Baculovirus-directed expression of the γ -subunit of phosphorylase kinase: purification and calmodulin dependence

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A recombinant baculovirus containing a cDNA encoding the γ -subunit of phosphorylase kinase from mouse skeletal muscle was constructed. Cultures of Sf-9 insect cells infected with the γ -baculovirus produce an intact and soluble γ -protein. A purification procedure is presented that yields a sample of γ -protein which is devoid of interfering enzyme activity and which is not associated with calmodulin from the insect cells. The isolated γ sample has a K_m for phosphorylase *b* of $36 (\pm 6, \text{S.E.M.}) \mu\text{M}$ at pH 8.2 and $140 (\pm 25) \mu\text{M}$ at pH 6.8. These values are similar to those reported for the activated phosphorylase kinase holoenzyme isolated from skeletal muscle tissue. However, the V_{max} of the baculovirus-expressed γ is 65 and 80% of that of the activated holoenzyme at pH 6.8 and 8.2 respectively. These

results indicate that one or more of the regulatory subunits α , β , or calmodulin stimulate the activity of the catalytic subunit γ in the activated holoenzyme. Addition of calmodulin to the baculovirus-expressed γ stimulates its activity 1.5–2.0 fold at pH 6.8 in both the presence and absence of calcium. At pH 8.2, calmodulin has only minor stimulatory effects. The stimulation by calmodulin at pH 6.8 results from an increase in the V_{max} of γ with little effect on its K_m . This result is unlike that for most calmodulin-stimulated kinases which bind calmodulin only in the presence of calcium and exhibit a decrease in their K_m upon binding calmodulin. The change in V_{max} of γ in the presence of calmodulin and in the absence of calcium presents a novel mechanism for the regulation of a calmodulin-stimulated kinase.

INTRODUCTION

The enzyme phosphorylase kinase (PhK) (EC 2.7.1.38) regulates glycogenolysis by the phosphorylation of glycogen phosphorylase *b*, converting phosphorylase *b* to its active *a*-form. The enzyme activity of PhK is regulated by cyclic AMP-dependent protein kinase (PKA) phosphorylation and by the binding of Ca^{2+} . These two regulatory mechanisms coordinate the hormonal signal for glycogenolysis with the Ca^{2+} signal for muscle contraction. The complex interactions between the four subunits α , β , γ and δ , which comprise the PhK holoenzyme, mediate the regulation by Ca^{2+} and PKA [1]. Two of the regulatory subunits, α and β , are substrates of PKA. Their phosphorylation increases enzyme activity principally at pH 6.8 with minor effects at pH 8.2. The third regulatory subunit, δ , is the Ca^{2+} -binding protein calmodulin [2,3]. Calmodulin is an integral subunit in PhK even in the absence of Ca^{2+} and mediates the Ca^{2+} dependence for PhK enzyme activity. The γ -subunit contains the ATP-binding site, substrate-binding site, and phosphotransferase activity.

The role of each subunit in the regulation of PhK activity has been investigated by isolating the individual subunits and then assaying activity in reconstitution experiments. Initially, denaturing solvents were used to dissociate the holoenzyme isolated from skeletal muscle and to purify the individual subunits [4–6]. These investigations identified the catalytic subunit as γ and identified a complex of γ with calmodulin as mediating a Ca^{2+} dependence for activity [7–9]. Studies with the denatured-renatured subunits from the holoenzyme are hampered because the denatured γ -subunit renatures poorly unless calmodulin is present and because the subunits are not accessible to site-directed mutagenesis. An alternative approach has been to express a γ -cDNA in cultured bacteria [10,11]. However, so far, the γ expressed in bacteria cultures is found principally in inclusion bodies and requires denaturing solvents to solubilize it.

The fraction of γ protein that is not in inclusion bodies is subject to proteolysis, and the primary form that can be isolated in the absence of denaturants is truncated and no longer exhibits a calmodulin interaction.

Because of the difficulties associated with expressing γ in bacteria systems, we selected an alternative approach using cultured insect cells and the baculovirus vector. The baculovirus/insect cell culture system is well-suited for manipulating and expressing cDNAs and is notably tolerant of foreign proteins. In this paper, we report the successful expression and partial purification of a full-length γ protein with high specific activity.

MATERIALS AND METHODS

Preparation and characterization of antibodies

Antibodies made against the γ -subunit from rabbit skeletal muscle were used to follow the purification of the expressed γ -protein, to quantify the amount of γ -protein, and to assay for the presence of γ -fragments. For the preparation of these antibodies, rabbit skeletal muscle PhK was electrophoresed in the presence of SDS on polyacrylamide gels. The separated α (and its oxidative-muscle isoform α'), β and γ bands were excised, pooled, and used for primary and booster injections into a single sheep. The α/α' , β and γ subunits were fractionated by gel electrophoresis to remove the calmodulin subunit and to remove some minor contaminants contained in the original PhK preparation. From the serum fraction, the anti- α/α' , β and γ antibodies were isolated by affinity chromatography on a PhK–Sephacrose matrix. From this sample of antibodies, a fraction enriched in anti- γ antibodies was prepared by an additional affinity chromatography on a γ -Sephacrose matrix. The resulting sample of anti- γ antibodies still recognizes the α/α' and β subunits although with lower avidity than the original antibody sample. This cross-reactivity may result from shared determinants among the

Figure 1 Immunoblot of a CNBr digest of the γ -subunit

The γ -subunit was isolated from PhK and subjected to CNBr digestion. The resulting fragments were electrophoresed on 18% (w/v) polyacrylamide gels in the presence of SDS and processed for immunoblotting with anti- γ antibodies. The positions of the 17.0 and 12.0 kDa molecular mass fragments generated by the CNBr digestion are illustrated.

subunits, but the possibility that anti- α and - β antibodies remain in the preparation cannot be disregarded. The PhK for these procedures was prepared following published procedures including DE-52 chromatography [1,12]. The γ -subunit was prepared from this PhK by molecular-sieve chromatography on Sephacryl S-300 (Sigma Chemical Co.) in the presence of SDS. The affinity matrices were prepared with crosslinked Sepharose-4B and activated with CNBr.

To determine whether the anti- γ antibodies recognized both N- and C-terminal determinants, we prepared an immunoblot of CNBr-digested γ . All the methionine residues in γ occur between amino acids 106 and 242. After CNBr digestion, the largest fragments are an N-terminal fragment (residues 1–106) with a molecular mass of 12.0 kDa and a C-terminal fragment (residues 243–387) with a molecular mass of 17.0 kDa [13]. All other fragments have a molecular mass of < 4.0 kDa and are not resolved in the polyacrylamide gel. The immunoblot of CNBr-digested γ is illustrated in Figure 1. The anti- γ antibodies recognize both the 12.0 and the 17.0 kDa molecular mass fragments. The 12.0 kDa fragment was electrophoretically transferred to an Immobilon-P membrane (Millipore), and the first 10 residues were sequenced to confirm that it was the N-terminal fragment. This demonstrates that the anti- γ antibodies are an effective reagent with which to detect the presence of potentially active γ -fragments that have lost their C-terminal calmodulin-binding domain.

The amount of γ in the extracts from infected Sf-9 cells was determined by immunoblotting using PhK as a standard and following published procedures [14]. The immunoblots were developed using peroxidase-conjugated anti-sheep antibodies and developed with the 'Renaissance' chemiluminescence reagents (Dupont NEN).

Kinase assay

In order to determine the enzymic activity of the baculovirus-expressed γ , a standard assay was used which measured $^{32}\text{PO}_4$ incorporation into phosphorylase *b* by trichloroacetic acid precipitation on filter paper [15]. In the absence of added calmodulin, either CaCl_2 (0.1 mM) or EGTA (0.1 mM) was included in the assay buffer with no significant difference. Incubations of 10 min were used but incorporation of $^{32}\text{PO}_4$ was linear up to 20 min.

Assays were performed at pH 6.8 or 8.2 as indicated. Phosphorylase *b* was isolated from rabbit skeletal muscle by published procedures [16] with an additional chromatography step on DE-52. The sample of phosphorylase was treated with activated charcoal and then dialysed against 25 mM Tris buffer, pH 7.6, containing Chelex-100 resin (Bio-Rad). After dialysis against Chelex, the sample was divided in half, and each half was dialysed against 25 mM Tris, 1.0 mM dithiothreitol (DTT), 0.1 mM EDTA, pH 6.8 or 8.2, for subsequent assay at the corresponding pH. For assays investigating the effects of calmodulin addition, the calmodulin sample was purified from bovine brain by chromatography on phenyl-Sepharose [17]. To assess the effects of calmodulin in the presence and absence of calcium, aliquots of calmodulin were dialysed against Chelex and then dialysed against either 0.1 mM CaCl_2 or 0.1 mM EGTA for later addition to assays at the corresponding condition.

Immunoprecipitation

For the immunoprecipitation of γ -protein, the anti- γ antibodies were crosslinked to Affi-Prep beads following the manufacturer's recommended procedure (Bio-Rad). In parallel, a sample of non-immune sheep IgG (Sigma Chemical Co.) at the same concentration as the anti- γ antibodies was crosslinked to an aliquot of the Affi-Prep beads. Both preparations of beads were washed in IP buffer (10 mM Tris, 50 mM NaCl, 0.05% (v/v) Tween-20, 0.5 mg/ml BSA, 1.0 mM DTT, pH 8.2). The sheep IgG Affi-Prep beads and the anti- γ beads were mixed in tubes in various proportions such that the concentration of anti- γ antibodies in each tube varied but the concentration of total IgG and the amount of Affi-Prep beads in each tube were approximately equal. The beads were concentrated by centrifugation, and were then suspended in IP buffer containing an aliquot of the baculovirus-expressed γ . After 1 h incubation at 4 °C, the beads were collected by centrifugation and equal volumes of the supernatant were assayed for γ activity at pH 8.2. The activity in each supernatant was normalized to the activity in the supernatant of the sample treated with only IgG-crosslinked beads (100% activity).

Construction of recombinant γ -baculovirus

The γ -cDNA prepared from mouse skeletal muscle RNA [18] was ligated into the pVL1393 plasmid [19] kindly provided by Dr. Max Summers at the Texas Agricultural Experimental Station. The resulting recombinant plasmid contained the γ -cDNA under the transcriptional control of the polyhedrin promoter. The first ATG start codon comes from the γ -cDNA whereas the polyadenylation site comes from the polyhedrin gene. The orientation and site of insertion were confirmed by sequencing over the junction regions.

The recombinant γ -pVL1393 plasmid was co-transfected with wild-type baculovirus DNA into Sf-9 cells using Lipofectin Reagent (Life Technologies) and Grace's media. Recombinant γ -baculovirus was identified by plaque lifts and was purified by three rounds of plaque purification [20]. Insertion of the γ -cDNA into the recombinant baculovirus was confirmed in a polymerase chain reaction using the recombinant baculovirus DNA as the target DNA and using primers to both internal sequences in the γ -cDNA and to sequences bordering the 5'-junction region.

Purification procedure

Initial characterization of uninfected Sf-9 cell extracts demonstrated the presence of both an endogenous protein recognized

Figure 2 Elution profiles from the chromatography on DE-52 cellulose, Sephacryl S-300 and calmodulin-Sepharose

Each elution profile is labelled corresponding to the matrix used in the chromatography. The positions where changes in buffer composition were made to effect either a change in the stringency of washing or the elution of γ are indicated. Fractions that were pooled from each chromatography elution and used in the next purification step are indicated.

by the anti- γ antibodies and an endogenous 'PhK-like' activity. The immunoreactive protein has molecular mass of approx. 76.0 kDa (see Figure 2). It was not difficult to separate the immunoreactive protein from the expressed γ protein by the purification

procedure reported in this text. The endogenous PhK-like activity does not correspond to the 76.0 kDa immunocrossreactive protein; rather, this activity must result from some other endogenous protein in uninfected Sf-9 cells that is not recognized by the antibodies. The endogenous activity has a K_m for phosphorylase *b* of 70 μ M and has chromatographic properties similar to those of the expressed γ -protein. However, the endogenous activity is not affected by either Ca^{2+} or calmodulin. These results indicate that, unlike the γ -protein, the endogenous Sf-9 enzyme does not bind calmodulin. We used this observation to separate the endogenous activity from the expressed γ -protein by incorporating an affinity column with a calmodulin-Sepharose matrix in the following purification procedure.

For the expression of γ -protein, suspension cultures of Sf-9 cells, adapted to media containing 0.5% fetal-calf serum, were infected with the γ -baculovirus. The cells were harvested 60–65 h after infection by centrifugation. The cell pellet was suspended in a hypotonic buffer (10 mM Tris, 0.2 mM EDTA, 1.0 mM EGTA, 5.0 mM DTT, 1.0 mM MgCl_2 , 0.1 mM phenylmethanesulphonyl fluoride, 10 μ g/ml leupeptin, and 1.0 μ g/ml pepstatin A, pH 8.0) at 1/20th the volume of the suspension culture, and was allowed to swell for 10 min at 4 °C before being ruptured by 10 passes with a Dounce homogenizer. The homogenate was diluted with 0.1 vol. of 500 mM Tris, 50% sucrose, pH 8.0, and was mixed by three additional passes with the Dounce pestle. This homogenate was centrifuged at 18000 g for 20 min. The resulting supernatant was transferred to fresh tubes and was centrifuged at 100000 g for 60 min. This high-speed supernatant was brought to 80 mM NaCl by the addition of a 5.0 M NaCl stock and was then loaded on a column of DE-52 cellulose (2.5 cm $\times$ 10 cm). The column was washed with buffer A (50 mM Tris, 80 mM NaCl, 1.0 mM EGTA, 0.2 mM EDTA, 1.0 mM MgCl_2 , 1.0 mM DTT, pH 7.6), and then the γ -protein was eluted with buffer A containing 250 mM NaCl.

The pooled γ -containing fractions from the DE-52 elution were precipitated by the addition of an equal volume of 90% saturated $(\text{NH}_4)_2\text{SO}_4$, collected by centrifugation, suspended in 3.0 ml of buffer A containing 100 mM NaCl, and were dialysed against the same buffer for 1 h. After dialysis, insoluble material was removed by a brief centrifugation, and the supernatant was loaded onto a column (1.5 cm $\times$ 95 cm) of Sephacryl S-300 (Pharmacia LKB Biotechnology Inc.). The column was eluted with buffer A containing 100 mM NaCl. Intact γ elutes shortly after the exclusion volume (see Figure 2). The elution volume of the γ peak indicates a molecular mass larger than that of a γ monomer and may indicate that a γ oligomer is the major form.

Fractions containing the γ -protein from the S-300 elution were pooled and were applied directly to a 10 ml column (0.9 cm $\times$ 15 cm) of heparin-Sepharose equilibrated in buffer A containing 100 mM NaCl. The column was washed with approximately three column volumes of equilibration buffer, and the γ -protein was eluted with buffer A containing 400 mM NaCl.

The sample of γ -protein eluted from the heparin-Sepharose column was dialysed overnight against buffer B (50 mM Tris, 100 mM NaCl, 0.5 mM CaCl_2 , 1.0 mM DTT, pH 8.2). After dialysis, the sample was applied to a column (2.5 cm $\times$ 5.0 cm) of calmodulin-Sepharose and was then washed with buffer B. This wash was followed by an additional wash with buffer B containing 250 mM NaCl. The γ -protein was eluted with buffer C (50 mM Tris, 500 mM NaCl, 1.0 mM EGTA, 1.0 mM DTT, pH 6.8) (see Figure 2). The components of buffer C which are necessary to elute effectively the bound γ are the low pH, the high salt, and the EGTA. Once the γ sample is loaded on the column, it can be washed with buffer B containing EGTA in place of the CaCl_2 . Under these conditions, the γ -protein elutes from the column

very slowly as a broad peak. This result indicates that the γ sample interacts with the calmodulin–Sepharose even in the absence of Ca^{2+} , although its affinity is reduced.

The sample of γ -protein obtained from the buffer C elution of the calmodulin–Sepharose column was dialysed against 50 mM Tris, 100 mM NaCl, 1.0 mM DTT, pH 7.6, with 1.0 g of Chelex-100 (Bio-Rad) included in the dialysis buffer, outside the dialysis bag. After an overnight dialysis, the dialysis bag was removed, rinsed in water, and placed in a second beaker for overnight dialysis against 50 mM Tris, 100 mM NaCl, 1.0 mM MgCl_2 , 0.2 mM EDTA, 0.2 mM EGTA, 2.0 mM DTT, pH 7.6. The dialysate was removed, and insoluble material was collected by centrifugation. The supernatant, containing the γ -protein, was concentrated by centrifugation in a Centrprep-30 concentrator (Amicon). For storage, the concentrated sample was mixed with an equal weight of glycerol and placed at -20°C .

RESULTS

The elution profiles from the chromatography steps are illustrated in Figure 2. Figure 3 presents the immunoblots of selected samples during the purification. Evident in the immunoblot of the DE-52 column load is the γ -protein and a protein of molecular mass ~ 76.0 kDa which originates from the Sf-9 cells. Also evident are several proteins which have a molecular mass higher than that of 76.0 kDa protein and which are weakly crossreactive with the antibodies. Elution on the DE-52 cellulose column enriches for both the 76.0 kDa protein and the γ -protein. The two can be separated by elution on the S-300 column. Chromatography on the heparin–Sepharose column does not significantly change the pattern of immunoreactive proteins, but it does remove a portion of the endogenous PhK-like activity originating from the Sf-9 cells (results not shown). The remaining PhK-like activity is removed by elution on the calmodulin–Sepharose column. For the sample of γ obtained from the calmodulin–Sepharose chromatography, both an immunoblot and silver-stained gel are shown. This is the γ sample used in all subsequent assays.

The final sample of γ prepared by the purification procedure is not absolutely pure. Several other proteins evident in the silver-stained gel shown in Figure 3 have comparable abundance to γ . Nevertheless, greater than 85% of the PhK activity in the γ sample is precipitated specifically by an affinity matrix with anti- γ antibodies, as demonstrated in Figure 4. Furthermore, in the absence of the substrate phosphorylase *b*, the amount of ^{32}P incorporated into the material precipitable by trichloroacetic acid is less than 4% of the γ activity in the presence of phosphorylase *b*. These results demonstrate that the purification procedure has removed most of the PhK-like activity originating from the Sf-9 cells and that the preparation contains no significant kinase activity other than γ . Further characterization has involved using the general phosphatase inhibitor NaF at 10 mM in the activity assay, and immunoblotting the γ sample after incubation in the assay buffer to detect proteolytic fragments. Those experiments revealed no interfering phosphatase activity or protease activity in the γ sample, and demonstrate that the γ preparation is sufficiently pure to investigate the enzymic properties of the γ -protein.

Figure 5 illustrates the curves for velocity versus phosphorylase substrate concentration for the baculovirus-expressed γ at pH 6.8 and 8.2. The same curves are obtained regardless of whether CaCl_2 up to 0.2 mM or EGTA up to 0.2 mM is included in the assay. To determine the V_{max} from these data in terms of mg γ protein, the amount of γ was quantified by immunoblots using

the PhK holoenzyme as a standard and the chemiluminescence method for staining [14]. A summary of the kinetic constants for the baculovirus-expressed γ is provided in Table 1, along with the reported values for some other forms of the enzyme. Comparison of these values indicates that the K_m for phosphorylase *b* of the baculovirus-expressed γ is similar to that of the PhK holoenzyme in its activated (i.e. phosphorylated) *a*-form and is similar to that of the γ -subunit isolated from the holoenzyme by denaturation and subsequently renatured. However, the V_{max} of the baculovirus-expressed γ is approximately 10-fold greater than that of the γ -subunit isolated by denaturation. This difference indicates that denatured γ is refractile to renaturation and indicates that the baculovirus-expressed

Figure 3 Immunoblots of selected samples from the purification procedure

PhK standard (PhK-Std.) from rabbit skeletal muscle is shown in the far left-hand lane. Lane A, an aliquot of the sample loaded on the DE-52 column; lane B, an aliquot of the pooled high-salt elution from the DE-52 column; lane C, an aliquot of the sample loaded on the S-300 column; lane D, an aliquot of the sample loaded on the calmodulin–Sepharose column; lanes E, the pooled eluted sample from the calmodulin–Sepharose column and the final γ sample. Lane E on the far right is a silver-stained gel; the other lanes are all immunoblots. The positions of the α -, β - and γ -subunits are indicated, as well as the position of the 76.0 kDa molecular mass protein.

Figure 4 Immunoprecipitation of γ activity from the baculovirus-expressed γ sample

Aliquots of the baculovirus-expressed γ were incubated with the indicated volumes of anti- γ -crosslinked AffiPrep beads. In addition, non-immune IgG-crosslinked AffiPrep beads were added to each aliquot to adjust the total volume of beads to 30 μl . This addition controls for non-specific absorption to the bead matrix. After incubation, the beads were collected by centrifugation, and the supernatants were assayed for γ activity.

Figure 5 Graph of activity versus phosphorylase *b* concentration at pH 6.8 (■) and at pH 8.2 (▲)

The data are fitted to the Michaelis–Menten equation.

γ is principally in an active configuration. Even though the baculovirus-expressed γ has a higher specific activity than other preparations of purified γ , it exhibits only 65 % of the specific activity of PhK_a at pH 6.8 and 80 % at pH 8.2. These differences between the activities of PhK_a and the baculovirus-expressed γ indicate either that 20–35 % of the baculovirus-expressed γ is in an inactive conformation or that the regulatory subunits α , β and calmodulin stimulate the basal activity of γ in the PhK_a holoenzyme. The second alternative implies that the activity of the baculovirus-expressed γ represents the basal activity of γ in the absence of the regulatory subunits. To investigate these possibilities, and to determine whether the baculovirus-expressed γ retains the ability to interact with calmodulin, the effect of adding calmodulin to the kinase assay was measured. Calmodulin was selected for these experiments because it is known to enhance the renaturation of denatured γ [9] and to increase its specific activity.

Because protein renaturation is generally a time-dependent process, the effect of calmodulin on γ activity was measured at various times. The γ sample was preincubated at 4 °C in the standard assay buffer supplemented with calmodulin. Aliquots were removed at various times, and the kinase reaction was

Figure 6 Changes in γ activity with varying times of preincubation with calmodulin

Aliquots of the γ sample were preincubated at the indicated times with 2.0 μ M calmodulin and with EGTA (○) or calcium (●) before assaying for kinase activity.

initiated by shifting the temperature to 30 °C and adding ATP with magnesium acetate. The results of this experiment at pH 6.8 with 2.0 μ M calmodulin in the presence and absence of calcium are illustrated in Figure 6. Calmodulin does stimulate γ activity under these conditions in a time-dependent manner. Maximum stimulation occurs after 4–5 h preincubation, and there is a slow decrease in activity after 6 h preincubation, particularly in the absence of calcium. At pH 8.2, in the presence or absence of calcium, there is only a marginal stimulation of γ activity ($\leq 10\%$) by calmodulin (results not shown). Because of the time dependence for calmodulin stimulation, subsequent experiments to determine concentration dependence and changes in the kinetic constants were done using a 5 h preincubation before initiation of the kinase reaction.

The dependence of γ activity on calmodulin concentration was determined at pH 6.8 in the presence and absence of calcium. The results are illustrated in Figure 7. In the presence of calcium, calmodulin stimulates γ activity approx. 2-fold. In the absence of calcium, the stimulation is slightly less, 1.5–2-fold. The biggest difference between the presence and absence of calcium is the concentration of calmodulin necessary for half-maximal activation ($A_{0.5}$). In the presence of calcium, $A_{0.5} = 3.5 \mu$ M whereas in the absence of calcium, $A_{0.5} = 9.2 \mu$ M. Differences in the

Table 1 Published kinetic constants for PhK in the presence of Ca²⁺ and their comparison with those of the γ -subunit isolated by various procedures

The kinetic constants for the ' γ baculovirus' sample are derived from the data in Figure 5 and replicate assays. Specific activity is expressed per mg of γ . The γ -subunit is 13% of the mass of the PhK holoenzyme. PhK_b is non-phosphorylated phosphorylase kinase; PhK_a is phosphorylated phosphorylase kinase. Data at pH 6.8 are from Newsholme and Walsh [21]. Data at pH 8.2 are from Kee and Graves [22], and Chan and Graves [8].

	pH 6.8		pH 8.2	
	K_m (μ M phosphorylase)	V_{max} (μ mol ³² P/min per mg)	K_m (μ M phosphorylase)	V_{max} (μ mol ³² P/min per mg)
PhK _b	67	1.2	190 ± 80	125 ± 18
PhK _a	125	73	60 ± 20	138 ± 15
γ Den/Ren*	116	6	80	8.5
γ baculovirus	140 ± 25	59 ± 10	36 ± 6	91 ± 16

* Data are from Kee and Graves [22] for γ purified by h.p.l.c. and subsequently renatured.

Figure 7 Changes in γ activity with varying calmodulin concentrations

The ordinate is the ratio of γ activity in the presence of the indicated calmodulin concentration to the activity in the absence of calmodulin. Each data point illustrated is the average of duplicates and some data from replicate assays are shown to illustrate the variability in the assay. ■, data in the presence of 0.1 mM calcium; □, data in the presence of 0.1 mM EGTA. Data were fitted to the illustrated curves by the P.Fit program (BIOSOFT, Fig. P Corp. Cambridge, U.K.). The dotted line is fitted to the data in the presence of EGTA and the solid line is fitted to the data in the presence of calcium.

Figure 8 Graph of activity versus phosphorylase *b* concentration in the presence of calmodulin

The data from three replicate assays are shown and fitted to a Michaelis–Menten curve. Each assay was performed in the presence of 8.0 μ M calmodulin and 0.1 mM CaCl_2 .

affinity of γ for calmodulin, depending on the presence or absence of calcium, have also been noted for γ prepared under denaturing conditions [23]. An interesting difference apparent in the data is that in the absence of calcium, the data fit an asymmetric sigmoid curve with a co-operativity factor of 1.5, whereas in the presence of calcium, the data fit a curve with a co-operativity factor of approx. 1.0. The co-operativity indicated in the absence of calcium suggests that the binding of calmodulin to one γ molecule increases the affinity of a neighbouring γ molecule for calmodulin. This effect may reflect the oligomerization state of the γ protein, as was also indicated by its elution on S-300. The absence of co-operativity in the presence of calcium may result from the higher affinity that γ has for calmodulin in

the presence of calcium, or it may indicate differences in the mechanism for interaction between γ and calmodulin depending on the presence or absence of calcium.

The calmodulin stimulation of γ activity cannot be fully explained by a renaturation of a portion of inactive γ . The stimulation by calmodulin occurs principally at pH 6.8, with only moderate stimulation ($\leq 10\%$) at pH 8.2. This would indicate that $< 10\%$ of the γ in the sample is in an inactive state. Furthermore, at pH 6.8, the stimulation of calmodulin in the presence of calcium results in a specific activity for γ that is slightly greater than that of the activated PhK holoenzyme. This would indicate that the interaction of calmodulin affects the kinetic properties of γ . Figure 8 presents the relationship between the velocity and phosphorylase *b* substrate concentration for the baculovirus-expressed γ at pH 6.8 in the presence of calcium and 8.0 μ M calmodulin; the results from three replicate experiments are shown. The average K_m value for the three experiments is $139 (\pm 17) \mu\text{M}$ ($\pm$ S.E.M.) and the average $V_{\max}$ is $120 (\pm 9) \text{ pmol PO}_4^{3-}/\text{min per ng of } \gamma$. This K_m value is not significantly different from that in Table 1 for the baculovirus-expressed γ in the absence of calmodulin; however, the $V_{\max}$ in the presence of calmodulin is 2-fold greater than that in the absence of calmodulin.

DISCUSSION

The observation that calmodulin can stimulate baculovirus-expressed γ even in the absence of calcium is consistent with previous reports which demonstrate a calcium-independent interaction between calmodulin and γ [5], and is consistent with the calcium-independent association of calmodulin in the holoenzyme. However, the co-operative effect on stimulation as a function of calmodulin concentration in the absence of calcium indicates that the association of γ and calmodulin may be mediated by interactions which differ in the presence and absence of calcium. The γ -protein contains two domains which, when assayed as synthetic polypeptides, bind calmodulin in the presence of calcium. Both domains are located in the C-terminal one-third of γ and are separated by only 15 amino acids [24]. Whether one or both of these domains are necessary for the calcium-independent interaction of γ with calmodulin is not known. However, the binding of calcium to calmodulin exposes a hydrophobic patch in each calmodulin head, and residues within these patches contribute to hydrophobic interactions between calmodulin and target sequences [25]. It is likely that in the absence of calcium, different residues are exposed in calmodulin. Thus γ may require the flexibility provided by two calmodulin binding sequences in order to accommodate the changes in calmodulin structure resulting from the presence or absence of calcium. Furthermore, the co-operative nature of the calmodulin interaction with γ is evidence that γ exists as an oligomer in the sample, exhibiting γ – γ interactions. This state could be representative of the holoenzyme which has the subunit structure $(\alpha, \beta, \gamma, \delta)_4$. Thus, in the holoenzyme, there is the potential for γ – γ interactions that could co-operatively interact to maintain calmodulin as an integral subunit in the absence of calcium.

Comparison of the kinetic constants in Table 1 with those from Figure 8 for the baculovirus-expressed γ with calmodulin indicates that the γ –calmodulin complex has kinetic constants similar to those of the activated holoenzyme. This finding indicates that the phosphorylated α - and β -subunits in the activated holoenzyme have little effect on the kinetic constants. However, the non-activated holoenzyme has a significantly reduced $V_{\max}$ and a moderately reduced K_m at pH 6.8. The observation that calmodulin does not induce such changes in the

baculovirus-expressed γ suggests that the non-phosphorylated α - and β -subunits are the major mediators of the decrease in $V_{\max}$ and K_m . Addition of the α - and β -subunits to a sample of denatured/renatured γ has been reported to inhibit its activity [26]. Whether the inhibition affects $V_{\max}$ and/or K_m was not reported. It is interesting that the phosphorylated α - and β -subunits were reported also to inhibit γ activity, albeit at higher concentrations. Why, then, does the γ -calmodulin complex not have a significantly higher specific activity than the activated holoenzyme? The answer to this discrepancy may be that calmodulin nullifies any inhibitory effects of the phosphorylated α - and β -subunits, perhaps by a direct interaction with those subunits or by altering their interactions with the γ -subunit. Studies with synthetic peptides of sequences in the α - and β -subunits are reported to bind calmodulin and to induce changes in the kinetic constants of the holoenzyme [27]. These studies indicate that there may be interactions between calmodulin and the α - and/or β -subunits in addition to the interactions between calmodulin and the γ -subunit. Other studies report that a sequence within the β -subunit can act as a pseudosubstrate and potentially can mediate autoinhibition of the holoenzyme [28]. The inhibitory properties of this sequence appear to be independent of the presence or absence of calmodulin.

Expression of γ in the *Bombyx mori* baculovirus system has been reported recently [14]. That system differs slightly from the one reported here and is not as accessible for large-volume cultures. However, the cell line used in the *Bombyx mori* system is reported not to have the endogenous PhK-like activity that is found in the Sf-9 cells. The sample of γ isolated from that system is reported also to have a specific activity of 60–70% of that of the activated holoenzyme, but its stimulation by calmodulin was not reported. The fact that similar specific activities for γ have been found in the two baculovirus expression systems indicates that this specific activity represents the basal activity of γ in the absence of the regulatory subunits, and thus, this system is a good source of γ for further investigations of its regulatory mechanisms.

The development of a recombinant γ -baculovirus expression system is an important step for the further investigation of the subunit interactions in PhK. The high specific activity of the γ -subunit provides a basal activity to which the regulatory subunits can be added, either singularly or in combination, to determine their role in the regulation of γ . By this approach, we can investigate the interactions which contribute to the complex regulation of the holoenzyme. Furthermore, by site-directed

mutagenesis, the determinants in γ that mediate the regulatory subunit interactions can be identified.

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