Robson, B., & Pain, R. (1976) *Biochem. J. 155*, 331-344.
Samuni, A. (1975) *Anal. Biochem. 63*, 17-26.
Schneider, C. H., & de Weck, A. L. (1966) *Helv. Chim. Acta 49*, 1701-1714.
Schuster, R., & Apfel, A. (1986) *Hewlett-Packard HPLC Application Note*, HP Publication No. 12-5954-6257.
Signal, I. S., Harwood, B. G., & Arentzen, R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7157-7162.
Sigal, I. S., Degrado, W, F., Thomas, B. J., & Petteway, S. R. (1984) *J. Biol. Chem. 259*, 5327-5332.
Waley, S. G. (1974) *Biochem. J. 139*, 789-790.
Waley, S. G. (1975) *Biochem J. 149*, 547-551.

Kinetic Mechanism of the Type II Calmodulin-Dependent Protein Kinase: Studies of the Forward and Reverse Reactions and Observation of Apparent Rapid-Equilibrium Ordered Binding†

Ann P. Kwiatkowski,‡,§ Charles Y. Huang,‖ and Marita M. King*,‡

Appendix: Various Cases of Rapid-Equilibrium Ordered Bireactant Mechanisms—Their Bases and Differentiation

Charles Y. Huang‖

Department of Chemistry and Ohio State Biochemistry Program, The Ohio State University, Columbus, Ohio 43210, and Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

Received May 8, 1989; Revised Manuscript Received August 18, 1989

ABSTRACT: The kinetic reaction mechanism of the type II calmodulin-dependent protein kinase was studied by using its constitutively active kinase domain. Lacking regulatory features, the catalytic domain simplified data collection, analysis, and interpretation. To further facilitate this study, a synthetic peptide was used as the kinase substrate. Initial velocity measurements of the forward reaction were consistent with a sequential mechanism. The patterns of product and dead-end inhibition studies best fit an ordered Bi Bi kinetic mechanism with ATP binding first to the enzyme, followed by binding of the peptide substrate. Initial-rate patterns of the reverse reaction of the kinase suggested a rapid-equilibrium mechanism with obligatory ordered binding of ADP prior to the phosphopeptide substrate; however, this apparent rapid-equilibrium ordered mechanism was contrary to the observed inhibition by the phosphopeptide which is not supposed to bind to the kinase in the absence of ADP. Inspection of product inhibition patterns of the phosphopeptide with both ATP and peptide revealed that an ordered Bi Bi mechanism can show initial-rate patterns of a rapid-equilibrium ordered system when a Michaelis constant for phosphopeptide, K_{ip}, is large relative to the concentration of phosphopeptide used. Thus, the results of this study show an ordered Bi Bi mechanism with nucleotide binding first in both directions of the kinase reaction. All the kinetic constants in the forward and reverse directions and the K_{eq} of the kinase reaction are reported herein. To provide theoretical bases and diagnostic aid for mechanisms that can give rise to typical rapid-equilibrium ordered kinetic patterns, a discussion on various sequential cases is presented in the Appendix.

The type II calmodulin-dependent protein kinase (CaM kinase II)[1] is a multifunctional enzyme found most prominently in the brain, where it appears to regulate a diverse number of physiological processes [for review see Nairn et al. (1985), Kennedy et al. (1987), Schulman and Lou, (1989), and Colbran et al. (1989a)]. Many laboratories, including ours, have studied the regulation of CaM kinase II activity by Ca^{2+}/calmodulin and autophosphorylation to elucidate molecular mechanisms by which this enzyme transforms transient Ca^{2+} signals into biochemical responses. Specific regulatory features have been identified: (1) Binding of Ca^{2+}/calmodulin to CaM kinase II removes an autoinhibitory effect which is attributed to an inhibitory region located between the catalytic and calmodulin-binding domains of the protein kinase (Payne et al., 1988; Colbran et al., 1989b). (2) Although calmodulin is not required for nucleotide binding to CaM kinase II, the activator increases the binding affinity (King, 1988; King et al., 1988). (3) Following binding of calmodulin, autophosphorylation of CaM kinase II is required to fully activate

† This work was supported by the National Science Foundation (DMB-8502706 and DMB-8816258).

* To whom correspondence should be addressed at the Department of Chemistry, The Ohio State University, 120 W. 18th Avenue, Columbus, OH 43210.
‡ The Ohio State University.
§ Present address: 823 Light Hall, Vanderbilt University, Nashville, TN 37232-0295.
‖ National Heart, Lung, and Blood Institute.

[1] Abbreviations: CaM kinase II, type II calmodulin-dependent protein kinase; EDTA, ethylenediaminetetraacetate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AMPPNP, adenosine 5′-(β,γ-imidotriphosphate); P-syntide, phosphorylated syntide.

0006-2960/90/0429-153$02.50/0

the kinase toward exogenous substrates (Kwiatkowski et al., 1988). (4) Autophosphorylation of threonine 286 and 287 of the α and β subunits, respectively, converts CaM kinase II to a Ca^{2+}/calmodulin-independent form (Miller et al., 1988; Thiel et al., 1988; Schworer et al., 1988). (5) Upon removal of Ca^{2+} from the system, this Ca^{2+}/calmodulin-independent kinase can modify newly exposed "inhibitory" sites, which seems to reduce kinase activity (Hashimoto et al., 1987) and to inhibit the ability of calmodulin to activate the kinase (Schulman & Lou, 1989; Colbran et al., 1989a). (6) Proteolytic cleavage of autophosphorylated CaM kinase II yields constitutively active kinase domains (Colbran et al., 1988; Kwiatkowski & King, 1989), while dephosphorylation of autophosphorylated enzyme by protein phosphatases restores its dependence on Ca^{2+}/calmodulin (LeVine et al., 1985; Miller & Kennedy, 1986; Lai et al., 1986; Lou et al., 1986; Saitoh et al., 1987).

To further elucidate molecular events leading to the phosphorylation of exogenous substrates by CaM kinase II, we have studied the kinetic mechanism of the protein kinase. An autonomous kinase domain of the enzyme and the peptide substrate syntide were used in these studies to simplify analyses and interpretations. According to initial-rate patterns for the forward and reverse reactions of CaM kinase II and the inhibition patterns obtained in product and dead-end inhibitor studies, CaM kinase II exhibits an ordered Bi Bi mechanism with nucleotide binding prior to peptide substrate. A rapid-equilibrium random mechanism has previously been suggested by Kuret and Schulman (1984) on the basis of partial kinetic results that, while consistent with a rapid-equilibrium random case with an abortive complex, could not exclude an ordered Bi Bi mechanism.

Experimental Procedures

Materials. [γ-^{32}P]ATP was purchased from ICN Biomedicals, Inc. The synthetic peptide substrate syntide (Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly-Leu-Pro-Gly-Lys-Lys) (Hashimoto & Soderling, 1987), which is analogous to phosphorylation site 2 of glycogen synthase, was obtained from Biosearch, Inc. TPCK-treated trypsin, soybean trypsin inhibitor, hexokinase, phenyl-Sepharose, and nucleotides were purchased from Sigma; Affi-Gel blue was from Bio-Rad Laboratories. Calmodulin was prepared from frozen bovine brain (Gopalakrishna & Anderson, 1982).

P-syntide was prepared by phosphorylation of syntide (0.5 mM) for 1 h at 25 °C in the presence of autophosphorylated enzyme (1 μM), $CaCl_2$ (0.5 mM), $MgCl_2$ (30 mM), EDTA (0.1 mM), Hepes buffer (50 mM, pH 7.5), ATP (3 mM), and calmodulin (10 μM); autophosphorylation of CaM kinase II was carried out for 5 min at 0 °C prior to the addition of the peptide substrate. Syntide and P-syntide were purified by HPLC using a reverse-phase C_{18} column and a gradient of acetonitrile/0.1% trifluoroacetic acid (v/v) and aqueous 0.1% trifluoroacetic acid (v/v). The fractions containing syntide or P-syntide were pooled, lyophilized to dryness, and redissolved in water. The concentrations of the peptides were determined by amino acid analysis (Biochemical Instrument Center, The Ohio State University). The peptides were at least 99% pure.

Preparation of Catalytic Domains of CaM Kinase II. CaM kinase II was isolated from frozen rat brain as described previously (Kwiatkowski & King, 1987). The enzyme was at least 90% pure and exhibited an apparent V_{max} of 2–4 μmol min^{-1} mg^{-1} with syntide. In preparation for proteolysis, CaM kinase II (0.4 mg/mL, 1 mg total protein) was autophosphorylated at 0 °C for 5 min in the presence of ATP (1 mM), calmodulin (30 μM), $CaCl_2$ (0.5 mM), $MgCl_2$ (10 mM), glycerol (10%), EDTA (0.1 mM), dithiothreitol (0.1 mM), and Hepes buffer (50 mM, pH 7.5). Following the addition of trypsin (2 μg/mL), proteolysis was carried out for 15 min at 25 °C. Trypsin inhibitor (20 μg/mL) was added to terminate the reaction. The active fragment was separated from calmodulin by chromatography over phenyl-Sepharose (0.5 mL bed volume) equilibrated with buffer (50 mM Hepes, pH 7.5, 0.1 mM EDTA, and 0.3 mM $CaCl_2$). The eluate was applied to a column of Affi-Gel blue (0.5 mL bed volume). The column was washed with buffer (50 mM Hepes, pH 7.5, 0.1 mM EDTA, and 0.3 mM $CaCl_2$), and the active kinase fragment was eluted with buffer containing 0.5 M NaCl. The concentration of the kinase was determined according to the method of Bradford (1976) using bovine serum albumin as a standard. Quantification of the kinase activity indicated recoveries of 46 and 75% in two separate experiments. To stabilize the active fragment, bovine serum albumin (1 mg/mL) was added before storage of the sample at −70 °C.

Table I: Summary of Kinetic Parameters

K_{ia} (μM) (5)	20 ± 8.1	K_p (μM) (4)	42 ± 23
K_a (μM) (5)	86 ± 36	K_p' (μM) (2)	56 ± 28
K_{ib} (μM) (5)	5.6 ± 2.3	k_r (s^{-1}) (4)	0.026 ± 0.011
K_b (μM) (5)	28 ± 21	k_1 (M^{-1} s^{-1}) (5)	(2.3 ± 0.9) × 10^5
k_f (s^{-1}) (5)	21 ± 14	k_{-1} (s^{-1}) (5)	4.6 ± 2.4
K_{iq}[a] (μM) (2)	51 ± 30	k_{-2}[d] (s^{-1})	0.026 ± 0.011
K_{iq}[b] (μM) (4)	57 ± 26	K_{eq}	3500 ± 300
K_{iq}[c] (μM) (2)	52 ± 17		

[a] From crossover points or initial velocity data. [b] From slope replot of reverse reaction. [c] From product inhibition studies. [d] Calculated from average values of k_r and k_{-1}. All the constants are defined in the text; the values are shown with standard deviations. The number of experiments averaged is shown in parentheses.

Measurements of Kinase Activity. (*a*) *Forward Reaction.* The kinase fragment was assayed for 1 min at 25 °C by measuring the transfer of $^{32}P_i$ from [γ-^{32}P]ATP to syntide. The reactions contained kinase (10 nM, on the basis of M_r = 30000), syntide (1–20 μM), [γ-^{32}P]ATP (5–100 μM), $MgCl_2$ (10 mM), bovine serum albumin (1 mg/mL), glycerol (10%), EDTA (0.1 mM), dithiothreitol (0.1 mM), and Hepes buffer (50 mM, pH 7.5). The incorporation of $^{32}P_i$ into syntide was measured by the filter paper method of Roskoski (1983). For inhibition studies, the kinase fragment (2 nM) was assayed in the presence of the indicated concentrations of ADP (0–200 μM), P-syntide (0–540 μM), or AMPPNP (0–400 μM).

(*b*) *Reverse Reaction.* To study the kinase reaction in the reverse direction (dephosphorylation of P-syntide), the transfer of $^{32}P_i$ from [^{32}P]P-syntide into ADP was coupled to the hexokinase reaction to remove [^{32}P]ATP from the system (Flockhart, 1983). The assay mixture contained the active kinase domain (0.2 μM, on the basis of M_r = 30000), [^{32}P]P-syntide (10–200 μM), ADP (25–200 μM), $MgCl_2$ (10 mM), hexokinase (10 units/mL), glucose (10 mM), bovine serum albumin (1 mg/mL), glycerol (10%), EDTA (0.1 mM), dithiothreitol (0.1 mM), and Hepes buffer (50 mM, pH 7.5). After 20 min at 25 °C, the reactions were stopped with acetic acid (33.3% v/v). The mixtures were applied to small columns of cellulose phosphate (0.15 mL bed volumes) which retained residual [^{32}P]P-syntide. [^{32}P]Glucose was eluted with water and quantified by liquid scintillation counting.

Data Analysis. The results were subjected to linear regression analysis of Lineweaver and Burk plots (Lineweaver & Burk, 1934). No weights were assigned to the data points. Linear regression analysis of secondary plots was used to calculate the kinetic parameters of Table I. The kinetic parameters of the initial velocity experiments were then used to calculate the common crossover points of the corresponding

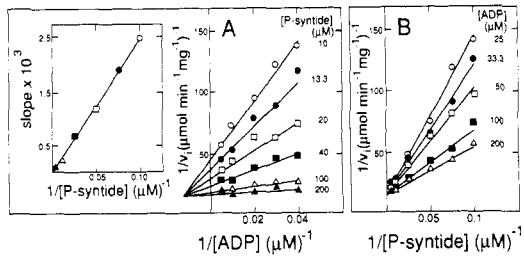


FIGURE 1: Initial-rate studies of the reverse reaction of CaM kinase II. (A) Double-reciprocal plots with ADP as the variable substrate at the indicated fixed levels of P-syntide. A replot of the slopes is shown in the inset. (B) Double-reciprocal plots with P-syntide as the variable substrate at the indicated fixed concentrations of ADP. The initial rates correspond to specific activities. The experiments were carried out and analyzed as discussed under Experimental Procedures.

primary double-reciprocal plots. By use of the calculated crossover points as a common value, the lines of the primary plots were redrawn by hand. It should be noted that these redrawn plots provided kinetic parameters that were almost indistinguishable from those obtained by linear regression analysis. Results of product and dead-end inhibition studies were analyzed exclusively by linear regression. The nomenclature was essentially that of Cleland (1963).

Results

Initial-Rate Studies. In the forward reaction (phosphorylation), when ATP was the variable substrate and syntide the varied fixed substrate, double-reciprocal plots yielded a family of lines intersecting to the left of the ordinate. When syntide was the variable substrate, a similar plot was obtained. These intersecting patterns indicated that the substrate binding mechanism for CaM kinase II was likely ordered or rapid-equilibrium random. In the reverse reaction (dephosphorylation), when ADP was the variable substrate and P-syntide the varied fixed substrate, a family of linear lines converging to the left of the ordinate was observed (Figure 1A). However, when P-syntide was the variable substrate, the lines obtained at different fixed levels of ADP seemed to converge on the ordinate (Figure 1B). The kinetic patterns shown in Figure 1 are typical of a rapid-equilibrium system in which the two substrates bind to the enzyme in an obligatory order; ADP binds first in this particular case. The rapid-equilibrium ordered mechanism was further supported by the observation that a replot of the slopes from Figure 1A versus 1/P-syntide passed through the origin (Figure 1A, inset).

Product Inhibition Studies in the Forward Direction. With variable concentrations of ATP and a constant, nonsaturating level of syntide, inhibition by the product ADP was found to be competitive. The competition was linear in the range 25–200 μM ADP. With syntide as the variable substrate at a constant level of ATP, inhibition by ADP was noncompetitive. Again, linear noncompetitive inhibition was evident from secondary plots. When P-syntide was used as the product inhibitor, with either ATP or syntide as the variable substrate, the inhibition pattern was essentially linear noncompetitive (Figure 2). Note that in the range 0–180 μM P-syntide the lines are practically parallel to one another, revealing the uncompetitive nature of inhibition by this product at low concentrations.

Product inhibition studies in the reverse direction of the kinase reaction were not performed because the presence of product inhibitors made the already slow reverse reaction even slower, thereby rendering activity measurements infeasible.

Dead-End Inhibitor Studies. If symmetrical reaction pathways are assumed for the forward and reverse directions,

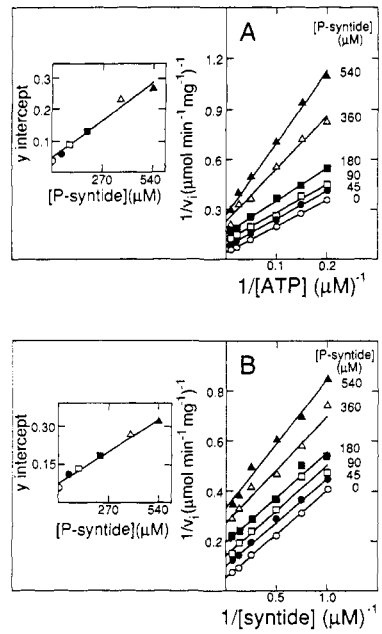


FIGURE 2: Product inhibition of CaM kinase II by P-syntide. (A) Inhibition by the product P-syntide with ATP as the variable substrate and a fixed, nonsaturating concentration of syntide (20 μM). (B) Inhibition by P-syntide with syntide as the variable substrate and a fixed, nonsaturating concentration of ATP (100 μM). The initial rates correspond to specific activities. Secondary plots of the y intercepts are shown as insets. These studies were performed and analyzed as described under Experimental Procedures.

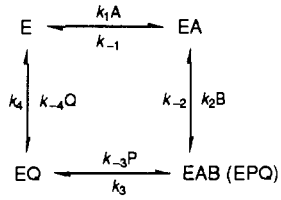
the product inhibition patterns reported above are consistent with two ordered mechanisms: the ordered Bi Bi mechanism, in which ATP is the first substrate to add to CaM kinase II, or the iso-Theorell–Chance mechanism, in which syntide is the first substrate. To differentiate these two possibilities, we used a known competitive inhibitor of ATP (Kwiatkowski & King, 1987), AMPPNP, to study its inhibition pattern against the other substrate, syntide. AMPPNP was found to be linearly noncompetitive with respect to syntide. The results indicate that ATP was the first substrate bound to CaM kinase II.

Discussion

The intersecting double-reciprocal plots observed in the forward reaction initial-rate studies are indicative of a sequential (rather than ping-pong) mechanism: an ordered substrate addition or a rapid-equilibrium random substrate binding. In the reverse direction (Figure 1), the initial-rate patterns indicate a rapid-equilibrium ordered mechanism with ADP binding first, followed by the binding of P-syntide. Thus, it appears that the overall mechanism is likely ordered binding in both forward and reverse directions except that the reverse binding steps are rapid. Product inhibition studies of the forward reaction, with the observation of P-syntide being noncompetitive to both ATP and syntide (Figure 2), allow one to exclude the rapid-equilibrium random mechanism and further strengthen the ordered binding case. However, a mechanism with ordered substrate addition in the forward direction and rapid-equilibrium, obligatory ordered in the reverse reaction requires that P-syntide by itself has no inhibitory effect since it cannot bind to CaM kinase II in the absence of ADP. This requirement contradicts the experimental results shown in Figure 2.

The apparent rapid-equilibrium ordered kinetic behavior shown in Figure 1 can be explained in a different way (see Appendix for a fuller discussion). The most likely explanation is that a Michaelis constant for P-syntide, K_{ip}, is large relative

Scheme I



to the concentration of P-syntide employed. Let us first examine the ordered Bi Bi case where A is ATP, B is syntide, P is P-syntide, and Q is ADP. In the reverse direction the initial-rate equation is given by

$$\frac{E_0}{v} = \frac{1}{k_r}\left[1 + \frac{K_p}{P} + \frac{K_q}{Q}\left(1 + \frac{K_{ip}}{P}\right)\right] \quad (1)$$

where

$$K_p = k_{-1}(k_{-2} + k_3)/k_{-3}(k_{-1} + k_{-2})$$

$$K_q = k_{-1}k_{-2}/k_{-4}(k_{-1} + k_{-2})$$

$$K_{ip} = k_4(k_{-2} + k_3)/k_{-2}k_{-3}$$

and

$$k_r = k_{-1}k_{-2}/(k_{-1} + k_{-2})$$

which is the catalytic rate constant in the reverse direction. If $K_{ip} \gg P$, i.e., $K_{ip}/P \gg 1$, then eq 1 reduces to

$$\frac{E_0}{v} = \frac{1}{k_r}\left(1 + \frac{K_p}{P} + \frac{K_qK_{ip}}{QP}\right) \quad (2)$$

which is a typical rapid-equilibrium ordered kinetic equation. In other words, if K_{ip} is much greater than the P-syntide concentrations, then a $1/v$ versus 1/P-syntide ($1/P$) plot will give rise to lines intersecting on the ordinate (or very close to the ordinate) because the intercept is unaffected by variations of Q. Another way of looking at this situation is to recall that in a $1/v$ versus $1/P$ plot, the x coordinate of the intersection point is $1/K_{ip}$. Therefore, when K_{ip} is large, $1/K_{ip}$ approaches zero and the intersection point will be very close to the ordinate.

The product inhibition experiments provide support that this situation, i.e., $K_{ip} \gg P$, is the cause for observing apparent rapid-equilibrium ordered kinetic patterns. When only P (P-syntide) is present, the product inhibition equation derived from Scheme I is

$$\frac{E_0}{v} = \frac{1}{k_f}\left[\left(1 + \frac{P}{K_p'}\right) + \frac{K_a}{A} + \frac{K_b}{B}\left(1 + \frac{P}{K_{ip}}\right) + \frac{K_{ia}K_b}{AB}\left(1 + \frac{P}{K_{ip}}\right)\right] \quad (3a)$$

where

$$K_a = k_f/k_1$$

$$K_{ia} = k_{-1}/k_1$$

$$K_b = k_4(k_{-2} + k_3)/k_2(k_3 + k_4)$$

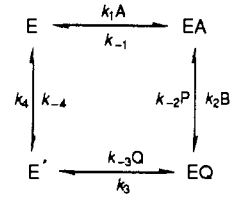
$$K_p' = (k_3 + k_4)/k_{-3}$$

and

$$k_f = k_3k_4/(k_3 + k_4)$$

which is the catalytic constant in the forward direction. If K_{ip}

Scheme II



$\gg P$, but $K_p' \simeq P$ (see Appendix for the required conditions), then eq 3a becomes

$$\frac{E_0}{v} = \frac{1}{k_f}\left(1 + \frac{P}{K_p'} + \frac{K_a}{A} + \frac{K_b}{B} + \frac{K_{ia}K_b}{AB}\right) \quad (3b)$$

Equation 3b predicts that if K_{ip} is large, at low levels of P the inhibition pattern of P versus either substrate A or B will become uncompetitive. This prediction is consistent with the parallel lines seen in Figure 2 at P-syntide concentrations up to 180 μM. At higher P-syntide concentrations, the inhibition should be noncompetitive as predicted by eq 3a. When only Q (ADP) is present, the product inhibition patterns are not affected by the magnitude of K_{ip}; i.e., ADP remains competitive to ATP and noncompetitive to syntide as observed.

It should be noted that the initial-rate and product inhibition patterns reported here can also occur in an iso-Theorell–Chance mechanism where A is syntide, B is ATP, P is ADP, Q is P-syntide, and the enzyme isomerizes between E and E′ forms. The orders of substrate binding and product release are reversed from those in Scheme I. The initial-rate equation for the reverse reaction has the expression

$$\frac{E_0}{v} = \frac{1}{k_r}\left(1 + \frac{K_p}{P} + \frac{K_q}{Q} + \frac{K_pK_{iq}}{QP}\right) \quad (4a)$$

Equation 4a also yields apparent rapid-equilibrium ordered reaction double-reciprocal plots if $K_{iq} \gg Q$ (same as $K_{ip} \gg P$ in eq 1):

$$\frac{E_0}{v} = \frac{1}{k_r}\left(1 + \frac{K_q}{Q} + \frac{K_{iq}K_p}{QP}\right) \quad (4b)$$

Similarly, when $K_{iq} \gg Q$, inhibition of P-syntide against ATP and syntide will appear uncompetitive.

To differentiate between the ordered Bi Bi and iso-Theorell–Chance cases, one only needs to find out which substrate is the first one to combine with the enzyme since ATP is A in the former case but ATP is B in the latter case. We chose the competitive inhibitor approach of Fromm and Zewe (1962) for achieving this end, using a known competitive inhibitor to ATP, AMPPNP (Kwiatkowski & King, 1987). In an ordered mechanism, whether Theorell–Chance or isomerization is involved, an inhibitor competitive to substrate A will be noncompetitive to substrate B, but an inhibitor competitive to substrate B will be uncompetitive to substrate A. The noncompetitive inhibition of AMPPNP against syntide we observed, therefore, demonstrates that the kinetic mechanism for CaM kinase II is most consistent with ordered Bi Bi as depicted in Scheme I. This conclusion is strengthened by the fact that other apparent rapid-equilibrium ordered mechanisms do not display the same product inhibition patterns (cf. Table II in the Appendix). It should be noted that Scheme I corresponds to the preferred pathway of substrate addition in the kinetic mechanism of CaM kinase II; whether syntide or a protein substrate binds to the enzyme in the absence of ATP must be determined through direct binding experiments.

Table II: Product Inhibition Patterns for Bireactant Mechanisms Displaying Apparent Rapid-Equilibrium Ordered Kinetics in the Reverse Reaction[a]

	forward reaction					reverse reaction				
		inhibition for the varied substrate[b]					inhibition for the varied substrate			
mechanism	product inhibitor	$K_{ip} \gg P$		$K_{iq} \gg Q$		product inhibitor	$K_{ip} \gg P$		$K_{iq} \gg Q$	
ordered[c]	P	UC	A	NC (UC)	A	A	comp (-)	P	NC (-)	P
		UC	B	NC	B		comp (-)	Q	comp	Q
	Q	comp	A	-	A	B	NC	P	NC (UC)	P
		NC (-)	B	-	B		NC (UC)	Q	NC (UC)	Q
isoordered[d]	P	UC	A	NC (UC)	A	A	NC (UC)	P	NC (UC)	P
		UC	B	NC	B		NC (UC)	Q	NC	Q
	Q	NC	A	UC	A	B	NC	P	NC (UC)	P
		NC (UC)	B	UC	B		NC (UC)	Q	NC (UC)	Q
Theorell–Chance[d]	P	-	A	NC (-)	A	A	comp	P	comp (-)	P
		-	B	comp	B		NC (-)	Q	comp (-)	Q
	Q	comp	A	-	A	B	comp (-)	P	NC (-)	P
		NC (-)	B	-	B		comp (-)	Q	comp	Q
iso-Theorell–Chance[c]	P	-	A	NC (-)	A	A	NC (UC)	P	NC (UC)	P
		-	B	comp	B		NC (UC)	Q	NC	Q
	Q	NC	A	UC	A	B	comp	P	comp (-)	P
		NC (UC)	B	UC	B		NC (-)	Q	comp (-)	Q
rapid-equilibrium random[e]	P	-	A			A	comp (-)	P		
		-	B				comp (-)	Q		
	Q	comp	A			B	comp (-)	P		
		comp (-)	B				comp (-)	Q		
+AP abortive complex	P	UC (-)	A			A	NC (-)	P		
		comp	B				comp	Q		
+BQ abortive complex	Q	comp	A			B	comp	P		
		NC (-)	B				NC (-)	Q		

[a] With exchanges A ⇌ Q, B ⇌ P, $K_{ip} \rightharpoonup K_{ib}$, and $K_{iq} \rightharpoonup K_{ia}$, the table is applicable to mechanisms with apparent REO features in the forward direction. [b] Notations used: comp, competitive; NC, noncompetitive; UC, uncompetitive; -, no effect. Parentheses indicate inhibition patterns that become different at saturating level of the fixed substrate. [c] Ordered, $K_{ip} \gg P$ case cannot be differentiated from iso-Theorell–Chance, $K_{iq} \gg Q$, case without the use of other methods (alternative substrate, competitive inhibitor, etc.). [d] Other techniques are required to identify A and B. [e] $K_{ip} \gg P$ and $K_{iq} \gg Q$ are the same since P and Q and A and B are not distinguishable. Also, true and apparent REO mechanisms yield identical patterns.

If asymmetrical reaction pathways for the forward and reverse directions are considered, the noncompetitive inhibition of P-syntide toward both ATP and syntide could also be explained by a hybrid mechanism of rapid-equilibrium random binding in the forward reaction and by ordered binding in the reverse reaction (Huang, 1979). The apparent rapid-equilibrium ordered reverse reaction still requires that $K_{ip} \gg P$, and the uncompetitive inhibition of ATP and syntide by low levels of P-syntide further requires $k_f \gg k_r$. To account for the noncompetitive inhibition of ADP with respect to syntide, however, an E–syntide–ADP abortive complex must be proposed. Since this complex can be formed from both the addition of ADP to E–syntide and addition of syntide to E–ADP, nonlinear inhibition by ADP and substrate inhibition by syntide are likely to occur. In the absence of such observations, the asymmetrical mechanism appears unlikely, though not excludable.

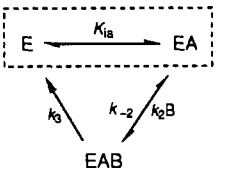
All the kinetic parameters defined in eq 1 and 3a are summarized in Table I. In addition, the equilibrium constant for the phosphorylation–dephosphorylation reaction has been calculated from the Haldane relationships:

$$K_{eq} = \frac{k_f K_p K_{iq}}{k_r K_{ia} K_b} = \frac{k_f K_p K_{iq}}{k_r K_a K_{ib}}$$

The K_{eq} so obtained, 3500 ± 300, clearly shows that the equilibrium, as expected, is greatly in favor of the phosphorylation reaction.

To avoid complications due to autophosphorylation and ligand-induced instability of the holoenzyme, we have used the autonomous kinase domain for our study of the kinetic mechanism of CaM kinase II. The kinetic mechanism of cAMP-dependent protein kinase also was investigated by using

Scheme III



the isolated catalytic subunit and a synthetic peptide (Kemptide) to circumvent problems caused by allosteric or substrate-directed effects. Whitehouse et al. (1983) concluded the mechanism of this protein kinase to be ordered Bi Bi, arguing mainly from the observation that the inhibitor protein was competitive to Kemptide but uncompetitive to ATP (Whitehouse & Walsh, 1983). Their data, likewise, cannot exclude the possibility of a hybrid rapid-equilibrium random and ordered Bi Bi mechanism mentioned above. Cook and co-workers (Cook et al., 1982; Kong & Cook, 1988) suggested a steady-state random (forward) and ordered (reverse) mechanism, arguing from the relative sizes of MgATP off-rate and catalytic rate constants. However, in a steady-state random mechanism, when linear reciprocal plots are obtained, product inhibitors and nonreactive substrate analogues, such as Ala-Kemptide, should behave as noncompetitive inhibitors. Such a mechanism, therefore, contradicts the observed competitive inhibition of ADP vs ATP, Ala-Kemptide vs Kemptide, etc. At higher levels of MgATP, nevertheless, Cook et al. (1982) suggested that there is a preference for MgATP binding to the enzyme first. Other workers have proposed a rapid-equilibrium random mechanism for phosphorylase kinase (Tabatabai & Graves, 1978) and myosin light chain kinase (Geuss et al., 1985). Thus, it seems that no uniform mech-

anism is operative for protein kinases.

Registry No. CaM kinase, 9026-43-1; 5′-ATP, 56-65-5; 5′-ADP, 58-64-0; syntide, 108334-68-5.

REFERENCES

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104–137.

Colbran, R. J., Fong, Y.-L., Schworer, C. M., & Soderling, T. R. (1988) *J. Biol. Chem. 263*, 18145–18151.

Colbran, R. J., Schworer, C. M., Hashimoto, Y., Fong, Y.-L., Rich, D. P., Smith, M. K., & Soderling, T. R. (1989a) *Biochem. J. 258*, 313–325.

Colbran, R. J., Smith, M. K., Schworer, C. M., Fong, Y.-L., & Soderling, T. R. (1989b) *J. Biol. Chem. 264*, 4800–4804.

Cook, P. F., Neville, M. E., Vrana, K. E., Hartl, F. T., & Roskoski, R., Jr. (1982) *Biochemistry 21*, 5794–5799.

Flockhart, D. A. (1983) *Methods Enzymol. 99*, 14–20.

Fromm, H. J., & Zewe, V. (1962) *J. Biol. Chem. 237*, 3027–3032.

Geuss, U., Mayr, G. W., & Heilmeyer, L. M. G., Jr. (1985) *Eur. J. Biochem. 153*, 327–334.

Gopalakrishna, R., & Anderson, W. B. (1982) *Biochem. Biophys. Res. Commun. 104*, 830–836.

Hashimoto, Y., & Soderling, T. R. (1987) *Arch. Biochem. Biophys. 252*, 418–425.

Hashimoto, Y., Schworer, C. M., Colbran, R. J., & Soderling, T. R. (1987) *J. Biol. Chem. 262*, 8051–8055.

Huang, C. Y. (1979) *Methods Enzymol. 63*, 54–84.

Kennedy, M. B., Bennett, M. K., Erondu, N. E., & Miller, S. G. (1987) *Calcium Cell Funct. 7*, 61–107.

King, M. M. (1988) *J. Biol. Chem. 263*, 4754–4757.

King, M. M., Shell, D. J., & Kwiatkowski, A. P. (1988) *Arch. Biochem. Biophys. 267*, 467–473.

Kong, C.-T., & Cook, P. F. (1988) *Biochemistry 27*, 4795–4799.

Kuret, J., & Schulman, H. (1984) *Biochemistry 23*, 5495–5504.

Kwiatkowski, A. P., & King, M. M. (1987) *Biochemistry 26*, 7636–7640.

Kwiatkowski, A. P., & King, M. M. (1989) *Biochemistry 28*, 5380–5385.

Kwiatkowski, A. P., Shell, D. J., & King, M. M. (1988) *J. Biol. Chem. 263*, 6484–6486.

Lai, Y., Nairn, A. C., & Greengard, P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4253–4257.

LeVine, H., Sahyoun, N. E., & Cuatrecasas, P. (1985) *Biochem. Biophys. Res. Commun. 131*, 1212–1218.

Lineweaver, H., & Burk, D. (1934) *J. Am. Chem. Soc. 56*, 658–666.

Lou, L. L., Lloyd, S. J., & Schulman, H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9497–9501.

Miller, S. G., & Kennedy, M. B. (1986) *Cell 44*, 861–870.

Miller, S. G., Patton, B. L., & Kennedy, M. B. (1988) *Neuron 1*, 593–604.

Nairn, A. C., Hemmings, H. C., Jr., & Greengard, P. (1985) *Annu. Rev. Biochem. 54*, 931–976.

Payne, M. E., Fong, Y.-L., Ono, T., Colbran, R. J., Kemp, B. E., Soderling, T. R., & Means, A. R. (1988) *J. Biol. Chem. 263*, 7190–7195.

Roskoski, R., Jr. (1983) *Methods Enzymol. 99*, 3–6.

Saitoh, Y., Yamamoto, H., Fukunaga, K., Matsukado, Y., & Miyamoto, E. (1987) *J. Neurochem. 49*, 1286–1292.

Schulman, H., & Lou, L. L. (1989) *Trends Biochem. Sci. 14*, 62–66.

Schworer, C. M., Colbran, R. J., Keefer, J. R., & Soderling, T. R. (1988) *J. Biol. Chem. 263*, 13486–13489.

Tabatabai, L. B., & Graves, D. J. (1978) *J. Biol. Chem. 253*, 2196–2202.

Thiel, G., Czernik, A. J., Gorelick, F., Nairn, A. C., & Greengard, P. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6337–6341.

Whitehouse, S., & Walsh, D. A. (1983) *J. Biol. Chem. 258*, 3682–3692.

Whitehouse, S., Feramisco, J. R., Casnellie, J. E., Krebs, E. G., & Walsh, D. A. (1983) *J. Biol. Chem. 258*, 3693–3701.

APPENDIX: VARIOUS CASES OF RAPID-EQUILIBRIUM ORDERED BIREACTANT MECHANISMS—THEIR BASES AND DIFFERENTIATION

In the rapid-equilibrium ordered (REO) mechanism for an enzyme-catalyzed reaction, the two substrates, A and B, bind to the enzyme in an ordered manner and the binding processes attain equilibrium rapidly:

$$E + A \overset{K_{ia}}{\rightleftharpoons} EA$$

$$EA + B \overset{K_b}{\rightleftharpoons} EAB \xrightarrow{k_3} E + P + Q$$

This mechanism has been treated by Segal et al. (1952) in which A is either an activator or the first substrate. The reciprocal initial-rate equation is

$$E_0/v = (1/k_3)[1 + (K_b/B) + (K_{ia}K_b/AB)] \quad (5)$$

Equation 5 illustrates the distinctive features observed with the REO mechanism: (1) In the $1/v$ versus $1/B$ plot, the lines obtained at different levels of A will intersect on the ordinate. (2) In the $1/v$ versus $1/A$ plot, the interaction point will be to the left of the ordinate, but the slopes of the lines, $K_{ia}K_b/k_3B$, when plotted against $1/B$, will generate a line that passes through the origin.

I want to point out that there are a number of conditions that can give rise to kinetic patterns typical of the REO mechanism. First, only the addition of the first substrate to enzyme needs to achieve equilibrium rapidly, as shown in Here the rapid-equilibrium segment is enclosed by dashed lines. The resultant rate equation from Scheme III

$$E_0/v = (1/k_3)[1 + ((k_{-2} + k_3)/k_2B) + (K_{ia}(k_{-2} + k_3)/k_2AB)] \quad (6)$$

is identical in form with eq 5 except that K_b is expressed as $(k_{-2} + k_3)/k_2$. This condition, however, does not alter any kinetic patterns predicted by the usual REO mechanism. Consequently, one needs only to focus on the apparent REO cases which can occur in various ordered and random reactions. The sequential mechanisms can be described by the general rate equation

$$E_0/v = (1/k_f)[1 + (K_a/A) + (K_b/B) + (K_{ia}K_b/AB)] \quad (7a)$$

which, in parallel fashion as has been shown in eq 1 and 2, reduces to the typical REO forms

$$E_0/v = (1/k_f)[1 + (K_b/B) + (K_{ia}K_b/AB)] \quad \text{if } K_{ib} \gg B \quad (7b)$$

and

$$E_0/v = (1/k_f)[1 + (K_a/A) + (K_{ia}K_b/AB)] \quad \text{if } K_{ia} \gg A \quad (7c)$$

Note that eq 7c describes a peculiar situation (similar to that of eq 4b) since a $1/v$ vs $1/A$ plot will yield lines converging on the ordinate, thereby making A, the first substrate in an ordered mechanism, look like the second substrate. Thus, caution should be exercised in using such a plot to identify the