Analysis by Mutagenesis of the ATP Binding Site of the γ Subunit of Skeletal Muscle Phosphorylase Kinase Expressed Using a Baculovirus System†

Jung-Hwa Lee,‡ Susumu Maeda,§ Karen L. Angelos,‡ Shizuo G. Kamita,§ Chidambaram Ramachandran,‡,∥ and Donal A. Walsh*,‡

Department of Biological Chemistry, School of Medicine, and Department of Entomology, University of California, Davis, California 95616

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ABSTRACT: Active γ subunit of skeletal muscle phosphorylase kinase has been obtained by expression of the rat soleus cDNA in a baculovirus system. The protein exhibited the expected pH 6.8/8.2 activity ratio of 0.6, and its activity was insensitive to Ca^{2+} addition, indicating that it was free γ subunit and not a γ subunit–calmodulin complex. It was stimulated approximately 2-fold by Ca^{2+}-calmodulin addition, demonstrating that it had retained high-affinity calmodulin binding. By site-directed mutagenesis, we have examined the role of six of the amino acids that constitute the consensus ATP binding site of the protein kinase, which in the γ subunit is represented by the sequence ^{26}Gly·Arg·Gly·Val·Ser·Ser·Val·Val^{33}. Changes were evaluated by the kinetic determination of the dissociation constants of γ-ATP, γ-ADP, γ-AMP·PCP, and γ-phosphorylase and the maximum catalytic activity. The mutants Ser^{26}-γ, Ser^{29}-γ, Phe^{30}-γ, and Gly^{31}-γ each exhibited an essentially identical dissociation constant for γ subunit phosphorylase, indicating that these mutations had not caused a global alteration in the protein structure but were limited to changes in the nucleotide binding site domain. Substitution of either Val^{33} (by Gly) or Gly^{28} (by Ser), two of the most conserved residues in all protein kinases, resulted in enzyme with marginally detectable activity. In noted contrast, the Ser^{26} mutant, which substituted the first glycine of the consensus glycine trio motif, and which is also very highly conserved, retained at least 25% of the enzymatic activity. The Gly^{31} substitution, which restored a glycine to a position characteristic for most protein kinases, had little overall effect upon the maximum rate of catalysis. Restoration of Ser^{30} to the more typical phenylalanine, which is present in most protein kinases, had minimal effect on catalysis. These data provide the first direct evaluation of the roles that different residues play within this consensus glycine trio/valine motif of the protein kinases, which up to now have only been surmised to be of importance because of their conservation. Two unexpected findings are that for one residue that is very conserved (Gly^{26}) there is some flexibility of substitution not apparent from the evolutionary conservation and that a second quite conserved residue in protein kinases (equivalent to Gly at position 31) does not produce a protein optimized for nucleotide binding.

Phosphorylase kinase is a central enzyme for the regulation of glycogenolysis (Pickett-Gies & Walsh, 1986). It has a complex structure, with a subunit composition of $\alpha_4\beta_4\gamma_4\delta_4$ and a molecular mass of 1300 kDa. The sequences of all four subunits are now known (Zander et al., 1988; Kilimann et al., 1988; Reimann et al., 1984). The regulation of phosphorylase kinase involves an elaborate network of interactions. The γ subunit contains the active catalytic site, and the δ subunit is identical to calmodulin but an intrinsic part of the holoenzyme. Both the α and β subunits are phosphorylated by the cAMP-dependent protein kinase, with that of the β subunit directly regulating activity and that of the α amplifying the effects of β subunit phosphorylation (Ramachandran et al., 1987). Both the α and β subunits are multiply phosphorylated by autophosphorylation, also leading to activation (Heilmeyer, 1991; King et al., 1983). Enzyme activity is also regulated by Ca^{2+}, both by interaction with the intrinsic δ subunit and via exogenous calmodulin. There are multiple binding sites for calmodulin, at least two high-affinity sites on γ (Dasgupta et al., 1989), one high-affinity site on β, and three additional sites, two on β and one on α, that are of intermediate if not high affinity (Newsholme et al., 1992; James et al., 1991). Altered calmodulin binding to one or more of these sites is involved not only in the Ca^{2+}-dependent regulation of enzyme activity but also in the mediation of the regulation of activity due to cAMP-dependent phosphorylation of the α and β subunits (Newsholme et al., 1992). A model accounting for these interactions has been recently proposed (Newsholme et al., 1992).

It seems quite unlikely that a full understanding of the interactions that occur in the regulation of phosphorylase kinase will be obtained until the holoenzyme can be reconstructed from the individual subunits. Partial complexes (αγδ and γδ) have been obtained by LiCl-promoted dissociation (Chan & Graves, 1982a–c), and both the free γ subunit and the γδ complex have been obtained by their separation as denatured protein followed by renaturation using calmodulin as a nucleating template (Kee & Graves, 1986, 1987; Paudel & Carlson, 1987, 1988). Free skeletal muscle γ, as a fusion protein, has also been obtained from the cDNA by expression in *Escherichia coli*; the initial product was inactive but was subsequently activated using calmodulin (Chen et al., 1989). We have been successful previously in directly expressing active γ in transfected 3T3 cells that produced a combination of the free γ subunit and a γ–calmodulin complex (Cawley et al., 1989). The amounts obtained by this system have not been sufficient for any extensive characterization. In this study, we report the expression of the free γ subunit using a BmNPV

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‡ Department of Biological Chemistry.

§ Department of Entomology.

∥ Current address: Center for Therapeutic Research, Merck Frosst Canada Inc. Pointe-Claire, Quebec, Canada.

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(*Bombyx mori*, nuclear polyhedrosis virus) baculovirus system [reviewed in Maeda (1989b)] to produce active protein.

We have used this system to obtain γ with mutations around the ATP binding site. As reviewed by Hanks et al. (Hanks et al., 1988; Hanks & Quinn, 1991), all protein kinases either contain the consensus sequence of Gly-X-Gly-X-X-Gly-X-Val or are very highly conserved in each of these residues. In phosphorylase kinase γ, the sequence conserved in rat, rabbit, and mouse (Chamberlain et al., 1987; Bender & Emerson, 1987; Cawley et al., 1988; da Cruz e Silva & Cohen, 1987) is ^{26}Gly·Arg·Gly·Val·Ser·Ser·Val·Val^{33}. A sequence of multiple glycines, similar to that observed for protein kinases, is found in a wide variety of proteins that bind nucleotides (Saraste et al., 1990). This sequence has been termed the "P-loop" on the basis of the following two properties: it is involved in the binding of the nucleotide phosphates, and it serves as the connecting loop between two regions of β sheet and/or α helix. X-ray crystallographic resolution of Ras (Wittinghofer & Pai, 1991; Pai et al., 1989) and adenylate kinase (Pai et al., 1977; Dreusicke & Schulz, 1986) with bound nucleotide analogues has identified in each case the mode of binding of the nucleotide to the P-loop. Despite the general conservation of the P-loop motif, however, the precise mode of nucleotide phosphate binding appears to differ between the different nucleotide binding proteins. X-ray crystallographic resolution of the structure of the cAMP-dependent protein kinase has confirmed that the P-loop of the glycine consensus sequence of protein kinases is involved in ATP binding but has yet to identify the exact mode of binding (Knighton et al., 1991a,b).[1] The differences in the consensus sequences of the P-loops in protein kinases, versus adenylate kinase and Ras, would predict, however, that there will also be some differences in how nucleotide phosphate is bound. Although the glycine trio/valine motif is a feature of all protein kinases, and is often used as one of the "landmarks" by which to identify a sequence as a protein kinase, there has to date been no direct evidence evaluating the essentiality of the constituents of this region for protein kinase activity. In this report we have examined the role of six residues of the P-loop of phosphorylase kinase γ subunit by determination of nucleotide interactions and enzymatic activity.

EXPERIMENTAL PROCEDURES

γ Subunit Transfer Vector Construction and Expression of Recombinant Baculovirus. The recombinant transfer vector pBPK7, containing the cDNA for the γ subunit of rat soleus muscle phosphorylase kinase (Cawley et al., 1988, 1989), was prepared by introducing an insert of pUC18-6$_1$ containing the γ subunit coding region after the polyhedrin gene promoter of the transfer vector pBK283 (Maeda, 1989a). To achieve this, pBK283 was cut with *Kpn*I, filled with Klenow, and then digested with *Sac*I, pUC18-6$_1$ was digested with *BamH*I, filled with Klenow, and digested with *Sac*I, and the fragment containing the γ coding region was then purified using a low-melting agarose gel. After ligation of the two fragments to form pBPK7, it was purified using Qiagen tips (Qiagen Inc.) and then cotransfected into BmN cells with BmNPV T3 isolate using calcium phosphate precipitation as detailed by Maeda (1989a). The cells were cultured for 5 days, and the culture fluid was used for isolation of the clones of recombinant virus containing the inserted γ subunit cDNA. The cell culture medium containing the recombinant virus was diluted in TC-100 medium (Maeda, 1989b) without fetal bovine serum so as to give ~80 PFU/96-well plate. The diluted medium was used to infect 5×10^6 cells attached to a 60-mm culture dish in a total volume of 5 mL. After 1 h of infection, the cells were suspended and 50 μL of the suspension was aliquoted to each well of a 96-well plate. Viral growth in each well was examined under the microscope after 4–5 days. The supernatant of a well of cells showing cytopathic effect but no polyhedrin inclusion bodies was then selected for further isolation by replating. To validate the construct, viral DNA was isolated as described by Maeda (1989a) from confluent cell monolayers (2.5×10^7 cells/150-mm dish and 15 mL of medium) infected with recombinant virus 62–72-h postinfection. Viral DNA was isolated by differential and sucrose gradient centrifugation and phenol/chloroform extraction as described (Maeda, 1989a). Its digestion with either *BamH*I, *Eco*RI, or *Hind*III gave the expected sized bands by Southern blotting techniques. To isolate expressed γ subunit, confluent BmN cell monolayers (2.5×10^7 cells/150-mm dish) were infected with the recombinant virus at a multiplicity of 3–5 PFU/cell. After a 48-h infection, cells were collected by centrifugation and stored at −80 °C until used for enzyme preparation. Two recombinant viruses, designated BmV-γ-1 and BmV-γ-2, each containing the intact γ subunit cDNA, have been used for the studies described; each gave identical results.

Construction of Transfer Vectors Containing Phosphorylase Kinase γ Subunit with Mutations in the ATP Binding Site. The plasmid pUC18-6$_1$, containing the phosphorylase kinase γ subunit cDNA, was cut with *Hind*III and self-ligated, making pUC18H, which has the 5′ untranslated region and the N-terminal region of the γ subunit up to the *Hind*III site (Figure 1). A synthetic adapter was ligated into the *Eco*RI–*Sma*I site which regenerated the lost 5′ coding region and put a new *Bgl*II site immediately in front of the ATG translation initiation site of the γ-cDNA. The adapter-containing plasmid (pUCES) was confirmed by digestion with the restriction enzymes *Bgl*II, *BamH*I, *Eco*RI, *Ban*II, *Nsi*I, and *Hind*III, each of which indicated a single site of digestion and the correct size. pUCES was then cut with *Ban*II and *Nsi*I, and synthetic oligomers, each of which would produce a single amino acid change in the ATP binding region of the γ subunit, were inserted between the *Ban*II–*Nsi*I site so as to regenerate the sequence of that part of the γ subunit with the single amino acid substitution. This strategy and the nomenclature of the resultant plasmids and proteins, as well as the sequence surrounding the amino acid substitution that would result, are summarized in Table I. The mutated plasmids (pUCES-Gly^{31}, etc.) were sequenced to confirm the mutated sequences. The *Hind*III fragment obtained from the original pUC18-6$_1$ was then ligated back into the *Hind*III site of the mutated plasmids, generating plasmids pPK-Gly^{31}, etc., containing the full sequence of the γ subunit but with the indicated ATP binding site mutations. Complete plasmids were selected by digestion with *Xho*I, which linearized the plasmid into a 4.1-kb fragment. The orientation of the insert was confirmed by double digestion with *Bgl*II and *Xho*I, which for a correct orientation produced fragments of 1.0 and 3.1 kb. The *Bgl*II–

[1] The structural resolution of a ternary complex of cAMP-dependent protein kinase–protein kinase inhibitor peptide–ATP has so far failed to clearly identify the location of the β- and γ-phosphates of ATP; hence the precise binding of the nucleotide by P-loop residues has not been identified (Knighton et al., 1991a). Additional difficulty with interpreting these latter studies is that although ATP was the added nucleotide, the high endogenous level of ATPase activity possessed by the protein kinase, although it would have been partially blocked by PKI (Whitehouse & Walsh, 1983), may well have been sufficient to hydrolyze all the ATP to ADP. This was not examined and so the solved structure may have been for the ADP rather than ATP ternary complex.

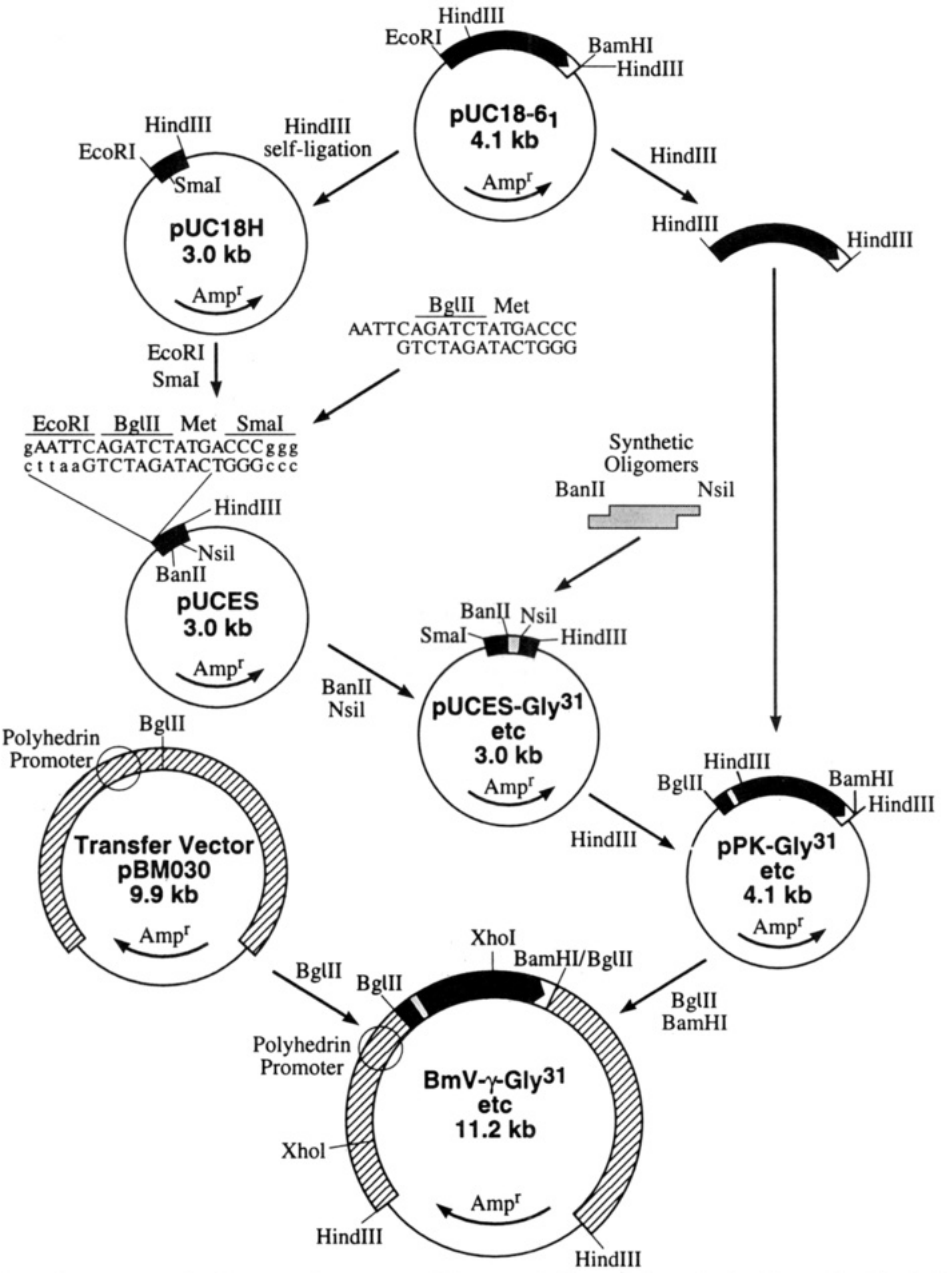

FIGURE 1: Construction of mutant γ subunit expression vectors. The strategy for use of synthetic oligonucleotides is illustrated in Table I.

*BamH*I fragment containing mutated DNA from each of these plasmids was then ligated into the *Bgl*II site of the pBM030 BmNPV transfer vector, and the correct insertion of the cDNA into the transfer vectors was confirmed by digestion with *Xho*I, which resulted in the expected fragments of 3.4 and 7.8 kb. Each of the recombinant vectors (BmV-γ-Gly^{31} etc.) was then cotransfected to get recombinant viruses, as described above with BmNPV T3 DNA, and selected by plaque assay according to Maeda (1989b). Insertion of the cDNA into the recombinant viruses was confirmed by a polymerase chain reaction of the viral DNAs using the polyhedrin gene 5′ and 3′ noncoding regions as primers. Each of the mutant viral DNAs yielded 1.5-kb fragments demonstrating that the isolated selected recombinants correctly contained the γ subunit DNA under the polyhedrin promoter. Expression of the mutant proteins in BmN cells was undertaken as described above for wild-type enzyme.

Cell Extraction and Partial Protein Purification. Harvested cells (~2.5 × 10^7) were suspended in 2 mL of an extraction buffer consisting of 20 mM Tris–HCl, pH 7.0, 0.2 mM EDTA, 45 mM β-mercaptoethanol, and 20 milliunits/mL aprotinin, also containing 2 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, and 0.1 mM tosylphenylalanine chloromethyl ketone, and then homogenized at 4 °C with a glass/Teflon homogenizer for 10 strokes. The centrifuged extract was added to 1-mL packed volume of DE52, equilibrated in the extraction buffer. After a 4-min incubation, the resin was washed twice with extraction buffer containing 0.11 M NaCl and then the enzyme was eluted with 0.72 mL of extraction buffer containing 0.5 M NaCl. This procedure resulted in a ~4-fold increase in specific activity and a near full recovery of activity. Glycerol was added to a final concentration of 50%, and the protein under these conditions maintained a stable activity for at least 5 days when stored at −20 °C.

Quantitation of Phosphorylase Kinase γ Subunit by Protein Immunoblotting. DE52 0.5 M salt eluates derived from the various BmN cell extracts (wild-type enzyme, mutants, and uninfected cells) and purified phosphorylase kinase standards were electrophoresed on 10% Laemmli gels (1970) and then

Table I: Generation and Nomenclature of γ Subunit Mutants

A. Nomenclature

recombinant viruses	sequence (23 ... 35)	protein product
BmV-γ-1 (Wild type)	.....E.I.L.G.R.G.V.S.S.V.V.R.R.....	γ
BmV-γ-Ser^{26}	.....E.I.L.**S**.R.G.V.S.S.V.V.R.R.....	Ser^{26}-γ
BmV-γ-Ser^{28}	.....E.I.L.G.R.**S**.V.S.S.V.V.R.R.....	Ser^{28}-γ
BmV-γ-Ser^{29}	.....E.I.L.G.R.G.**S**.S.S.V.V.R.R.....	Ser^{29}-γ
BmV-γ-Phe^{30}	.....E.I.L.G.R.G.V.**F**.S.V.V.R.R.....	Phe^{30}-γ
BmV-γ-Gly^{31}	.....E.I.LG.R.G.V.S.**G**.V.V.R.R.....	Gly^{31}-γ
BmV-γ-Gly^{33}	.....E.I.L.G.R.G.V.S.S.V.**G**.R.R.....	Gly^{33}-γ
Comparison Sequence of cAMP-dependent Protein Kinase	.....G.T.G.S.F.G.R.V.....	

B. Oligonucleotide Strategy for Mutation

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                BanII                                                NsiI
                CGAGCC....CAAGGAGATCCTGGGCAGGGGAGTCAGCAGCGTGGTCAGGAGATGCA....TT
γ Sequence      GC....TCGGGTTCCTCTAGGACCCGTCCCCTCAGTCGTCGCACCAGTCCTCT....ACGTAA
                      E  P  K  E  I  L  G  R  G  V  S  S  V  V  R  R

                CGAGCC....CAAGGAGATCCTGGGCAGGGGAGTCATTAGCGTGGTCAGGAGATGCA....TT
Phe30-γ Sequence GC....TCGGGTTCCTCTAGGACCCGTCCCCTCAGTAATCGCACCAGTCCTCT....ACGTAA
                      E  P  K  E  I  L  G  R  G  V  F  S  V  V  R  R
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transferred to nitrocellulose BA85 (Schleicher and Schuell) for 1 h at 0.2 A followed by 2 h at 0.5 A, in 25 mM Tris/192 mM glycine, pH 8.3/10% methanol (Towbin et al., 1979) using a Hoefer Transphor apparatus. The resulting blots were blocked for 1 h in 6% casein/50 mM HEPES, pH 6.8 ["blocking buffer" (Gillespie & Hudspeth, 1991)], prior to treatment with primary antibody. This antibody was raised in guinea pig against phosphorylase kinase $\gamma_{342-366}$ peptide (Newsholme et al., 1992), which was initially conjugated to *N*-succinimidyl *S*-acetylthioacetate (Pierce) modified ovalbumin (Sigma) using maleimidohexanoyl-*N*-hydroxysuccinimide ester (Boehringer Mannheim). The $\gamma_{342-366}$ peptide represents one of the two high-affinity calmodulin binding sites on the γ subunit (Dasgupta et al., 1989; Newsholme et al., 1992), and the sequence is absolutely conserved between rat and rabbit skeletal muscle phosphorylase kinase (Cawley et al., 1988; Reimann et al., 1984). Blots were incubated for 16 h at 4 °C in primary antibody and then for 2 h at 25 °C with biotinylated goat anti-guinea pig IgG (Sigma), followed by 30 min at 25 °C with ExtrAvidin–peroxidase (Sigma), each diluted in blocking buffer. After each treatment the membranes were washed three times for 10 min each at 25 °C in 0.1% casein/0.05% Tween-20 (Pierce)/50 mM Hepes, pH 6.8. Following a 5-min incubation in 100 mM Tris, pH 7.6/100 mM NaCl, duplicate blots were treated for 1 min with enhanced chemiluminescence reagent (ECL; Amersham) and chemiluminescence was detected using Kodak XAR-2 film with exposure times of from 2 to 20 s. The intensities of the bands corresponding to the γ subunit were quantitated relative to standards on a Model 300A computing densitometer (Molecular Dynamics). An example of such an analysis is illustrated in Figure 2. A band equivalent to the γ subunit of the pure skeletal muscle enzyme was obtained with the extracts from the BmV-γ infected cells and each of the six mutants that was totally absent in both mock infected cells and cells infected with wild-type virus without γ. The amount of γ obtained was very similar for all of the forms and only varied within a ~2-fold range. Other bands were detected with all of the cells, whether infected or not, the most prominent of which ran slightly faster than γ. These likely represent calmodulin binding proteins from the baculovirus, since the antibody was produced against one of the calmodulin binding segments of γ. These were present in about equal amounts in all cells and were not detectable with preimmune serum (not shown). This method has allowed the quantitation of the γ subunit in partially purified extracts. Between the different mutants, the amounts ranged from 0.2 to 0.4% of the total soluble protein. From this quantitation, we have been able to determine and compare the activities of the wild-type and mutant enzymes per mole of γ-protein.

Assay of Phosphorylase Kinase and Kinetic Analyses. For standard determinations, phosphorylase kinase was assayed at pH 8.2 or pH 6.8 as described previously (Angelos et al., 1987), except that the $CaCl_2$ concentration was 0.6 mM and no glycogen was added, and with 10 mM magnesium acetate, 3 mM [γ-^{32}P]ATP, and 51 μM phosphorylase *b*. Phosphorylase *b* was prepared as described previously (Sul et al., 1983). For all assays, enzyme was diluted in extraction buffer containing 12.5% glycerol/125 mM NaF.

For the determination of the kinetic constants for ATP and phosphorylase, the assays were performed at pH 7.6 with a reaction containing 28 mM HEPES/42 mM Tris–HCl/0.1 mM $CaCl_2$, with varying phosphorylase (8.6–120 μM) and varying ATP (20–360 μM), each being varied over at least an 8-fold range in any experiment. Total free Mg was maintained at 7 mM by varying Mg in proportion to the amount

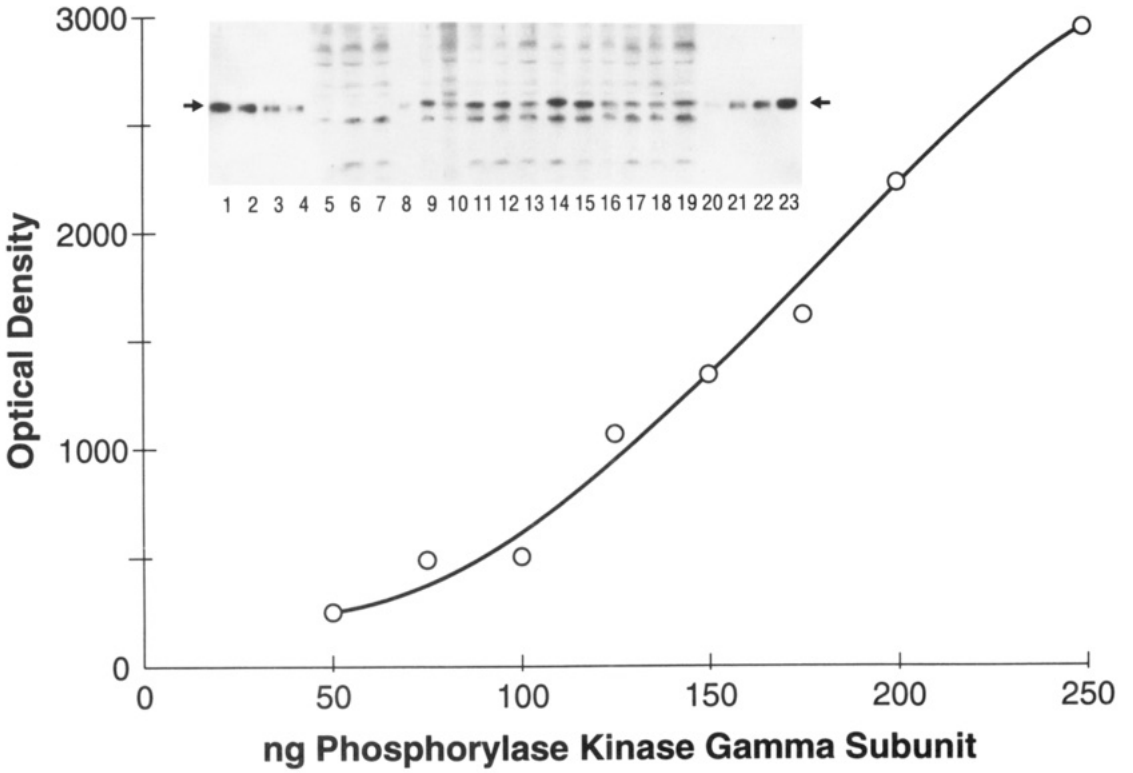
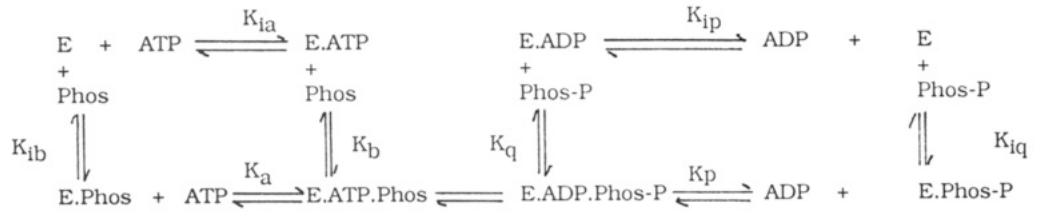


FIGURE 2: Protein immunoblotting of expressed γ subunit and mutants. Experimental methods are detailed under Experimental Procedures. The graph shows a standard curve derived using pure rabbit skeletal muscle phosphorylase kinase by scanning of the immunoblot. The inset shows a typical immunoblot: lanes 1–4, 8, and 20–24 contained 250, 175, 125, 75, 50, 50, 100, 150, and 200 ng of rabbit skeletal muscle phosphorylase kinase, respectively; lane 5, mock infected cells; lanes 6 and 7, cells infected with virus without γ. Remaining lanes were from the extracts of cells infected with the following: lanes 9 and 10, BmV-γ-1 (wild type); lanes 11 and 12, BmV-γ-Ser^{26}; lane 13, BmV-γ-Ser^{28}; lane 14, BmV-γ-Ser^{29}; lanes 15 and 16, BmV-γ-Phe^{30}; lanes 17 and 18, BmV-γ-Gly^{31}; lane 19, BmV-γ-Gly^{33}.

Scheme I: Rapid Equilibrium Random Reaction Mechanism for γ Subunit



of added ATP. All kinetic assays were for 3 min or less, during which period less than 20% of the limiting substrate was consumed. For each enzyme form, initial rates were determined at five concentrations of ATP, each at five concentrations of phosphorylase (varied according to the enzyme form being determined). Each reaction rate was determined in duplicate. For each enzyme form each experimental set of 25 data points, obtained with varying ATP and phosphorylase concentrations, was repeated at least three times, and for these at least two different enzyme preparations were employed. Studies with both phosphorylase kinase holoenzyme (Tabatabai & Graves, 1976) and free γ subunit (Farrar & Carlson, 1991) have indicated that the reaction mechanism for this enzyme is rapid equilibrium random, as illustrated by Scheme I. The relationships of the kinetic constants for the rapid equilibrium kinetic mechanism are given by eq 1, and by the relationship $K_{ib} = K_{ia}K_b/K_a$.

$$v = \frac{V_{max}}{1 + K_a/[A] + K_b/[B] + K_{ia}K_b/[A][B]} \quad (1)$$

The kinetic constants (K_{ia}, K_{ib}, K_a, and K_b), and standard deviations for these constants, were determined by normalizing the enzyme activity for all of the data sets for each enzyme form and then curve fitting the entire data for each enzyme form using the curve-fitting program provided by Sigma Plot 4.1 (Jandel Scientific, Corte Madera, CA). This curve fitting is based upon the Marquardt–Levenberg algorithm, and all data points were weighted equally. The total number of data points used to measure each set of kinetic constants was thus at least 75, each determined in duplicate. The small standard deviations obtained reflect the reproducibility between the data sets. Because of the magnitude of the values of K_a and K_b, the data for Ser^{26}-γ could not be suitably analyzed by this procedure. For this form of mutant enzyme, the K_{ia} and K_{ib} values were determined graphically from the point of intersection of the double-reciprocal plots for five experiments and the standard deviations determined from the mean of these points. These values were then substituted into eq 1 for a solution of the limiting values for K_a and K_b.

For determinations of K_i values for ADP and AMP·PCP, the same reaction conditions were used as described above for the determination of the other kinetic constants, using five concentrations of ATP, at least four concentrations of nucleotide inhibitor (0.1–2.5 mM, depending upon the enzyme and nucleotide), and 70 μM phosphorylase. Enzyme inhibition data were analyzed by fitting the data to eq 2 for mixed noncompetitive inhibition (where K_{is} and K_{ii} are the competitive and noncompetitive inhibition constants, respectively) and

$$v = \frac{V_{max}}{(1 + [I]/K_{is})\, K_m/[S] + [I]/K_{ii}} \quad (2)$$

comparing the significance fit (F test) with that obtained by using the equations for competitive, noncompetitive, and uncompetitive inhibition. Inhibition by ADP and AMP·PCP was competitive for all enzyme forms. The K_i value for each was determined from the average of at least three equivalent experiments (unless stated), utilizing at least two preparations of enzyme with each enzyme form. The standard deviation was determined from this calculation of the average K_i value.

RESULTS AND DISCUSSION

Properties of Baculovirus Expressed Phosphorylase Kinase γ Subunit. Two recombinant viruses were constructed, BmV-γ-1 and BmV-γ-2, each containing the fully intact cDNA for the γ subunit of phosphorylase kinase. BmN cells, infected with either virus showed a prompt response with the production of active enzyme, with maximum expression after ~48 h of infection (Figure 3a). Neither mock-infected BmN cells nor cells infected with wild-type virus showed significant phosphorylase phosphorylating activity, indicating the absence of a "phosphorylase kinase-like" enzyme in these cells and also that the source of enzyme activity was the γ subunit cDNA from the recombinant virus. Phosphorylase phosphorylation, even with crude cell extracts, was linearly dependent upon both assay time (Figure 3b) and protein concentration. Activity was totally dependent upon phosphorylase addition to the assay, indicating the absence in BmN cells of significant levels of endogenous substrates for the γ subunit. On the basis of the known activity of purified phosphorylase kinase, the γδ complex, and reconstituted γ subunit at pH 8.2, the specific activity of the expressed γ subunit in the initial extracts corresponds to approximately 0.1–0.2% of the soluble protein of the BmN cell extracts (Pickett-Gies & Walsh, 1986; Chan & Graves, 1982b; Kee & Graves, 1987). A ~4-fold purification of the expressed γ subunit was achieved by batch adsorption and elution from DEAE, as described in Experimental Procedures, and this is the preparation used for all subsequent studies described. These values correspond well with the amount of γ subunit protein determined by immunoblotting (Figure 2). The protein obtained by this procedure maintained stable activity for at least 5 days when stored at −20 °C. Cleanup by a variety of procedures did result in further purification, but in all cases the resultant protein was markedly unstable. Partially purified γ subunit exhibited a pH 6.8/8.2 activity ratio of 0.54–0.65; this is identical to that obtained with protein obtained by expression in 3T3 cells (Cawley et al., 1988) or following renaturation (Kee & Graves, 1986) and contrasts with the nonactivated holoenzyme (pH 6.8/8.2 activity ratio, 0.02–0.05) or the γδ complex [pH 6.8/8.2 activity ratio, 0.9; Chan and Graves (1982c)]. The activity of the partially purified γ subunit was unaffected by EGTA addition (0.4 mM), indicating that it was free γ and not associated with calmodulin (potentially contributed by the BmN cells). Activity was increased from 70 to 110% by the addition of 0.17 μM calmodulin (+Ca^{2+}). This degree of stimulation is identical to that observed with either renatured γ (Kee & Graves, 1986) or γ expressed in 3T3 cells (Cawley et al. 1989) and the response was maximal at this concentration of calmodulin, indicating that the γ subunit has retained a high calmodulin affinity. With holophosphorylase kinase (containing intrinsic calmodulin), Ca^{2+} stimulates enzymatic activity between 20- and 40-fold. As discussed in detail elsewhere (Newsholme et al., 1992), it is apparent that the interaction between the γ and δ subunits is modified substantially in the holoenzyme by the presence of the α and β subunits; hence free γ subunit (plus added calmodulin) and the holoenzyme are stimulated to quite different extents by added Ca^{2+}.

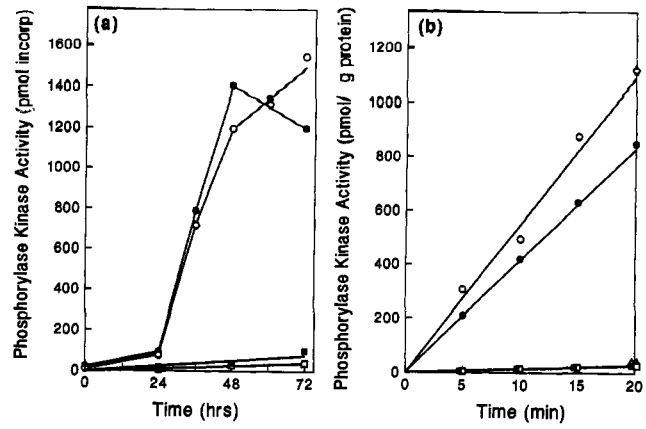


FIGURE 3: Characteristics of γ subunit expression. (a) Time course of γ production by BmN cells. Cells were infected and extracted at the indicated times, and phosphorylase kinase activity was determined as described in Experimental Procedures. (b) Dependence of expressed γ subunit activity on incubation time and phosphorylase addition. Phosphorylase kinase assays were performed either with the complete reaction mixture with either mock infection (■) or infection with wild-type virus (□), BmV-γ-1 (●), or BmV-γ-2 (○), or with phosphorylase omitted from the assay with BmV-γ-1 (▲) or BmV-γ-2 (△) infected cells. Infection time for (b) was 48 h. Assay time for (a) was 10 min.

The size of the expressed γ subunit was examined by both gel filtration and sucrose gradient ultracentrifugation (Figure 4a,b). By both procedures the protein exhibited a molecular mass of between 160 and 180 kDa, markedly larger than the expected size of a single γ subunit (45 kDa) and the size of monomeric protein identified by immunoblotting (Figure 2). One possible explanation of this observation might be that the γ is present as a tetramer, reflective of the polymeric nature of the holoenzyme; this remains to be further evaluated.[2] It is possible that the ability to form a multimeric species accounts for formation of active enzyme, which was obtained here with the baculovirus expression system and in 3T3 cells (Cawley et al., 1989).

Kinetic Analysis of γ Subunit with Mutations in the ATP Binding Site. Six recombinant viruses were constructed, designated as BmV-γ-Ser^{26}, BmV-γ-Ser^{28}, BmV-γ-Ser^{29}, BmV-γ-Phe^{30}, BmV-γ-Gly^{31}, and BmV-γ-Gly^{33} (Table I), whose expression would lead to the indicated amino acid substitutions, each of which are in the ATP binding site identified as a key motif of all protein kinases (Hanks et al., 1988). The mutations at sites 26 and 28 were designed to evaluate the role of the first two glycine moieties in the consensus sequence Gly-X-Gly-X-X-Gly-X-Val. The third glycine of the normal consensus trio is missing in the γ subunit, being replaced by serine; in this case, the mutation strategy was to evaluate substitution by the more frequently occurring glycine. Mutation at site 33 was designed to test the role of the very highly conserved valine. Mutations at sites 29 and 30 (and also 31) each substituted into the γ subunit sequence the residue present in an equivalent position in the ATP binding site of the catalytic subunit of the cAMP-dependent protein

[2] Of note, the γ subunit expressed in 3T3 cells (Cawley et al., 1989) has a size (~180 kDa) very similar to that seen here with the baculovirus expression system (Cawley, K. C., and Walsh, D. A., unpublished observations).

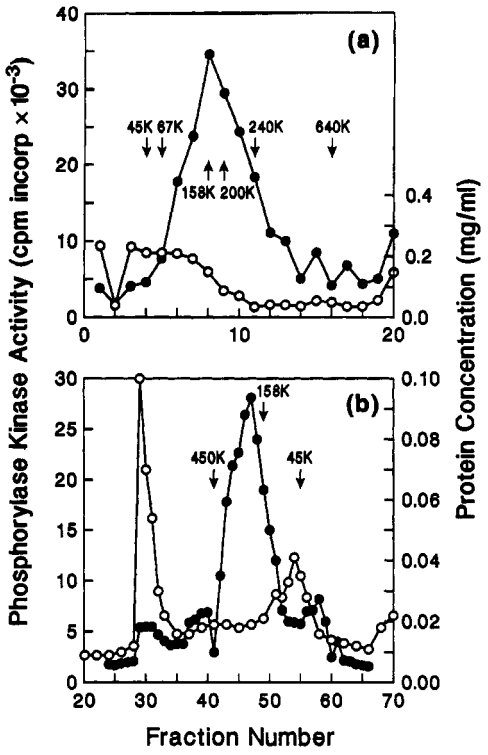


FIGURE 4: Size characteristics of expressed γ subunit. Partially purified extract from BmN cells infected with BmV-γ-1 either was applied to a 5–20% sucrose gradient and centrifuged at 200000g for 4 h in a Beckman TL-100 ultracentrifuge (a) or was applied to a Superose 12 FPLC column (b). The buffer in each case was 20 mM Tris–HCl, pH 7.0, 0.2 mM EDTA, 45 mM β-mercaptoethanol and 20 milliunits/mL aprotinin, except that the Superose column buffer also contained 0.2 M NaCl. Arrows denote molecular weight standards. Symbols: ●, phosphorylase kinase activity; ○, protein concentration.

kinase (Table I), whose 3D structure is now available for comparison (Knighton et al., 1991a,b).

With the exception of recombinants BmV-γ-Ser^{28} and BmV-γ-Gly^{33}, the infection of BmN cells with each of the γ subunit mutant viruses resulted in active enzyme ranging in activity (per microgram of cellular protein) from 25 to 100% of that obtained with wild-type enzyme. Each of the mutant proteins was purified by batch adsorption and eluted from DEAE with characteristics similar to the wild-type enzyme, each exhibited a pH 6.8/8.2 activity ratio similar to that observed with wild-type enzyme, and each showed a similar responsiveness to EGTA and calmodulin. Initial velocity patterns, varying both ATP and phosphorylase *b*, were determined for the wild-type γ subunit and the γ subunit mutants Ser^{26}-γ, Ser^{29}-γ, Phe^{30}-γ, and Gly^{31}-γ. This was not possible with either BmV-γ-Ser^{28} or BmV-γ-Gly^{33} because of the latter's very low specific activities. The wild-type and four mutants exhibited closely similar kinetic patterns with the reciprocal plots intersecting in the negative quadrants, compatible with a sequential reaction mechanism and from which the K_{ia} and K_{ib} values could be determined from the points of intersection. Kinetic constants for all except Ser^{26}-γ were calculated in accord with eq 1 by curve fitting the entire data set from at least three separate experiments for each protein species as described in Experimental Procedures. The small standard deviations obtained reflects the consistency obtained between kinetic assays. These kinetic constants are reported in Table II. The kinetic evaluation for Ser^{26}-γ could not be obtained by this procedure because of the high values for K_a and K_b. For this form, each set of data was analyzed graphically by double-reciprocal plots. From the points of intersection obtained by this procedure, K_{ia} and K_{ib} values were determined, and with these values, limiting lower levels of K_a and K_b were estimated by their substitution into eq 1. For all samples, maximum velocities were calculated using the determination of γ-protein concentration by immunoblotting (Figure 2).

Studies with both the holoenzyme complex (Tabatabai & Graves, 1976) and the renatured γ subunit (Farrar & Carlson, 1991) have indicated that phosphorylase kinase exhibits a rapid equilibrium random reaction mechanism. With such a reaction mechanism the K_{ib} value obtained is equal to the dissociation constant for the E·phosphorylase complex (Scheme I; Experimental Procedures). The K_{ib} values for each of these mutant γ subunits and the wild-type enzyme were very closely similar (Table II). This provides good evidence that these mutations did not significantly alter the binding site for phosphorylase and thus in all likelihood did not induce global changes of structure in the protein. Changes induced by these mutations are thus quite likely restricted simply to the environment of the ATP binding site.

Both BmV-γ-Ser^{28} and BmV-γ-Gly^{33} had only marginally detectable levels of activity (about double the blank values). These levels were approximately 2% that of the wild-type protein, and the Ser^{28}-γ protein had a slightly higher activity than the Gly^{33}-γ mutant. With both mutants, soluble γ subunit protein, purified through DEAE, was readily detectable by immunoblotting (Figure 2) and was present with each in amounts similar to wild-type γ enzyme and the other mutants. It is apparent that with both of these mutants their expression as soluble proteins was successful, but that the specific substitutions virtually eliminated phosphotransferase activity. A glycine at the position equivalent to Gly^{28} is very highly conserved in all protein kinases, being present at this position in 197 of the 198 protein kinases in the protein kinase data bank (Hanks & Quinn, 1991). A glycine within the P-loop motif at this position has also been found to be essential for normal activity of the cellular homologue of the GTP-dependent Ras protein. In Ras, its substitution by an amino acid such as valine leads to a diminished GTPase activity (Weintraub et al., 1991; Milburn et al., 1990; Barbacid, 1987), although GTP binding is maintained, resulting in a Ras oncogene protein that is permanently activated because GTP is only slowly hydrolyzed. This glycine thus appears to be critical for terminal phosphate transfer in Ras, and a similar role in protein kinases could readily account for the greatly diminished phosphorylase phosphorylation activity of the Ser^{28}-γ mutant. In Ras, the peptidyl amide of the glycine equivalent to glycine-28 in γ directly hydrogen bonded to the terminal nucleotide phosphodiester (Wittinghofer & Pai, 1991); thus it may not be surprising that even a slight increase in size at this site (i.e., to that of a seryl residue as tested here) would not be tolerated.

The absence of activity of the mutant of γ with the valine substituted at position 33 is also of particular interest. In the region equivalent to positions 32 and 33 in γ there are some striking differences in amino acid conservation between the protein kinases and most other nucleotide binding proteins that contain the P-loop structure (Saraste et al., 1990). With many of the nucleotide binding proteins, other than the protein kinases, the terminal glycine of the motif (equivalent to position 31 in γ) is very frequently followed by a basic residue (i.e., equivalent of position 32 in γ). This basic residue is most frequently a lysine, and structural studies have indicated that its side-chain amine interacts with the β- and γ-phosphates of ATP (Saraste et al., 1990; Pai et al., 1989). In these latter proteins there is little to no conservation of the next amino

Table II: Kinetic Parameters of the γ Subunit of Phosphorylase Kinase and ATP Binding Site Mutants[a]

protein	n	K_{ia} (μM)	K_{ib} (μM)	K_a (μM)	K_b (μM)	$K_{i\ ADP}$ (μM)	$K_{i\ AMP\cdot PCP}$ (μM)	V_{max} (pmol min^{-1} (pmol of γ)$^{-1}$)
γ	4	170 ± 33	24 ± 6	280 ± 55	40 ± 9	135 ● 15	490 ± 80	2100
Ser^{26}-γ	5	390 ± 120	36 ± 10	>2000	>250	300 ± 15	1000 ± 50	~500
Ser^{28}-γ		nd[b]	nd	nd	nd	nd	nd	<60[c]
Ser^{29}-γ	3	150 ± 30	34 ± 5	30 ± 5	7 ± 1	21	367	570
Phe^{30}-γ	5	160 ± 50	40 ± 10	380 ± 158	90 ± 40	160 ± 15	600 ± 80	1100
Gly^{31}-γ	3	450 ± 160	26 ± 4	125 ± 20	7 ± 2.5	76 ± 6	1400 ± 170	2500
Gly^{33}-γ		nd	nd	nd	nd	nd	nd	<40[c]

[a] The kinetic constants K_{ia}, K_{ib}, K_a, and K_b are as defined in Scheme I and were obtained from the number of anlayses indicated by *n*. K_i values were determined from three separate analyses, except for the $K_{i\ ADP}$ and $K_{i\ AMP\cdot PCP}$ values for Ser^{29}-γ, which were from two and one anlayses, respectively. Standard deviations for K_{ia}, K_{ib}, K_a, and K_b values were determined by curve fitting as described in Experimental Procedures, except for Ser^{26}-γ, where the K_{ia} and K_{ib} values were first determined from reciprocal plots. Standard deviations for K_i values were determined from the three separate determinations. V_{max} values were determined based upon concentrations of γ subunit determined by immunoblotting. [b] nd, not determined. [c] Apparent V_{max} values were determined from assays with 10 mM ATP and 51 μM phosphorylase with γ subunit concentration determined by immunoblotting.

acid in the sequence (i.e., that equivalent to the position in γ of Val^{33}). With protein kinases, however, the situation is distinctly different. In the 198 protein kinases in the protein kinase data bank, there are 16 different amino acids in the position equivalent to residue 32 of the γ subunit, with the most abundant being glutamate (37), lysine (37), valine (22), threonine (20), and arginine (20). In contrast, the valine present at position 33 in γ is conserved in >95% of all protein kinases (Hanks & Quinn, 1991). In the cAMP-dependent protein kinase, this valine is at the interface of the flexible P-loop and the second of the five β strands that form the core of one of the two lobes of the protein (Knighton et al., 1991a,b). Clearly the substitution by a glycine at this position markedly affects activity, possibly by introducing a flexibility into the structure that cannot be tolerated during catalysis. Alternatively, it may eliminate some critical interaction of the side chain of valine to maintain structure and/or contribute to the conformation required for catalysis. The very low level of activity of Ser^{28}-γ and Gly^{33}-γ prohibited any further analysis other than an estimate of their apparent maximum activities (Table II).

Marked changes in K_{ia}, K_a, and K_b for the interaction of the substrates and the V_{max} of the reaction were observed in several of the other γ subunit mutants. The K_{ia} value obtained with a rapid equilibrium random mechanism is a direct measure of the dissociation constant for the E·ATP complex (Scheme I; Experimental Procedures), and therefore, this value provides the most direct indication of an alteration in binding of ATP. K_a and K_b values, as illustrated in Scheme I, reflect the interactions necessary to form the ternary E·ATP·phosphorylase complex from either E·phosphorylase or E·ATP, respectively. Both of these latter values would be influenced by changes that directly affect the binding of either substrate, such as for example the alteration of a recognition determinant that directly interacts with that substrate. In addition, these values would also reflect potential differences between the proteins in their response to the binding of the first substrate and the consequential conformational changes that may be promoted by this binding and may be essential for optimal binding of the second substrate. Changes in conformation upon the binding of the first and second substrates that are critical for catalysis have not been explored for phosphorylase kinase, but have been documented for the cAMP-dependent protein kinase (Reed et al., 1985; Mitchell et al., 1990). The effects of mutations in the nucleotide binding site of the γ subunit were also examined by an evaluation of the kinetics of inhibition by the product ADP and the ATP analogue AMP·PCP. Both ADP and AMP·PCP were competitive inhibitors versus ATP for all of the forms of γ subunit; the K_i values for the wild-type γ subunit and for each of the γ subunit mutants were obtained by three separate determinations and are summarized in Table II. The K_i value for ADP determined from the inhibition studies would equal K_{ip} as depicted in Scheme I.

Glycine-26 is the first of the trio of glycines of the ATP binding site motif (Hanks et al., 1988) of the γ subunit. Its conversion to serine (in Ser^{26}-γ) diminished the affinity of ATP, ADP, and AMP·PCP by 2–2.5-fold (Table II). These changes were further associated with large increases in both K_a and K_b and a ~75% reduction in V_{max}, reflecting a quite diminished capacity to form the E·ATP·phosphorylase complex and thus have productive catalysis. The absence of this first glycine of the trio appears to both diminish nucleotide binding and impede the conformational changes likely to be necessary for optimal ternary complex formation. The role of this glycine appears to be directly related to nucleotide binding, either as a recognition determinant for the β- or α-phosphate or in allowing for the optimal conformation of the P-loop for binding or catalysis. Although these data suggest that a glycine at this position in the ATP binding site of protein kinases appears quite preferable, it is obviously not essential. This is of considerable interest because this glycine is conserved in 191 of the 198 protein kinases in the protein kinase data bank (Hanks & Quinn, 1991) and is in fact more conserved than Val^{33}, whose substitution, as we have shown above, results in near total inactivation. It is not apparent from these data as to what evolutionary pressures have resulted in such a very high level of conservation of this glycine, spanning from plants to unicellular organisms to higher vertebrates, given that with its substitution by serine the enzyme exhibited good affinity for both protein and nucleotide substrates and a quite high level of phosphotransferase capacity.

In phosphorylase kinase γ subunit, the third of the trio of glycines of the standard ATP binding site motif is replaced by serine (position 31). This third glycine of the trio shows more variation than the first two of the motif. It is conserved in 169 of the 198 protein kinases in the data bank (Hanks & Quinn, 1991), but is only replaced by either serine (21 cases) or alanine (8 cases) and not by any other amino acid. In a mutant form of the insulin receptor, substitution of the glycine at this equivalent position by a valine results in a very marked reduction in tyrosine kinase activity (Odawara et al., 1989). Mutant Gly^{31}-γ was constructed to examine the effects of a change back to the consensus. Restoration of a glycine at this location in fact diminished the affinity of both ATP and AMP·PCP by ~3-fold. In contrast, the ADP affinity of Gly^{31}-γ was approximately double and there was a 2-fold decrease in K_a and a 6-fold decrease in K_b. The decreased affinity for ATP of Gly^{31}-γ was apparently offset by the decrease in K_b such that overall ternary complex formation

was favored with a shift in equilibrium of E + ATP + phosphorylase → E·ATP·phosphorylase by ~2-fold. The resultant V_{max}, in likely consequence, was not affected. The switch from glycine to serine in position 31 in the γ subunit thus does not appear to greatly affect the overall phosphotransferase activity, even though it affects some of the parameters along the reaction pathway and produces protein with less than the maximal nucleotide affinity. Possibly there is some advantage in the interchange of serine and glycine at this position in different kinases that relates to nucleotide availability.

Mutant Phe^{30}-γ substituted a phenylalanine for a serine, mimicking the sequence present in the ATP binding site of the cAMP-dependent protein kinase. In protein kinases, the equivalent to position 30 of γ is quite highly conserved as either phenylalanine or tyrosine with these residues present in 149 and 33 of the 198 identified protein kinases, respectively (Hanks & Quinn, 1991). Interestingly, this tyrosine in cdc2 kinase is a site of regulatory phosphorylation (Gould et al., 1991; Norbury et al., 1991). The next most prominent amino acid found in this position is serine, and this occurs in seven protein kinases including the γ subunit of phosphorylase kinase. Possibly this serine could also be a site of regulatory phosphorylation, although this has not been reported. It would be an interesting phenomenon if this residue was generally phenylalanine, except when tyrosine or serine were present to allow for regulation. All together, phenylalanine, tyrosine, and serine occur in 189 out of the 198 identified protein kinases, so this site is quite highly conserved. In the P-loop structure, the side chain of the residue at this position would be directed away from the side to which the nucleotide binds. It is thus ideally suited to be available for covalent regulation occurring separately from catalysis, but then influencing catalysis. Overall, the replacement of serine at position 30 in γ by phenylalanine, creating the more consensus sequence, had relatively mild consequences. No significant changes in the affinities of ATP, AMP·PCP, and ADP (Table II) or in ternary complex formation occurred; however, the V_{max} was somewhat compromised and decreased by about half. These results are compatible with the side chain of the residue at this position having a minimal role in the actual phosphotransferase reaction.

The remaining site tested was position 29, which is a serine in the cAMP-dependent protein kinase and a valine in γ, but in protein kinases overall there is no conservation at this position (Hanks & Quinn, 1991). (Seventeen different amino acids occupy this site in the 198 proteins in the protein kinase data bank.) Mutant Ser^{29}-γ substituted a serine for a valine to mimic the site in the cAMP-dependent protein kinase. This substitution had more effects than might be expected. Although it had minimal to no effect on the affinity of either ATP or AMP·PCP (Table II), it caused a marked increase in the formation of the ternary E·ATP·phosphorylase complex, as reflected by the 8- and 6-fold decreases in K_a and K_b values, respectively, but in contrast, caused a 6-fold increase in the affinity of ADP and diminished V_{max} by ~75%. The clear distinction between the absence of an effect of this substitution on either ATP or AMP·PCP binding and its marked effect upon ADP binding is of note. One explanation of these data would be that, following the binding of nucleotide substrate, the subsequent conformational changes that occur during catalysis result in a conformation that now binds nucleotide more strongly, diminishing the rate of reaction and lowering the rate of overall catalysis. A serine rather than a valine appears to favor these conformational changes, perhaps in a protein substrate specific manner.

These studies begin an evaluation of the role that different residues play within the consensus glycine trio/valine P-loop motif of protein kinases, especially with respect to those residues where there is a high degree of conservation. The trio of glycines of all P-loop-containing proteins appear to have a central role in the binding of nucleotide phosphates although the roles appear often to be different in different nucleotide binding species. With adenylate kinase, the amide nitrogens of at least two of the glycines of this trio, together with those of other residues in the sequence, form an anion hole for the binding of the terminal phosphate of ATP as it is transferred to AMP (Dreusicke & Schulz, 1986). In the GTP binding Ras protein, the amide nitrogens of the glycine equivalents of Gly^{28} and Ser^{31} of the γ subunit are hydrogen bonded to the oxygens of the β- and γ-phosphates of GTP (Wittinghofer & Pai, 1991; Pai et al., 1989). Although the P-loop is a common feature of nucleotide binding proteins, there are some noted differences in the actual consensus sequences between the various types of enzymes (Hanks et al., 1988; Saraste et al., 1990) and therefore some important differences in the specific roles of the constituent amino acids. The P-loops in adenylate kinase, Ras, and EF-Tu, for example, connect a β strand and an α helix (Pai et al., 1977, 1989; Dreusicke & Schulz, 1986; Jurnak, 1985), with the positive dipole moment of the α helix contributing to neutralizing the negative charges of the nucleotide phosphates (Hol, 1985). In contrast, in the cAMP-dependent protein kinase, the P-loop connects two regions of β sheet (Knighton et al., 1991a,b). The latter studies of the cAMP-dependent protein kinase clearly indicate that the P-loop plays some role in nucleotide binding but these X-ray crystallographic studies have yet to precisely define that role.[1] The equivalent of Val^{33} in γ is very highly conserved in protein kinases but not in other P-loop nucleotide binding sites. Its substitution by glycine in these current studies was not tolerated, suggesting a major role for this residue in kinase activity that does not arise for the other nucleotide binding sites. One possibility would be that this valine plays a key role in the structure of the kinase, since the P-loop links different secondary structural elements between kinases (two β sheets) than occur with other nucleotide binding proteins (α helix to β sheet). Also of note is the difference in spacing between the glycines in protein kinases as compared to many other nucleotide binding proteins. The glycine trio/valine motif has become one of the landmarks for identification of protein kinases. Of these four residues it is of interest that for two of them there is apparently extremely little latitude for substitution whereas the other two, despite being highly conserved, show a much greater flexibility of constituency at this site. To date we have the crystal structure of one protein kinase (Knighton et al., 1991a,b) but remaining to be understood is what part of the reaction mechanism of phosphotransferase catalysis this structure represents. The studies presented here have begun to specifically define the limitations on some of the residues critically involved in catalysis, helping to lead to a more coherent picture of the catalytic process.

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