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A hypothesis concerning diffusion-limited protein–ligand interactions

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Abstract

A simple assumption allows the prediction of the numerical value for a 'universal' limiting kinetic rate for wholly diffusion-limited reactions between small neutral molecules and macromolecules. This prediction is compared with appropriate experimental data for binding of ligands to myoglobin and to enzymes. It is shown that in the absence of electrostatic effects, this limit is approached but not exceeded. The model also makes very specific predictions concerning the viscosity and temperature dependence of such reactions.

Keywords: Kinetics; Binding; Oxygen; Myoglobin; Enzymes

1. Introduction

The binding of small ligands to macromolecules is a part of many of the most important biochemical processes. In addition to the myriad examples of enzyme–substrate interaction, there are also a large number of cases in which small molecules are simply bound to proteins or nucleic acids in a reversible fashion. The prototype for such reactions, and the one for which the most detailed information is available, is the binding of oxygen to hemoglobin and myoglobin. Although in most cases the total binding reaction is a complex process involving a number of steps, it almost always begins with a simple diffusional encounter between the ligand and the macromolecule.

Although the basic theory of diffusional encounters extends back to Smoluchowski's work in the early part of the 20th century [1], only recently have theoretical analyses been developed to describe the binding of small ligands to specific sites on macromolecules, and only very recently have data of the kind needed to really test such theories become available.

It is my intent in this paper to show how appropriate theoretical expressions can be reasonably combined to predict a practical upper limit to the second-order rate constant for this kind of biologically relevant encounter, and to compare this prediction with the most appropriate experimental data.

2. Theoretical encounter rates

Treatment of diffusion-controlled bimolecular reactions is traditionally couched in terms of the Smoluchowski theory [1]. For two molecules of radii r_A and r_B, with diffusion coefficients D_A and

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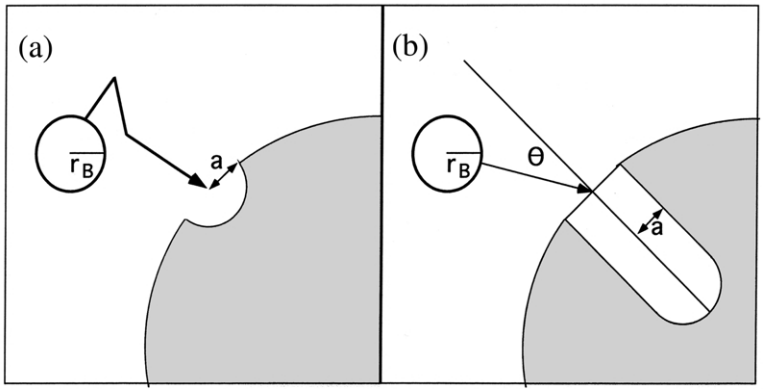

Fig. 1. Models for ligand–protein interaction. (a) Diffusional encounter of small molecule (radius r_B) with a macromolecule that has on its surface a binding site of radius a. (b) Diffusional entry into a pocket with opening of radius a, carrying the binding site. It is easily shown that the maximum angle of approach (θ) is given by $\cos\theta = r_B/a$. If $r_B = a$, only the unlikely approach along $\theta = 0$ can lead to entry.

D_B, the rate constant for encounters is predicted to be:

$$k_e = \frac{4\pi N}{1000}(D_A + D_B)(r_A + r_B) \quad (1)$$

here D and r are expressed in cgs units, and k_e in $M^{-1}\,s^{-1}$. N is Avogadro's number. Eq. (1) is quite unsuitable for interactions between proteins and small ligands, as can be shown by a simple calculation. Consider the well-studied reaction between oxygen and myoglobin at 25 °C. For the protein, we have $D_A = 1.2\times10^{-6}$ $cm^2\,s^{-1}$ [2], which yields a Stokes' radius of $r_B = 1.7\times10^{-7}$ cm. The diffusion coefficient of oxygen is $D_B = 1.9\times10^{-5}$ $cm^2\,s^{-1}$ [3]. This would give a Stokes' radius of $r_B = 1.3\times10^{-8}$ cm. Although the use of Stokes' law is only approximate for so small a molecule as oxygen, the result is in accord with expected molecular dimensions, and in any event has little effect on the result. The calculation predicts $k_e = 2.8\times10^{10}$ $M^{-1}\,s^{-1}$, approximately three orders of magnitude larger than the observed values.

The reasons for this discrepancy, as we shall see later, are several. We may begin, however, by noting the inappropriateness of Eq. (1) itself. Eq. (1) calculates for *any* encounter between B and A, whereas only ligand contact over a small fraction of the surface of myoglobin (the opening to the active site) can lead to binding. This problem has been addressed by a number of workers (see, e.g. [4–10]). A simplest model [7,8] represents the 'target' on the surface of the protein by a circular patch of radius a (Fig. 1a). Then, k_e is predicted to be

$$k_e \cong \frac{4N}{1000} D_B a \quad (2)$$

where dimensions are as in Eq. (1). Here it is assumed that $D_A \ll D_B$. It seems not to have been previously noted that a very simple result can be obtained in this case if we express D_B in terms of the Stokes' radius r_B,

$$D_B = \frac{RT}{N 6\pi\eta r_B} \quad (3)$$

and combine Eq. (2) and Eq. (3):

$$k_e = \frac{RT}{1500\pi\eta}\left(\frac{a}{r_B}\right) \quad (4)$$

Now, if the site exists on the protein surface and can hold B, its radius a cannot be appreciably less than r_B. On the other hand, if binding is to be both effective and selective, a should not be much larger than r_B. We might expect, then, that evolutionary constraints would have dictated that $a \cong r_B$. The Stokes' radius seems appropriate here

in that it represents the effective radius of a molecule interacting dynamically with a molecular medium. Given this assumption, the rate constant for encounters with such a site should *always* be approximated by

$$k_e \cong \frac{RT}{1500\pi\eta} \tag{5}$$

At 25°, in water, this yields the value

$$k_e \cong 6\times 10^8\ \mathrm{M}^{-1}\ \mathrm{s}^{-1} \tag{6}$$

A more realistic model, that seems to describe many enzymes and transport proteins, assumes that the binding site is recessed in the protein, accessible by a tunnel with an opening of radius a. This model has also been analyzed by Shoup et al. [7] with the result

$$k_e = \frac{2N}{1000} D_B \{|a - r_B| + (a - r_B)\} \tag{7}$$

where k_e now represents the rate of potentially effective encounters with the entry. If $a < r_B$, $k_e = 0$; the ligand cannot enter. Even if $a = r_B$, the probability of entering is vanishingly small, for only a trajectory exactly normal to the surface will suffice (Fig. 1b). In fact, for entry to be a frequent event, we must have $a > r_B$, in which case Eq. (7) becomes

$$k_e = \frac{4N}{1000} D_B (a - r_B) \tag{8}$$

As pointed out in [7], the effective radius of the entrance is not a, but $(a - r_B)$. Again, using Eq. (3) for D_B

$$k_e = \frac{RT}{1500\pi\eta}\left(\frac{a - r_B}{r_B}\right) \tag{9}$$

If the effective hole radius equals the Stokes' radius, we again find $k_e = RT/1500\pi\eta$, which is exactly the same as Eq. (5). Thus, the result is independent of details of the model.

The analysis presented above predicts a remarkably simple result, and it is important to point out its limitations. First any electrostatic effects which might either aid or hinder the approach of the ligand to the protein site have been neglected. That these can be significant is shown, for example, by studies of fumarase [4] acetylcholinesterase [11], and superoxide dismutase (SOD) [12] where pronounced enhancement of the rate is found for negatively charged substrates at low ionic strength.

More generally, the second-order rate constants *observed* for small molecule–protein interactions will be lower than predicted by Eq. (5) because the observable process (binding) often represents the second or later rate-limiting step in a multistep process, of which encounter with the protein is only the first. Furthermore, even if binding is not rate-limiting, access to the active site itself may require passing additional energy barriers, or 'gates', any of which may be rate-limiting [9,10,13,14]. Given these caveats, it is nevertheless of interest to compare the model with experimental data.

3. Comparison with experimental results

In comparing experimental results with such a model, it is important to note that what can be most easily observed, the overall second-order rate constant (k') for *binding* of a ligand to active site does not, in general, correspond to the rate constant (k_e) for diffusional encounter with the site or its gate. Nor does a potentially effective encounter, as described above, guarantee entry into the active site pocket; because the opening may be periodically blocked by gating by amino acid residues or water molecules [9,10,13,14]. A closer approximation to k_e than is k' will be given by k'_{entry}, which measures the rate of entry into the active site pocket.

3.1. Globins

An example in which this kind of information has been obtained in detail is the recombination of O_2 or NO with mutant myoglobins following flash photolysis [14]. In this study, Scott et al. have modeled the overall binding reaction as shown in Fig. 2. If the steady-state concentrations of B and C are small and $\mathrm{d[B]}/\mathrm{d}t$, $\mathrm{d[C]}/\mathrm{d}t \cong 0$, then the observed second-order constant is given by

$$k' = k'_{\mathrm{entry}} \frac{k_1}{k_1 + k_2} \tag{10}$$

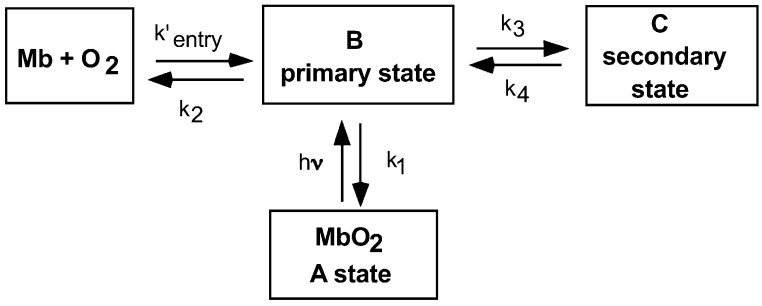

Fig. 2. The model of ligand binding to myoglobin proposed by Scott et al. [14]. Entry of the ligand into the active site pocket (B state) is described by k'_{entry}; it may be followed by binding (k_1) (A state) or release (k_2). The occupancy of other, non-binding sites (C state), is assumed to be very small.

If $k_1 \gg k_2$, virtually every entry into the binding site results in an observable binding. In fact, this is the case for NO binding to myoglobin, in which case $k' \cong k'_{entry}$. However, with O_2 as ligand k' is usually several-fold smaller than k'_{entry}, for k_1 is not sufficiently high to make k_2 negligible.

The comprehensive study by Scott et al. [14] provides for the first time k'_{entry} values for a ligand binding to a set of mutant variants of a single protein. The most interesting, from the point of view of this paper, are those mutants in which amino acid residues in the first shell of distal residues have been modified from the wild type. This is because it is bulky amino acid side chains in this region, especially F43 and H64, that can act as gates, periodically blocking access to the binding site and therefore reducing k'_{entry} below the value it would have were the gate permanently open [14]. Results for O_2 binding by the wild type and 20 such mutants are depicted in a bar graph in Fig. 3. Also shown are k' values for NO binding by the same group of mutants. The result is striking: whenever and only when the critical distal residues are replaced by amino acids with very small side chains (G, A, V) do k'_{entry} values approach the theoretical limit given in Eq. (6) (vertical line in figure). On the other hand, when very bulky residues (like Trp) are substituted, the rate becomes much lower than for wild type. Thus, the results are wholly consistent with a diffusion-limited process modified by a 'gating' mechanism, whereby diffusional access to the binding site is controlled by a molecular gate. The more bulky the gate, the greater the departure of k'_{entry} from the theoretical limiting value.

A search through the myoglobin–hemoglobin data for values of k'_{entry} that exceed 6×10^8 $M^{-1}\,s^{-1}$ has yielded only one qualified example: the cyanoglobin of *Nostoc commune* [15] exhibits $k'_{entry} \cong 1.3\times10^9$ $M^{-1}\,s^{-1}$, roughly twice the limit proposed. Given the approximations involved in the analysis, and the experimental uncertainty in kinetic data, a factor of 2 does not seem especially significant.

3.2. Enzymes

The binding of small substrates or analogs by enzymes should also follow the rules set forth

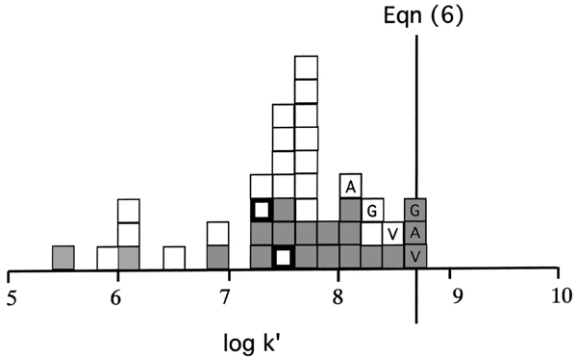

Fig. 3. A bar graph showing the distribution of log k'_{entry} values (O_2, grey) and log k' values (NO, open) for a number of mutant myoglobins [14]. Results for the wild-type protein are heavily outlined. The symbols G, A, V indicate mutants in which H64 has been replaced by gly, ala, or val, respectively.

above. However, in most cases the analysis of such reactions has not been carried out with as much detail as in the binding of oxygen to myoglobin, and the relationship of the observed second-order rate constants (usually obtained from k_{cat}/K_M) to actual k'_{entry} values is usually unclear. There are a few 'very fast' enzymes for which more detailed information is available (e.g. see [16]). A number of these do exhibit values for k_{cat}/K_M clustering approximately 10^8–10^9 $M^{-1}\,s^{-1}$. However, interpretation of these data is complicated by the fact that many of these enzymes interact with charged substrates, and 'electrostatic steering' may give rise to rate enhancement above the diffusion limit [4,11,12,17]. Because 'gating' (see above) and electrostatic steering can modify the observed rate in opposite directions, it is very difficult to make comparisons in these cases with the equations derived here. For example, triose phosphate isomerase (TIM) and SOD are both enzymes that bind small, negatively charged substrates and have shown evidence for diffusion control [18,19]. TIM from various species exhibit k_{cat}/K_M values from 1.0 to 8.4×10^8 $M^{-1}\,s^{-1}$, exactly in the range predicted by Eq. (5). However, this may be in part due to electrostatic steering, as suggested by Wade et al. [17]. SOD is one of the fastest enzymes known with k_{cat}/K_M as large as 4×10^9 $M^{-1}\,s^{-1}$ at low ionic strength, pH 7 [17]. This is clearly electrostatically enhanced, for at high pH where the positive charges responsible for steering have been neutralized, an ionic strength independent value of approximately 2×10^8 $M^{-1}\,s^{-1}$ is observed. Again, this is in the range predicted by Eq. (5).

Some very recent data for the binding of a peptide substrate to thrombin have been interpreted in a manner that allows a calculation of k_{entry} [20]. This gives a value, for the 'fast form' of the enzyme, of 6.5×10^8 $M^{-1}\,s^{-1}$, in excellent agreement with Eq. (6). However, this coincidence should be regarded with caution, for the apparent activation energy is larger than we would expect for a purely diffusion-controlled reaction (see below).

Thus, although the data are more difficult to interpret, it seems that in the absence of strong electrostatic steering, substrate binding by very efficient enzymes conforms to the same behavior exhibited by myoglobins and hemoglobins.

4. Dependence of the limiting rate on viscosity and temperature

It is obvious from Eq. (5) that the diffusion-controlled rate for the binding of a small ligand to a protein will be inversely proportional to the viscosity of the medium. Such a proportionality can, of course, be deduced from the original Smoluchowski treatment, and has long been used as a test for diffusion-control (see [18,20] for recent examples). The usual analysis involves variation of the viscosity by addition of a substance like glycerol or sucrose and graphing (k'_0/k') vs. (η/η_0) where the zeros denote the reference solvent, water. (See [21] for problems with this approach if the process is not *wholly* diffusion controlled.) Eq. (5) presents a more stringent test, for a plot of the absolute value of the rate constant versus the reciprocal of the absolute viscosity should have the slope $RT/1500\pi$. One general difficulty with any such viscosity–variance test is that one must assure that addition of the viscosity-enhancing agent does not in itself modify the reaction. (See [18], for a very careful analysis.)

In a similar fashion, a wholly diffusion-controlled protein–small molecule reaction should exhibit a particular, well defined temperature dependence. We calculate, as for the usual Arrhenius plot, the quantity $\mathrm{d}\ln k/\mathrm{d}(1/T) = -E_a/R$. From Eq. (5):

$$\ln k = \ln\frac{R}{1500\pi} + \ln T - \ln\eta \tag{11}$$

$$\frac{\mathrm{d}\ln k}{\mathrm{d}(1/T)} = \frac{\mathrm{d}\ln T}{\mathrm{d}(1/T)} - \frac{\mathrm{d}\ln\eta}{\mathrm{d}(1/T)} = -T - \frac{\mathrm{d}\ln\eta}{\mathrm{d}(1/T)} \tag{12}$$

Taking water viscosity data from [22], we find that a graph of $\ln\eta$ vs. $1/T$ is nearly linear, with a slope of 2.2×10^3 K. At room temperature then, we expect $\mathrm{d}\ln k/\mathrm{d}(1/T) \cong -2.5\times10^3$ K $= -(E_a/R)$ which gives $E_a \cong 5.0\times10^3$ cal mol^{-1}. Thus, for small molecule–macromolecule reactions exhibit-

ing a true diffusion limit, the apparent activation energy will be approximately 5 kcal mol^{-1}. This value has nothing to do with 'barriers' to reaction; it simply represents an energy cost for diffusion transport. It can, in fact, be identified with the quantity that has been termed the 'viscous heat' [23].

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