Theory of Allosteric Effects in Serine Proteases

Enrico Di Cera, Karl-Peter Hopfner, and Quoc D. Dang
Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110 USA

ABSTRACT The classical Botts-Morales theory for the action of a modifier on the catalytic properties of an enzyme has been extended to deal with allosteric effects in serine proteases. The exact analytical solution derived for the linkage scheme at steady state provides a rigorous framework for the study of many biologically relevant systems, including enzymes activated by monovalent cations and cofactor-controlled protease-zymogen interactions in blood coagulation. When the enzyme obeys Michaelis-Menten kinetics, the exact solution of the kinetic linkage scheme simplifies considerably. Of particular importance for practical applications is a simple equation expressing the dependence of the specificity constant of the enzyme, k_{cat}/K_m, on the concentration of the modifier, from which the equilibrium binding constant for the formation of the enzyme-modifier complex can be estimated. Analysis of the allosteric changes in thrombin activity induced by thrombomodulin and Na^+ in terms of this equation yields accurate determinations of the equilibrium binding constants for both effectors.

INTRODUCTION

In a landmark paper published more than 40 years ago, Botts and Morales derived an analytical solution for the steady-state properties of an enzyme influenced by the action of a modifier (Botts and Morales, 1953). They considered the following kinetic scheme:

$$\begin{array}{ccc} & {}^{0}k_1[S] \quad {}^{0}k_2 & \\ E \rightleftharpoons ES & \longrightarrow & E + P \\ & {}^{0}k_{-1} & \\ {}^{E}k_{-L} \uparrow\downarrow {}^{E}k_L[L] & & {}^{ES}k_{-L} \uparrow\downarrow {}^{ES}k_L[L] \\ & {}^{1}k_1[S] \quad {}^{1}k_2 & \\ EL \rightleftharpoons ELS & \longrightarrow & EL + P \\ & {}^{1}k_{-1} & \end{array} \qquad (1)$$

where E is the enzyme, S the substrate, L is the modifier or allosteric effector, and P is the product of the reaction. The various kinetic rate constants pertain to substrate binding and dissociation (k_1 and k_{-1}), effector binding and dissociation (k_L and k_{-L}), and catalytic conversion of the substrate (k_2). In the absence of effector, as well as under saturating conditions ([L] $\rightarrow \infty$), Scheme 1 simplifies into the familiar Michaelis-Menten scheme:

$$E \underset{k_{-1}}{\overset{k_1[S]}{\rightleftharpoons}} ES \xrightarrow{k_2} E+P \qquad (2)$$

for which the velocity of product formation at steady state is given by the expression

$$v = \frac{d[P]}{dt} = e_T \frac{k_{cat}[S]}{K_m + [S]} \qquad (3)$$

where e_T is the total concentration of active enzyme, whereas $k_{cat} = k_2$ and $K_m = (k_{-1} + k_2)/k_1$ are the Michaelis-Menten constants. The effect of the modifier can be assessed by comparison of the Michaelis-Menten constants in the absence and presence of saturating concentrations of L. However, this does not allow resolution of the kinetic rate constants pertaining to L in Scheme 1, which is often sought in experimental studies. This information is embodied by the expression for the velocity v for finite [L] as follows (Botts and Morales, 1953):

$$v = \frac{d[P]}{dt} = e_T \frac{\alpha[S] + \beta[S]^2}{1 + \gamma[S] + \delta[S]^2} \qquad (4)$$

where α, β, γ, and δ are rather elaborate functions of the kinetic rate constants in Scheme 1 and [L]. The presence of the modifier makes the velocity of product formation quadratic in [S]. Although the enzyme has a single active site, it is no longer expected to obey simple Michaelis-Menten kinetics. This does not imply cooperativity in the usual (equilibrium) sense. At steady state, the order of the polynomial expressions involving [S] is set by the number of enzyme-substrate intermediates rather than the number of substrate-binding sites (Botts and Morales, 1953; King and Altman, 1956; Hill, 1977). The simple Scheme 2 always leads to Michaelis-Menten kinetics (Eq. 3) because it contains only one enzyme-substrate complex. In general, the expression for v will contain polynomials of order N in [S], if there are N substrate-bound intermediates in the kinetic scheme. The same argument applies to the allosteric effector, L, whose concentration [L] enters the definition of the rate equations with an exponent that can be as high as the number of effector-bound intermediates in the kinetic scheme.

Botts and Morales derived the exact analytical solution for product formation in Scheme 1 in the general case and under special conditions where the enzyme obeys Michaelis-Menten kinetics. Scheme 1, however, depicts the simplest possible kinetic mechanism for substrate hydrolysis by

Received for publication 26 July 1995 and in final form 26 September 1995.

Address reprint requests to Dr. Enrico Di Cera, Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, 660 S. Euclid Ave., Box 8231, St. Louis, MO 63110-1093. Tel.: 314-362-4185; Fax: 314-362-7183; E-mail: enrico@caesar.wustl.edu.

0006-3495/96/01/174/08 $2.00

an enzyme and may not be realistic in practice. Serine proteases (Perona and Craik, 1995) provide a pertinent example where scheme 1 oversimplifies the kinetic mechanism of hydrolysis. Many of these enzymes are controlled specifically and allosterically by monovalent cations (Suelter, 1970; Orthner and Kosow, 1978; Steiner et al., 1980; Wells and Di Cera, 1992), or by specific cofactors, as seen in the blood coagulation cascade (Esmon, 1989; Mann et al., 1990; Beck, 1991). In all of these cases, a theoretical treatment of allosteric effects at steady state demands a necessary extension of the original Botts-Morales scheme. In this study we describe such an extension and apply the results to the analysis of thrombin interaction with its two physiologically important allosteric effectors, thrombomodulin and Na^+.

ANALYTICAL SOLUTION OF THE KINETIC LINKAGE SCHEME FOR SERINE PROTEASES

The simplest and most widely accepted formulation of the mechanism of hydrolysis of amide and ester bonds by serine proteases is

$$E \underset{k_{-1}}{\overset{k_1[S]}{\rightleftharpoons}} ES \xrightarrow{k_2} EP \xrightarrow{k_3} E + P' \qquad (5)$$

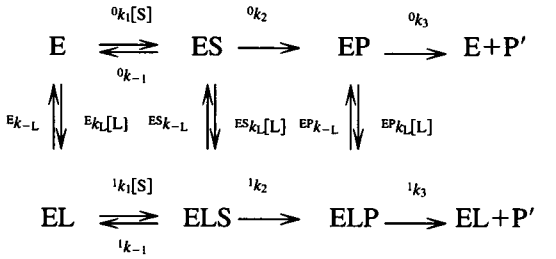
k_2 and k_3 are the acylation and deacylation rate constants, and k_1 and k_{-1} are the rate constants for binding and dissociation of the substrate, S. The deacylation rate constants also include the rate constant of product diffusing away from the active site, whichever is rate-limiting. The difference with Scheme 2 is in the addition of the deacylation step that follows the formation of the acyl intermediate. Acylation and deacylation are assumed to be irreversible processes, because the reverse reactions are extremely slow and can be neglected. The simpler Scheme 2 is recovered when k_3 is much faster than k_2. The notion that this condition applies in general to the hydrolysis of amide bonds by serine proteases (Fersht, 1985) is not supported by experimental data. In the case of thrombin, acylation or deacylation may be rate-limiting in the hydrolysis of amide bonds, depending on the allosteric state of the enzyme (Wells and Di Cera, 1992). The Michaelis-Menten parameters accessible to experimental measurements at steady state depend on the individual rate constants as follows:

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \qquad (6a)$$

$$\frac{k_{cat}}{K_m} = \frac{k_1 k_2}{k_{-1} + k_2} \qquad (6b)$$

The specificity constant, k_{cat}/K_m, is the same as for Scheme 2, and k_{cat} depends on both the acylation and deacylation rate constants.

In the presence of an allosteric effector, L, binding to a single site of the enzyme, Scheme 5 turns into the following more complex scheme:



(7)

The various kinetic rate constants pertaining to the substrate have the same meaning as in Scheme 5, with the suffix 0 or 1 denoting the ligation state relative to the effector. The other rate constants reflect effector binding to and dissociation from the various enzyme intermediates. Scheme 7 is an extension of the Botts-Morales Scheme 1, which is obtained as a special case by dropping the deacylation step.

To obtain the analytical solution for the velocity of product formation in Scheme 7, it is necessary to calculate all allowable fluxes in the scheme. This was done using the diagram method introduced by Hill (1977), which is a graphical implementation of the King-Altman algorithm (King and Altman, 1956). The solution depends on the probabilities of finding the intermediates EP and ELP at steady state, which are derived as the ratios of the fluxes populating these intermediates relative to all possible fluxes in the scheme. There are over 100 such fluxes, each of which is the product of five rate constants and [L] and [S] raised to a power equal to the number of effector-bound and substrate-bound intermediates involved in the particular flux pathway. The complexity of the analytical solution does not stem from the enumeration of these fluxes, which is done using matrix algebra on a computer, but rather from the resulting algebraic expressions that contain hundreds of terms. These terms can be grouped to yield the following expression for product formation:

$$v = \frac{d[P']}{dt} = e_T k_{cat} \frac{(\alpha + [S])[S]}{\beta + \gamma[S] + [S]^2} \qquad (8)$$

which is analogous to Eq. 4. The various parameters in Eq. 8 can be expressed as

$$\alpha = \frac{C_0 + C_1[L] + C_2[L]^2 + C_3[L]^3}{A_0 + A_1[L] + A_2[L]^2} \qquad (9a)$$

$$\beta = \frac{D_0 + D_1[L] + D_2[L]^2 + D_3[L]^3}{B_0 + B_1[L] + B_2[L]^2} \qquad (9b)$$

$$\gamma = \frac{E_0 + E_1[L] + E_2[L]^2 + E_3[L]^3}{B_0 + B_1[L] + B_2[L]^2} \qquad (9c)$$

and depend on the various rate constants in Scheme 7 and [L]. k_{cat} is given by the expression

$$k_{cat} = \frac{A_0 + A_1[L] + A_2[L]^2}{B_0 + B_1[L] + B_2[L]^2} \qquad (10)$$

As in the case of the Botts-Morales Scheme 1, the velocity of product formation in Eq. 8 is quadratic in [S].

The explicit expressions for the coefficients in Eqs. 9a–c were derived using the symbolic algebraic language Mathematica (Wolfram Research, Inc.), running on a Hewlett Packard Apollo9000/730. The kinetic scheme was mapped into a connected graph, and all possible fluxes were generated using the King-Altman algorithm from the properties of the connectivity matrix of the graph (Gould, 1988). Each flux generated in vectorial form was converted into its analytical form using the symbolic algebraic language. The resulting algebraic expressions were then simplified with Mathematica until they could be handled analytically to give the final results:

These results were scrupulously checked with Mathematica to match, term by term, the original solutions given in the expanded form. The consistency of the results was also checked in the limits $[L] \to 0$ and $[L] \to \infty$, in which Scheme 7 is expected to obey Michaelis-Menten kinetics with k_{cat} and K_m given by Eqs. 6a, b. Algebraic manipulations of Eqs. 8–15 in those limits confirm this expectation. The analytical expressions found earlier by Botts and Morales (1953) for the simpler Scheme 1 are obtained from the expressions above as a special case in the limit where the deacylation rate constants become much faster than the acylation rate constants.

$$A_0 = {}^0k_2{}^0k_3[{}^1k_2{}^{EP}k_{-L} + {}^{ES}k_{-L}({}^{EP}k_{-L} + {}^1k_3)] \tag{11a}$$

$$A_1 = {}^0k_3{}^1k_2{}^{ES}k_L({}^1k_3 + {}^{EP}k_{-L}) + {}^0k_2{}^1k_3{}^{EP}k_L({}^1k_2 + {}^{ES}k_{-L}) \tag{11b}$$

$$A_2 = {}^1k_2{}^1k_3{}^{ES}k_L{}^{EP}k_L \tag{11c}$$

$$B_0 = ({}^0k_2 + {}^0k_3)[{}^1k_2{}^{EP}k_{-L} + {}^{ES}k_{-L}({}^{EP}k_{-L} + {}^1k_3)] \tag{12a}$$

$$B_1 = ({}^0k_2{}^{EP}k_L + {}^0k_3{}^{ES}k_L)({}^1k_2 + {}^1k_3) + {}^{ES}k_{-L}{}^{EP}k_L({}^0k_2 + {}^1k_3) + {}^{ES}k_L{}^{EP}k_{-L}({}^1k_2 + {}^0k_3) \tag{12b}$$

$$B_2 = {}^{ES}k_L{}^{EP}k_L({}^1k_2 + {}^1k_3) \tag{12c}$$

$$C_0 = {}^0k_1{}^0k_2{}^0k_3{}^Ek_{-L}({}^{EP}k_{-L} + {}^1k_3)({}^{ES}k_{-L} + {}^1k_{-1} + {}^1k_2) \tag{13a}$$

$$\begin{aligned} C_1 = {}&{}^0k_2{}^0k_3{}^Ek_L{}^1k_1({}^{EP}k_{-L} + {}^1k_3)({}^{ES}k_{-L} + {}^1k_2) + {}^0k_1{}^0k_2{}^{EP}k_L{}^Ek_{-L}{}^1k_3({}^{ES}k_{-L} + {}^1k_{-1} + {}^1k_2) \\ &+ {}^0k_3{}^1k_2({}^0k_1{}^{ES}k_L{}^Ek_{-L} + {}^0k_{-1}{}^1k_1{}^Ek_L)({}^{EP}k_{-L} + {}^1k_3) \end{aligned} \tag{13b}$$

$$C_2 = {}^Ek_L{}^{ES}k_L{}^0k_3{}^1k_1{}^1k_2({}^{EP}k_{-L} + {}^1k_3) + {}^Ek_L{}^{EP}k_L{}^0k_2{}^1k_1{}^1k_3({}^{ES}k_{-L} + {}^1k_2) + {}^{EP}k_L{}^1k_2{}^1k_3({}^0k_1{}^{ES}k_L{}^Ek_{-L} + {}^0k_{-1}{}^1k_1{}^Ek_L) \tag{13c}$$

$$C_3 = {}^Ek_L{}^{ES}k_L{}^{EP}k_L{}^1k_1{}^1k_2{}^1k_3 \tag{13d}$$

$$D_0 = {}^0k_3{}^Ek_{-L}({}^0k_{-1} + {}^0k_2)({}^{EP}k_{-L} + {}^1k_3)({}^{ES}k_{-L} + {}^1k_{-1} + {}^1k_2) \tag{14a}$$

$$\begin{aligned} D_1 = {}&{}^0k_3{}^Ek_L({}^0k_{-1} + {}^0k_2)({}^{EP}k_{-L} + {}^1k_3)({}^{ES}k_{-L} + {}^1k_{-1} + {}^1k_2) \\ &+ {}^Ek_{-L}[{}^0k_3{}^{ES}k_L({}^1k_{-1} + {}^1k_2)({}^{EP}k_{-L} + {}^1k_3) + {}^1k_3{}^{EP}k_L({}^0k_{-1} + {}^0k_2)({}^{ES}k_{-L} + {}^1k_{-1} + {}^1k_2)] \end{aligned} \tag{14b}$$

$$D_2 = {}^0k_3{}^Ek_L{}^{EP}k_L({}^0k_{-1} + {}^0k_2)({}^{ES}k_{-L} + {}^1k_{-1} + {}^1k_2) + {}^{ES}k_L({}^1k_{-1} + {}^1k_2)[{}^0k_3{}^Ek_L({}^{EP}k_{-L} + {}^1k_3) + {}^1k_3{}^{EP}k_L{}^Ek_{-L}] \tag{14c}$$

$$D_3 = {}^1k_3{}^Ek_L{}^{ES}k_L{}^{EP}k_L({}^1k_{-1} + {}^1k_2) \tag{14d}$$

$$E_0 = {}^0k_1{}^Ek_{-L}({}^0k_2 + {}^0k_3)({}^{EP}k_{-L} + {}^1k_3)({}^{ES}k_{-L} + {}^1k_{-1} + {}^1k_2) + {}^0k_3{}^1k_1({}^0k_{-1} + {}^0k_2)[{}^{EP}k_{-L}({}^{ES}k_{-L} + {}^1k_2) + {}^1k_3{}^{ES}k_{-L}] \tag{15a}$$

$$\begin{aligned} E_1 = {}&{}^0k_1{}^0k_2{}^{EP}k_L({}^Ek_{-L} + {}^1k_3)({}^{ES}k_{-L} + {}^1k_{-1} + {}^1k_2) + {}^1k_1{}^0k_3{}^Ek_L({}^0k_{-1} + {}^0k_2)({}^{EP}k_{-1} + {}^1k_2 + {}^1k_3) \\ &+ {}^0k_1{}^0k_3{}^{ES}k_L[{}^Ek_{-L}({}^{EP}k_{-L} + {}^1k_2) + {}^1k_{-1}{}^{EP}k_{-L} + {}^1k_3({}^Ek_{-L} + {}^1k_{-1} + {}^1k_2)] \\ &+ {}^1k_1({}^0k_2 + {}^0k_3)[{}^Ek_L{}^{ES}k_{-L}({}^{EP}k_{-L} + {}^1k_3) + {}^1k_2{}^{EP}k_{-L}({}^Ek_L + {}^{ES}k_L)] \\ &+ {}^1k_3{}^{EP}k_L[{}^0k_{-1}{}^1k_1{}^{ES}k_{-L} + {}^0k_1{}^Ek_{-L}({}^1k_{-1} + {}^1k_2)] + {}^1k_2{}^{EP}k_{-L}({}^0k_1{}^Ek_{-L}{}^{ES}k_L + {}^0k_{-1}{}^1k_1{}^Ek_L) \end{aligned} \tag{15b}$$

$$\begin{aligned} E_2 = {}&({}^1k_2 + {}^1k_3)[{}^0k_1{}^{ES}k_L{}^{EP}k_L{}^Ek_{-L} + {}^1k_1{}^Ek_L({}^0k_2{}^{EP}k_L + {}^0k_3{}^{ES}k_L + {}^0k_1{}^{EP}k_L)] \\ &+ {}^1k_1{}^Ek_L[{}^{ES}k_{-L}{}^{EP}k_L({}^0k_2 + {}^1k_3) + {}^{ES}k_L{}^{EP}k_{-L}({}^0k_3 + {}^1k_2)] + {}^0k_1{}^1k_3{}^{ES}k_L{}^{EP}k_L({}^1k_{-1} + {}^1k_2) \end{aligned} \tag{15c}$$

$$E_3 = {}^1k_1{}^Ek_L{}^{ES}k_L{}^{EP}k_L({}^1k_2 + {}^1k_3) \tag{15d}$$

SPECIAL CONDITIONS FOR MICHAELIS-MENTEN KINETICS

It should be pointed out that not all rate constants in Scheme 7 are independent, because detailed balance demands

$$ {}^{0}k_{1}\,{}^{1}k_{-1}\,{}^{E}k_{-L}\,{}^{ES}k_{L} = {}^{0}k_{-1}\,{}^{1}k_{1}\,{}^{E}k_{L}\,{}^{ES}k_{-L} \tag{16} $$

Notwithstanding this constraint among the kinetic rate constants, the exact solution for the steady-state velocity of product formation in Scheme 7 remains of extraordinary complexity and is hopeless to apply in practice. The value of such a solution, however, stems from the possibility of deriving analytical expressions for the kinetic properties of the enzyme under any condition of interest. Of particular importance in practical applications is the condition where the enzyme obeys Michaelis-Menten kinetics, even in the presence of the allosteric effector. In this case, the definition of v only involves the first power of [S] (see Eq. 3). Scheme 7 obeys Michaelis-Menten kinetics in two nontrivial cases: 1) when binding and dissociation of the effector occur on a time scale much faster than any other process, or 2) when the rate constants obey a particular condition.

The first case is known as pseudo-equilibrium and is typically encountered in the study of enzymes activated by monovalent cations (Suelter, 1970). Formal treatments of these effects have appeared for thrombin (Wells and Di Cera, 1992) and activated protein C (Steiner and Castellino, 1985). In this case, the effector and the substrate interact with the enzyme with similar second-order rate constants, but with widely different equilibrium constants. Good substrates typically bind to enzymes in the micromolar range, whereas monovalent cations bind in the millimolar range. The first-order rate constant resulting from the product of the second-order rate constant and the concentration of the ligand is therefore three orders of magnitude faster for the effector. Likewise, the dissociation rate constant is much faster for the effector, because of the difference in binding affinity. This leaves the manifold pertaining to the interactions of the effector with the enzyme in a regime of pseudo-equilibrium (Hill, 1977). Hence, the probability of finding the enzyme in a given configuration depends solely on the equilibrium distribution of the species free and bound to the effector. Scheme 7 "contracts" into Scheme 5 and Eq. 8 simplifies into Eq. 3, where the rate constants pertaining to the substrate are averages over the various manifolds of enzyme intermediates in pseudo-equilibrium, so that (Wells and Di Cera, 1992)

$$ k_1 = \frac{{}^{0}k_1 + {}^{1}k_1 K_E[L]}{1 + K_E[L]} \tag{17a} $$

$$ k_{-1} = \frac{{}^{0}k_{-1} + {}^{1}k_{-1} K_{ES}[L]}{1 + K_{ES}[L]} \tag{17b} $$

$$ k_2 = \frac{{}^{0}k_2 + {}^{1}k_2 K_{ES}[L]}{1 + K_{ES}[L]} \tag{17c} $$

$$ k_3 = \frac{{}^{0}k_3 + {}^{1}k_3 K_{EP}[L]}{1 + K_{EP}[L]} \tag{17d} $$

$K_E = {}^{E}k_L/{}^{E}k_{-L}$, $K_{ES} = {}^{ES}k_L/{}^{ES}k_{-L}$, and $K_{EP} = {}^{EP}k_L/{}^{EP}k_{-L}$ are the equilibrium association constants for the effector binding to the E, ES, and EP intermediates. The relevant expressions for the Michaelis-Menten parameters are

$$ k_{cat} = \frac{k_2 k_3}{k_2 + k_3} = ({}^{0}k_2 + {}^{1}k_2 K_{ES}[L])({}^{0}k_3 + {}^{1}k_3 K_{EP}[L]) \div \{({}^{0}k_2 + {}^{1}k_2 K_{ES}[L])(1 + K_{EP}[L]) + ({}^{0}k_3 + {}^{1}k_3 K_{EP}[L])(1 + K_{ES}[L])\} \tag{18a} $$

$$ \frac{k_{cat}}{K_m} = \frac{k_1 k_2}{k_{-1} + k_2} = \frac{({}^{0}k_1 + {}^{1}k_1 K_E[L])({}^{0}k_2 + {}^{1}k_2 K_{ES}[L])}{[{}^{0}k_{-1} + {}^{0}k_2 + ({}^{1}k_{-1} + {}^{1}k_2)K_{ES}[L]](1 + K_E[L])} \tag{18b} $$

Although Eq. 18b contains terms quadratic in [L], it simplifies into an expression that is first order in [L] under many conditions of interest. When the catalytic steps are much slower than any other rate in Scheme 7, the substrate itself enters a regime of pseudo-equilibrium. In this case, the concentration of any intermediate in the kinetic linkage scheme can be derived from the equilibrium partition function of the system. The expression for k_{cat} remains unchanged, and the specificity constant in Eq. 18b becomes

$$ \frac{k_{cat}}{K_m} = \frac{k_1 k_2}{k_{-1}} = \frac{({}^{0}k_1 + {}^{1}k_1 K_E[L])({}^{0}k_2 + {}^{1}k_2 K_{ES}[L])}{({}^{0}k_{-1} + {}^{1}k_{-1} K_{ES}[L])(1 + K_E[L])} = {}^{0}K_S \frac{{}^{0}k_2 + {}^{1}k_2 K_{ES}[L]}{1 + K_E[L]} \tag{19} $$

where ${}^{0}K_S = {}^{0}k_1/{}^{0}k_{-1}$ is the equilibrium binding constant for the substrate. Equation 19 follows directly from the condition of detailed balance in Eq. 16. When the substrate dissociates from the enzyme with a rate constant much slower than acylation, Eq. 18b becomes

$$ \frac{k_{cat}}{K_m} = k_1 = \frac{{}^{0}k_1 + {}^{1}k_1 K_E[L]}{1 + K_E[L]} \tag{20} $$

When substrate dissociation and acylation occur on a comparable time scale then

$$ {}^{0}k_{-1} + {}^{0}k_2 + ({}^{1}k_{-1} + {}^{1}k_2)K_{ES}[L] \approx f({}^{0}k_{-1} + {}^{1}k_{-1} K_{ES}[L]) \approx g({}^{0}k_2 + {}^{1}k_2 K_{ES}[L]) \tag{21} $$

where f and g are constants. Hence,

$$ \frac{k_{cat}}{K_m} \approx \frac{1}{f}\frac{{}^{0}k_2 + {}^{1}k_2 K_{ES}[L]}{1 + K_E[L]} \approx \frac{1}{g}\frac{{}^{0}k_1 + {}^{1}k_1 K_E[L]}{1 + K_E[L]} \tag{22} $$

and again Eq. 18b simplifies into an expression that contains only first-order terms in [L] (see also Eq. 31, below). Only when ${}^{0}k_{-1} \gg {}^{1}k_{-1}K_{ES}[L]$ or ${}^{0}k_2 \gg {}^{1}k_2 K_{ES}[L]$ the foregoing simplifications do not hold and a plot of k_{cat}/K_m versus [L] is not hyperbolic. In all other cases, the pseudo-equilibrium regime for effector binding to the

enzyme leads to an expression that is first order in [L] with good approximation.

The second case leading to Michaelis-Menten kinetics is when the effector binds with comparable or higher affinity compared to the substrate, but the rate constants in Scheme 7 are constrained by special conditions. Mathematically, Eq. 8 turns into Eq. 3 when

$$\alpha(\alpha - \gamma) + \beta = 0 \tag{23}$$

Solution of Eq. 23 demands

$${}^0k_1\,{}^1k_3{}^{E}k_{-L}{}^{EP}k_L = {}^0k_3\,{}^1k_1{}^{E}k_L{}^{EP}k_{-L} \tag{24a}$$

$${}^0k_2\,{}^1k_3{}^{ES}k_{-L}{}^{EP}k_L = {}^0k_3\,{}^1k_2{}^{ES}k_L{}^{EP}k_{-L} \tag{24b}$$

These constraints, along with detailed balance in Eq. 16, give

$$\frac{{}^1k_{-1}}{{}^0k_{-1}}K_{ES} = \frac{{}^1k_1}{{}^0k_1}K_E = \frac{{}^1k_3}{{}^0k_3}K_{EP} = \frac{{}^1k_2}{{}^0k_2}K_{ES} \tag{25}$$

When Eq. 25 is obeyed for all of the terms involved, the resulting velocity of product formation follows Michaelis-Menten kinetics. It should be noted that Eq. 25 implies that

$$\frac{{}^1k_{-1}}{{}^0k_{-1}} = \frac{{}^1k_2}{{}^0k_2} \tag{26}$$

which is the condition for Michaelis-Menten kinetics found earlier by Botts and Morales (1953) for Scheme 1. Given the constraints in Eq. 25, the relevant expressions for the kinetic parameters are

$$k_{cat} = \frac{{}^0k_2 + {}^1k_2K_{ES}[L]}{{}^0k_2/{}^0k_{cat} + ({}^1k_2/{}^1k_{cat})K_{ES}[L]} \tag{27a}$$

$$\frac{k_{cat}}{K_m} = \frac{{}^0k_{cat}/{}^0K_m + ({}^1k_{cat}/{}^1K_m)K_E[L]}{1 + K_E[L]} \tag{27b}$$

where

$${}^jk_{cat} = \frac{{}^jk_2\,{}^jk_3}{{}^jk_2 + {}^jk_3} \tag{28a}$$

$$\frac{{}^jk_{cat}}{{}^jK_m} = \frac{{}^jk_1\,{}^jk_2}{{}^jk_{-1} + {}^jk_2} \tag{28b}$$

with $j = 0, 1$. Again, the specificity constant in Eq. 27b depends solely on the first power of [L]. It should be pointed out that although Eq. 27b does not depend on the deacylation rate constants in Scheme 7, its validity depends on Eq. 25, which puts a constraint on these constants. The fundamental difference between Scheme 7 and the simpler Botts-Morales Scheme 1 is that the condition for Michaelis-Menten kinetics imposes far more constraints in Scheme 7 (Eq. 25) than Scheme 1 (Eq. 26).

THE k_{cat}/K_m VERSUS [L] PLOT

In practical applications, the question often arises as to whether the binding constant K_E, reflecting the interaction of the effector with the enzyme, can be derived in a simple manner from the analysis of steady-state data. A remarkable consequence of the analysis shown in the previous section bears directly on this question. When the enzyme obeys Michaelis-Menten kinetics in the presence of the effector, information on the equilibrium binding constant K_E for the formation of the enzyme-effector complex can be derived from a plot of k_{cat}/K_m versus the effector concentration [L]. The expression to be used in practice is

$$\frac{k_{cat}}{K_m} = \frac{s_0 + s_1K_E[L]}{1 + K_E[L]} \tag{29}$$

where s_0 and s_1 are the limiting values of the specificity constant in the absence and under saturating concentrations of effector. Although not of general validity, Eq. 29 holds under a wide variety of conditions encompassing Eqs. 19, 20, 22, and 27b. These equations are in fact identical to Eq. 29 after simple transformations. If the plot of k_{cat}/K_m versus [L] is hyperbolic, as implied by Eq. 29, then the enzyme obeys Michaelis-Menten kinetics (This statement has the value of a conjecture until a rigorous mathematical proof is provided. When Eq. 29 holds, the enzyme obeys Michaelis-Menten kinetics. The reverse, however, is not necessarily true.) and K_E can be derived from the plot as the inverse of the concentration of effector where the value of k_{cat}/K_m is half-way between its asymptotic values s_0 and s_1.

It should be pointed out that Eq. 29 is, in general, a good approximation to Eq. 18b. In fact, using detailed balance Eq. 18b can be rewritten as

$$\begin{aligned}\frac{k_{cat}}{K_m} &= {}^0K_S\frac{({}^0k_{-1} + {}^1k_{-1}K_{ES}[L])({}^0k_2 + {}^1k_2K_{ES}[L])}{[{}^0k_{-1} + {}^0k_2 + ({}^1k_{-1} + {}^1k_2)K_{ES}[L]](1 + K_E[L])} \\ &= \frac{s_0 + s_1K_E[L]}{1 + K_E[L]}\frac{1 + ({}^1k_{-1}/{}^0k_{-1})K_{ES}[L]}{1 + [({}^1k_{-1} + {}^1k_2)/({}^0k_{-1} + {}^0k_2)]K_{ES}[L]}\end{aligned} \tag{30}$$

A Taylor expansion of the rational expression involving the dissociation rate constants leads to

$$\begin{aligned}\frac{k_{cat}}{K_m} = \frac{s_0 + s_1K_E[L]}{1 + K_E[L]}\Bigg\{1 + \left(\frac{{}^1k_{-1}/{}^0k_{-1} - {}^1k_2/{}^0k_2}{1 + ({}^0k_{-1}/{}^0k_2)}\right)K_{ES}[L] \\ \times \sum_{i=0}^{\infty}(-1)^i\left(\frac{{}^1k_{-1} + {}^1k_2}{{}^0k_{-1} + {}^0k_2}\right)^iK_{ES}^i[L]^i\Bigg\}\end{aligned} \tag{31}$$

The correction to Eq. 29 is proportional to the difference between two terms reflecting the relative effect of L on the rate constants for dissociation and acylation. This correction will be small in general if these rate constants are of comparable magnitude, which is usually the case, or are affected to the same extent by L. When the special condition 26 is obeyed, the correcting term in Eq. 31 vanishes.

APPLICATIONS

We now test the accuracy of Eq. 29 in two cases of biological relevance. Thrombin is an allosteric enzyme for which Scheme 7 describes many linked interactions pertaining to its function in the blood. Among these, the allosteric transitions induced by thrombomodulin and Na^+ are of particular importance and are considered below.

In the blood, thrombomodulin enhances the specificity of the anticoagulant slow form of thrombin (Wells and Di Cera, 1992; Dang et al., 1995) toward protein C, leading to the activation of a potent inhibitor of the prothrombinase complex responsible for the generation of thrombin from prothrombin (Esmon, 1989). The effect of thrombomodulin is allosteric and mediated by a site distinct from the active site (Mathews et al., 1994). Thrombomodulin also enhances the specificity of the slow form of thrombin toward small chromogenic substrates; the results are shown in Fig. 1. The hydrolysis of p Glu-Pro-Arg-*p*-nitroanilide (S2366) in the presence of this effector obeys Michaelis-Menten kinetics. It is unlikely that this results from fast equilibration of thrombomodulin with thrombin, because binding of S2366 to the enzyme occurs with a second-order rate constant in the range of 10^7 M^{-1} s^{-1}, and the concentration of this substrate in the assay is three orders of magnitude higher than that of the effector. Furthermore, the catalytic rate constants for substrate conversion are on the order of 100 s^{-1} and cannot be much slower than the rate constants pertaining to substrate or effector binding and dissociation. The condition in Eq. 25 is more likely to hold. (The validity of Eq. 25 is of course within experimental error, as is the condition for Michaelis-Menten kinetics. The range of validity of Eq. 25 as a function of experimental error in the steady-state determinations of substrate hydrolysis is difficult to quantify analytically.) Analysis of the data in Fig. 1 in terms of Eq. 29 yields a value of $1/K_E$ = 21 ± 5 nM, which is in satisfactory agreement with the value of 14 ± 2 nM determined independently from quite lengthy competition experiments of thrombomodulin and hirudin binding to thrombin (Dang et al., 1995). The use of Eq. 29 will simplify enormously the study of the energetics of thrombomodulin-thrombin interaction.

Binding of Na^+ to a site located more than 15 Å away from the catalytic triad (Di Cera et al., 1995) allosterically switches thrombin from the anticoagulant slow form to the procoagulant fast form, this effect being of central importance to the function of the enzyme in hemostasis (Dang et al., 1995). The switch also enhances the specificity of thrombin toward H-D-Phe-pipecolyl-Arg-*p*-nitroanilide (S2238), as shown by the data in Fig. 2. These data were taken from the original study of Wells

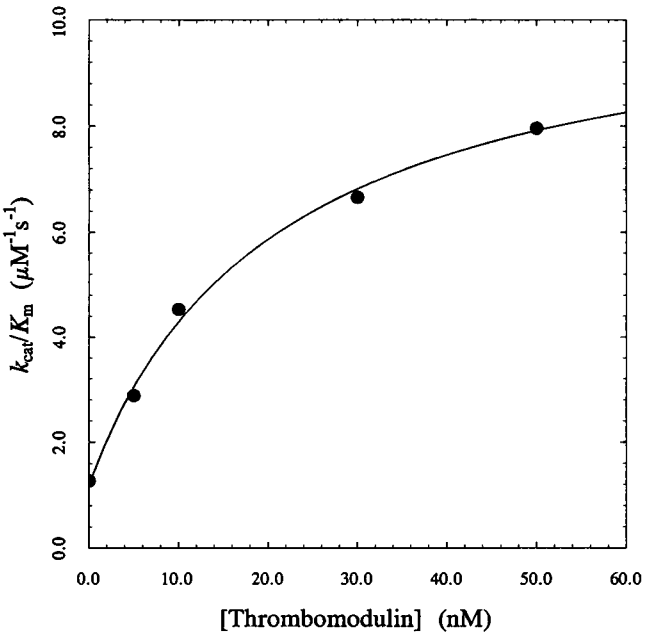


FIGURE 1 Effect of thrombomodulin on the specificity constant for the hydrolysis of S2366 by thrombin. Experimental conditions are: 500 pM human α-thrombin, 5 mM Tris(hydroxymethyl)aminomethane, 0.1% poly(ethylene)glycol, pH 8.0, 25°C, 0.2 M ChCl. The continuous line was drawn according to Eq. 29 in the text, with best-fit parameter values: s_0 = 1.2 ± 0.2 μM^{-1} s^{-1}, s_1 = 11 ± 1 μM^{-1} s^{-1}, $1/K_E$ = 21 ± 5 nM. Human α-thrombin was purified and tested for activity as described previously (Wells and Di Cera, 1992; Dang et al., 1995). Rabbit lung thrombomodulin of the highest purity was purchased from Hematologic Technologies (Essex Junction, VT). The chromogenic substrate S2366 was purchased from Chromogenix (Molndal, Sweden). The specificity constant for the hydrolysis of S2366 was determined from analysis of progress curves as described previously (Dang et al., 1995).

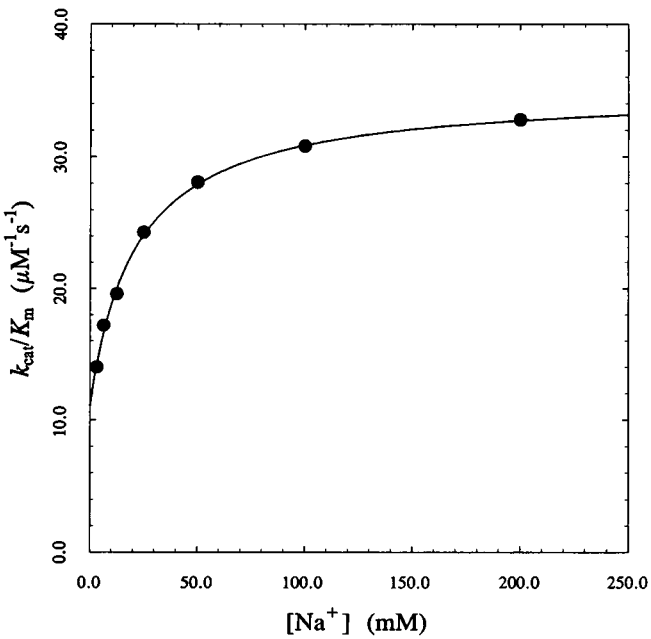


FIGURE 2 Effect of Na^+ on the specificity constant for the hydrolysis of S2238 by thrombin. Experimental conditions are: 3 nM human α-thrombin, 5 mM Tris(hydroxymethyl)aminomethane, 0.1% poly(ethylene)glycol, pH 8.0, 25°C, I = 0.2 M kept constant with ChCl. The continuous line was drawn according to Eq. 29 in the text, with best-fit parameter values: s_0 = 11.1 ± 0.6 μM^{-1} s^{-1}, s_1 = 35 ± 1 μM^{-1} s^{-1}, $1/K_E$ = 21 ± 2 mM. The chromogenic substrate S2238 was purchased from Chromogenix (Molndal, Sweden). The data were taken from Wells and Di Cera (1992).

and Di Cera (1992), where the slow and fast forms of thrombin were first discovered. In that study, the equilibrium binding constant of Na^+ was determined from extensive analysis of hundreds of steady-state determinations collected as a function of [Na^+] and [sucrose] using the viscogenic method, and also from direct equilibrium titrations of the intrinsic fluorescence of the enzyme. The hydrolysis of S2238 always obeys Michaelis-Menten kinetics as a result of the fast equilibration of Na^+ with thrombin. Analysis of the data in Fig. 2 in terms of Eq. 29 yields a value of $1/K_E = 21 \pm 2$ mM, which is in excellent agreement with the value of 22 ± 2 derived previously (Wells and Di Cera, 1992). Also in this case, use of Eq. 29 simplifies the study of the energetics of the interaction of thrombin with a physiologically important allosteric effector.

CONCLUSION

The exact solution of the linkage scheme for allosteric effects in serine proteases at steady state provides a much-needed framework for the analysis of effects involving enzymes that are activated by monovalent cations (Suelter, 1970) and are active in blood coagulation (Mann et al., 1990). A rigorous determination of the parameters involved in Scheme 7 demands measurements of the kinetic rate constants pertaining to the substrate in the absence and under saturating concentrations of the effector, followed by application of Eqs. 8–15 in the analysis of data collected as a function of [L]. This may represent a formidable, if not impossible task if the enzyme does not obey Michaelis-Menten kinetics. When the condition for Michaelis-Menten kinetics holds, Eq. 29 provides a simple and accurate expression for deriving the binding constant of the effector from measurements of the specificity constant of the enzyme. Because these measurements are relatively easy to carry out, Eq. 29 will come in quite handy in practical applications. We have tested and proved the validity of this equation with the analysis of two paradigmatic interactions that have been studied in great detail by independent and quite elaborate procedures (Wells and Di Cera, 1992; Dang et al., 1995). The study of these interactions is now greatly simplified by the analysis based on Eq. 29 introduced here.

The treatment based on Eq. 29 presented in this study represents a significant improvement over the treatment of allosteric effects at steady state based on the pseudo-equilibrium approximation (Segel, 1975). Because the simple Eq. 29 holds even in the absence of pseudo-equilibrium, the often unrealistic assumptions invoked under the pseudo-equilibrium approximation are no longer required if the enzyme obeys Michaelis-Menten kinetics. Although this approximation is valid in some cases (e.g., effect of monovalent cations on enzyme activity), there is no reason to impose it a priori whenever the enzyme obeys Michaelis-Menten kinetics, nor is there a practical need to invoke it when Eq. 29 applies. The pseudo-equilibrium approximation has been invoked in myriad cases without justification. For example, in the study of blood clotting factors, the approximation has been used in the analysis of the interaction of factor Xa with factor Va, leading to enhancement of the cleavage of prothrombin (Morrison, 1983; Krishnaswamy et al., 1987), as well as in the analysis of the thrombomodulin-induced enhancement of the cleavage of protein C by thrombin (Le Bonniec et al., 1992). The magnitude of the specificity constant for the catalytic conversion of protein C by thrombin (Dang et al., 1995) indicates that binding of this substrate may be diffusion-controlled and occurs on a time scale that is at least as fast as that for binding of thrombomodulin. The origin of Michaelis-Menten kinetics in the hydrolysis of protein C by thrombin in the presence of thrombomodulin is therefore due to the validity of Eq. 25 and not to pseudo-equilibrium. This may well be the case for many other allosteric enzymes obeying Michaelis-Menten kinetics.

This work was supported in part by National Institutes of Health Research grant HL49413, NSF Research grant MCB94-06103, and by grants from the American Heart Association and the Monsanto-Searle Company. E. D. C. is an Established Investigator of the American Heart Association and Genentech.

REFERENCES

Beck, W. S. 1991. Hematology. MIT Press, Cambridge, MA.

Botts, J., and M. Morales. 1953. Analytical description of the effects of modifiers and of multivalency upon the steady state catalyzed reaction rate. *Trans. Faraday Soc.* 49:696–707.

Dang, Q. D., A. Vindigni, and E. Di Cera. 1995. An allosteric switch controls the pro-coagulant and anti-coagulant activities of thrombin. *Proc. Natl. Acad. Sci. USA.* 92:5977–5981.

Di Cera, E., E. R. Guinto, A. Vindigni, Q. D. Dang, Y. Ayala, M. Wuyi, and A. Tulinsky. 1995. The Na^+ binding site of thrombin. *J. Biol. Chem.* 270:22089–22092.

Esmon, C. T. 1989. The roles of protein C and thrombomodulin in the regulation of blood coagulation. *J. Biol. Chem.* 264:4743–4746.

Fersht, A. R. 1985. Enzyme Structure and Mechanism. Freeman and Co., New York.

Gould, R. 1988. Graph Theory. Benjamin/Cummings Publishing Co., Menlo Park, CA.

Hill, T. L. 1977. Free Energy Transduction in Biology. Academic Press, New York.

King, E. L., and C. Altman. 1956. A schematic method of deriving the rate laws for enzyme-catalyzed reactions. *J. Phys. Chem.* 50:1375–1378.

Krishnaswamy, S., W. R. Church, M. E. Nesheim, and K. G. Mann. 1987. Activation of human prothrombin by human prothrombinase. *J. Biol. Chem.* 252:3291–3299.

Le Bonniec, B. F., E. R. Guinto, and C. T. Esmon. 1992. Interaction of des-ETW with antithrombin III, the Kunitz inhibitors, thrombomodulin and protein C. *J. Biol. Chem.* 267:19341–19348.

Mann, K. G., M. E. Nesheim, W. R. Church, P. Haley, and S. Krishnaswamy. 1990. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood.* 76:1–16.

Mathews, I. I., K. P. Padmanabhan, A. Tulinsky, and J. E. Sadler. 1994. Structure of a nonadecapeptide of the fifth EGF domain of thrombomodulin complexed with thrombin. *Biochemistry.* 33:13547–13552.

Morrison, S. A. 1983. Kinetics of activation of human prothrombin. Use of a fluorescein-labeled derivative to obtain kinetic constants as a function of factor V concentration and activation state. *Biochemistry.* 22: 4053–4061.

Orthner, C. L., and D. P. Kosow. 1978. The effect of metal ions on the amidolytic activity of human factor Xa (activated Stuart-Prower factor). *Arch. Biochem. Biophys.* 185:400–406.

Perona, J. J., and C. S. Craik. 1995. Structural basis of substrate specificity in the serine proteases. *Protein Sci.* 4:337–360.

Segel, I. H. 1975. Enzyme Kinetics. John Wiley and Sons, New York.

Steiner, S. A., G. N. Amphlett, and F. J. Castellino. 1980. Stimulation of the amidase and esterase activity of activated bovine plasma protein C by monovalent cations. *Biochem. Biophys. Res. Commun.* 94:340–347.

Steiner, S. A., and F. J. Castellino. 1985. Kinetic mechanism for stimulation by monovalent cations of the amidase activity of the plasma protease bovine activated protein C. *Biochemistry.* 24:609–617.

Suelter, C. H. 1970. Enzymes activated by monovalent cations. *Science.* 168:789–795.

Wells, C. M., and E. Di Cera. 1992. Thrombin is a Na^+-activated enzyme. *Biochemistry.* 31:11721–11730.