Site-Directed Mutants of Glycogen Phosphorylase Are Altered in Their Interaction with Phosphorylase Kinase[†,‡]

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ABSTRACT: Glycogen phosphorylase is found in resting muscle as phosphorylase *b*, which is inactive without AMP. Phosphorylation by phosphorylase kinase (PhK) produces phosphorylase *a*, which is active in the absence of AMP. PhK is the only kinase that can phosphorylate phosphorylase *b*, which in turn is the only physiological substrate for PhK. We have explored the reasons for this specificity and how these two enzymes recognize each other by studying site-directed mutants of glycogen phosphorylase. All mutants were assayed for changes in their interaction with a truncated form of the catalytic subunit of phosphorylase kinase, $\gamma(1-300)$. Five mutations (R69K, R69E, R43E, R43E/R69E, and E501A), made at sites that interact with the amino terminus in either phosphorylase *b* or *a*, showed little difference in phosphorylation by $\gamma(1-300)$ compared to wild-type phosphorylase *b*. Five mutations, made at three sites in the amino-terminal tail of phosphorylase (K11A, K11E, I13G, R16A, and R16E), however, produced decreases in catalytic efficiency for $\gamma(1-300)$, compared to that for phosphorylase *b*. R16E was the poorest substrate for $\gamma(1-300)$, giving a 47-fold decrease in catalytic efficiency. The amino terminus, and especially Arg 16, are very important factors for recognition of phosphorylase by $\gamma(1-300)$. A specific interaction between Lys 11 of phosphorylase and Glu 110 of $\gamma(1-300)$ was also confirmed. In addition, I13G and R16A were able to be phosphorylated by protein kinase A, which does not recognize native phosphorylase.

Glycogen phosphorylase (GP)[1] is an essential enzyme in muscle carbohydrate metabolism. Using inorganic phosphate, it removes glucose from the nonreducing ends of glycogen, producing glucose-1-phosphate. In this way, it frees stored glucose for use in the production of ATP. GP is activated by the allosteric binding of AMP (*1*, *2*) or by phosphorylation of its Ser 14 residue by phosphorylase kinase (PhK) (*3*−*5*). Phosphorylation converts phosphorylase *b* (phos. *b*), which is inactive without AMP, to phos. *a*, which is active in the absence of AMP (*6*−*8*). PhK itself is activated in response to increased levels of cytosolic Ca^{2+} released during muscle contraction or to hormonal stimuli which lead to an increase in the cytosolic cyclic AMP (cAMP) concentration, such as epinephrine binding to β-adrenergic receptors (reviewed in refs *9* and *10*). Glycogenolysis is therefore activated as the muscle cell requires energy.

While many kinases, such as cAMP-dependent protein kinase (PKA) and MAPK/Erk, have broad substrate specificity (*11*, *12*), the substrate specificity of PhK is very limited. PhK has been shown to have a few substrates in vitro, but its only known in vivo substrate is phos. *b* (*9*). In addition, phos. *b* is not phosphorylated by any other kinase, in vivo or in vitro. Their recognition of each other, then, must be very specific. GP and its regulatory enzymes have been studied longer than any others and have been used as a model system for the study of other enzymatically interconvertible systems (*13*), yet we have little knowledge of how these proteins recognize each other. Our goal in these studies was to investigate some of the possible factors that may influence the specificity of glycogen phosphorylase and phosphorylase kinase for each other.

We approached this problem from the substrate side, creating site-directed mutants of GP and looking for changes in their interactions with PhK. PhK is a very large enzyme, with a total molecular mass of 1.2×10^6 Da, and 16 subunits with an $(\alpha,\beta,\gamma,\delta)_4$ composition (*9*). Because of this complexity, we have chosen to use a truncated version of the catalytic subunit of PhK, the γ subunit. This form, $\gamma(1-300)$, is missing the 86-residue C-terminal regulatory region, but the kinase core is completely intact. Huang et al. (*14*) found that this 1−300 form had a higher V_{max} than the full-length γ subunit, but their K_ms for phos. *b* were identical, indicating that the first 300 residues should comprise the major portion of the catalytic subunit that interacts with GP.

For this study, we have constructed 10 mutants of rabbit muscle glycogen phosphorylase (RMGP) at six different residues. The residues that were mutated were chosen on the basis of either previous studies of PhK substrate

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[1] Abbreviations: PhK, phosphorylase kinase; AMP, adenosine 5′-monophosphate; phos. *b*, phosphorylase *b*; phos. *a*, phosphorylase *a*; GP, glycogen phosphorylase; RMGP, rabbit muscle glycogen phosphorylase; BSA, bovine serum albumin; PKA, cAMP-dependent protein kinase, protein kinase A; TCA, trichloroacetic acid; 9−18 peptide, residues 9−18 of GP.

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specificity using peptides or information from the crystal structures of both GP (*15*, *16*) and PhK γ(1−300) (*17*, *18*). Three residues have been mutated in the N-terminal tail, which contains the region directly around the phosphorylatable Ser 14. These residues are Lys 11 in the P−3 position, Ile 13 in the P−1 position, and Arg 16 in the P+2 position. All of these when changed in a peptide comprised of the N-terminal residues 9−18, 9−18 peptide, produced poor substrates for PhK (*19*−*22*). The other three mutation sites are Arg 43 on the Cap′ and Arg 69 on the α2 helix, which have been shown to form salt bridges with the serylphosphate of phos. *a*, and Glu 501, whose side chain shields the Ser 14 phosphorylation site in phos. *b* (*16*).

The peptide studies that have been carried out gave us valuable information about PhK's primary structure requirements in the phosphorylatable region, but they do not reflect the binding of the three-dimensional folded protein substrate to the kinase. The effects of the mutations are likely to be quite different in the whole proteins than in the peptides. This is indeed what we found. The mutants are all recognized by γ(1−300) differently than is phos. *b*. Some of the mutants are even able to be phosphorylated by PKA, which does not phosphorylate phos. *b* (*19*). In addition, we present data to support previously reported information about a specific residue−residue interaction between γ(1−300) and GP.

EXPERIMENTAL PROCEDURES

Materials. The plasmids RMGP-Nde53 and RMGP-pTacTac, containing the cDNA of rabbit skeletal muscle phosphorylase, along with mutagenesis primers for mutants R16A, R69K, and E501A, were generous gifts of R. Fletterick (University of California, San Francisco, CA). All other mutagenesis and DNA sequencing primers were synthesized in the Iowa State University DNA Facility on an Applied Biosystems 3948 Nucleic Acid Synthesis and Purification System. *Escherichia coli* 25A6 cells were kindly provided by Genentech (South San Francisco, CA). Oyster glycogen type II and the protein kinase catalytic subunit were purchased from Sigma (St. Louis, MO). Chelating Sepharose Fast Flow and DEAE-Sepharose Fast Flow were from Amersham Pharmacia (Piscataway, NJ).

Mutagenesis. Mutants R16A, R69K, and E501A were made using the Kunkel method for single-stranded DNA (*23*, *24*) in the RMGP-Nde53 plasmid. These mutants were constructed to add unique restriction sites by conservative mutation for quicker screening of mutants. All other mutants (K11A, K11E, I13G, R16E, R43E, R69E, and R43E/R69E) were constructed using the QuikChange Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA), also in the RMGP-Nde53 plasmid. Mutants of PhK γ(1−300) (γE61K, γE62K, and γE65K) were also constructed by the QuikChange method in the plasmid pUC118-γ(1−300). Sequencing of plasmids for confirmation of mutagenesis was performed by the Iowa State University DNA Facility on an ABI Prism 377 DNA sequencer.

Protein Expression and Purification. Expression of recombinant GP and mutant forms was carried out in the pTacTac expression vector in *E. coli* strain 25A6, according to the method developed by Browner et al. (*25*). Purification of recombinant wild-type phosphorylase and mutants was carried out by chromatography on a Cu^{2+}-chelated Sepharose column, followed by a DEAE-Sepharose column to remove the remaining contaminating proteins (*26*). Final samples were concentrated in Millipore Ultrafree-15 centrifugal filter units, dialyzed into 40 mM β-glycerophosphate and 5 mM DTT (pH 6.8) (40/5 buffer), and stored at 4 °C. Phosphorylase purified in this manner was 95% pure, as judged by SDS−PAGE. Phosphorylase *b* was purified from rabbit skeletal muscle (*27*) and recrystallized at least three times. It was then dialyzed into a 50% 40/5 buffer (pH 6.8)/50% glycerol mixture and stored at −20 °C. Concentrations of phosphorylase samples were determined spectrophotometrically at 280 nm, using the extinction coefficient of 1.32 cm^2/mg of phosphorylase (*28*, *29*). The phosphorylase kinase catalytic subunit, γ(1−300), and mutant forms, γE110K, γE61K, γE62K, and γE65K, were expressed and purified as previously described (*14*, *30*) and stored in 50% glycerol at −20 °C.

Phosphorylation Assays. Time courses of phosphorylation were determined at 2 mg/mL phosphorylase, 1 mM [γ-^{32}P]-ATP, 10 mM $MgCl_2$, and 2 mM DTT in 50 mM PIPES/50 mM Tris buffer (pH 8.2). Reaction mixtures were incubated at 30 °C for 30 min with γ(1−300) (0.05 μg/mL), γE110K (0.1 μg/mL), or buffer for blanks. Reaction mixtures with γE61K, γE62K, and γE65K were incubated at 30 °C for 10 min, with γ concentrations of 0.5 μg/mL. All forms of γ were diluted in the presence of 1 mg/mL BSA for stability. Progress of reactions was monitored by incorporation of ^{32}P from [γ-^{32}P]ATP. Aliquots (10 μL) were removed from the reaction mixture at various times and spotted on Whatman 31ET paper. Papers were washed for 30 min each in 10% TCA/1% pyrophosphate, 5% TCA/1% pyrophosphate, and 5% TCA, dried, and counted by liquid scintillation counting. Assays with PKA were carried out at 4 mg/mL substrate protein, 0.5 unit/μL protein kinase catalytic subunit, 1 mM ATP, 10 mM $MgCl_2$, and 2 mM DTT in 50 mM HEPES/50 mM Tris buffer (pH 6.5) at 30 °C. For ^{32}P incorporation assays, 10 μL aliquots were spotted at various times, washed, and counted as described above. To measure the increase in phosphorylase activity, 6 μL aliquots were removed at various times and diluted to 0.02 mg/mL GP in cold 40/5 buffer (pH 6.8). Phosphorylase activity was then assayed at 10 μg/mL in the presence or absence of 1 mM AMP, using the method of Krebs et al. (*31*).

Kinetics. Kinetic analyses were performed at five different concentrations of substrate phosphorylase, designating the molecular mass as 195 kDa, the weight of the GP dimer. γ(1−300) was used at concentrations from 0.015 to 0.1 μg/mL, to permit 1−2% total phosphorylation of phosphorylase, as monitored by the level of incorporation of ^{32}P. γE110K was similarly used at concentrations from 0.05 to 0.5 μg/mL, and both enzymes were diluted in BSA as described above. Assays also contained 1 mM [γ-^{32}P]ATP, 10 mM $MgCl_2$, and 3.25 mM total DTT in 50 mM PIPES/50 mM Tris buffer (pH 8.2). Reaction mixtures (30 μL) were incubated at 30 °C for 3 min, when 15 μL aliquots were spotted on paper, washed, and counted as described above. Data were analyzed with the EnzymeKinetics program (Trinity Software).

RESULTS

Rationale for Mutagenesis. The phosphorylase mutants are shown in Table 1 and compared to the sequence of wild-

Table 1: Sequence Changes of GP Mutants in Comparison to Wild-Type Phosphorylase *b*

wild-type phos. *b*[a–c]	K[9]-R-K-Q-I-**S**-V-R-G-L[18]
K11A	K[9]-R-A-Q-I-**S**-V-R-G-L[18]
K11E	K[9]-R-E-Q-I-**S**-V-R-G-L[18]
I13G	K[9]-R-K-Q-G-**S**-V-R-G-L[18]
R16A	K[9]-R-K-Q-I-**S**-V-A-G-L[18]
R16E	K[9]-R-K-Q-I-**S**-V-E-G-L[18]
wild-type phos. *b*	T[38]-L-V-K-D-R-N-V-A-T[47]
R43E	T[38]-L-V-K-D-E-N-V-A-T[47]
wild-type phos. *b*	V[64]-G-R-W-I-R-T-Q-Q-H[73]
R69K	V[64]-G-R-W-I-K-T-Q-Q-H[73]
R69E	V[64]-G-R-W-I-E-T-Q-Q-H[73]
wild-type phos. *b*	P[497]-G-L-A-E-I-I-A-E[505]
E501A	P[497]-G-L-A-A-I-I-A-E[505]

[a] Numbers next to residues denote the residue number in GP. [b] The boldface **S** represents the Ser 14 phosphorylation site. [c] Mutated residues are underlined.

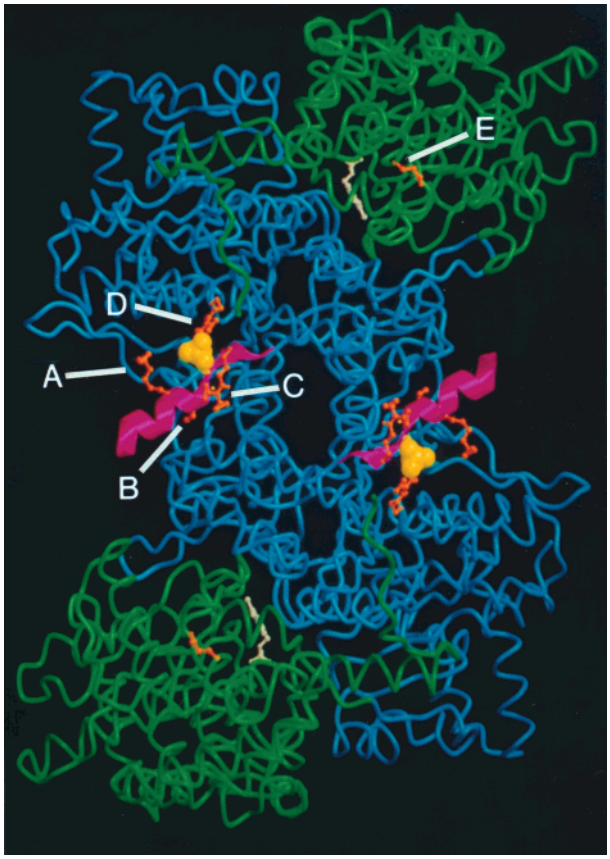

FIGURE 1: Crystal structure of phosphorylase *a* highlighting mutation sites. The N-terminal domain of each monomer is shown in blue, with the C-terminal domain in green. The 3_{10} helical N-terminus is shown as a magenta ribbon, with the phosphoserine in yellow. Mutated residues are shown as orange ball-and-stick models and labeled as follows: (A) Lys 11, (B) Ile 13, (C) Arg 16, (D) Arg 69, and (E) Glu 501 (Arg 43 not shown) (photo courtesy of R. Fletterick and P. Hwang).

type phos. *b*. The location of each residue (except for Arg 43) in the structure of phos. *a* is shown in Figure 1. Mutants of residues in the N-terminal tail of GP are shown in the top portion of the table. These residues were chosen on the basis of previous peptide studies which indicated Lys 11, Ile 13, and Arg 16 were very important for phosphorylation of the peptides by phosphorylase kinase (*19*−*22*). For Lys 11, which has been shown in a peptide to have a specific

Table 2: Kinetic Constants for γ(1−300) with Wild-Type Phosphorylase *b* and GP Mutants

phosphorylase variant	k_{cat} (s^{-1})	K_m (μM)	catalytic efficiency k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	fraction of catalytic efficiency with phos. *b*
phos. *b*	26.4 ± 2.3	10.0 ± 1.2	2.64	1.00
K11A	23.8 ± 0.1	20.0 ± 0.5	1.19	0.45
K11E	17.8 ± 0.7	38.8 ± 2.5	0.458	0.17
I13G	15.3 ± 0.1	27.7 ± 1.5	0.552	0.21
R16A	16.1 ± 0.1	46.7 ± 1.3	0.345	0.13
R16E	11.8 ± 0.2	210.0 ± 8.9	0.056	0.021
R69K	32.9 ± 1.4	11.4 ± 1.0	2.88	1.09
R69E	33.6 ± 1.6	12.7 ± 0.6	2.64	1.00
E501A	33.6 ± 0.7	18.0 ± 2.0	1.86	0.71

interaction with Glu 110 of γ (*32*), we have both removed and reversed the positive charge, making K11A and K11E. These mutations may impair this interaction and reduce the level of phosphorylation by γ(1−300). Ile 13 has been changed to a Gly, as peptide data showed a requirement for a large apolar group in this position (*19*, *20*). In addition, Gly is known to be a helix breaker and may disrupt the secondary structure of the N-terminus (*33*, *34*), which may also be an important factor for γ recognition. Alteration of Arg 16 in peptides produced the most drastic reductions in the levels of phosphorylation, making it a very important residue for recognition. We have both removed and reversed the charge of this residue (R16A and R16E) to see if the effects are as drastic in the intact protein.

Mutants of Arg 43 and Arg 69, in the middle of the table, were made on the basis of information from the crystal structures of phos. *a* which show that these two arginines are involved in tight interactions with the serine phosphate (*15*, *16*). By making a conservative (R69K) and three charge-reversed mutations (R43E, R69E, and R43E/R69E), we may see if the binding of the serine phosphate is an important part of the kinase reaction mechanism and if one of these arginines is more important than the other. We may also see if Arg 69 resides in a proposed second area for binding with γ. Mutation of Glu 501 is shown at the bottom of Table 1. The side chain of Glu 501 acts to shield the Ser 14 side chain in phos. *b* (*16*). Mutating this residue to Ala might remove this shielding effect and perhaps allow more rapid phosphorylation of this Ser.

Interaction of Mutants with γ(1−300). All phosphorylase mutants were first assayed in a time course of phosphorylation with γ(1−300). Two charge-reversal mutations, K11E and R16E (data not shown), had the lowest rates of phosphorylation of all the mutants, with R16E having 20-fold less phosphate incorporated than phos. *b* at 30 min. The interaction of the mutants with γ(1−300) was further studied by determining the kinetic constants, K_m and k_{cat}. These constants were determined from Lineweaver−Burk plots (not shown), and the results are shown in Table 2. With three mutant forms of GP, R69K, R69E, and E501A, γ(1−300) exhibited a higher k_{cat} than with phos. *b*. Each of these mutants also showed a higher K_m for binding to γ(1−300), however. The k_{cat}/K_m ratio is a measure of the catalytic efficiency of an enzyme with a particular substrate. If these ratios are compared for phos. *b* and the three mutants listed above, the mutants' higher K_ms compensate for their higher k_{cat}s. The catalytic efficiency of γ(1−300) for both R69

mutants is equal to that for phos. *b*, while that for E501A is 1.3-fold lower. Not shown in the table are the data for R43E and R43E/R69E, which were made and assayed after the rest of the mutants. These mutants were similar to the other R69 and E501A mutants, with k_{cat}s and K_ms slightly increased compared to those of phos. *b*. The catalytic efficiency of γ(1−300) for R43E and R43E/R69E was 1.3- and 1.5-fold lower, respectively, than that for phos. *b*.

Mutations in the N-terminal tail of GP produced poorer substrates for γ(1−300) than those elsewhere on the protein. With the K11 mutations, along with I13G, γ(1−300) had reduced catalytic efficiencies, due to both reductions in k_{cat} and increases in K_m. With the worst substrate of these three, K11E, γ(1−300) exhibited a 5.8-fold decrease in catalytic efficiency. Substitution of the Arg 16, known to be a very important site for PhK recognition of the 9−18 peptide, had the largest effects on recognition and phosphorylation by γ(1−300). For both R16A and R16E, γ(1−300) showed minor reductions in k_{cat}, 1.6- and 2.2-fold, respectively, versus phos. *b*. However, these mutants also had the highest K_ms of any, R16A showing a 4.7-fold increase and R16E showing a 21-fold increase in K_m. These values translate into a 7.6-fold decrease in catalytic efficiency for γ(1−300) with R16A and a very large 47-fold decrease with R16E.

Interaction of GP Mutants with a Mutant of γ(1−300), γE110K. It was shown previously that the Glu 110 residue of γ(1−300) interacted specifically with residue Lys 11 in the 9−18 peptide (*32*). A mutant of γ(1−300), γE110K, phosphorylated 9−18 peptide very poorly, but phosphorylated 9−18 peptide with a Lys 11 to Glu substitution almost as well as γ(1−300) phosphorylated 9−18 peptide. Using the GP mutants K11A and K11E, we attempted to see if these charge removals and reversals would function in the GP protein they way they did in the peptide studies. Figure 2A demonstrates the phosphorylation of phos. *b* by either γ(1−300) or γE110K (each at 0.1 μg/mL). In the short time course, γ(1−300) phosphorylated phos. *b* to 62% of its maximum in 10 min while γE110K phosphorylated it to only 0.8%. The charge-reversal mutation in γE110K virtually eliminates its phosphorylation of phos. *b*. When γE110K was tested with the K11A and K11E mutants of GP, however, it was able to significantly phosphorylate them in 10 min (Figure 2B). K11A and K11E exhibited 14 and 21% of maximal phosphate incorporation by γE110K, respectively. In contrast, GP mutants R16A and R16E were phosphorylated even less than phos. *b* by γE110K (data not shown), indicating the increased extent of interaction with K11A and K11E is not due to mutagenesis alone.

The kinetics of these phosphorylations were then determined, and the results are shown in Table 3. The E110K mutation in γ(1−300) affects both the K_m and k_{cat} for phosphorylation of phos. *b*. The k_{cat} of γE110K is 11 times lower than that of γ(1−300) for phos. *b*, while the K_m is 30 times higher. The catalytic efficiency of γE110K is 330 times lower than that of γ(1−300) for phos. *b* (Tables 2 and 3). This charge-reversal mutation clearly has a detrimental effect on the phosphorylation of phos. *b*. Both mutations of Lys 11 of GP, however, dramatically improve phosphorylation by γE110K. With both K11A and K11E, γE110K has an increased k_{cat} and a decreased K_m compared to those with phos. *b*. The catalytic efficiencies for γE110K with these GP mutants are 35- and 60-fold higher, respectively, than

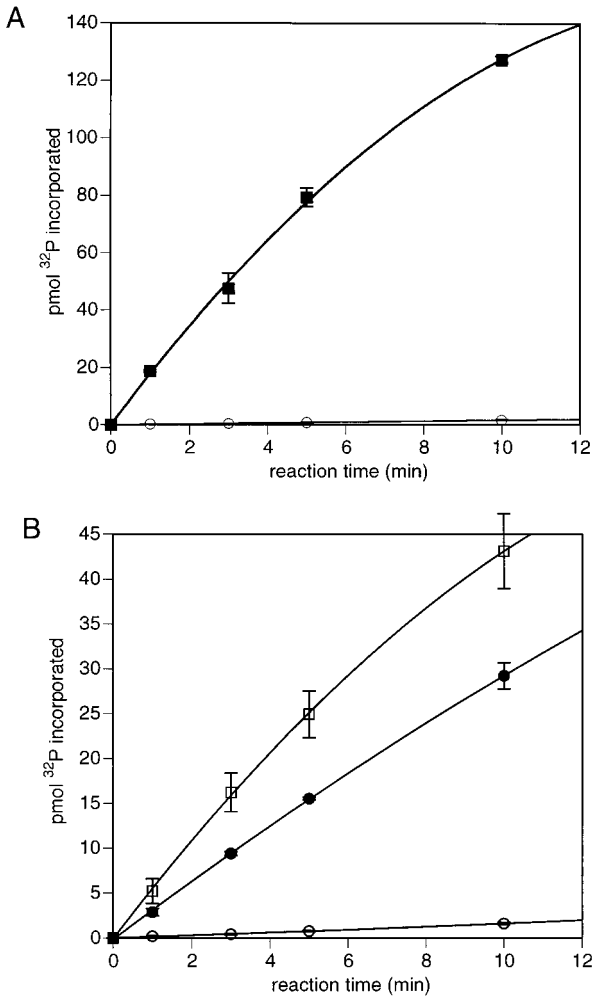

FIGURE 2: Time courses of phosphorylation of phos. *b*, K11A, and K11E by PhK γ(1−300) or γE110K. Phosphorylase (2 mg/mL) was incubated with either γ(1−300) or γE110K (each at 0.1 μg/mL). Phosphorylation is reported as picomoles of ^{32}P incorporated into phosphorylase. (A) Phosphorylation of phos. *b* by γ(1−300) (■) or γE110K (○). (B) Phosphorylation of phos. *b* (○), K11A (●), and K11E (□) by γE110K.

that with phos. *b*. In addition, when compared to its phosphorylation by γ(1−300), K11E is a slightly better substrate for γE110K (Tables 2 and 3). These data support the previous evidence for a specific interaction between Glu 110 of γ and Lys 11 of phos. *b*.

Does Arg 16 Interact with a Stretch of Acidic Residues in γ? Arg 16 of phosphorylase resides in the P+2 position. Very few other kinase substrates have a basic residue in this position, while we have seen that it is very important for the recognition of GP by PhK. Therefore, there may be a specific residue-to-residue interaction between the Arg 16 and perhaps an acidic residue of γ. When compared to the sequence of PKA, the γ sequence has a unique insert containing three glutamic acids: Glu 61, Glu 62, and Glu 65 (*35*). The crystal structure of truncated γ shows these Glu residues are located in a flexible region near the substrate binding site (*17*). Therefore, we predicted that one or more of these acidic residues might be able to interact with Arg 16 (*36*). To test this possibility, three charge-reversal mutations of γ(1−300) were constructed: E61K, E62K, and E65K. These mutants were tested with phos. *b* and the R16A and R16E mutants of GP, to look for any differences in

Table 3: Kinetic Constants for γE110K with Wild-Type Phosphorylase *b* and GP Mutants, K11A and K11E

phosphorylase variant	k_{cat} (s^{-1})	K_m (μM)	catalytic efficiency k_{cat}/K_m (s^{-1} μM^{-1})	fraction of catalytic efficiency of γ(1−300) with phos. *b*	fraction of catalytic efficiency of γE110K with phos. *b*
phos. *b*	2.37 ± 0.39	295.1 ± 46	0.008	0.003	1.0
K11A	12.6 ± 1.24	44.7 ± 7.0	0.283	0.107	35.4
K11E	18.4 ± 2.35	38.1 ± 4.8	0.483	0.183	60.4

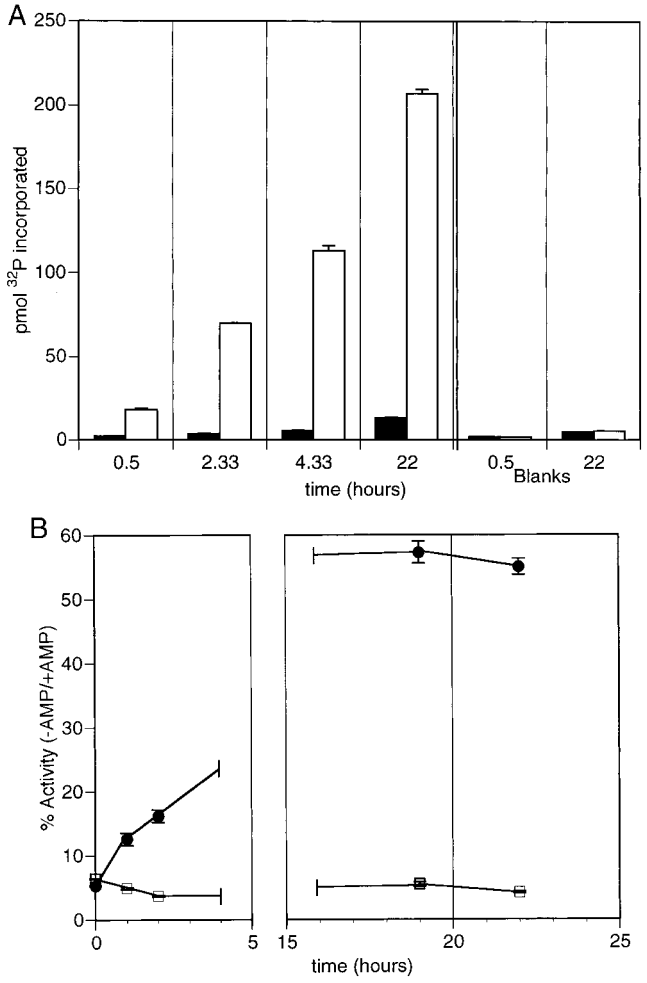

FIGURE 3: Time courses of phosphorylation by PKA. Phosphorylase *b* (4 mg/mL) (A) or R16A (4 mg/mL) (A and B) was incubated with either 0.5 unit/μL PKA or 2 mM DTT for blanks and controls. (A) Aliquots were removed and counted at various times, and phosphorylation of phos. *b* (black) and R16A (white) is reported as picomoles of ^{32}P incorporated. (B) Aliquots of R16A from PKA (■) and control reaction mixtures (□) were removed at various times and diluted to 20 μg/mL phosphorylase. Diluted samples were then assayed with and without 1 mM AMP. Phosphorylation is reported as percent activity without AMP vs with AMP.

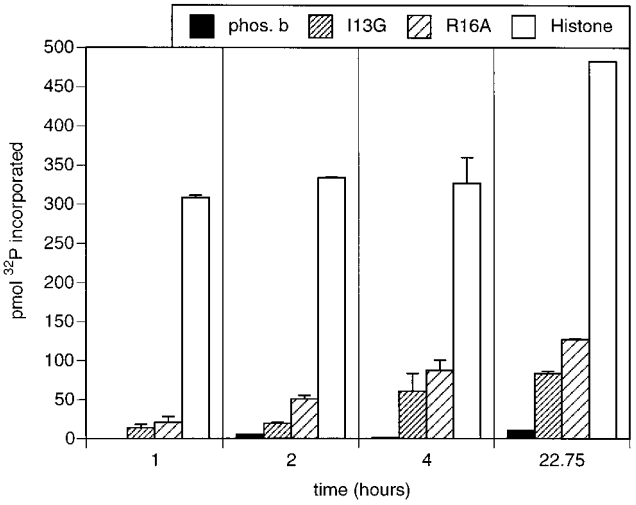

FIGURE 4: Time courses of phosphorylation of phos. *b*, I13G, R16A, and histone by PKA. Each protein substrate (4 mg/mL) was incubated with 0.5 unit/μL PKA. Phosphorylation is reported as picomoles of ^{32}P incorporated.

phosphorylation. Each of the mutant γs had reduced activity with wild-type phos. *b*, but each phosphorylated phos. *b* better than either R16A or R16E (data not shown). These data do not support an interaction between Arg 16 of GP and this region of PhKγ.

Interaction of GP Mutants with Protein Kinase A. It has long been known that phos. *b* is not a substrate for PKA (*19*, *37*). PKA was able to phosphorylate the GP 9−18 peptide, although poorly (*19*). By substitution of the Arg 16 residue with Ala, however, the peptide became a significant substrate for PKA (*20*). An experiment was carried out to see if the Arg 16 to Ala mutation in the entire GP protein could be phosphorylated by PKA. Figure 3A shows that incubation of R16A with PKA for 22 h indeed resulted in a substantial amount of ^{32}P incorporated into this mutant. The incorporated phosphate translates to 50% phosphorylation of R16A at 22 h, while phos. *b* had very little incorporation above the blank. The possibility existed, however, that the phosphorylation of R16A by PKA did not occur on the same Ser that is phosphorylated by PhK, Ser 14. To test this, PKA phosphorylation of R16A was monitored by measuring increase in phos. *a* activity (Figure 3B). The ratio of phosphorylase activity without AMP to activity with AMP increased over time, reaching 55% at 22 h. This indicated that phos. *a* was indeed being produced and, therefore, that PKA was phosphorylating R16A at Ser 14.

In the peptide studies with PKA, another variant of 9−18 peptide, with Gly substituted for Ile 13, was able to be phosphorylated (*20*). The I13G mutant was therefore also tested with PKA (Figure 4). I13G was able to be phosphorylated to a significantly greater extent than phos. *b*, although it was a slightly poorer substrate than R16A. All GP variants are compared to histone, a known substrate for PKA. In a separate experiment, R16E was tested as a substrate for PKA. R16E was slightly phosphorylated by PKA, becoming 7% phosphorylated in 22 h, compared to 0.5% for phos. *b* and 43% for R16A (data not shown).

DISCUSSION

The studies with peptides that were performed previously in this lab were very important. They were useful in determining that the primary structure of phosphorylase in the phosphorylatable region was a major factor for its specificity for phosphorylase kinase. We also used them as a guide for the mutagenesis done here. The substitutions

introduced into the N-terminal peptides dramatically affected their phosphorylation by PhK. We therefore felt it would be beneficial to determine if the same residue substitutions had similar effects in the entire GP protein, since we finally had the capability to do so. The mutations introduced outside the N-terminus were in areas that we believed might be other sites of interaction between phos. *b* and γ(1−300), based on information from their crystal structures.

The results that we obtained, summarized in Table 2, were surprising in some ways. Almost all the mutants showed some change in K_m and k_{cat}, but overall, the changes were much less severe than what was seen with the peptides. For mutants of residues outside the phosphorylation site, R43E, R69K, R69E, R43E/R69E, and E501A, there is little difference in their phosphorylation by γ(1−300) compared to phos. *b*. The K_ms of these mutants were only slightly higher than that of phos. *b*. For E501A, we did not know what to expect, as this area of the GP surface has never been studied. In addition to its side chain shielding the Ser 14, Glu 501 forms part of an acidic patch on the surface of the monomer (*38*), on which the highly basic N-terminus rests in phos. *b*. It was reasoned that changing this residue to Ala might remove this shielding, and/or weaken the electrostatic interactions between the acidic patch and the N-terminus, perhaps making the N-terminus more readily accessible to the kinase. This may indeed be the case, as the k_{cat} of phosphorylation of this mutant is increased. The slight increase in K_m may be due to a slight change in the conformation of E501A (to be discussed in another paper), but overall, this shielding of Ser 14 is not a major factor for the interaction of phos. *b* and γ(1−300).

Arg 43 and Arg 69 are interesting residues, with both side chains forming salt bridges to the Ser 14 phosphate group in phos. *a* (*15*, *16*). We hypothesized that the tight interaction of these basic groups with the phosphate might be a necessary step in the phosphorylation mechanism, perhaps to help release the kinase. Alteration of one or both of these residues, then, might elicit a decrease in the k_{cat} of the reaction. We see now that this is probably not the case, as both R69K and -E, R43E, and the double R43E/R69E mutants all elicited k_{cat}s for γ(1−300) that were higher than that for phos. *b*. Instead, it may be that formation of the complex with the phosphate reduces the k_{cat} of phosphorylation of wild-type GP, and possible disruption of the complex by replacement of the arginines would then speed the reaction slightly.

Another aspect of Arg 69 about which we speculated was its possible function as a secondary binding site for PhK. PhK can phosphorylate peptides of the N-terminus of phos. *b*, but the K_m for this phosphorylation is 50 times higher than that for phos. *b* (*21*). This indicates that there are other parts of phosphorylase besides the N-terminus that contribute to the binding and recognition by PhK. A cyanogen bromide cleavage fragment of phosphorylase, containing the first 91 residues (including Arg 69), has a K_m that is much lower than that of an N-terminal peptide (residues 5−18), and much closer to that of phos. *b*. Most of the residues needed for binding, then, may reside in the first 91 residues of phosphorylase, yet may be comprised of at least two different sites in this region (*21*).

Dasgupta and Blumenthal (*39*) pointed out a sequence homology between the region of GP around Arg 69 and the PhK5 autoinhibitory segment on the C-terminus of the γ subunit. PhK13, the other C-terminal autoinhibitory segment of γ, is highly homologous to residues 9−18 of GP. Peptides of PhK5 and PhK13 can bind simultaneously and synergistically to inhibit PhK. Because these two segments seem to bind together to different sites of PhK, we reasoned that the two homologous regions of GP might also interact with PhK at the same sites. Here we see no significant changes in K_m, however, for the binding of R69K and R69E to γ(1−300). Arg 69 cannot be ruled out as a second area of interaction with holoenzyme PhK, though, as it is possible that the interaction may be with one of the other subunits. This is consistent with the observations that inhibition by PhK5 versus phos. *b* is noncompetitive for γ(1−300) (*32*), but is competitive for holoenzyme PhK (*39*).

The mutations of residues in the N-terminus were expected to have greater effects on recognition and phosphorylation by γ(1−300) than those on the body of GP. While they did exhibit larger changes in K_m and k_{cat} than the other mutations, we were surprised that the changes were not larger still. In most of the previous peptide studies with substitutions of Lys 11, Ile 13, and Arg 16, the major effects were large decreases in V_{max}, with usually minor increases in K_m (*19*−*22*). In this work, we saw that the effects on k_{cat} were quite small. K11A showed the slightest decrease in k_{cat} (1.11-fold), while R16E showed the greatest (2.24-fold). These values are much lower than the values seen with the peptides, for example, a 5-fold V_{max} decrease for K11A (*19*, *20*) and 10−66-fold decreases for R16A (*19*, *21*). The presence of the entire protein in our studies may ameliorate the effects of residue changes on k_{cat} that are so dramatic in the peptides.

For the most part, the K_m increases that are seen with the N-terminal mutations are small as well, with the exception of R16E. Most are on the order of what was seen with each residue change in the peptides. The Arg 16 residue is worth discussing at length. From the many previous studies, it was clear that PhK has a definite requirement for a basic residue in the P+2 position of its substrate. When this Arg was changed to a nonpolar group, such as Ala or Gly, the peptides produced large decreases in V_{max} and small increases in K_m (*19*−*21*). Removing only the positive charge from the Arg 16 in the entire phos. *b* protein, by enzymatically converting the Arg to a Citrulline, also slowed phosphorylation (*40*). The charge reversal to Glu in a peptide could not be phosphorylated at all (*22*). In this work, the R16E mutant could be phosphorylated, but is a very poor substrate for γ(1−300). Its 21-fold increase in K_m helped decrease the catalytic efficiency for γ(1−300) 47-fold. This requirement for Arg at P+2 by PhK may be one of the major factors that makes it the only kinase to act on GP, as few other known kinases have been shown to have a basic residue in this position of their substrate consensus sequences.

For the determination of specificity requirements between phos. *b* and PhK, it would be interesting to know if there are any direct residue−residue interactions. For γ(1−300), we know of two such specific residue interactions, Glu 110 with Lys 11 of GP and Glu 153 with Gln 12 of GP. These interactions have been determined with peptides and mutant forms of γ(1−300) (*32*). Further support for this work was a crystal structure of γ(1−300) with a peptide substrate. An Arg was positioned in place of the Lys 11, but this Arg showed an ion pair with Glu 110 of γ (*18*). We therefore used the same charge-reversal mutant of γ(1−300) used by

Huang et al., γE110K, with our GP mutants K11A and K11E to further confirm this interaction (Figure 2 and Table 3). While the γE110K had a catalytic efficiency 330-fold lower than γ(1−300) for phos. *b*, the mutant γ could phosphorylate the K11A and -E mutants quite well. The K11E mutant was a slightly better substrate for γE110K than γ(1−300), which would support a specific interaction between these two residues.

We investigated another possible residue−residue interaction between Arg 16 and a glutamic acid of γ(1−300). The insertion of the 60s loop in γ(1−300) compared to other kinases and the three glutamates there presented an interesting idea to pursue. It is very possible that one or all of these residues may interact with the P+2 Arg in the γ(1−300) substrate. Our data could not confirm such an interaction, but do not rule it out. It is possible that more than one of these Glu residues is involved with the Arg 16, and mutating one residue at a time has little effect. It is also possible that the lysines substituted for the Glu residues are not large enough to mimic the interaction in the charge-reversal experiment. In any case, we saw no increase in the extent of phosphorylation of R16A and R16E by the γE61K, E62K, and E65K mutants as we did with the corresponding Lys 11 mutants and γE110K.

Because GP is such a specific substrate for PhK, we investigated the GP mutants as possible substrates for another kinase, protein kinase A. Many studies have been performed comparing the substrate specificity of these two kinases (*19*, *20*, *41*, *42*). No modifications of phos. *b*, in terms of adding effectors or modifying the PLP group, or modification of reaction conditions, could make it a substrate for PKA, yet the 5−18 N-terminal peptide could be phosphorylated (*19*). While this peptide was still a poor substrate for PKA, changing the Arg 16 to Ala dramatically improved the peptide as a substrate (*20*). Change of the Ile 13 to Gly in the peptide also allowed moderate phosphorylation by PKA. By using these GP mutants with PKA, we could see if this phosphorylation was limited to short peptides, or if the entire mutant proteins could serve as substrates.

Here we found that the R16A mutant of GP could indeed be phosphorylated to a significant extent by PKA and that this phosphorylation was on Ser 14 (Figure 3). The I13G was also a moderate substrate for PKA (Figure 4), but R16E was only minimally phosphorylated. It appears, then, that the primary structure of the N-terminus is the major negative determinant preventing the phosphorylation of GP by PKA. These GP mutants cannot be called good substrates for PKA, as they were phosphorylated to much less of an extent than histone, and at a much slower rate. They required 22 h to achieve 20−50% phosphate incorporation, as opposed to just 1 h for histone. The fact that this amount of phosphate was incorporated at all, however, is still significant.

The aim of this research was to better understand the reasons for the specificity between glycogen phosphorylase and phosphorylase kinase. We find that the residues around the phosphorylation site are all important in contributing to the interaction of GP with PhK γ(1−300), with Arg 16 probably being the most important of these. None of them alone, however, is the only contributing determinant. All of them taken together, in addition to the conformational structure of glycogen phosphorylase, make GP the best substrate for PhK.

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