Kinetic Characterization of the Calmodulin-Activated Catalytic Subunit of Phosphorylase Kinase†

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ABSTRACT: The phosphorylase kinase holoenzyme from skeletal muscle is composed of a catalytic and three different regulatory subunits. Analysis of the kinetic mechanism of the holoenzyme is complicated because both the natural substrate phosphorylase *b* and also phosphorylase kinase itself have allosteric binding sites for adenine nucleotides. In the case of the kinase, these allosteric sites are not on the catalytic subunit. We have investigated the kinetic mechanism of phosphorylase kinase by using its isolated catalytic γ-subunit (activated by calmodulin) and an alternative peptide substrate (SDQEKRKQISVRGL) corresponding to the convertible region of phosphorylase *b*, thus eliminating from our system all known allosteric binding sites for nucleotides. This peptide has been previously employed to study the kinetic mechanism of the kinase holoenzyme before the existence of the allosteric sites on the regulatory subunits was suspected [Tabatabai, L. B., & Graves, D. J. (1978) *J. Biol. Chem. 253*, 2196–2202]. This peptide was determined to be as good an alternative substrate for the isolated catalytic subunit as it was for the holoenzyme. Initial velocity data indicated a sequential kinetic mechanism with apparent K_m's for MgATP and peptide of 0.07 and 0.47 mM, respectively. MgADP used as product inhibitor showed competitive inhibition against MgATP and noncompetitive inhibition against peptide, whereas with phosphopeptide as product inhibitor, the inhibition was competitive against both MgATP and peptide. The initial velocity and product inhibition studies were consistent with a rapid equilibrium random mechanism with one abortive complex, enzyme–MgADP–peptide. The substrate-directed, dead-end inhibitors 5′-adenylyl imidodiphosphate and Asp-peptide, in which the convertible Ser of the alternative peptide substrate was replaced with Asp, were competitive inhibitors toward their like substrates and noncompetitive inhibitors toward their unlike substrates, further supporting a random mechanism, which was also the conclusion from the report cited above that used the holoenzyme.

Phosphorylase kinase (EC 2.7.1.38) couples muscle contraction with energy production via glycogenolysis–glycolysis by catalyzing the Ca^{2+}-dependent phosphorylation and activation of glycogen phosphorylase *b*. The holoenzyme of this kinase is a tetramer of $\alpha\beta\gamma\delta$ protomers [for a review, see Pickett-Gies and Walsh (1986)]. The γ-subunit is catalytic and is homologous to the catalytic domains of most other protein kinases (Krebs, 1986; Hanks et al., 1988; Hanks, 1991). The α-, β-, and δ-subunits are regulatory, with δ being an intrinsic molecule of tight-binding calmodulin.

In 1978, Tabatabai and Graves reported the kinetic mechanism of the holoenzyme. In that study the tetradecapeptide SDQEKRKQISVRGL, hereafter referred to as S-peptide,[1] was used as the phosphoryl acceptor instead of phosphorylase *b*, thus avoiding complications caused by the binding of adenine nucleotides and divalent cations to phosphorylase [for a review, see Graves and Wang (1972)]. This peptide corresponds to the convertible region of the N-terminus of phosphorylase *b*, in which the single site of phosphorylation by phosphorylase kinase is Ser-14 of phosphorylase, which corresponds to Ser-10 of the S-peptide. Previous work with this peptide had shown that it was an acceptable alternative model substrate for the phosphorylase kinase holoenzyme in that it mimicked phosphorylase *b* in its phosphorylation by the kinase, e.g., identity of Ser phosphorylated, pH and Ca^{2+} dependence, and hysteresis in the rate of product formation (Tessmer & Graves, 1973; Carlson et al., 1975). On the basis of steady-state initial velocity studies and the use of substrate analogues as competitive inhibitors, Tabatabai and Graves (1978) concluded that the kinetic mechanism for phosphorylation of S-peptide by the kinase holoenzyme was random Bi-Bi.

At the time that this earlier kinetic study was performed, it was not known that phosphorylase kinase has at least one additional adenine nucleotide binding site on its β-subunit distinct from its catalytic site on the γ-subunit. The existence of this allosteric site is evident from affinity labeling (Gulyaeva et al., 1977; King & Carlson, 1982; King et al., 1982), direct binding studies (Cheng et al., 1985, 1988), and the ability of low concentrations of ADP to stimulate the ATP-dependent phosphotransferase reaction (Cheng et al., 1985; Cheng & Carlson, 1988). Moreover, ATP also binds tightly to this allosteric site (Cheng et al., 1988; Cheng & Carlson, 1988). Posing additional complications, affinity labeling evidence suggests that there may be a second adenine nucleotide binding site on the β-subunit (Gulyaeva et al., 1977), and it has been argued that the α-subunit may also have a binding site for ATP (Zaman et al., 1989). Because of these reports of additional nucleotide binding sites on the regulatory subunits, which could potentially complicate initial velocity patterns and their interpretations, we decided that a reinvestigation of the kinetic mechanism of phosphorylase kinase was in order.[2] To elim-

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[1] Abbreviations: BSA, bovine serum albumin; S-peptide, Ser-Asp-Gln-Glu-Lys-Arg-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu; P-peptide, S-peptide phosphorylated at Ser-10; D-peptide, Asp substituted at position 10 of S-peptide; AMPPNP, 5′-adenylyl imidodiphosphate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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inate from our system all known nucleotide-binding sites other than the catalytic site, we have used as enzyme the isolated catalytic γ-subunit (activated by calmodulin) with the S-peptide previously employed by Tabatabai and Graves (1978) as phosphoryl acceptor. This report also further characterizes the renaturation and activation of the γ-subunit by calmodulin, compares the influence of free Mg^{2+} on the activities of the γ-subunit and γ-calmodulin complex, and characterizes the S-peptide as an alternative model substrate for the γ-calmodulin complex.

Experimental Procedures

Proteins and Enzymes. Bovine brain calmodulin (P2277) and BSA (A9647) were from Sigma. Nonactivated phosphorylase kinase was purified from the white skeletal muscle of New Zealand White rabbits through the DEAE-cellulose step (Cohen, 1973), dialyzed against 50 mM Hepes, 10% sucrose, 0.2 mM EDTA, and 1 mM dithiothreitol, pH 6.8, and stored frozen at −80 °C until used. The γ-subunit of phosphorylase kinase was isolated according to Paudel and Carlson (1987). Phosphorylase *b* was isolated from rabbit skeletal muscle as described (Fischer & Krebs, 1958), and residual AMP was removed with activated charcoal (Sigma, C-4386).

Peptides. The synthetic tetradecapeptide substrate corresponding to the convertible region of phosphorylase *b* (S-peptide, SDQEKRKQISVRGL) was a gift of Dr. Thomas J. Fitzgerald of St. Jude Children's Research Hospital, Memphis, TN, and also was prepared by the Molecular Resource Center of the University of Tennessee, Memphis. D-Peptide, which possesses aspartic acid at position 10 of S-peptide, was prepared by the Macromolecular Structure Analysis Facility of the University of Kentucky. These peptides were further purified over a Cm-cellulose (Whatman, CM-52) column equilibrated with 10 mM ammonium acetate, pH 5.5, and eluted with a 0.0–0.3 M NaCl gradient in the same buffer. We found the cation-exchange chromatography to be much more effective than reversed-phase HPLC in purification of these basic, hydrophilic peptides. The amino acid compositions of the purified S- and D-peptides, determined respectively by Dr. Thomas J. Lukas of Vanderbilt University and by the Harvard Microchemistry Facility, Cambridge, MA, were entirely as predicted, as was the mass of the D-peptide in mass spectrometry, also performed at the Harvard facility.

The phosphorylated tetradecapeptide (P-peptide) at Ser-10 was synthesized enzymatically using phosphorylase kinase in a reaction mixture of S-peptide (1 mM), ATP (5 mM), Mg^{2+} (15 mM), Ca^{2+} (0.2 mM), EGTA (0.1 mM), dithiothreitol (0.1 mM), Hepes (100 mM, pH 8.2), and phosphorylase kinase (2.6 mg) in a volume of 120 mL at 30 °C for 4 h. The product phosphopeptide was separated from ATP, ADP, and unreacted S-peptide using Cm-cellulose column chromatography. ATP and ADP did not bind to the Cm-cellulose column, whereas phosphopeptide, identified by carrying out the reaction with [γ-^{32}P]ATP, bound less tightly than unreacted S-peptide. The purified phosphopeptide was desalted on a Sephadex G-15 column to remove salt and any adventitiously bound nucleotide and then lyophilized.

Determination of Protein and Peptide Concentrations. The concentrations of bovine brain calmodulin and the γ-subunit of phosphorylase kinase were determined using the Bio-Rad protein assay (Bio-Rad) with BSA as the standard. Concentrations of phosphorylase kinase and phosphorylase *b* were determined spectrophotometrically using the appropriate absorbance indexes (Cohen, 1973; Kastenschmidt et al., 1968). The concentration of S-peptide was determined by amino acid analysis. We found that the concentrations of S-peptide determined by amino acid analysis or by spectrophotometric absorbance at 210 nm using BSA as standard were equivalent within experimental error (±10%). Consequently, the spectrophotometric method was used to determine the concentrations of stock solutions of D-peptide and P-peptide.

Renaturation of the γ-Subunit. (*A*) *As the γ-Calmodulin Complex.* The isolated γ-subunit in 8 M urea, 0.1 M H_3PO_4, and 0.1 mM EDTA, pH 3.3, was renaturated according to the method of Kee and Graves (1986) with some modifications, paying particular attention to maintaining the low temperature and the presence of BSA. The standard renaturation mixture of 100-μL volume at 0 °C contained the following: γ-subunit (10 μg/mL), calmodulin (100 μg/mL), BSA (1.66 mg/mL), urea (0.8 M), H_3PO_4 (10 mM), EDTA (0.1 mM), Ca^{2+} (0.5 mM), dithiothreitol (0.3 mM), and Hepes (90 mM, pH 8.0). The urea and phosphoric acid were carried over with the isolated γ-subunit. For the kinetic studies shown in Figures 2 and 3, as well as in related kinetic studies, the concentrations of γ and calmodulin in the renaturation were doubled. Any other variations from the standard renaturation mixture are noted in the appropriate figure legends. Maximal activity was achieved after 5 h of renaturation and was stable for up to 4 days, after which the activity declined. The isolated γ-subunit in 8 M urea was routinely renatured at 0 °C overnight in the standard renaturation mixture. The γ-subunit renatured in this manner had a molar specific activity at pH 8.2 from 25 to 75% of that of the γ-subunit within the phosphorylase kinase hexadecameric holoenzyme, which is similar to the specific activity observed by Kee and Graves (1986) for the renatured γ-calmodulin complex. When the otherwise standard renaturation was carried out in the absence of dithiothreitol, activity was not observed in subsequent assays.

As Kee and Graves (1986) discussed, the maintenance of low temperature during the renaturation process seems to allow the unfolded γ-subunit to refold efficiently in the presence of Ca^{2+}/calmodulin. When the concentration of urea in which the γ-subunit was stored prior to renaturation was decreased from 8 M to less than 5.8 M with no change in the concentrations of H_3PO_4 or EDTA, renaturation failed to occur. When the isolated γ-subunit in 8 M urea, 0.1 M H_3PO_4, and 0.1 mM EDTA, pH 3.3, was dialyzed stepwise against subsequent 6, 4, and 2 M urea solutions, again with no change in the H_3PO_4 or EDTA concentrations, the γ-subunit precipitated at a concentration of urea less than 4 M but greater than 2 M.

(*B*) *As the Free γ-Subunit.* When the γ-subunit was renatured in the absence of calmodulin, as in the experiment shown in Figure 1, the components in the 55-μL renaturation mixture were γ-subunit (0.42 mg/mL), BSA (1.4 mg/mL), urea (0.73 M), dithiothreitol (9.3 mM), H_3PO_4 (9 mM), EDTA (9 μM), and Hepes (80 mM, pH 8). Renaturation was carried out for 24 h at 0 °C.

Enzymatic Activity Assays. The assays relied on the incorporation of ^{32}P into phosphorylase *b* or S-peptide using phosphocellulose paper and the washing method of Cook et al. (1982). The individual data points shown in Figures 1–3 are the average of simultaneous duplicate assays. All experiments were performed at least twice, each time with duplicate assays, and those experiments shown in Figures 1 and 2 were performed at least 3 times.

(*A*) *γ-Calmodulin Complex.* The renatured γ-calmodulin complex was diluted 10-fold with buffer (100 mM Hepes, 0.2

[2] A preliminary report of this work has been published in abstract form (Farrar & Carlson, 1988).

mM dithiothreitol, 0.5 mM Ca^{2+}, 0.1 mM EDTA, and 1 mg/mL BSA, pH 7). One part of this diluted mixture was added to 3 parts of the assay mixture to start the reaction. The final concentrations in the standard assay were γ-subunit (0.25 μg/mL), calmodulin (2.5 μg/mL), BSA (0.3 mg/mL), Ca^{2+} (0.5 mM), Mg^{2+} (8.3 mM), [γ-^{32}P]ATP (0.11 mM), S-peptide (24 μM), EDTA (25 μM), EGTA (75 μM), dithiothreitol (0.2 mM), urea (20 mM), H_3PO_4 (0.25 mM), and Hepes (110 mM, pH 7.0). The urea and phosphoric acid were carried over with the isolated γ-subunit. The reaction was carried out at 30 °C, and P-peptide formation was quantified using the above phosphocellulose paper assay. Any deviations from this standard assay are noted in the appropriate figure legends.

The assays for the initial velocity, product, and dead-end inhibition studies were performed slightly differently. The γ-calmodulin complex was renatured using the standard renaturation mixture except that the amounts of the γ-subunit and calmodulin were increased to 20 and 200 μg/mL, respectively. The renatured complex was diluted prior to assay and assayed according to the standard protocol described above for the γ-calmodulin complex, except, of course, that the concentrations of substrates were systematically varied and the concentrations of γ and calmodulin were doubled over the standard assay. For the product inhibition by ADP, the concentration of ADP was varied from 18 to 150 μM with ATP concentration varied from 27 to 214 μM (S-peptide fixed at 165 μM) and with S-peptide concentration varied from 21 to 165 μM (ATP concentration fixed at 124 μM). For product inhibition by P-peptide, the concentration of P-peptide was varied from 0.7 to 10.4 mM with ATP concentration varied from 12.5 to 100 μM (S-peptide concentration fixed at 61 μM) and with S-peptide concentration varied from 50 to 400 μM (ATP concentration fixed at 25 μM). For the dead-end inhibition study with AMPPNP (Boehringer Mannheim Biochemicals), the concentration of AMPPNP was varied from 0.2 to 0.8 mM with ATP concentration varied from 12.5 to 100 μM (S-peptide concentration fixed at 150 μM) and with S-peptide concentration varied from 50 to 400 μM (ATP concentration fixed at 25 μM). In the assay mixtures, virtually all of the ATP and ADP were in the form of their Mg^{2+} chelates (O'Sullivan & Smithers, 1979). The assay reaction mixtures were preincubated for 2 min at 30 °C before the reactions were started with the γ-calmodulin complex and were carried out at 30 °C for 6 min. Product formation was linear with time and amount of enzyme, and less than 2% of the limiting substrate was consumed during the reactions.

(*B*) *Free γ-Subunit.* The renatured γ-subunit was initially diluted 2-fold with a buffer containing 0.2 mM dithiothreitol/100 mM Hepes (pH 8). One part of this diluted renaturation mixture was then added to three parts of assay mixture to start the reaction. The final concentrations in the assay at 30 °C were γ-subunit (53 μg/mL), BSA (0.17 mg/mL), urea (90 mM), EGTA (33 μM), dithiothreitol (1.7 mM), Hepes (115 mM, pH 8), [γ-^{32}P]ATP (50 μM), and S-peptide (2.43 mM).

Analyses of Kinetic Data. Rate data were initially plotted in double-reciprocal fashion to visually estimate the form of the rate equation being followed. The data were then analyzed using the FORTRAN programs described by Cleland (1979), which make weighted least-squares fits to the appropriate equations. If a question existed as to which equation was most appropriate, fits were made to each possible equation, and the standard errors of the generated kinetic constants and the residual variances were compared to determine the best fit. The initial velocity data were fitted to the equation for a two substrate sequential mechanism:

$$v = VAB/(K_aB + K_bA + AB + K_{ia}K_b) \quad (1)$$

Product and dead-end inhibition data were fitted to the equations for competitive inhibition (eq 2) or noncompetitive inhibition (eq 3). The definitions of the various kinetic

$$v = VA/[K_a(1 + I/K_{is}) + A] \quad (2)$$

$$v = VA/[K_a(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (3)$$

constants used in the above equations and in Table I are as follows: K_a and K_b are the Michaelis constants for substrates A and B, respectively, K_{ia} and K_{ib} are the dissociation constants for A and B from their respective binary complexes with the enzyme, and K_{is} and K_{ii} are inhibition constants that represent the values of I that double the slope and intercept terms, respectively, in double-reciprocal plots (Cleland, 1963).

Results and Discussion

Characterization of S-Peptide as an Alternative Substrate for the γ-Calmodulin Complex. It has been previously shown that the tetradecapeptide (S-peptide) corresponding to the convertible region of phosphorylase *b* mimics phosphorylase *b* in its reaction with the phosphorylase kinase holoenzyme. The phosphorylation of either substrate by phosphorylase kinase showed a similar pH dependence, Ca^{2+} requirement, lag in the rate of product formation, and sensitivity to activation state of the kinase, plus the same seryl residue was phosphorylated in each substrate (Tessmer & Graves, 1973; Carlson et al., 1975). With the kinase holoenzyme at pH 8.2, the K_m value for the S-peptide was 4.4-fold greater than that for phosphorylase, and the V_{max} with the peptide was 19% of that with phosphorylase (Tessmer et al., 1977). The V_{max}/K_m ratio was 23 times greater for phosphorylase than for the peptide. In order to perform kinetic studies using the S-peptide as a substrate for the isolated catalytic subunit of phosphorylase kinase, it was first necessary to prove that the S-peptide also mimicked phosphorylase *b* in this simpler system.

When the catalytic γ-subunit was renatured with increasing amounts of calmodulin, its abilities to phosphorylate phosphorylase *b* and S-peptide increased in parallel, with approximately 10 μM calmodulin producing half-maximal phosphorylation with either substrate. Unlike the phosphorylase kinase holoenzyme, there was no lag in the time course of either phosphorylase or S-peptide phosphorylation by the free γ-subunit or by the γ-calmodulin complex. Peptide maps of tryptic digests of S-peptide phosphorylated by the phosphorylase kinase holoenzyme and by the γ-calmodulin complex were identical when analyzed by high-voltage electrophoresis and autoradiography, indicating that the γ-calmodulin complex phosphorylates the same seryl residue in S-peptide as does phosphorylase kinase. Finally, with the γ-calmodulin complex at pH 8.2, we found that the K_m value for the S-peptide was 10.2-fold greater than that for phosphorylase, whereas the V_{max} with the peptide was 67% of that with phosphorylase. With the γ-calmodulin complex, the V_{max}/K_m ratio was 15 times greater for phosphorylase than for the peptide, which is equivalent to the results cited above that were previously determined for the holoenzyme acting on the two substrates (Tessmer et al., 1977). The sum of these data indicates that the S-peptide is as good an alternative substrate for the γ-calmodulin complex as it is for the phosphorylase kinase holoenzyme.

Mg^{2+} Requirements. With the phosphorylase kinase holoenzyme, free Mg^{2+} ions are stimulatory in that they enhance activity when included in assays at a concentration exceeding

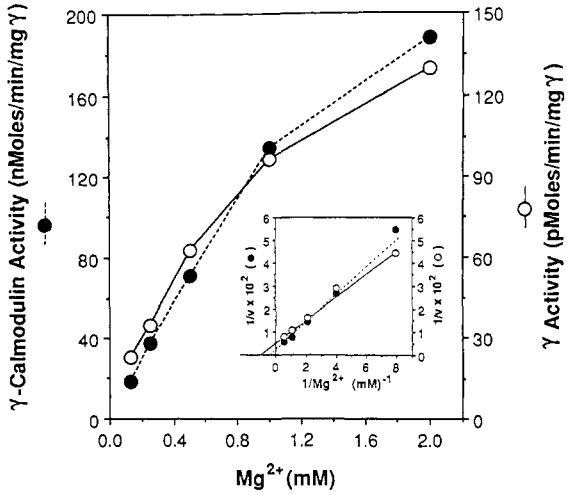

FIGURE 1: Stimulation by Mg^{2+} of S-peptide phosphorylation by γ and γ-calmodulin at 30 °C and pH 8. The free γ-subunit and the γ-calmodulin complex were renatured according to the standard renaturation protocols, except that in the case of the complex the concentrations of γ and calmodulin were doubled over the standard. The phosphorylation assays with the γ-calmodulin complex (●) were carried out for 6 min and included γ-subunit (0.5 μg/mL), calmodulin (5.1 μg/mL), [γ-^{32}P]ATP (50 μM), and S-peptide (0.4 mM); all other reaction components were as described for the standard activity assay for the complex, except that the pH was 8 instead of 7. The phosphorylation assays with the free γ-subunit (○) were carried out for 79 min as described under Experimental Procedures. Because the γ-calmodulin complex has a much greater catalytic activity than the free γ-subunit, it was necessary to use different concentrations of γ and S-peptide, as well as different times, for the two assays. The concentrations of Mg^{2+} in the assays as $MgCl_2$ are indicated on the abscissa. The amount of Mg^{2+} necessary for half-maximal activity was determined by nonlinear regression using the Enzfitter program (Elsevier-Biosoft). Inset: Double-reciprocal plot of the data.

that of ATP (Chelala & Torres, 1968; Villar-Palasi & Wei, 1970; Clerch & Huijing, 1972; Kilimann & Heilmeyer, 1982; Singh et al., 1982), activate the enzyme synergistically with Ca^{2+} in a time-dependent manner during incubations prior to assays (King & Carlson, 1981), and cause conformational changes associated with activation to occur in the β-subunits (Trempe & Carlson, 1987). It is not known which subunit, or subunits, is responsible for binding Mg^{2+} and initiating these stimulatory effects. It is known that calmodulin has the ability to bind Mg^{2+} and that Mg^{2+} induces new Ca^{2+}-binding sites within the holoenzyme (Kilimann & Heilmeyer, 1977); however, it is far from clear whether Mg^{2+} exerts all, or any, of its stimulatory effects by binding to the enzyme's δ-subunit (calmodulin).

To gain insight into this question, we compared the Mg^{2+} requirements of the free γ-subunit and γ-calmodulin complex. The S-peptide was used as substrate for these assays because phosphorylase *b* also binds Mg^{2+} (Graves & Wang, 1972). As is shown in Figure 1, both the γ-subunit and the γ-calmodulin complex were activated by concentrations of Mg^{2+} well in excess of the concentration of ATP, which was only 50 μM in this particular experiment. The amount of Mg^{2+} required to give half-maximal activity of γ and γ-calmodulin was 1.1 and 2.7 mM, respectively. Stimulation of the free γ-subunit by 11 mM Mg^{2+} was not influenced by the addition of 0.5 mM Ca^{2+} (data not shown). A similar activating effect of Mg^{2+} on the isolated γ-subunit using phosphorylase as substrate has been previously observed by Kee and Graves (1987). Because the free γ-subunit is activated by Mg^{2+} and because the Mg^{2+} stimulation profiles for γ and γ-calmodulin are similar (Figure 1), it is possible that activation by Mg^{2+} of the holoenzyme is effected, at least in part, through the γ-subunit. This

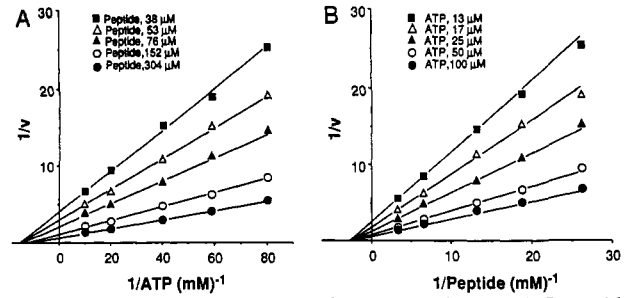

FIGURE 2: Initial velocity patterns for γ-calmodulin with S-peptide and ATP concentrations varied. (A) ATP as the variable substrate and S-peptide as the fixed variable substrate; (B) S-peptide as the variable substrate and ATP as the fixed variable substrate.

conclusion must remain tentative because the possibility of indirect effects mediated through quaternary interactions causes an inherent risk, especially in questions concerning regulation, in predicting the properties of a particular subunit within a holoenzyme from the behavior manifested by the free, isolated form of that same subunit. It is somewhat surprising that with the catalytic subunit of cAMP-dependent protein kinase, which shares a relatively high homology with the γ-subunit, free Mg^{2+} is inhibitory rather than stimulatory (Armstrong et al., 1979; Cook et al., 1982).

Studies on the Kinetic Mechanism of the γ-Calmodulin Complex. Because the observed activity of the γ-calmodulin complex was about 3 orders of magnitude greater than that of the free γ-subunit at the various concentrations of Mg^{2+} tested in Figure 1, we elected to use the complex for all of the kinetic studies that follow. As was pointed out under Experimental Procedures, the molar specific activity at pH 8.2 of the renatured γ-subunit within the γ-calmodulin complex approaches that of the γ-subunit within the native $(\alpha\beta\gamma\delta)_4$ hexadecamer from which it was initially isolated. Several possibilities could explain the low detectable activity of free γ compared to γ-calmodulin. It is possible that the activity of the renatured γ-subunit is intrinsically very low but is dramatically stimulated by calmodulin. On the other hand, it is possible that γ by itself renatures to only a very slight extent and that its full renaturation requires the presence of calmodulin, perhaps serving as a template. Alternatively, elements of each of these two extremes could contribute to the difference in the measured specific activities of renatured γ and γ-calmodulin. At this time, we do not know which possibility is most responsible for the observed difference.

Double-reciprocal plots of the initial velocities of the γ-calmodulin complex at pH 7.0 with varied ATP and S-peptide concentrations gave intersecting patterns (Figure 2A,B), indicative of a sequential reaction mechanism. When the same studies were performed at pH 8.2, essentially the same pattern was obtained, but with slightly larger apparent Michaelis constants. The kinetic constants derived from these initial velocity studies are tabulated in Table I(A,B). The value of 110 μM for the K_m of MgATP at pH 8.2 agrees well with the reported value of 98 μM by Kee and Graves (1987) for the free γ-subunit acting on phosphorylase. Although in their kinetic study with the holoenzyme Tabatabai and Graves (1978) observed upward curvature at high concentrations of ATP in double-reciprocal plots with either phosphorylase or S-peptide as fixed variable cosubstrate, we observed only linear behavior with variable ATP concentration in similar plots with the γ-calmodulin complex as enzyme (Figure 2A).

In order to differentiate among the various possible bisubstrate sequential mechanisms, product inhibition studies were performed using ADP and P-peptide. ADP as product

Table I: Summary of Kinetic Data for the γ-Calmodulin Complex[a]

	substrate	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_{ia} (μM)	K_{ib} (mM)
(A) kinetic constants from initial velocity data (pH 7.0)	MgATP	0.07 ± 0.01	3.0 ± 0.3	56 ± 6	
	S-peptide	0.47 ± 0.08			0.36 ± 0.05
(B) kinetic constants from initial velocity data (pH 8.2)	MgATP	0.11 ± 0.03	4.3 ± 0.6	105 ± 8	
	S-peptide	0.68 ± 0.13			0.64 ± 0.08

	inhibitor	varied substrate	inhibition pattern	K_{is} (mM)	K_{ii} (mM)
(C) kinetic constants from product inhibition	MgADP	MgATP	competitive	0.029 ± 0.002	
	MgADP	peptide	noncompetitive	0.076 ± 0.007	0.14 ± 0.03
	P-peptide	MgATP	competitive	5.9 ± 0.4	
	P-peptide	peptide	competitive	7.1 ± 0.5	
(D) kinetic constants from dead-end inhibition	AMPPNP	MgATP	competitive	0.40 ± 0.02	
	AMPPNP	peptide	noncompetitive	0.48 ± 0.05	0.72 ± 0.16
	D-peptide	MgATP	noncompetitive	1.9 ± 0.2	4.0 ± 1.1
	D-peptide	peptide	competitive	1.5 ± 0.1	

[a] The meaning of the various constants is defined under Experimental Procedures following the rate equations.

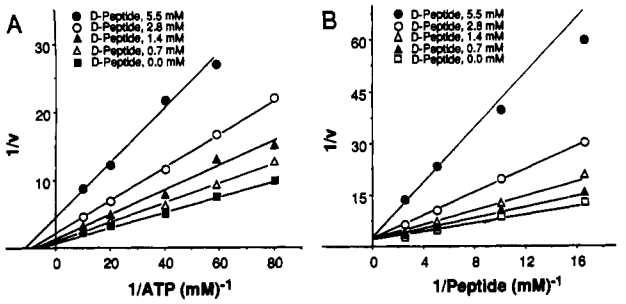

FIGURE 3: Dead-end inhibition by D-peptide of the γ-calmodulin complex. (A) ATP as the variable substrate with the concentration of S-peptide fixed at 150 μM. Insufficient D-peptide was available to determine the velocity at the highest concentration of D-peptide and lowest concentration of ATP. (B) S-Peptide as the variable substrate with the concentration of ATP fixed at 25 μM.

inhibitor showed competitive inhibition versus ATP and noncompetitive inhibition versus S-peptide. When P-peptide was used as the product inhibitor, it was found to be competitive with respect to both ATP and S-peptide. The apparent kinetic constants derived from these product inhibition studies are listed in Table I(C). The competitive K_i for MgADP of 29 μM is half that reported by Kee and Graves (1987) for inhibition of phosphorylase conversion using the free γ-subunit. The product inhibition data are consistent with a rapid equilibrium random mechanism with an abortive ternary complex of enzyme–peptide–ADP (Segel, 1975).

To substantiate this kinetic mechanism, competitive inhibitors were used to study substrate binding order (Fromm, 1979). For the rapid equilibrium random mechanism, a competitive inhibitor for either substrate will act as a noncompetitive inhibitor for the other, whereas for the ordered mechanism, a competitive inhibitor for the first substrate to add will be noncompetitive relative to the second substrate, and a competitive inhibitor for the second substrate will be uncompetitive with respect to the first. The substrate analogues that we elected to use as competitive inhibitors were AMPPNP and D-peptide, in which the convertible seryl residue was replaced with an aspartyl group. Although the interactions within the active site of peptide analogue competitive inhibitors may not always be as predictable as those that occur with less complex competitive inhibitors (Whitehouse et al., 1983; Salerno et al., 1990), the results that we obtained with this pair of competitive inhibitors did corroborate our conclusion derived from the product inhibition data. We found that AMPPNP was competitive against ATP and noncompetitive against S-peptide and, likewise, that D-peptide was competitive against S-peptide and noncompetitive against ATP (Figure 3). The apparent kinetic constants derived from these dead-end inhibition studies are listed in Table I(D). It should be noted that the concentrations of the fixed substrates used in these dead-end inhibition studies were nonsaturating. The data obtained with these competitive inhibitors support the conclusion that the kinetic mechanism of the catalytic subunit of phosphorylase kinase is rapid equilibrium random bi-bi. This conclusion is in agreement with that obtained for the holoenzyme by Tabatabai and Graves (1978) relying on initial velocity and dead-end inhibition data. Apparently the additional nucleotide binding sites on the holoenzyme did not obscure the kinetic behaviour of the enzyme's catalytic γ-subunit. It may be relevant, however, that the upward curvature observed at high concentrations of MgATP in Lineweaver–Burk plots with the holoenzyme (Tabatabai & Graves, 1978) was not observed in this study with the γ-calmodulin complex.

The kinetic behavior of several protein kinases that are homologous to the catalytic domain of the γ-subunit has also been studied in detail. For myosin light-chain kinase, Geuss et al. (1985) proposed that the forward reaction, product inhibition, and reverse reaction data were, as with the γ-subunit, most consistent with a rapid equilibrium random Bi-Bi mechanism with a dead-end complex of enzyme–ADP–light chain. For the type II calmodulin-dependent protein kinase, Kwiatkowski et al. (1990) have reported that for both the forward and reverse reactions the kinetic mechanism is ordered Bi-Bi with nucleotides binding first to the free enzyme. For the forward reaction of the catalytic subunit of cAMP-dependent protein kinase, both a random mechanism (Cook et al., 1982; Kong & Cook, 1988) and an ordered mechanism with nucleotide binding first (Whitehouse & Walsh, 1983; Whitehouse et al., 1983; Salerno et al., 1990) have been proposed. Thus, for these four homologous protein kinases, the kinetic mechanism of their reactions in the physiological direction is sequential (either ordered or random) and not ping-pong.

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