Diffusion modifies the connectivity of kinetic schemes for multisite binding and catalysis

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The simplest way to describe the influence of the relative diffusion of the reactants on the time course of bimolecular reactions is to modify or renormalize the phenomenological rate constants that enter into the rate equations of conventional chemical kinetics. However, for macromolecules with multiple inequivalent reactive sites, this is no longer sufficient, even in the low concentration limit. The physical reason is that an enzyme (or a ligand) that has just modified (or dissociated from) one site can bind to a neighboring site rather than diffuse away. This process is not described by the conventional chemical kinetics, which is only valid in the limit that diffusion is fast compared with reaction. Using an exactly solvable many-particle reaction-diffusion model, we show that the influence of diffusion on the kinetics of multisite binding and catalysis can be accounted for by not only scaling the rates, but also by introducing new connections into the kinetic scheme. The rate constants that describe these new transitions or reaction channels turn out to have a transparent physical interpretation: The chemical rates are scaled by the appropriate probabilities that a pair of reactants, which are initially in contact, bind rather than diffuse apart. The theory is illustrated by application to phosphorylation of a multisite substrate.

multisite phosphorylation | escape and capture probabilities | splitting probability | diffusion-influenced rate constants | ultrasensitivity

For bimolecular reactions in solution, the formalism of chemical kinetics is valid only in the limit that the reactants come together many times before reacting. This means that the intrinsic reaction rate must be slower than the rate at which the partners diffuse together. Starting with the seminal work of Smoluchowski (1), it has been shown that the relative diffusion of the reactants, even in a macroscopically homogeneous solution, can lead to deviations from the predictions of conventional chemical kinetics. For example, for reversible reactions, the concentrations decay to their equilibrium values not exponentially, but rather as a power law (2, 3). However, for biochemically relevant concentrations, such effects are small. Even in the crowded environment of a cell, although the total concentration of all macromolecules is of course high, the concentrations of specific molecules that can react with each other are typically low. Although it is interesting and challenging to develop a theory of reversible diffusion-influenced reactions that is accurate at all times and concentrations, in many cases the concentrations are so low that all one has to do is to replace the phenomenological rate constants by their diffusion-influenced values.

In this paper we consider a class of reactions involving macromolecules with multiple sites where, even at low concentrations, it is not enough to replace the rate constants with diffusion-influenced ones. Our interest in such problems was stimulated by the important work of Takahashi et al. (4) on the role of diffusion in a dual phosphorylation–dephosphorylation cycle that can exhibit ultrasensitive (5) and bistable behavior (6). Based on many-particle stochastic simulations, it was shown that slowing down diffusion can speed up response and lead to the loss of ultrasensitivity and bistability (4). These results were attributed to "spatio-temporal correlations between the enzyme and the substrate molecules." The physical idea is illustrated in Fig. 1 for dual phosphorylation. For the sake of simplicity, we have assumed that the binding and catalytic sites are the same and adopted a reference frame where the substrate is fixed. After modifying the first site, the enzyme dissociates and (*i*) diffuses away and another enzyme binds and modifies the second site (lower pathway) or (*ii*) binds to the second site and modifies it (upper pathway). In other words, in the upper pathway both sites are phosphorylated by the same enzyme molecule, whereas in the lower pathway the sites are phosphorylated by different molecules. In the fast diffusion limit, only the lower pathway is in play. In the phosphorylation field, the mechanism is called distributive when the enzyme and substrate dissociate after each modification, whereas in a processive mechanism all sites are phosphorylated before dissociation (7, 8). Thus, when the rate of diffusion is finite, the mechanism inevitably contains both distributive and processive features (4, 9, 10).

The goal of this paper is to develop a simple theory that can quantitatively describe such phenomena for physically relevant concentration ranges without having to resort to many-particle computer simulations. In a nutshell, not only does one need to replace the existing phenomenological rate constants by their diffusion-influenced counterparts, but also one has to introduce new transitions into the kinetic scheme. The rates of these new reaction channels are determined by the probability that a reactant released from one site binds to another rather than diffusing away. Even for complex geometries, such capture and escape probabilities can be found by numerically solving or simulating only a time-independent, two-particle problem.

The outline of the paper is as follows. We start with arguably the simplest model of multisite modification that can be solved exactly on the many-particle level. In this model, the lifetime of the enzyme–substrate complex is assumed to be so short that a site can be modified when the enzyme simply comes in contact with the substrate. When the enzyme concentration is sufficiently low, it is found that the exact time dependence of the concentrations is well described by ordinary rate equations corresponding to the

Significance

Repeated modification of a multisite protein plays a key role in cell signaling. After an enzyme catalyzes a reaction at one site, it can modify another site before diffusing away. This physical process, which is amplified in the crowded environment of the cell, has been recently shown by stochastic simulations of hundreds of particles to dramatically change the response of a biochemical network. Here we show that the influence of diffusion on multisite protein modification can be described in a much simpler way, by introducing new connections into the kinetic scheme with rates depending on the probability that an enzyme–substrate pair diffuses apart rather than reacts. Our approach is applicable to complex reaction networks.

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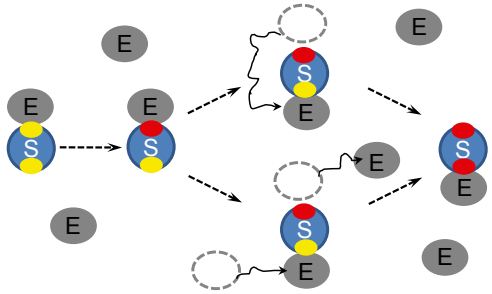

Fig. 1. A substrate *S* with two sites (yellow) that can be modified (red) by an enzyme *E* at concentration [*E*]. Lower pathway: After modifying one site, the enzyme diffuses away and another enzyme binds and phosphorylates the unmodified site. Upper pathway: The enzyme that has just modified one site dissociates, binds to the unmodified site, and then phosphorylates it.

standard kinetic scheme with the crucial difference that new transitions have been allowed. The corresponding rate constants turn out to have such a simple physical interpretation that the formalism can be readily generalized to treat more realistic cases (e.g., intermediate bound complexes, enzyme reactivation, and binding to inequivalent sites). The rate constants in the modified kinetic schemes can be expressed in terms of the capture and escape probabilities of an isolated pair of reactants. In simple cases, these can be found by solving a time-independent equation subject to the appropriate boundary conditions and, for realistic geometries, by simulating the dynamics of an isolated pair of reactants. Illustrative calculations for dual phosphorylation show that our formalism reproduces the speeding up of the response discovered using stochastic many-particle simulations (4).

Theory

We begin by showing how diffusion leads to the appearance of new reaction channels in the context of a simple exactly solvable model. The final results turn out to be so intuitively plausible that they can be understood without following their derivation. Consider a substrate with N sites that can be irreversibly modified by an enzyme. If the lifetimes of the enzyme–substrate complexes are sufficiently short, this process can be described within the framework of chemical kinetics by the scheme

$$S_i + E \xrightarrow{\kappa_i} S_{i+1} + E, \qquad [1]$$

where $i = 0,1,\ldots,N-1$. Here S_i denotes the substrate with i modified sites and κ_i is the phenomenological rate constant for modifying a site on the substrate with i sites already modified. The corresponding rate equations, in matrix form, are

$$\frac{d}{dt}[\boldsymbol{S}(t)] = -\boldsymbol{K}_{CH}[E][\boldsymbol{S}(t)], \qquad [2]$$

where $[\boldsymbol{S}(t)]$ is a vector of concentrations with components $[S_i(t)]$, $i = 0,\ldots,N$, and $\boldsymbol{K}_{CH}$ is an $(N+1)\times(N+1)$ rate matrix whose only nonzero elements are $[\boldsymbol{K}_{CH}]_{i,i} = -[\boldsymbol{K}_{CH}]_{i+1,i} = \kappa_i$, $i = 0,1,\ldots,N-1$.

To see how diffusion influences the kinetics of this reaction, we adopt arguably the simplest nontrivial many-particle model. We consider a single impenetrable spherical substrate uniformly surrounded by M spherical enzymes in volume V. The substrate is static. The enzymes diffuse with diffusion constant D and do not interact with each other. When an enzyme comes in contact with the substrate (i.e., the distance between their centers is R irrespective of orientation), the transition $S_i \to S_{i+1}$ can occur with the reaction rate constant κ_i. In this model, modification occurs without forming an enzyme–substrate complex, because we have assumed that the lifetime of such a complex is so short that it can be ignored. Let $P_i(r_1,\ldots,r_M,t)$ be the probability density that at time t i sites are modified and the enzymes are located at distances $r_1,\ldots,r_M$ from the substrate. The vector of these probabilities satisfies the M-particle diffusion equation

$$\frac{\partial}{\partial t}\boldsymbol{P} = D\sum_{m=1}^{M}\nabla_{r_m}^2\boldsymbol{P}, \qquad r_m \geq R, \qquad [3]$$

where $\nabla_r^2 = r^{-2}(\partial/\partial r)r^2\partial/\partial r$. The reaction is described by imposing boundary conditions obtained by balancing the diffusive and chemical fluxes at contact:

$$4\pi R^2 D\frac{\partial}{\partial r_m}\boldsymbol{P} = \boldsymbol{K}_{CH}\boldsymbol{P}, \qquad r_m = R, \qquad [4]$$

where $m = 1,2,\ldots,M$. The substrate concentrations are given by $[S_i(t)]/[S_{tot}] = \int_V P_i\, d\boldsymbol{r}_1 \ldots d\boldsymbol{r}_M$ when $M, V \to \infty$ in such a way that $M/V = [E]$ (i.e., the thermodynamic limit). Here $[S_{tot}] = \sum_{i=0}^{N}[S_i(t)]$.

The different components of $\boldsymbol{P}$ are coupled only by the boundary conditions in Eq. **4**. However, by diagonalizing the rate matrix $\boldsymbol{K}_{CH}$, we can decouple them and reduce the solution of the many-particle problem to N one-particle problems. Let $\boldsymbol{T}$ be the transformation matrix that diagonalizes $\boldsymbol{K}_{CH}$ (i.e., $\boldsymbol{K}_{CH}\boldsymbol{T} = \boldsymbol{T}\boldsymbol{\Lambda}_{CH}$, where $\boldsymbol{\Lambda}_{CH}$ is the diagonal matrix with elements κ_i, $i = 0,\ldots,N-1$ and $\kappa_N = 0$). Then it can be shown that (*Supporting Information*)

$$[\boldsymbol{S}(t)] = \boldsymbol{T}\boldsymbol{F}(t)\boldsymbol{T}^{-1}[\boldsymbol{S}(0)]$$
$$F_{ij}(t) = \lim_{\substack{M,V\to\infty \\ M/V=[E]}}\left(\int_V g_i(r,t)\frac{d\boldsymbol{r}}{V}\right)^M\delta_{ij}, \qquad [5]$$

where $g_i(r,t)$ satisfies $\partial g_i/\partial t = D\nabla_r^2 g_i$, with the boundary condition $4\pi R^2 D\partial g_i/\partial r = \kappa_i g_i$ at $r = R$. Initially, $g_i(r,0) = 1$. Differentiating Eq. **5** with respect to time and taking the thermodynamic limit, it can be shown that

$$\frac{d}{dt}[\boldsymbol{S}(t)] = -\boldsymbol{K}(t)[E][\boldsymbol{S}(t)]$$
$$\boldsymbol{K}(t) = \boldsymbol{T}\boldsymbol{\Lambda}(t)\boldsymbol{T}^{-1}, \qquad [6]$$

where $\boldsymbol{\Lambda}(t)$ is a diagonal matrix with elements

$$k_i(t) = -\int_R^\infty \frac{\partial g_i(r,t)}{\partial t}d\boldsymbol{r} = \kappa_i\left(\epsilon_i + (1-\epsilon_i)e^{\tau_i}\mathrm{erfc}\sqrt{\tau_i}\right), \qquad [7]$$

where $i = 0,\ldots,N-1$, $k_N(t) = 0$, $d\boldsymbol{r} = 4\pi r^2\, dr$, $\tau_i = Dt/(\epsilon_i R)^2$, $\epsilon_i = k_D/(\kappa_i + k_D)$, and $k_D = 4\pi DR$ is Smoluchowski's diffusion-limited rate constant. As $D \to \infty$, $k_i(t) \to \kappa_i$, which are in fact the eigenvalues of $\boldsymbol{K}_{CH}$ for the kinetic scheme shown in Eq. **1**. Thus, in the fast diffusion limit, Eq. **6** reduces to Eq. **2**, as it must. It should be noted that, except when $n = 1$, the new rate matrix $\boldsymbol{K}(t)$ is not the chemical kinetics rate matrix $\boldsymbol{K}_{CH}$ with κ_i being replaced by $k_i(t)$.

At times longer than the characteristic diffusion time ($t \gg R^2/D$), the time-dependent rate coefficients in Eq. **7** approach constant values, $\lim_{t\to\infty}k_i(t) = \kappa_i k_D/(\kappa_i + k_D) = \Lambda_{ii}(\infty)$, and the rate matrix in Eq. **6** approaches a constant matrix, $\boldsymbol{K}(\infty)$. In the limit of low concentrations, when the effective volume fraction is much less than unity, the rate equation Eq. **2** with the

diffusion-influenced rate matrix $\boldsymbol{K}(\infty)$ replacing $\boldsymbol{K}_{CH}$ becomes a good approximation for $t \gg R^2/D$.

The crucial point is that $\boldsymbol{K}(\infty)$ is not simply $\boldsymbol{K}_{CH}$ with the chemical rate constants κ_i replaced by the diffusion-influenced ones, $\kappa_i k_D/(\kappa_i + k_D)$. Rather, is it given by

$$\begin{aligned}\boldsymbol{K}(\infty) &= \boldsymbol{T}\boldsymbol{\Lambda}(\infty)\boldsymbol{T}^{-1} = \boldsymbol{T}\boldsymbol{\Lambda}_{CH} k_D (\boldsymbol{\Lambda}_{CH} + k_D \boldsymbol{I})^{-1}\boldsymbol{T}^{-1} \\ &= \boldsymbol{K}_{CH} k_D (\boldsymbol{K}_{CH} + k_D \boldsymbol{I})^{-1},\end{aligned} \quad [8]$$

where we have used $\boldsymbol{\Lambda}_{CH} = \boldsymbol{T}^{-1}\boldsymbol{K}_{CH}\boldsymbol{T}$. The new rate matrix $\boldsymbol{K}(\infty)$ has finite elements that were zero in $\boldsymbol{K}_{CH}$. Consequently, the kinetic scheme that corresponds to the rate matrix $\boldsymbol{K}(\infty)$ has new connections (or reaction channels) that were absent in the original chemical kinetic scheme. These new transitions appear due to diffusion and, as shown below, the corresponding rates have a simple physical interpretation.

It should be mentioned that the reversible reaction $S_i + E \rightleftharpoons S_{i+1} + E$ can also be solved exactly. All of the above equations hold when $\boldsymbol{K}_{CH}$ contains both forward and reverse rate constants, except Eq. **7**, where κ_i must be replaced by the eigenvalues of the corresponding rate matrix $\boldsymbol{K}_{CH}$.

Results and Discussion

Consider first how diffusion affects the kinetics of single-site modification, $S_0 + E \to S_1 + E$. In this case, the above results are identical to those obtained from the Smoluchowski–Collins–Kimball theory of irreversible diffusion-influenced reactions (11). The decay of the concentration of S_0 is not single-exponential, but it is given by $[S_0(t)]/[S_{tot}] = \exp(-[E]\int_0^t k_0(t')dt')$, where the time-dependent rate coefficient $k_0(t)$ is given in Eq. **7**. At short times ($t \ll R^2/D$), $k_0(t)$ approaches the chemical rate constant κ_0. As time increases, the rate coefficient decreases because the enzymes must diffuse to the substrate in order for reaction to occur. When $t \gg R^2/D$, $k_0(t)$ approaches the diffusion-influenced (often called steady-state) rate constant $k_0(\infty) = \kappa_0 k_D/(\kappa_0 + k_D)$, where $k_D = 4\pi DR$ is the rate constant in the diffusion-controlled limit when either diffusion is slow ($D \to 0$) or reaction is fast ($\kappa_0 \to \infty$).

The diffusion-influenced rate constant, $k_0(\infty)$, has a simple physical interpretation. Imagine an isolated pair of reactants (e.g., S_0 and E), initially in contact, that can irreversibly react. If one waits enough, there are only two possible outcomes: (*i*) Reaction has occurred (E is "captured" by S_0) and (*ii*) the reactants diffuse apart never to see each other again (E has "escaped" to infinity). For isotropic contact reactivity, the escape probability is $\epsilon_0 = k_D/(\kappa_0 + k_D)$, whereas the capture probability is $1 - \epsilon_0 = \kappa_0/(\kappa_0 + k_D)$. When diffusion is fast, the substrate and enzyme are much more likely to separate rather than react, so $\epsilon_0 \to 1$. When diffusion is slow, a contact pair is much more likely to react, so $\epsilon_0 \to 0$. In terms of this quantity, the diffusion-influenced rate constant can be written either as $k_0(\infty) = \kappa_0\epsilon_0$ [the chemical (contact) rate constant is reduced by the escape probability] or, perhaps more intuitively, as $k_0(\infty) = k_D(1 - \epsilon_0)$ (the diffusion-limited rate constant is reduced by the probability that a contact pair eventually reacts). Escape and capture probabilities will play a central role in the rest of the paper. As shown by Onsager (12) in the context of ion recombination, they can be obtained by solving a time-independent problem (13).

The simplest example where diffusion leads to a change in the connectivity of the kinetic scheme is two-site modification, $S_0 + E \to S_1 + E \to S_2 + E$. Naively, it seems that one could just renormalize the chemical rate constants as shown in Fig. 2*A* [i.e., $\kappa_i \to \kappa_i k_D/(\kappa_i + k_D) \equiv \kappa_i\epsilon_i$, $i = 0, 1$]. However, the rate matrix in Eq. **8** actually corresponds to the kinetic scheme shown in Fig. 2*B*. The "naive" diffusion-influenced rate constant for the transition S_0 to S_1 ($k_0(\infty) = \kappa_0\epsilon_0$) is reduced by the escape probability ϵ_1 of the S_1 and E contact pair. In addition, there is a new direct transition between S_0 and S_2 with a rate constant equal to $k_0(\infty)$ times the capture probability, $1 - \epsilon_1$, of the S_1 and E contact pair. These results can be easily understood. At the instant S_0 has been modified, S_1 and E are in contact. The probability that the enzyme diffuses away is ϵ_1 and the probability that it hangs around and modifies the substrate again is $1 - \epsilon_1$. The first scenario is just the one depicted by the reaction $S_0 + E \to S_1 + E$ in the original scheme. In the second scenario, the same enzyme modifies the substrate twice before diffusing away. This new mechanism, which is only possible because the rate of diffusion is finite, can be approximately treated within the framework of chemical kinetics by allowing the direct transition $S_0 + E \to S_2 + E$. The relative importance of these two channels (i.e., the splitting probability) is determined by the escape probability of the S_1 and E contact pair (ϵ_1).

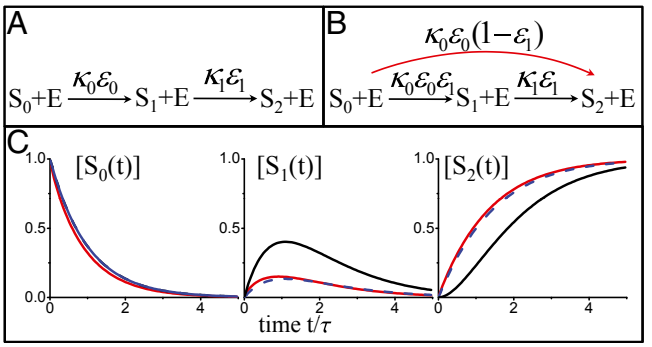

Fig. 2. Effect of diffusion on the kinetics of a simple model of dual modification of a substrate by an enzyme. (*A*) The standard kinetic scheme in which the phenomenological rate constants (κ_0, κ_1) have been scaled by the escape probabilities of contact pairs. (*B*) Our kinetic scheme with an additional transition. (*C*) Comparison of the exact kinetics (red lines, Eq. **S21** in *Supporting Information*) with the predictions of scheme A (black lines, Eq. **S23** in *Supporting Information* with $\kappa_i \to \kappa_i\epsilon_i$) and B (dashed blue lines, Eq. **S24** in *Supporting Information*). Time is normalized to $\tau = (\kappa_0\epsilon_0[E])^{-1}$. $\kappa_0 = 4k_D$, $\kappa_1 = 2k_D$, $4\pi R^3[E]/3 = 0.01$, $D = 1$, $R = 1$, $[S_{tot}] = 1$.

Fig. 2*C* shows how the new transition in the kinetic scheme influences the time dependence of the concentrations. Because S_0 and S_2 are now directly connected, the population of S_1 is smaller and the concentration of S_2 increases faster than that predicted by the "naive" kinetic scheme in Fig. 1*A*. The faster response of the fully modified substrate becomes more pronounced as the number of sites increases (Fig. S1). For the low concentration used (effective volume fraction $4\pi R^3[E]/3 = 0.01$) it can be seen that the exact kinetics are almost the same as those predicted by the scheme with the extra connection shown in Fig. 2*B*. As the concentration increases, the difference increases (Fig. S2) so that any theory based on rate equations with time-independent rate constants eventually becomes inadequate.

Two-Site Phosphorylation: Michaelis–Menten Mechanism. Using the exactly solvable model, we have demonstrated that the effect of diffusion can be accounted for by (*i*) replacing the bimolecular rate constants by diffusion-influenced ones and (*ii*) introducing new reaction channels into the kinetic scheme. In the low-concentration limit, for times longer than the diffusion time, the new kinetic scheme describes the kinetics of a simple model of site modification with high accuracy. We will now use physical arguments based on the insights gained from the exactly solvable model to extend these results to more general models of enzyme catalysis. The results presented below are derived in *Supporting Information* using an approximate low-concentration theory.

Consider the double phosphorylation of a substrate described by the Michaelis–Menten mechanism (scheme in Fig. 3*A*). In contrast to the kinetic scheme in Eq. **1**, here the enzyme E and the substrate with i modified sites, S_i, bind and form the complex S_iE, which has a finite lifetime. It is assumed that catalysis and the

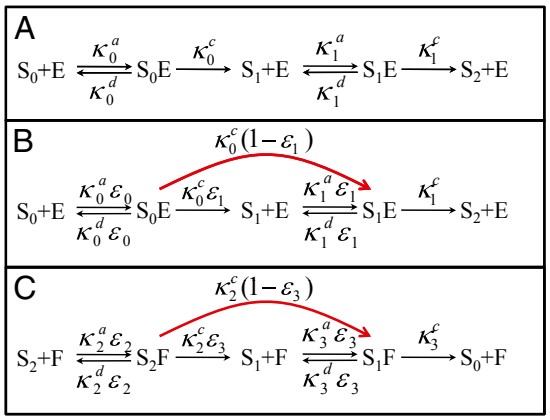

Fig. 3. Conventional and diffusion-modified kinetic schemes for two-site phosphorylation and dephosphorylation. (*A*) A substrate *S* with two sites is phosphorylated by a kinase *E* via the Michaelis–Menten mechanism. (*B*) Kinetic scheme that describes the influence of diffusion. Not only are the chemical rates scaled by the escape probabilities (ε's), but a new transition appears (red arrow) because the same enzyme can phosphorylate the substrate twice before diffusing away. ϵ_i ($i=0, 1$) is the probability that an enzyme, initially in contact with a substrate with *i* modified sites, diffuses away rather than binds to the substrate. (*C*) Corresponding scheme for dephosphorylation by a phosphotase *F*.

release of the substrate occur in one step and that no more than one enzyme binds to the substrate. In the microscopic model of this reaction, the enzyme and substrate diffuse with the relative diffusion coefficient $D=D_E+D_S$. When they come in contact, the complex S_iE can be formed with the association rate constant κ_i^a. The complex can dissociate (with the dissociation rate constant κ_i^d) and form a contact pair of S_i and E. Alternatively, the substrate in the complex can be modified (with the catalytic rate constant κ_i^c), resulting a contact pair of S_{i+1} and E. As before, we assume that the molecules are spherical and uniformly reactive, so that the escape probability, ϵ_i, of the contact pair of S_i and E is given by $\epsilon_i=k_D/(\kappa_i^a+k_D)$.

We now change the kinetic scheme in Fig. 3*A* to take diffusion into account (Fig. 3*B*; the corresponding rate equations, Eq. **S42**, are given in *Supporting Information*). The diffusion-influenced rate constant for the association of S_0 and E to form S_0E is given by $\kappa_0^a\epsilon_0$, where ϵ_0 is the escape probability of S_0 and E contact pair. The dissociation rate constant of S_0E to form S_0+E is the product of the dissociation rate constant κ_0^d and the escape probability of the S_0 and E contact pair, $\kappa_0^d\epsilon_0$. The physical reason for this is that a contact pair cannot be considered to be really dissociated because it can quickly associate again. Full dissociation occurs only when the enzyme and substrate separate completely. It should be noted that the equilibrium constant for binding $\kappa_0^a\epsilon_0/\kappa_0^d\epsilon_0=\kappa_0^a/\kappa_0^d$ is independent of diffusion, as it must be, because the equilibrium constant is a thermodynamic quantity. These expressions for the renormalized association and dissociation rates have been known at least since the paper of Schurr (14) and their interpretation in terms of escape probabilities at least since the work of Shoup and Szabo (15).

To understand the influence of diffusion on the catalytic step, consider the fate of the contact pair of S_1 and E formed from the complex S_0E. This pair can separate with probability ϵ_1 or the enzyme can bind again to form the complex S_1E with probability $1-\epsilon_1$. Thus, the rate corresponding to the catalytic channel in the scheme shown in Fig. 3*A* is reduced by ϵ_1 and a new channel, which directly connects the two complexes, S_0E and S_1E, appears with rate constant $\kappa_0^c(1-\epsilon_1)$. The physical reason for this new connection is that, after modifying the substrate, the enzyme and substrate do not always diffuse apart but can stay close to each other and bind again. Thus, diffusion naturally introduces an element of processivity into a distributive mechanism. No new parameters are needed in this case beyond those that renormalize the rate constants in the original kinetic scheme. When diffusion is fast, all of the escape probabilities approach unity and the modified kinetic scheme in Fig. 3*B* reduces to that in Fig. 3*A*. As diffusion slows down, a new transition or reaction channel appears corresponding to the process where after phosphorylating one site the enzyme rebinds and modifies the other.

The generalization to more than two sites is straightforward: The rate constant for the catalytic step $S_iE\rightarrow S_{i+1}+E$ is $\kappa_i^c\epsilon_{i+1}$, whereas the new transition between adjacent complexes $S_iE\rightarrow S_{i+1}E$ occurs at a rate $\kappa_i^c(1-\epsilon_{i+1})$, where ϵ_{i+1} is the escape probability of a contact pair involving E and S_{i+1}.

Dual Phosphorylation–Dephosphorylation Cycle. In addition to phosphorylation by a kinase, consider dephosphorylation of a two-site substrate by a phosphotase. The influence of diffusion on the kinetics of this cycle was recently studied using stochastic many-particle simulations based on a clever Green's function algorithm (4). It was found that slowing down diffusion can speed up response and decrease untrasensitivity.

We now show that the theory developed in this paper predicts this behavior. The diffusion-modified kinetic schemes for this problem are shown in Fig. 3 *B* and *C*. The corresponding differential equations for the concentrations are given in Eq. **S45** in *Supporting Information*. The concentration of the doubly phosphorylated substrate relative to its steady-state value is shown in Fig. 4 as a function of time for various diffusion coefficients. It can be seen that the response at first becomes faster as the diffusion constant is decreased. The escape probabilities and the rate constants that appeared in the conventional chemical kinetic scheme all decrease as diffusion slows down because the contact pairs are less likely to separate than bind. This is why one naively expects the response to slow down as the diffusion coefficient becomes smaller. However, the rate constants for the new transitions, because they are proportional to the capture probabilities, increase as diffusion slows down. The competition between these two opposing effects leads to an optimal diffusion coefficient that minimizes the response time.

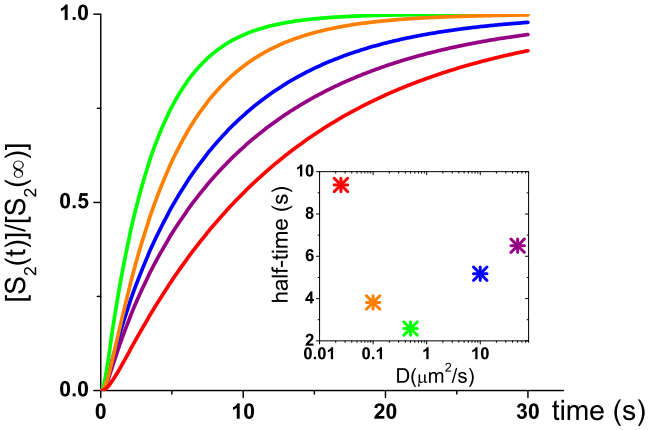

Fig. 4. Time dependence of the concentration of the doubly phosphorylated substrate for different diffusion coefficients. The kinetics were obtained by solving numerically the ordinary differential equations (Eq. **S45** in *Supporting Information*) that correspond to the modified schemes in Fig. 3 *B* and *C*. Initially, the substrates are in the unphosphorylated form. The diffusion coefficients are (in square micrometers per second) 50 (purple), 10 (blue), 0.5 (green), 0.1 (orange), and 0.025 (red). The other parameters, taken from ref. 4, are $\kappa_0^a=\kappa_2^a=0.027\ nM^{-1}s^{-1}$, $\kappa_1^a=\kappa_3^a=0.056\ nM^{-1}s^{-1}$, $\kappa_0^d=\kappa_2^d=1.35\ s^{-1}$, $k_1^d=k_3^d=1.73\ s^{-1}$, $k_0^c=k_2^c=1.5\ s^{-1}$, $k_1^c=k_3^c=15\ s^{-1}$, $R=5nm$, $[E_{tot}]=[F_{tot}]=30\ \mu m^{-3}$, and $[S_{tot}]=120\ \mu m^{-3}$. (*Inset*) The half-time (i.e., the time at which $[S_2]$ reaches half of its steady-state value) as a function of the diffusion coefficient.

The kinetic scheme with additional reaction channels in Fig. 3 *B* and *C* can also predict the steady-state concentrations of diffusing reactants in a dual phosphorylation cycle. As diffusion slows down, the flux through the new connection increases. Consequently, the steady-state input–output curve becomes more shallow and less sensitive to the variation of the kinase/phosphotase concentration, as shown in Fig. S3 and in agreement with ref. 4.

Enzyme Reactivation Time. After catalytic modification, an enzyme may be inactive and require some time to become active again. In phosphorylation, kinase reactivation is related to the release of ADP, and binding of ATP and has been shown to play an important role in the kinetics (4, 16). In the framework of chemical kinetics, this effect can be described by replacing the scheme in Fig. 3*A* by ($i = 0, 1$):

$$S_i + E \underset{\kappa_i^d}{\overset{\kappa_i^a}{\rightleftharpoons}} S_iE \xrightarrow{\kappa_0^c} S_{i+1} + E^*, \quad E^* \xrightarrow{k^\star} E. \qquad [9]$$

How does diffusion affect this kinetic scheme? As shown in *Supporting Information*, when $[E] \gg [E^\star]$, for times longer than both the diffusion time (R^2/D) and the reactivation time $(1/k^\star)$, the diffusion-modified kinetic scheme has the same connectivity as that shown in Fig. 3*B* with the important difference that the ϵ_1 's that enter the catalytic step [i.e., $\kappa_0^c\epsilon_1$ and $\kappa_0^c(1-\epsilon_1)$] are replaced by $\epsilon_1^\star$. This new quantity is the probability that the enzyme, which is initially in the inactive form $E^\star$ in contact with the singly phosphorylated substrate S_1, diffuses away rather than reactivates and binds to the substrate. If the lifetime of $E^\star$ is infinite, then the enzyme always escapes because it cannot react with S_1 and thus $\epsilon_1^\star = 1$. Consequently, when the reactivation time is much longer than the diffusion time, the new reaction channel does not contribute and simply renormalizing the rates in the standard kinetic scheme is adequate.

For uniformly reacting species, $\epsilon_1^\star$ can be found as follows. Let $\mathcal{E}^\star(r)$ [or $\mathcal{E}(r)$] be the escape probability of an isolated pair when initially the distance between $E^\star$ [or E] and S_1 is r. We are interested in $\epsilon_1^\star = \mathcal{E}^\star(R)$. As $r \to \infty$, both $\mathcal{E}^\star(r)$ and $\mathcal{E}(r)$ approach unity. $\mathcal{E}(r)$ satisfies $D\nabla_r^2\mathcal{E} = 0$ subject to the partially reactive boundary condition at contact, $4\pi R^2 D\partial\mathcal{E}/\partial r|_{r=R} = \kappa_1^a\mathcal{E}(R)$ (15). $\mathcal{E}^\star(r)$, however, satisfies $D\nabla_r^2\mathcal{E}^\star - k^\star\mathcal{E}^\star + k^\star\mathcal{E} = 0$ subject to the reflecting boundary condition at contact, $\partial\mathcal{E}^\star/\partial r|_{r=R} = 0$, because $E^\star$ cannot bind to the substrate. Solving these equations, we find

$$\epsilon_1^\star = \frac{k_D}{\kappa_1^a + k_D}\left(1 + \frac{\kappa_1^a}{k_D\left(1 + \sqrt{k^\star R^2/D}\right)}\right). \qquad [10]$$

When the lifetime of $E^\star$ is very short $(k^\star \to \infty)$, then $\epsilon_1^\star \to \epsilon_1 = k_D/(\kappa_1^a + k_D)$ and the diffusion-modified kinetic scheme in Fig. 3*B* is recovered. However, when $k^\star \to 0$, $\epsilon_1^\star \to 1$ and so $E^\star$ always escapes before reactivating and binding to the unmodified site. No new transition appears in the kinetic scheme. Thus, the rate constant of the new reaction channel changes from zero to $\kappa_0^c\kappa_1^a/(\kappa_1^a + k_D)$ as the ratio of the diffusion time to the reactivation time increases.

Anisotropic Reactivity. We now generalize the theory to handle two sites that, although equivalent, are located in different places on the surface of the substrate. In addition we remove the assumption in the Michaelis–Menten mechanism that catalysis and product release occur in the same step and explicitly consider the reversible binding of the enzyme to the modified sites. We start, as before, with the conventional chemical kinetics scheme, which is shown in Fig. 5*A* when all of the ε's are 1 and the q's are zero. We denote the nonmodified site (yellow) by "n" and the modified site (red) by "m" so that, for example, κ_{1m}^a is the association rate constant for binding

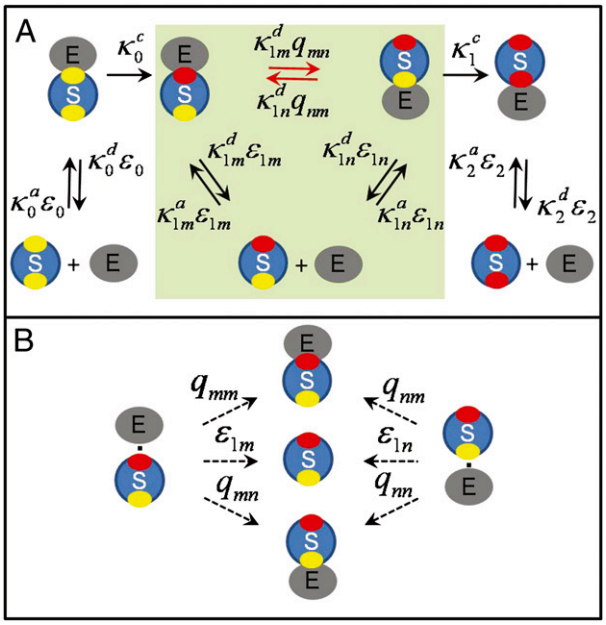

Fig. 5. Two-site modification with anisotropic reactivity. (*A*) A substrate with nonmodified (yellow or "n") and modified (red or "m") sites in the presence of free enzyme (lower row) can form a variety of complexes (upper row). The rate constants in the conventional kinetic scheme (black arrows) are scaled by the escape probabilities, whereas those of the new transitions (red arrows) are proportional to the probability of starting at one site and being captured by the other. (*B*) Definition of escape (ε's) and capture (q's) probabilities. The enzyme, initially in contact with one site, can bind to the same site (q_{nn}, q_{mm}), diffuse away (ϵ_{1n},ϵ_{1m}), or bind to the other site (q_{nm}, q_{mn}). Because of detailed balance, $\kappa_{1n}^a q_{nm} = \kappa_{1m}^a q_{mn}$.

of the enzyme to the modified site of a substrate with one modified and one nonmodified site.

The diffusion-modified kinetic scheme for this problem is shown in Fig. 5*A*. Note that the catalytic rate constants do not change here. The escape probability ϵ_0 is the probability that the enzyme, initially in contact with one of the sites, escapes rather than binds. The most interesting results are shown in the shaded portion of Fig. 5*A* and depend on the escape and capture probabilities defined in Fig. 5*B*. To understand the definition of these probabilities, consider an isolated pair of an enzyme and a substrate in which one of the two sites has been modified. Suppose that the enzyme is initially in contact with the phosphorylated ("m" for modified) site (see the left-hand side of Fig. 5*B*). Such a pair can have only three fates: (*i*) The enzyme can rebind to the "m" site with probability q_{mm}, or (*ii*) it can diffuse away into the bulk with escape probability ϵ_{1m}, or (*iii*) it can bind to the other ("n") site with capture probability q_{mn}. Clearly $\epsilon_{1m} + q_{mm} + q_{mn} = 1$. The case when the enzyme is initially in contact with the "n" site is analogous (see the right-hand side of Fig. 5*B*).

With these definitions, the rate constants in the shaded portion of Fig. 5*A* have a transparent physical interpretation. The rates corresponding to the new transitions (red arrows) are the product of the dissociation rate constant (κ_{1m}^d or κ_{1n}^d) and the probability that the enzyme, starting near one site, binds to the other (q_{mn} or q_{nm}). This new pathway corresponds to the upper mechanism shown schematically in Fig. 1. The association and dissociation rate constants for the transitions that were present in the conventional chemical kinetic scheme (black arrows) are scaled by the appropriate escape probabilities in such a way that the equilibrium constants do not depend on diffusion.

For anisotropic reactivity, the escape and capture probabilities must be obtained numerically by solving differential equations

subject to the appropriate boundary conditions or, for more complex models, by stochastic simulations. Because this is a two-particle problem, it is a lot easier than calculating the time dependence of the concentrations by simulating the reactive dynamics of hundreds of particles. We note for the sake of completeness that for isotropic reactivity simple expressions for these quantities can be found. In the isotropic model, the contact pairs on the left and right sides of Fig. 5*B* are assumed to be equivalent, so $\epsilon_{1m} = \epsilon_{1n} = k_D/(\kappa^a_{1m} + \kappa^a_{1n} + k_D)$, $q_{mm} = q_{nm} = \kappa^a_{1m}/(\kappa^a_{1m} + \kappa^a_{1n} + k_D)$, and $q_{nn} = q_{mn} = \kappa^a_{1n}/(\kappa^a_{1m} + \kappa^a_{1n} + k_D)$.

In the limit that the dissociation rate constants of the complexes between the enzyme and the modified site are very large ($\kappa^d_{1m},\ \kappa^d_2 \to \infty$), the kinetic scheme in Fig. 5*A* reduces to that in Fig. 3*B* with one important difference: ϵ_1 in the rates $\kappa^c_0\epsilon_1$ and $\kappa^c_0(1-\epsilon_1)$ must be replaced by ϵ^c_1, which is the escape probability of the enzyme initially in contact with the modified site. Because now the enzyme does not bind to the modified site, ϵ^c_1 is different from ϵ_1 that scales κ^a_1 and κ^d_1 and is the escape probability of the enzyme initially in contact with the reactive, nonmodified site.

Ligand Binding to Inequivalent Sites. From the shaded portion of Fig. 5*A*, it is clear that the enzyme E can be regarded as a "ligand" that binds to two inequivalent sites (red and yellow). Thus, our diffusion-modified scheme with extra connections also describes ligand binding when the complexes are distinguishable. The extra transitions describe processes in which a ligand dissociates from one site and binds to another without diffusing away. Although we explicitly considered only two sites, the generalization to many sites is straightforward.

Concluding Remarks

We have shown that the influence of the relative diffusion of the reactants on the kinetics of multisite catalysis can be described by introducing new connections or transitions into the conventional kinetic scheme in addition to using diffusion-influenced rate constants. These new pathways or reaction channels appear because an enzyme, after modifying one site on a substrate, can modify another site before the enzyme–substrate pair diffuses apart. It is remarkable that the rate constants in the new kinetic scheme depend on diffusion only through the escape and capture probabilities of isolated enzyme–substrate pairs. These probabilities depend on the enzyme–substrate interaction potential, the precise definition of the reaction zone, and on other factors such as rotational diffusion, but the connectivity of the diffusion-modified kinetic scheme does not. Thus, the influence of molecular crowding can be treated by solving the pair problem with the appropriate potential of mean force and relative diffusion coefficient. For realistic microscopic models, where the escape and capture probabilities must be found numerically, this is a tremendous simplification because one can now simulate the reactive dynamics of two rather than hundreds of molecules to find the time dependence of the concentrations.

The theory presented here is valid at times longer than the diffusion time when concentrations are low. The fastest relaxation time of the diffusion-modified kinetic scheme must be much longer than the characteristic diffusion time (R^2/D), which is typically on the submicrosecond time scale. When the concentrations are large, so that three-particle correlations are important, or when the lifetime of the bound state is comparable to the diffusion time, one can no longer use the rate equations of chemical kinetics, even in their modified forms. It is possible to extend this theory by using the self-consistent relaxation time formalism that we developed and applied to simple reactions such as $A + B \rightleftharpoons C$ and $A + B \rightleftharpoons C + D$ (17), where it is exact at both short and long times. For a substrate with a single catalytic site, this formalism predicts that, as a result of diffusion, the Lineweaver–Burk plot becomes nonlinear at high concentrations (18, 19). When there are multiple sites, the complexity of the theory increases dramatically, and thus there must be compelling reasons for carrying out such a generalization. For the concentrations and parameters used by Takahashi et al. (4) in the many-particle simulations of the mitogen-activated protein kinase cascade, we fully expect that the approach presented here, based on rate equations corresponding to kinetic schemes with renormalized rate constants and additional connections, would accurately describe the influence of diffusion on ultrasensitivity and bistability.

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