effect also increases the activation energy for the "on" reaction of CO and makes the transition state for CO less reactant-like than it is for O_2 and NO, because the CO—Fe bond in the transition state must be formed to a greater extent to pay for the distortion of the protein. Even if both electronic and steric factors contribute to decreasing the rate of CO binding to the free chains, the implications for the kinetics of the reaction of CO with hemoglobin are the same.

This distortion becomes even more significant in the reactions with isocyanides which react more slowly than smaller ligands. The free energy required to bring a ligand close enough to the iron to commence bond formation increases with its bulkiness, and unfavorable interactions occurring before the transition state is reached increase the activation energy for the "on" reaction. Moreover, to pay for the distortion of the protein, the iron–ligand bond must be formed to a significant extent before the transition state is realized. Thus, the bulkier the ligand, the more product-like the transition state becomes. This is consistent with the decrease in binding constants being reflected primarily in the decrease of the "on" rates (Table 1). However, the magnitudes of the "on" and equilibrium constants of isocyanides do not entirely reflect the importance of the distortion of the heme pocket, because it is energetically favorable to transfer the organic part of an isocyanide from water into the hydrophobic environment of the pocket. Thus, these constants are larger than should be expected from steric consideration alone.

Kinetics of hemoglobin

In hemoglobin kinetics it is important to understand the differences between the "on" and "off" rate constants for the first and last reaction steps. The Perutz stereochemical mechanism (1, 2) for cooperative ligand binding is based on the structures of the two quaternary conformations: the liganded (oxy) and unliganded (deoxy). The deoxy quaternary structure is more stable in the absence of ligand whereas the oxy is more stable in the presence of ligand. However, if the COOH-terminal

residues of the subunits, which participate in stabilizing interactions (i.e., salt bridges), are modified or eliminated, even in the absence of ligand the oxy quaternary structure becomes the more stable. When the first ligand binds to hemoglobin, a part of the ligand–iron bond energy is used to break or loosen the salt bridges associated with the subunit. This is accomplished by the iron moving into the plane of the porphyrin ring and thus triggering a chain of conformational events that culminates in the breakage of the salt bridges. After enough of the salt bridges have been perturbed, the conformational equilibrium is shifted to the intrinsically (i.e., in the absence of salt bridges) more stable oxy quaternary structure. In this quaternary structure, the stabilizing salt bridges assocated with the remaining unliganded chains are absent and thus the last ligand binds with higher affinity.

In the simplest terms, the first ligand is bound more weakly than the last because a larger part of the free energy arising from bond formation is required for the conformational changes triggered by the motion of the iron. This mechanism explains why NO and bulky isocyanides such as BuNC bind less cooperatively to hemoglobin (6, 10, 16) than does O_2. In the reaction of bulky isocyanides, so much of the free energy resulting from formation of the metal–ligand bond must be used to pay for the steric distortion of the heme pocket that not enough is left to perform all the conformational changes needed to move the iron completely into the porphyrin plane. On the other hand, NO does not experience severe steric hindrance in the pocket but the bond between the iron and the proximal histidine in the NO complex is too weak and elastic (29) to communicate the motion of the iron effectively to the rest of the protein.

We can now combine these ideas with the insights gained from single-chain kinetics. If the transition state is reactant-like, the indirect conformational changes triggered by the motion of the iron have not yet occurred at the time this state is reached and thus its energy is insensitive to the conformation of the protein. On the other hand, if the transition state occurs later along the reaction path, the iron has already moved toward the heme plane by a significant amount so that the energy of the transition state is influenced by the conformation of the protein. The more costly it is to move the iron, the higher is that energy. Now consider again the data in Table 2.

The high affinity of NO for hemoglobin and its fast "on" rate point to a reactant-like transition state. Therefore, we expect the "on" rate constants for the first and last reactions to be approximately the same and cooperativity to show up in changes in the "off" rates. For O_2 the situation is similar although not so extreme, the transition state being largely but not entirely reactant-like ($\alpha = 0.1$), so that the greater binding constant of the last oxygenation step would also imply some increase in the "on" rate constant (see Eq. 7). We have seen that the CO transition state is expected to lie further along the reaction path than the transition states of NO and O_2, so that the parameter α is larger (0.4) and an increase in the binding constants implies both a decrease in the "off" rate constants and an increase in the "on" rates (see Eqs. 7 and 8). Similarly, a more product-like transition state is expected with BuNC because of the steric hindrance in the heme pocket encountered before bonding can begin. In the limit of a completely product-like transition state ($\alpha = 1$), cooperativity should manifest itself in an increase in the "on" rates only. The α chains, which react much more slowly with BuNC than the β chains, are expected to have the more product-like transition state and their "on" rates do indeed increase but their "off" rates remain the same.

Two further implications of these ideas should be mentioned. For a very bulky ligand such as tertiary butyl isocyanide we expect cooperativity to manifest itself almost exclusively in the increase of the "on" rates. Allosteric effectors such as 2,3-diphosphoglycerate which bind strongly to unliganded hemoglobin but hardly at all to the liganded form are expected to alter the first "off" rate constant when the transition state is essentially reactant-like whereas the first "on" rate constant should be primarily changed when the transition state lies further along the reaction path. This is true for O_2 (13); no reliable data are available on the effect of diphosphoglycerate on the kinetics of CO and the isocyanides. Significant changes in the first "on" rate should be observed for these ligands.

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