Kinetic Analysis of Multisite Phosphorylation Using Analytic Solutions to Michaelis–Menten Equations

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Phosphorylation-induced expression or modulation of a functional protein is a common signal in living cells. Many functional proteins are phosphorylated at multiple sites and it is frequently observed that phosphorylation at one site enhances or suppresses phosphorylation at another site. Therefore, characterizing such cooperative phosphorylation is important. In this study, we determine a temporal progress curve of multisite phosphorylation by analytically integrating the Michaelis–Menten equations in time. Using this theoretical progress curve, we derive the useful criterion that an intersection of two progress curves implies the presence of cooperativity. Experiments generally yield noisy progress curves. We fit the theoretical progress curves to noisy progress curves containing 4% Gaussian noise in order to determine the kinetics of the phosphorylation. This fitting correctly identifies the sites involved in cooperative phosphorylation. © 2002 Elsevier Science Ltd. All rights reserved.

Introduction

Functional proteins in living cells are expressed or modulated often by phosphorylation. Many functional proteins are phosphorylated at multiple sites. In such proteins in general, phosphorylation at different sites is not completely independent. It is frequently observed that phosphorylation at one site enhances or suppresses phosphorylation at another site (Roach, 1991). This cooperative phosphorylation may enable elaborate control of functional proteins in living cells. Therefore, characterizing cooperativity in multisite phosphorylation will help us understand how living cells function. In this study, we provide a novel theoretical tool to quantify cooperative phosphorylation on the basis of the progress curve analysis of biochemical reactions.

Let us consider a protein that is phosphorylated at multiple sites by a certain kinase. Cooperativity in phosphorylation between sites modifies temporal progress of phosphorylation at each site. We predict this modification from the progress curve obtained by integrating the Michaelis–Menten equations incorporating cooperativity. By fitting the theoretical progress curves to experimentally obtainable progress curves, we quantify cooperativity in multisite phosphorylation. In order to show that this procedure works in practice, we prepare noisy progress curves that would be obtained in
experiments and demonstrate that the fitting procedure correctly identifies the sites responsible for cooperative interactions.

For complex reactions in general, however, Michaelis–Menten equations cannot easily be integrated. In this study, we change a variable to linearize the system of differential equations and obtain the complete system of solutions to the Michaelis–Menten equations for an arbitrary number of phosphorylation sites. From the analytic progress curves, we can derive a simple criterion for the presence of cooperativity: if two progress curves intersect each other, two corresponding sites are phosphorylated cooperatively.

In the second section, we study the simplest multisite phosphorylation, that is the two-site phosphorylation, and obtain the theoretical progress curves. We then derive the criterion for the presence of cooperativity. Subsequently, we extend the discussion to \( N \)-site phosphorylation. In the third section, we demonstrate that fitting of theoretical progress curves to experimental ones actually determines a site responsible for cooperative phosphorylation. The last section is devoted to discussion.

### Theory

**DERIVATION OF MICHAELIS–MENTEN EQUATIONS—THE TWO-SITE PHOSPHORYLATION CASE**

Assume that protein \( S \) has two different phosphorylation sites with respect to kinase \( E \). Kinase \( E \) can be bound to either one of the two phosphorylation sites on \( S \) to form an intermediate complex \( (ES)_1 \) or \( (ES)_2 \) (Fig. 1). As kinase \( E \) is unbound from it, the first or the second site of the substrate is phosphorylated to produce \( S_1 \) or \( S_2 \). Kinase \( E \) can be bound either to the first phosphorylation site of \( S_2 \) or to the second phosphorylation site of \( S_1 \) to form an intermediate complex \( (ES)_12 \) or \( (ES)_21 \). Subsequently, kinase \( E \) is unbound from the complex to produce fully phosphorylated substrate \( S_{12} \). Note that our notation is organized such that the subscript to \( S \) indicates the site that has been phosphorylated, and the subscript to a parenthetical term indicates the site to which \( E \) is bound.

![Diagram of Two-Site Phosphorylation](image)

**Fig. 1.** Two-site phosphorylation. The simplest example of multisite phosphorylation. Substrate \( S \) has two phosphorylation sites with respect to kinase \( E \). The kinase binds to one of the two sites (---), then phosphorylates the site (----) at rates specified by \( k_i \) and \( k_{ij} \). Letter “\( P \)” indicates a phosphate molecule.

The entire reactions constituting the two-site phosphorylation are given as

\[
E + S \xrightarrow{k_{i0}} (ES)_1, \quad (ES)_1 \xrightarrow{k_1} E + S_1,
\]

\[
E + S \xrightarrow{k_{i2}} (ES)_2, \quad (ES)_2 \xrightarrow{k_2} E + S_2,
\]

\[
E + S_1 \xrightarrow{k_{12}} (ES)_12, \quad (ES)_12 \xrightarrow{k_{12}} E + S_{12},
\]

\[
E + S_2 \xrightarrow{k_{21}} (ES)_21, \quad (ES)_21 \xrightarrow{k_{21}} E + S_{12}.
\]  

(1)

The rate equations describing these reactions are as follows:

- \[
\frac{d[S]}{dt} = - (\lambda_{i01} + \lambda_{i02})[E][S] + \lambda_{i01}(ES)_1 + \lambda_{i02}(ES)_2,
\]

- \[
\frac{d[(ES)_1]}{dt} = \lambda_{i01}[E][S] - (\lambda_{20} + k_1)[(ES)_1],
\]

- \[
\frac{d[(ES)_2]}{dt} = \lambda_{i02}[E][S] - (\lambda_{20} + k_2)[(ES)_2],
\]

- \[
\frac{d[S_1]}{dt} = k_1[(ES)_1] + \lambda_{201}[(ES)_12] - \lambda_{1a}[E][S_1],
\]

- \[
\frac{d[S_2]}{dt} = k_2[(ES)_2] + \lambda_{201}[(ES)_21] - \lambda_{1b}[E][S_1],
\]

- \[
\frac{d[(ES)_12]}{dt} = \lambda_{1a}[E][S_1] - (\lambda_{a1} + k_{12})[(ES)_12],
\]

- \[
\frac{d[(ES)_21]}{dt} = \lambda_{2b}[E][S_2] - (\lambda_{a2} + k_{21})[(ES)_21].
\]  

(2)

The steady-state condition of the intermediate states, which is used to derive the conventional
Michaelis–Menten equation, now reads
\[ d[(ES)_j]/dt = d[(ES)_j]/dt = 0 \quad (i,j = 1,2). \] (3)

With newly defined constants, \( B_1 = z_0/(\gamma_{01} + k_i) \), \( B_{12} = \lambda_{1a}/(\lambda_{1a} + k_{12}) \), and \( B_{21} = \lambda_{2b}/(\lambda_{2b} + k_{21}) \), this condition is written as \([ES_i] = B_i[E][S]\) and \([ES_j] = B_j[E][S]\). These equations reduce eqn (2) to the following three equations:
\[ d[S]/dt = -(k_1B_1 + k_2B_2)[E][S], \] (4)
\[ d[S_1]/dt = [E](k_1B_1[S] - k_1B_{12}[S_1]), \] (5)
\[ d[S_2]/dt = [E](k_2B_2[S] - k_1B_{21}[S_2]). \] (6)

The r.h.s.’s of eqns (4)–(6) are written as functions of \([S], [S_1]\) and \([S_2]\) by expressing \([E]\) with these three concentrations:
\[ E_{tot} = [E] + [ES_1] + [ES_2] + [ES_12] \]
\[ = [E](1 + B_1[S] + B_2[S] + B_{12}[S_1] + B_{21}[S_2]), \] (7)

where \( E_{tot} \) is the total concentration of enzyme \( E \). Therefore, eqns (4)–(6) constitute a closed set of equations that determine the temporal evolutions of \([S], [S_1]\) and \([S_2]\).

We note here that setting \([S_2] = 0\) and \( k_2 = k_2B_2 = k_{12}B_{12} = k_{21}B_{21} = 0 \) in eqns (4)–(7) reduces them to the conventional Michaelis–Menten equation, \( d[S_1]/dt = E_{tot}k_1[S]/([S] + 1/B_1) \). We also note that the steady-state condition, that is, eqn (3), holds when the substrate concentration dominates the enzyme concentration as in the case of the conventional Michaelis–Menten formalism. Therefore, we need to ensure that the substrate dominance is satisfied when we design a biochemical experiment for examining cooperative phosphorylation by using the present method.

SOLUTIONS TO MICHAELIS–MENTEN EQUATIONS FOR MULTISITE PHOSPHORYLATION—THE TWO-SITE PHOSPHORYLATION

In the following, we integrate the Michaelis–Menten equations, eqns (4)–(6). Dividing eqns (5) and (6) by eqn (4) and using the formula \( (d[S_1]/dt)/(d[S]/dt) = d[S_1]/d[S] \), we obtain the following equations in which \([E]\) is eliminated:
\[ d[S_1]/d[S] = -(F_1[S] - f_1[S_1])/[S], \] (8)
\[ d[S_2]/d[S] = -(F_2[S] - f_2[S_2])/[S], \] (9)

where we set \( F_i = k_iB_i/h, f_1 = k_{12}B_{12}/h \) and \( f_2 = k_{21}B_{21}/h \) with \( h = k_1B_1 + k_2B_2 \).

Note that in eqns (8) and (9), two independent kinetic parameters, \( k \) and \( B \), always appear in combination \( kB \), and they always appear in dimensionless form \( F_i \) or \( f_i \).

On the other hand, using eqn (7), eqn (4) is transformed to
\[ \frac{d[S]}{dt} = -E_{tot}h[S]/(1 + (B_1 + B_2)[S] + B_{12}[S_1] + B_{21}[S_2]). \] (10)

Note that this equation involves \( B \)'s without \( k \)'s, unlike eqns (8) and (9). Thus, the Michaelis–Menten equations [eqns (4)–(6)] describing the two-site phosphorylation are now equivalently transformed to eqns (8)–(10).

Equations (8) and (9) do not involve a time variable, hence they determine mutual relations between concentrations \([S], [S_1]\) and \([S_2]\). We call them mutuality equations. The mutuality equations written as
\[ \left( -\frac{d}{d[S]} + f_i \right) [S_i] = F_i[S] \quad (i = 1,2) \] (11)
are transformed to a linear differential equation using the change of variable \( y = -\ln ([S]/S_{tot}) \), where \( S_{tot} \) is the total substrate concentration. Equation (11) is readily integrated to give
\[ [S_i] = \frac{F_i}{1 - f_i} (S_{tot}^{-1} - f_i[S] - [S]) \quad (i = 1,2). \] (12)

Equation (10) is transformed to
\[ -E_{tot}h[S][S_1]/d[S] = 1 + h'[S] + f'_1[S_1] + f'_2[S_2], \] (13)

where the newly defined parameters, \( f'_1 = B_{12}, f'_2 = B_{21} \) and \( h' = B_1 + B_2 \) are analogous to but different from \( f_1, f_2 \) and \( h \), respectively. Since the r.h.s. of eqn (13) is definitely positive, the equation determines a one-to-one correspondence between \( t \) (0 ≤ \( t < \infty \)) and \([S] \) (\( S_{tot} \geq [S] > 0 \)). After inserting eqn (12) into eqn (13), we
can integrate eqn (13) under the initial condition $[S] = S_{tot}$ at $t = 0$ and obtain the one-to-one correspondence explicitly:

$$
t = \frac{1}{E_{tot}} \left[ -\ln([S]/S_{tot}) + h'(S_{tot} - [S]) + \sum_{i=1}^{2} \frac{f_i^1}{1 - f_i} \left( \frac{1}{f_i} (S_{tot} - S_i - f_i[S]) - (S_{tot} - [S]) \right) \right].$$

(14)

Equation (12), together with eqn (14), provides analytic solutions to the Michaelis–Menten equations as a parametric representation of parameter $[S]$. By calculating the values of $t$, $[S_1]$, $[S_2]$, and $[S_{12}] = S_{tot} - [S_1] - [S_2]$ for each value of $[S]$ using eqns (12) and (14), we obtain Fig. 2 which shows how $[S_1]/S_{tot}$, $[S_2]/S_{tot}$, and $[S_{12}]/S_{tot}$ evolve with time. In Fig. 2, kinetic parameters are set such that site 1 is phosphorylated faster than site 2; therefore, $[S_1]$ increases more rapidly than $[S_2]$. However, the concentrations of singly phosphorylated substrates, $S_1$ and $S_2$, eventually begin to decrease because they are further phosphorylated. Each substrate is finally converted to $S_{12}$ under a phosphatase-free condition.

In eqn (12), $[S]$ was represented as a function of $[S]$ (mutual representation). However, $[S]$ can also be represented as a function of $t$ ($t$ representation) by first inverting eqn (14) to write $[S]$ as a Taylor expansion in $t$ and inserting it into eqn (12). We later show that each representation has its own advantages.

**Observable Quantity**

The temporal progress of multisite phosphorylation has been measured experimentally using $^{32}$P-autoradiography (Sellers et al., 1983) and $^{31}$P-NMR spectroscopy (Hirai et al., 2000). These methods measure the degree of phosphorylation on the basis of a local change occurring at each phosphorylation site. Therefore, $[S_{12}]$ and $[S_1]$ cannot be measured independently. They are measured inclusively. Therefore, the quantities to be measured in experiments are $O_1 = [S_1] + [S_{12}]$ and $O_2 = [S_2] + [S_{12}]$.

**Criterion for the Presence of Cooperativity—the Two-site Phosphorylation Case**

Two progress curves, $O_1(t)$ and $O_2(t)$, intersect each other in some cases [see Fig. 3(A)] but not...
in other cases [see Fig. 3(B)]. What causes this difference?

We determine the condition for the intersection using mutual representations of $O_1$ and $O_2$; that is, eqn (12) (Note that whether $O_1$ and $O_2$ intersect or not is independent of representation.)

If we assume the absence of cooperativity we have $k_i = k_{ji}$ and $B_i = B_{ji}$ (recall the definitions of $k_{ji}$ and $B_{ji}$ shown in Fig. 1). This leads to $f_i = 1 - F_i$, and eqn (12) is simplified to $[S] = S_{tot}^{1-F_i}[S]^{f_i} - [S]$. Therefore, we have

$$O_1(t([S])) - O_2(t([S])) = [S_1] - [S_2] = S_{tot}^{F_1}[S]^{1-F_1} - S_{tot}^{F_2}[S]^{1-F_2}$$

$$- S_{tot}^{F_i}[S]^{1-F_i} = S_{tot}^{F_2}[S]^{1-F_2}(1 - ([S]/S_{tot})^{F_2-F_i}).$$

Except in the case where the two curves are identical ($F_1 = F_2$), eqn (15) does not become
zero for $0 < [S] < S_{\text{tot}}$. This implies that the two progress curves $O_1(t)$ and $O_2(t)$ never intersect in the absence of cooperativity. In other words, if two progress curves intersect, cooperative interaction is present.

We note that the inverse proposition is not true. In a certain case, cooperative interaction is present without intersection of progress curves. In order to determine whether cooperativity is present or not in such a case, we need to quantify the difference $B_i - B_j$ by fitting analytic progress curves $O_i(t)$ to the experimentally obtained progress curves. Nevertheless, the simple criterion derived above will help to detect cooperativity in practical experimental situations.

**Solutions to Michaelis–Menten Equations for Multisite Phosphorylation—General Case**

Having considered the case of two-site phosphorylation, we now generalize the discussion to $N$-site phosphorylation. The Michaelis–Menten equations in this general case are written as follows:

$$\frac{d[S]}{dt} = -[E] \sum_n k_n B_n [S]. \quad (16)$$

$$\frac{d[S_i]}{dt} = [E] \left( k_i B_i [S] - \sum_{n \neq i} k_n B_n [S] \right),$$

$$\frac{d[S_{ij}]}{dt} = [E] \left( k_{ij} B_{ij} [S_i] + k_{ji} B_{ji} [S_j] - \sum_{n \neq i,j} k_{ijn} B_{ijn} [S_{ij}] \right),$$

$$\frac{d[S_{ijk}]}{dt} = [E] \left( k_{ijk} B_{ijk} [S_{ij}] + k_{kij} B_{kij} [S_{ik}] + k_{jik} B_{jik} [S_{jk}] - \sum_{n \neq i,j,k} k_{ijnk} B_{ijnk} [S_{ijk}] \right), \quad \ldots. \quad (17)$$

Here, $k_{ijk}$ and $B_{ijk}$ are, respectively, a phosphorylation rate constant and the inverse Michaelis–Menten constant with respect to the $k$-th phosphorylation site of $S_{ij}$ ($S_{ij}$ refers to the substrate whose sites $i$ and $j$ have been phosphorylated). Other parameters are defined in an analogous manner.

Dividing eqn (17) by eqn (16) removes the time variable and $[E]$, and provides the mutual representation in the same manner as in the previous case. Hence, we can solve this set of equations analytically again, see Appendix A.

The observable quantity is defined as the summation of concentrations of a substrate in any state whose site $i$ is phosphorylated,

$$O_i = [S] + \sum_{j \neq i} [S_{ij}] + \sum_{j < k \neq i} [S_{ijk}] + \cdots + [S_{12\ldots N}]. \quad (18)$$

When all the phosphorylation sites are independent and there is no cooperativity, expressions for $[S]$, $[S_{ij}]$ and so on are markedly simplified, and all but two terms in $O_1 - O_2$ are removed. We finally obtain

$$O_1 - O_2 = S_{\text{tot}}^f \sum_{i=1}^{N-1} [S_{tot}]^{f_{134\ldots N}} - S_{\text{tot}}^f \sum_{N=2}^{f_{234\ldots N}} [S_{tot}]^{f_{234\ldots N}}$$

$$= SF_{\text{tot}}^f [S]^1 - SF_{\text{tot}}^f [S]^N$$

$$= SF_{\text{tot}}^f [S]^1 - SF_{\text{tot}}^f (1 - ([S]/[S_{\text{tot}}])^{F_2 - F_1}),$$

which does not become zero for $0 < [S] < [S]_{\text{tot}}$. Therefore, the two progress curves $O_1(t)$ and $O_2(t)$ never intersect. We have thereby proved the criterion that if any two progress curves intersect, cooperativity is present in general cases. Figure 4 shows theoretical progress curves thus obtained, with the absence and presence of cooperativity in the case of four-site phosphorylation.

**Determination of Kinetic Parameters by Fitting of Progress Curves**

By fitting analytic progress curves to experimentally obtainable progress curves, we can
efficiently determine the values of kinetic parameters of a reaction. Here, we demonstrate the actual fitting procedure using noisy progress curves data obtained by adding Gaussian noise to analytically calculated progress curves.

**Two Representations of Progress Curves**

We use the mutual representation for the fitting because of the following two advantages. First, mutual representation is mathematically simpler than \( t \) representation that involves the
Taylor expansion in $t$. Second, the mutual representation involves less redundant parameters. As we mentioned immediately after the derivation of eqns (8)–(10), the mutuality equations involve kinetic parameters only in combination $kB$. In contrast, $t$ representation additionally involves parameter $B$. When the value of $B$ changes due to cooperativity, the value of $kB$ is also expected to change. This is because it is unnatural to assume that $kB$ remains unchanged because the change of $k$ exactly compensates for the change of $B$.

For this reason, monitoring only one of $kB$ and $B$ is sufficient to detect the cooperativity. We note, however, that $t$ representation becomes necessary when we address a more specific question, such as which of $k$ and $B$ mainly changes its value upon a cooperative interaction.

**Actual Fitting**

Here, we demonstrate the actual fitting procedure of progress curves. For convenience in numerical analysis, we regard observable quantities $O_1, O_2, \ldots, O_N$ as functions of $y = -\ln([S]/S_{tot})$ instead of $[S]$ itself. We assume that sets of values for $(y; O_1, O_2, \ldots, O_N)$ are obtained at $K$ different points in time to give $(y_k^{obs}, O_1^{obs}, O_2^{obs}, \ldots, O_N^{obs}); \ k = 1, 2, \ldots, K$. Upon fitting of the theoretical progress curves to experimental data, the best-fit values for $F_i, F_{ij}, F_{ijk}, \ldots$, are determined by determining the minimum of squared error $\chi^2$ defined by

$$\chi^2 = \chi^2(F_i, F_{ij}, \ldots) = \sum_{i=1}^{N} \sum_{k=1}^{K} (O_i(y_k^{obs}; F_i, F_{ij}, \ldots) - O_{ik}^{obs})^2 / \sigma_{ik}^2,$$

where $\sigma_{ik}$ is the expected error of $O_{ik}^{obs}$. Since we know $\chi^2$ to be a function of $F_i, F_{ij}, F_{ijk}, \ldots$, analytically, we use derivatives of $\chi^2$ with respect to $F_i, F_{ij}, F_{ijk}, \ldots$ to determine the minimum of $\chi^2$ effectively. Figure 5 shows the fitting to the noisy progress curves in the three-site phosphorylation.

Table 1 lists predicted values of kinetic parameters for the best-fit curves shown in Fig. 5. The predicted values fall in 95.4% ($\sigma = 2$) confidence intervals around the

**Fig. 5.** Fitting the theoretical progress curves to noisy progress curves. Under the condition that site-3 phosphorylation is enhanced 15-fold due to site-1 phosphorylation, analytic progress curves expressed as a function of $y = -\ln([S]/S_{tot})$ were calculated. Gaussian noise with $\sigma = 4\%$ was added to each data point to obtain noisy progress curves for site 1 ($\bigcirc$), site 2 ($\blacksquare$) and site 3 ($\triangle$), which simulate experimental data. We pretended to be ignorant of the true values for $F_i, F_{ij}$ and $F_{ijk}$, and attempted to predict the values by the nonlinear regression described in Appendix B. Thus the obtained best-fit curves (---) are superimposed on the noisy progress curves. Predicted values of parameters are listed in Table 1.
corresponding true values, demonstrating that this method works well in practice.

**Discussion**

**RELATION TO PREVIOUS STUDIES**

Analytic progress curves have so far been obtained for various reaction schemes. Schönhheyder (1952) and Duggleby & Morrison (1977) obtained analytic progress curves under the condition that a reaction product functions as an inhibitor of an enzyme. Alberty & Koerber (1957) and Walter (1963) obtained analytic progress curves for a catalytic reaction when it cannot be regarded as a one-way reaction. Walter (1963), Darvey & Williams (1964), Duggleby & Morrison (1977), Boeker (1984, 1985) and Duggleby & Wood (1989) obtained analytic progress curves when more than one substrate binds to an enzyme. Orsi & Tipton (1979) obtained analytic progress curves when an inhibitor is present and its concentration is much higher than the enzyme concentration, while Szedlacsek et al. (1990) obtained analytic progress curves when the two concentrations are comparable. Duggleby (1986) obtained an analytic progress curve when an enzyme gradually becomes inactive. Tsou (1988), Topham (1990) and Wang & Zhao (1997) also obtained analytic progress curves of reactions involving an enzyme being inactivated and an inhibitor or activator. Di Cera et al. (1996) obtained an analytic progress curve of an allosteric catalytic reaction. For more exhaustive citations of previous studies, refer to Duggleby (1995).

All the reaction schemes reported in these papers share the common feature that no catalytic reaction is subsequent to other catalytic reactions. For instance, in a reaction scheme with an allosteric enzyme as shown in Fig. 6, none of the three catalytic reactions (dotted lines) are subsequent to other catalytic reactions. We call such reaction schemes a parallel scheme. In contrast, multisite phosphorylation analysed in the present study is non-parallel because the catalytic reaction $ES_1 \rightarrow E + S_{12}$ was subsequent to the catalytic reaction $ES \rightarrow E + S_1$ (Fig. 1). Sequential degradation of a protein by a protease gives another example of a non-parallel scheme (Fig. 7). We believe that the significance of the progress curve analysis is even greater in a non-parallel scheme than in a parallel scheme for the following reason. In a non-parallel scheme, information on catalytic reactions subsequent to another catalytic reaction (e.g. $ES_1 \rightarrow E + S_{12}$) is

<table>
<thead>
<tr>
<th>Parameter values determined by the best fit of progress curves*</th>
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<tbody>
<tr>
<td>$F_1$</td>
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<tr>
<td>True values</td>
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<tr>
<td>Predicted values</td>
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*The best-fit parameter values were determined by the procedure described in the legend to Fig. 5. Due to the imposed condition, only the parameters appearing in Table 1 (except $F_3 = 1 - F_1 - F_2$) are independent.
absent in the initial reaction rate. Therefore, we must use progress curve analysis to examine all the catalytic reactions in a scheme. Meanwhile, in a parallel scheme such as that shown in Fig. 6, information on all the catalytic reactions is present in the initial reaction rate. Therefore, in principle, we can examine catalytic reactions by using the initial rate analysis although it may be more advantageous to use progress curve analysis under many other conditions.

Analytic progress curves have been obtained for various parallel schemes as described above. However, to our knowledge, no analytic progress curve has ever been obtained for any non-parallel schemes. This study, by providing the first example of analytic progress curves for a non-parallel scheme, paves the way for an investigation on widely diverse non-parallel schemes for which experimental data have been obtained (Dent et al., 1989; Girault et al., 1989; Wettenhall et al., 1991, 1992; Teleman et al., 1995; DePaoli-Roach et al., 1983).

COMPARISON OF COOPERATIVITY IN MULTISITE PHOSPHORYLATION AND AN ALLOSTERIC REACTION

Cooperativity in an allosteric enzyme (Segel, 1991) contrasts sharply with that in multisite phosphorylation. Comparison of Figs 6 and 1 clarifies that roles of an enzyme and substrate are interchanged. An enzyme molecule has multiple binding sites for substrate molecules in the former case, while a substrate molecule has multiple binding sites for enzyme molecules in the latter case. Although these two cases seem symmetric, they are not because substrate and enzyme concentrations are largely imbalanced ([S] ≫ [E]) in many physiological conditions. Under the substrate dominance, two or more substrate molecules can coexist on an enzyme molecule (Fig. 6) from the beginning of the reaction. Therefore, the cooperativity operating between two bound molecules can be quantified with the initial rate analysis called the Hill plot (Segel, 1991). On the other hand, in the multisite phosphorylation, not more than one enzyme molecule can bind to a substrate molecule at the beginning of the reaction. Enzyme molecules can only bind to a substrate molecule in turn. This induces a sequential catalytic reaction, and cooperativity operating in such a reaction can only be quantified by using the progress curve analysis.

ROBUSTNESS OF RESULTS AGAINST MODULATORY FACTORS

An advantage of the progress curve analysis based on the mutual representation introduced here is that it is robust against effects such as product inhibition and change in pH, which may sometimes unavoidably occur as a reaction progresses and may invalidate the use of the Michaelis–Menten equation (Duggleby, 1994).

The rate of the catalytic reaction, $E + S \leftrightarrow ES \rightarrow E + S^*$, is $V = kE_{tot}[S]/(1 + B[S] + B'[I])$ instead of $V = kE_{tot}[S]/(1 + B[S])$ if competitive inhibitor $I$ can bind to $E$ and inactivate it. This change in the reaction rate, $V = k[E]S = kB[E][S]$, results from the reduced availability of $E$, which is quantified from the conservation law, $E_{tot} = [E] + [ES] + [EI] = [E](1 + B[S] + B'[I])$ (Stryer, 1995). From this argument, we find that the competitive inhibitor modifies $t$ representation of progress curves through $[E]$, but it does not modify mutual representation of progress curves because $[E]$ is eliminated to derive mutual representation. For uncompetitive, noncompetitive or mixed inhibitor, another term $[ESI]$ is
Fig. 8. Reaction scheme that explains the pH dependence of a catalytic reaction. A catalytic reaction proceeds when an enzyme molecule binds to one but not to two hydrogen ions.

added to the conservation law (Stryer, 1995). However, since the corresponding modification is limited to \([E]\), mutual representation is again unaffected. Similar argument applies to the effect of pH. The presence of the optimal pH in an enzymatic reaction has been explained by the reaction scheme in Fig. 8 (Voet & Voet, 1990),

\[
\frac{-d[S]}{dt} = h[EH^+]\cdot [S]/K_{\text{M}} + [S] + k_{12}[H^+] + k_{12}[H^+] + [S]
\]

This factor results in the bell-shaped dependence of the reaction rate on pH. Therefore, the Michaelis–Menten equations for the two-site phosphorylation with the pH effect incorporated are written as

\[
\frac{d[S]}{dt} = -(k_{12}B_{12} + k_{21}B_{21})[EH^+]\cdot [S],
\]

\[
\frac{d[S]}{dt} = [EH^+]\cdot [k_{12}B_{12}B_{12}[S] - k_{12}B_{12}B_{12}[S]],
\]

\[
\frac{d[S]}{dt} = [EH^+]\cdot [k_{21}B_{21}[S] - k_{21}B_{21}[S]]
\]

If we divide the last two equations by the first equation, \([EH^+]\) is eliminated. Hence, a progress curve in the mutual representation is independent of pH. Similarly, we can also show that the progress curves in mutual representation are unchanged even when the enzyme is inactivated.

The key properties leading to the independence of mutual representation of various modulatory factors are as follows: (1) the temporal changes of substrate concentrations are always a linear combination of the concentrations of complexes containing enzyme \(E\) and \([E]\), which dictates modulatory effects, is factored out under the steady-state condition and is eliminated to derive mutual representation. In this way, progress curve analysis based on mutual representation is robust against factors that would emerge and affect the reaction.

**HOW TO MEASURE \([S]\)**

Although we used \([S]\) as an independent variable in the fitting procedure, \([S]\) cannot be directly measured since \([S] = S_{\text{tot}} - ([S]_{\text{in}} + O_2)\) is not an observable quantity. However, we can show a practical indirect method for measuring \([S]\) as follows.

Let us assume that we have protein \(U\) that has a single phosphorylation site for kinase \(E\). In fact, for most kinases of interest, such a simple substrate protein is commercially available for the purpose of testing kinase activity. We consider what happens when we add \(U\) into the reaction mixture in which the multisite phosphorylation of \(S\) by \(E\) is in progress. Since \(U\) is phosphorylated by the simple reaction scheme \(E + U \leftrightarrow EU \rightarrow k'E + U*\), the unphosphorylated form of \(U\) decreases according to the simple Michaelis–Menten equation,

\[
\frac{d[U]}{dt} = -k'[EU] = -k'B'[E][U]
\]

Here, \(k'\) and \(B'\) are a rate constant and an inverse Michaelis–Menten constant, respectively. Unlike \(S\), \(U\) is either in one of the two states, phosphorylated or not. Therefore, \([U]\) is an observable quantity.

Addition of \(U\) changes the expression of \([E]\) in terms of \([S]\), \([S_i]\) and \([S_j]\) [see eqn (7)] because the conservation law now includes a new term \([EU]\). This change, however, does not modify the mutuality equations because \([E]\) is eliminated upon its derivation. Therefore, the progress curves of multisite phosphorylation, if expressed in \([S]\), remain unchanged even after the addition of \(U\).

If we divide eqn (20) by eqn (4), we obtain \(d[S]/d[U] = h[S]/(k'B'[U]).\) This is solved to give \(U/[U]_{\text{tot}} = ([S]/[S]_{\text{tot}})\) with \(\lambda = k'B'/h\), which relates \([S]\) to an observable quantity \([U]\), enabling indirect measurement of \([S]\).
In this paper, we have only considered multisite phosphorylation catalysed by a single kinase. However, some functionally important proteins are catalysed by two or more enzymes (Roach, 1991). If different enzymes act almost independently, our formulation is still applicable. However, if the actions of the enzymes interfere significantly with each other, the key step of eliminating \([E]\) to derive the mutualty equations fails. An alternative approach to such cases is now being explored.

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REFERENCES


APPENDIX A

Here we explain that a set of Michaelis–Menten equations, eqns (16) and (17), which describes multisite phosphorylation with an arbitrary number of phosphorylation sites can be solved analytically in the same manner as in the case of the two-site phosphorylation.

If we divide eqn (17) by eqn (16), we obtain mutuality equations:

\[
\begin{align*}
\left[-[S]\frac{d}{ds} + f_i\right][S_i] &= F_i[S], \\
\left[-[S]\frac{d}{ds} + f_{ij}\right][S_{ij}] &= F_{ij}[S_i] + F_{ji}[S_j], \\
\left[-[S]\frac{d}{ds} + f_{ijk}\right][S_{ijk}] &= F_{ijk}[S_{ij}] + F_{jik}[S_{jk}] + F_{kij}[S_{ki}], \\
&
\end{align*}
\]

\(\text{(A.1)}\)

The conservation law of the enzyme determines \([E]\) as a function of substrate concentrations:

\[
[E] = E_i/\left(1 + \sum_n B_n[S] + \sum_{i \neq i} B_{in}[S_i] + \cdots\right).
\]

\(\text{(A.2)}\)

Combining eqns (16) and eqn (A.2), we obtain the equation relating \(t\) and \([S]\) as

\[
\frac{dt}{ds} = 1 + h'[S] + \sum_i f'_i[S_i] + \sum_{i < j} f'_{ij}[S_{ij}] + \cdots + \sum_{i_1 < \cdots < i_N-1} f'_{i_1\cdots i_N-1}[S_{i_1\cdots i_N-1}].
\]

\(\text{(A.3)}\)

Here, the newly introduced notations are defined as follows:

\[
\begin{align*}
h &= \sum_{n=1}^N k_n B_n, \\
h' &= \sum_{n=1}^N B_n, \\
F_i &= k_i B_i/h, \\
F_{ij} &= k_{ij} B_{ij}/h, \\
f_i &= \sum_{n(\neq i)} k_{in} B_{in}/h, \\
f'_{ij} &= \sum_{n(\neq i)} B_{in}, \\
F_{ijk} &= k_{ijk} B_{ijk}/h, \\
f_{ij} &= \sum_{m(\neq i,j)} k_{ijn} B_{ijn}/h, \\
f'_{ij} &= \sum_{m(\neq i,j)} B_{ijn}, \\
&
\end{align*}
\]

\(\text{(A.4)}\)

In our notation, \(S_{12}\) denotes the substrate phosphorylated at sites 1 and 2 in this order, while \(S_{21}\) denotes the substrate phosphorylated in the reverse order. It is theoretically possible that the conformation of \(S_{12}\) differs from that of \(S_{21}\). However, since there are not many known examples of this case, we restrict ourselves to the case of \(S_{12} = S_{21}\) in this study. Therefore, \(B_{ijk} = B_{jik}\) and \(k_{ijk} = k_{jik}\). Constants with more subscripts are understood to have similar symmetry. This is expressed as \(F_{ijk} = F_{jik}, f_{ij} = f_{ji}, g_{ij} = g_{ji}\), and so on. As in the case of two-site phosphorylation, the parameters appearing in the mutuality equations include \(k\) and \(B\) only in combination \(kB\), while eqn (A.3) include...
B without k. The mutuality equation, eqn (A.1), can be integrated with the initial condition, [S] = [S_{ij}] = \cdots = 0 at [S] = S_{tot}, by linearizing them by changing variable \( y = -\ln([S]/S_{tot}) \). Solutions are written using function \( \alpha(a, b; x) = (x^b - x^a)/(a - b) \) as follows:

\[
[S_i] = S_{tot} F_i \alpha(1, f_i; [S]/S_{tot}),
\]

\[
[S_{ij}] = S_{tot} F_i F_j (\alpha(f_i, f_{ij}; [S]/S_{tot}) - \alpha(1, f_{ij}; [S]/S_{tot})) + (p.t.),
\]

\[
[S_{ijk}] = S_{tot} F_i F_j F_k (\alpha(f_i, f_{ijk}; [S]/S_{tot}) + \alpha(f_i, f_{ijk}; [S]/S_{tot}) + \alpha(1, f_{ijk}; [S]/S_{tot})) + (p.t.),
\]

\[
[S_{12\cdots n}] = S_{tot} F_1 \cdots F_{1\cdots n-1} + \alpha(1, f_{1\cdots n}; [S]/S_{tot}) + (p.t.),
\]

\[
(S_{12\cdots n}) + \alpha(1, f_{1\cdots n}; [S]/S_{tot}) = \frac{\alpha(1, f_{1\cdots n-1} - f_{1\cdots n-2}, f_{1\cdots n-2}; [S]/S_{tot})}{(f_{1\cdots n-2} - f_{1\cdots n-1}) \cdots (f_1 - f_{1\cdots n-1}) (1 - f_{1\cdots n-1})} \sum_{k=1}^{n} \alpha(f_{1\cdots n-1} - f_i) \cdots (f_{12} - f_i) (1 - f_i)
\]

The notation \((p.t.)\) indicates the addition of all the possible permutations of the first term with respect to its subscripts.

Thus, the concentration of each phosphorylated substrate is written as a linear summation of powers of [S]. Inserting eqn (A.5) into eqn (A.3) and solving eqn (A.3), we obtain the one-to-one relation \( t = t([S]) \) explicitly. In this way, the Michaelis–Menten equations for general multisite phosphorylation were integrated.

**APPENDIX B**

Here we explain how to obtain the best fit curves for the noisy progress curves in Fig. 5. With the analytic expression for \( O_i(\gamma_i^{obs}, F_i, F_{ij}, \cdots) \), we calculate derivatives \( \partial \chi^2(F_i, F_{ij}, \cdots)/\partial F_i, \partial \chi^2(F_i, F_{ij}, \cdots)/\partial F_{ij} \), and so on to determine the appropriate amount of change of each parameter that is necessary to reduce the squared error (steepest descent method). We perform this calculation iteratively until \( \chi^2 \) is minimized. The Levenberg–Marquardt algorithm (Press et al., 1992) was used in the numerical calculation. In order to avoid numerical instability caused by too many free parameters, we adopted the following strategy. We performed the fitting under three possible conditions: (1) site 1 is the source of cooperativity, i.e. site 1, but not sites 2 or 3, causes changes in kinetic parameters, (2) site 2 is the source of cooperativity, and (3) site 3 is the source of cooperativity. Subsequently, we found the best among the three fittings. Under condition (3) the algorithm did not converge. Under conditions (1) and (2), the algorithm converged and \( \chi^2 \) was minimized to \( \chi^2 = 190 \) and 290, respectively. The fitting under condition (1) yielded the values listed in Table 1 (predicted values).