Articles

Structural Features Contributing to Complex Formation between Glycogen Phosphorylase and Phosphorylase Kinase†

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Received January 26, 1999; Revised Manuscript Received June 2, 1999

ABSTRACT: A polyclonal antibody was generated against a peptide corresponding to a region opposite the regulatory face of glycogen phosphorylase *b* (P-*b*), providing a probe for detecting and quantifying P-*b* when it is bound to its activating kinase, phosphorylase kinase (PhK). Using both direct and competition enzyme-linked immunosorbent assays (ELISAs), we have measured the extent of direct binding to PhK of various forms of phosphorylase, including different conformers induced by allosteric effectors as well as forms differing at the N-terminal site phosphorylated by PhK. Strong interactions with PhK were observed for both P-*b*′, a truncated form lacking the site for phosphorylation, and P-*a*, the phosphorylated form of P-*b*. Further, the binding of P-*b*, P-*b*′, and P-*a* was stimulated a similar amount by Mg^{2+}, or by Ca^{2+} (both being activators of PhK). Our results suggest that the presence and conformation of P-*b*'s N-terminal phosphorylation site do not fully account for the protein's affinity for PhK and that regions distinct from that site may also interact with PhK. Direct ELISAs detected the binding of P-*b* by a truncated form of the catalytic γ subunit of PhK, consistent with the necessary interaction of PhK's catalytic subunit with its substrate P-*b*. In contrast, P-*b*′ bound very poorly to the truncated γ subunit, suggesting that the N-terminal phosphorylatable region of P-*b* may be critical in directing P-*b* to PhK's catalytic subunit and that the binding of P-*b*′ by the PhK holoenzyme may involve more than just its catalytic core. The sum of our results suggests that structural features outside the catalytic domain of PhK and outside the phosphorylatable region of P-*b* may both be necessary for the maximal interaction of these two proteins.

Glyogen phosphorylase-*b* (P-*b*,[1] EC 2.4.1.1) plays an important role in carbohydrate energy metabolism (*1*). The activity of P-*b* is regulated by interconversion between different conformational states modulated by various allosteric effectors and by its reversible phosphorylation by phosphorylase kinase (PhK) to form phosphorylase *a* (P-*a*) (reviewed in refs *2*−*4*). The allosteric effectors control the properties of P-*b* by preferentially stabilizing either the so-called active (R) or inactive (T) states of the enzyme. Although many studies have characterized the conformational changes induced by these effectors and the interactions between effectors, the question of whether the different conformations of P-*b* induced by effectors interact differently with PhK remains largely unresolved.

PhK (EC 2.7.1.38), another complex regulatory enzyme in the cascade activation of glycogenolysis, is a hexadecameric oligomer with $(\alpha\beta\gamma\delta)_4$ subunit stoichiometry, with δ being an intrinsic molecule of calmodulin (reviewed in ref *5*). The Ca^{2+}-dependent phosphorylation and activation of P-*b* by PhK couples muscle contraction with energy production through an increased level of glycogenolysis. PhK is the only known kinase that can convert P-*b* to P-*a*, and does so by phosphorylating a single seryl residue (Ser-14) within the N-terminal region of each monomer of the dimeric

† This work was supported by National Institutes of Health Grant DK32953.

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[1] Abbreviations: Ab-R13, polyclonal antibody against the peptide corresponding to residues 727−748 of glycogen phosphorylase; AMP-PNP, 5′-adenylyl imidodiphosphate; AP, alkaline phosphatase; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Fab, monovalent antigen-binding fragment; Fab-R13, Fab derived from Ab-R13; γ_{300}, catalytically active fragment comprising 300 N-terminal residues of the γ subunit of PhK; IgG, immunoglobulin G; $K_{d'}$, apparent dissociation constant describing binding between PhK and glycogen phosphorylase as determined by ELISAs; mAb, monoclonal antibody; mAb157, mAb against the C-terminal region of the α subunit of PhK; MAPS, multiple-antigen peptide system; PAGE, polyacrylamide gel electrophoresis; P-*a*, phosphorylase *a*; P-*b*, phosphorylase *b*; P-*b*′, phosphorylase *b*′, an active, truncated form of glycogen phosphorylase lacking the 16 N-terminal residues; PhK, $(\alpha\beta\gamma\delta)_4$ oligomeric phosphorylase *b* kinase holoenzyme; PK-A, cAMP-dependent protein kinase; S-peptide, tetradecapeptide corresponding to residues 5−18 of P-*b*; STI, soybean trypsin inhibitor.

10.1021/bi9901836 CCC: $18.00
Published on Web 07/01/1999

P-*b*. The catalytic subunit of cAMP-dependent protein kinase (PK-A) is homologous to the catalytic γ subunit of PhK and phosphorylates an array of protein substrates, with the β subunit of PhK being among the best (*6*); however, PK-A cannot phosphorylate P-*b* (*7*). Although PhK can also phosphorylate a few other substrates in vitro, including its own α and β subunits, P-*b* is by far its best substrate (*8*). The structural basis for the specificity of PhK is not completely understood.

The phosphorylation of synthetic peptides by PhK has been used extensively to elucidate its specificity for P-*b*. A peptide corresponding to residues 5−18 of P-*b*, and thus containing the convertible Ser-14, can be phosphorylated by PhK with a V_{max} that is $^1/_2$ that of P-*b*; however, its K_M is 75-fold greater than that of P-*b* (*9*). Paradoxically, this peptide, which will be called the S-peptide, is an equally good substrate for PK-A (*9*−*11*), even though the parent P-*b* is not. The fact that the S-peptide does not preserve the specificity toward PhK shown by P-*b* may be due in part to its small size, because a 99-residue peptide generated by CNBr digestion of P-*b* is also a substrate for PhK (with a K_M lower than that of the S-peptide), but not for PK-A (*12*). The tertiary structure of P-*b* may also be important in its recognition by PhK, because denatured P-*b*, in contrast to native, is phosphorylated by PK-A (*13, 14*). These previous findings indicate that features responsible for the specificity of PhK for P-*b* are not preserved in the S-peptide, and have suggested to various researchers that regions outside of the phosphorylation site of P-*b* may be important in its interaction with PhK (*5, 15, 16*).

In this study, we have investigated the interaction between PhK and P-*b* not by phosphorylation, but by direct binding. Because of the large size of PhK (1.3×10^6 Da) and P-*b* (1.95×10^5 Da), quantification of their interaction is problematic. The binding assays used herein were adapted from an enzyme-linked immunosorbent assay (ELISA) approach that was previously developed to investigate the effect of the activation of PhK on its affinity for P-*b* (*17*). The current study focuses on the structural features of phosphorylase that influence its binding to PhK. One feature that was evaluated was the role of the N-terminal phosphorylatable region of phosphorylase in its interaction with PhK. Three forms of phosphorylase differing in the N-terminal region were compared in their interactions with PhK: P-*b*, P-*a*, and a truncated form, P-*b*′, lacking the 16 N-terminal residues, including the phosphorylation site. Another feature of phosphorylase that was evaluated was whether different conformational states of P-*b* induced by its allosteric effectors, such as the activator AMP, interact differently with PhK. Finally, the potential contribution to the binding of P-*b* by the regulatory subunits of PhK was investigated using a truncated γ subunit of PhK, consisting of only its catalytic domain.

EXPERIMENTAL PROCEDURES

Reagents. Bio-gel A-5M (200−400 mesh) was from Bio-Rad. Sephadex G-100 resin was from Pharmacia Biotechnology. Trypsin, soybean trypsin inhibitor (STI), STI−agarose, substrate tablets for alkaline phosphatase, and bovine serum albumin (BSA) were from Sigma.

Proteins and Enzymes. The monoclonal antibody (mAb) specific for the α subunit of PhK, mAb157 (*18*), was generated and used in ELISAs as described previously (*17*). The goat anti-mouse immunoglobulin G−alkaline phosphatase (IgG-AP) conjugate and the goat anti-rabbit IgG-AP conjugate were from Southern Biotechnology, Inc. The γ_{300} of PhK, an expressed truncated form of the γ subunit containing only the 300 N-terminal residues, was generously provided by D. Graves of Iowa State University (Ames, IA) and L. Johnson of the University of Oxford (Oxford, U.K.).

P-*b* and PhK were isolated and their concentrations determined as described previously (*17*). The molecular weights used in calculations were 1.3×10^6 for PhK, 1.95×10^5 for P-*b*, and 3.45×10^4 for γ_{300}. The concentration of γ_{300} was determined by the Bio-Rad assay with BSA as the standard, and those of Ab-R13 and its monovalent antigen-binding fragment (Fab) were determined with rabbit IgG as the standard.

P-*a* was generated through P-*b* conversion by PhK. The final concentrations in the phosphorylation reaction were as follows: 4.5 mg/mL P-*b*, 3 μg/mL PhK, 75 mM Hepes (pH 8.5), 0.3 mM $CaCl_2$, 10 mM $Mg(CH_3CO_2)_2$, 0.1 mM EDTA, 0.1 mM DTT, 25 mM NaF, and 1.5 mM ATP. The reaction proceeded for 3 h at 30 °C and was terminated by 50% $(NH_4)_2SO_4$ precipitation. Precipitated P-*a* was collected by centrifugation, resuspended in a buffer containing 50 mM β-glycerophosphate (pH 6.8), 50 mM β-mercaptoethanol, and 0.1 M glucose, and then separated at room temperature from the PhK by passage over a Bio-gel A-5M column pre-equilibrated with the above glycerophosphate buffer.

To generate P-*b*′, purified P-*a* was subjected to limited trypsin digestion at 30 °C for 12.5 min in a mixture containing 1.55 mg/mL P-*a*, 4.2 μg/mL trypsin (45:1 molar ratio), 47 mM β-glycerophosphate, 47 mM β-mercaptoethanol, 24 mM Tris, 50 mM glucose 6-phosphate, and 0.1 M glucose, with a final pH of approximately 7.5. Dombradi et al. (*19*) reported that glucose 6-phosphate appeared to be more effective than glucose in limiting proteolysis of P-*a* primarily to its N-terminal region; therefore, both glucose 6-phosphate and glucose were included in our tryptic digestion of P-*a*, which resulted in only one dominant fragment. In our hands, previously published procedures for generating P-*b*′ resulted in small amounts of proteolysis at secondary sites. The proteolysis was stopped by addition of STI (50-fold molar excess over the trypsin), followed by 50% $(NH_4)_2SO_4$ precipitation. The precipitate was again collected through centrifugation, resuspended in 25 mM Hepes (pH 6.8)/0.1 mM EDTA/1 mM dithiothreitol/0.1 M glucose, and purified over a Sephadex G-100 column at 4 °C. The pH of the pooled fractions containing P-*b*′ was raised to approximately 7.5 with 0.75 M Hepes (pH 8.5), and the solution was applied to an STI−agarose column, pre-equilibrated with the above buffer, to remove any remaining trypsin. This last purification step was deemed necessary because a small amount of residual trypsin activity could rapidly activate PhK. The flow-through was collected and subsequently concentrated using a Centricon-30 device.

The concentrations of P-*a* and P-*b*′ were matched by the Bio-Rad protein assay against P-*b*.

Protein samples were analyzed by SDS−polyacrylamide gel electrophoresis (PAGE) on mini-slab gels as described previously (*17*).

Activity Assays for P-b, P-b′, and P-a. The activities of P-*b*, P-*b*′, and P-*a* were assayed at 30 °C in the direction of

glycogen synthesis essentially as described previously (*20*). The final concentrations in the assay were as follows: 10 μg/mL P-*b*, P-*b*′, or P-*a*, 16 mM glucose 1-phosphate, and 1% glycogen, with or without 1 mM AMP. The chromophoric complex of the product phosphate produced was determined at 760 nm, and was within the linear range for all conditions and forms of phosphorylase that were tested.

PhK Activity Assays. Measurement of the extent of phosphorylation of different forms of phosphorylase by PhK was performed at 30 °C using a phosphocellulose paper assay (*21*). The reaction was initiated by addition of PhK to the reaction mixture containing all the remaining components. The final concentrations in the assay were as follows: 1 mg/mL P-*b* (or P-*a* or P-*b*′), 0.1 μg/mL PhK, 50 mM Hepes (pH 8.2), 0.3 mM $CaCl_2$, 10 mM $Mg(CH_3CO_2)_2$, 0.1 mM EDTA, 0.1 mM DTT, and 1.5 mM [γ-^{32}P]ATP (NEN-DuPont). To test for contaminating PhK activity in the P-*a* and P-*b*′ preparations, 1 mg/mL P-*a* or P-*b*′ was added instead of PhK to phosphorylate P-*b* in the same mixture as described above, except the concentration of P-*b* was 0.5 mg/mL.

To test the influence of the Fab fragment of the anti-phosphorylase antibody (Fab-R13) on the phosphorylation of P-*b* by PhK, P-*b* was preincubated at room temperature for 30 min with an excess amount of Fab-R13 (30 times the molar concentration of the P-*b* monomer) or the buffer for Fab-R13 (as the control). Then the remaining components of the reaction, except PhK, were added. The activity assay was initiated by addition of the kinase to the reaction mixture at 30 °C. The final concentrations of the components in the assay were as follows: 50 mM Hepes (pH 8.2), 0.2 mg/mL P-*b*, 10 mM $Mg(CH_3CO_2)_2$, 0.3 mM $CaCl_2$, 0.1 mM dithiothreitol, 0.1 mM EDTA, 1.5 mM [γ-^{32}P]ATP, 0.1 μg/mL PhK, with or without 1.54 mg/mL Fab-R13, and 2 mg/mL BSA. The inclusion of BSA was to compensate for possible kinase stabilization by the large excess of Fab-R13 in the reaction mixture, which was not present in the control set.

Generation and Characterization of Anti-P-b Antibodies in Rabbits. The peptide selected as the antigen for generating anti-phosphorylase antibodies was a 22-mer, termed Back 4, corresponding to residues 727−748 of phosphorylase (NAQEYYDRIPELRQIIEQLSSG; 2602 Da) (*22*). To increase the immunogenicity of this peptide, a multiple-antigen peptide system (MAPS) was used to synthesize the peptide for use in immunization. The MAPS involves synthesis of tetrameric or octameric peptides on a small, radially branched lysine core, which is immunogenically inert. This results in a large molecule that has a high molar ratio of peptide antigen to lysine core and that is often immunogenic without the need for conjugation to a carrier protein (*23*, *24*). In our case, the octameric MAPS peptide of Back 4 was used as the antigen without conjugation to a carrier, and was kindly synthesized by M. Dockter of the Molecular Resource Center of the Department of Microbiology (University of Tennessee at Memphis).

The generation of anti-P-*b* antibodies in two rabbits was carried out by Rockland, Inc., as described previously (*25*). ELISAs were used to screen for the presence in test bleed sera of polyclonal antibodies capable of recognizing peptide antigen and P-*b*, plated at 5 μg/mL for antigen peptide or 10 μg/mL for P-*b*. The general procedure for this ELISA was similar to that using mAb157, except that the preimmune bleed (as control) or test bleed from the same rabbit was added instead of mAb157, and that goat anti-rabbit IgG-AP conjugate (0.4 μg/mL) was used to detect rabbit antibody. Serum from rabbit 13 exhibited strong and specific binding to P-*b* even in the first test bleed screening, so production bleeds were collected from this rabbit and used in future experiments, with the antibody being termed Ab-R13.

The specificity of Ab-R13 was tested by comparing its binding to plated PhK (5 μg/mL) or P-*b* (12 μg/mL). To test if P-*b* that was bound to plated PhK could still be recognized by Ab-R13, P-*b* (12 μg/mL) diluted in 50 mM Hepes (pH 6.8)/4 mM EGTA (buffer A) was added to wells with PhK plated (prepared by washing once and blocking as described above). For controls, buffer A was added to plated P-*b* or plated PhK. After washing three times, Ab-R13 in 50 mM Hepes (pH 6.8)/4 mM EGTA/1% BSA (1:1000 dilution) was allowed to bind for 1 h. The remaining steps were performed as described above for the ELISAs. At high Ab concentrations, Ab-R13 was found to bind nonspecifically to PhK (data not shown); however, the extent of this nonspecific binding could be reduced by including 0.5% Triton X-100 in the Ab-R13 dilution buffer and the washes following Ab-R13's binding step. This did not affect the specific binding of Ab-R13 to P-*b* (data not shown). Therefore, 0.5% Triton X-100 was used in all binding assays, but only in the steps described above.

Generation and Purification of Fab-R13. After 25-fold dilution with a buffer containing 6 mM Na_2HPO_4/5 mM NaH_2PO_4/138 mM NaCl (pH 7.6), the crude rabbit serum was loaded onto a pre-equilibrated Protein A column (4 mL bed volume) and eluted with 100 mM sodium citrate (pH adjusted to 4.3). The IgG-R13 fractions were pooled, neutralized with 2 M Tris (pH unadjusted), and concentrated using a Centricon-30 device. Fab-R13 was generated from purified IgG-R13 by papain digestion using a Fab preparation kit (Pierce catalog no. 44885) and purified by passage over a protein A column.

Direct and Competition ELISAs. The direct and competition ELISAs with mAb 157, which measured the extent of binding of PhK to P-*b*, P-*b*′, or P-*a*, were performed and analyzed as described previously (*17*). At least four independent measurements were taken with two different preparations of PhK, P-*b*, P-*b*′, and P-*a*.

The general prodedures of both ELISAs using the anti-P-*b* Ab-R13 were carried out and binding data analyzed as previously described for mAb157 (*17*), with the differences noted below. At least four independent measurements were made with two different preparations of PhK and P-*b*. For the direct ELISA, PhK (3 μg/mL) was plated instead of P-*b*. After blocking, varied concentrations of P-*b*, with or without effectors, were added to the plate and incubated for 1 h. The amount of P-*b* bound to the plated PhK was detected by adding a near-saturating amount of rabbit anti-P-*b* Ab-R13 (1:1000 dilution of the crude serum). The bound Ab-R13 was in turn detected as described above for the screening of rabbit sera. When effectors were tested for their influence on binding, they were included during the binding of P-*b* and were also present throughout all subseqent steps in both the control and plus effector sets. This corrected for their possible influence on the detection system in both the control and effector sets, which were performed simultaneously on

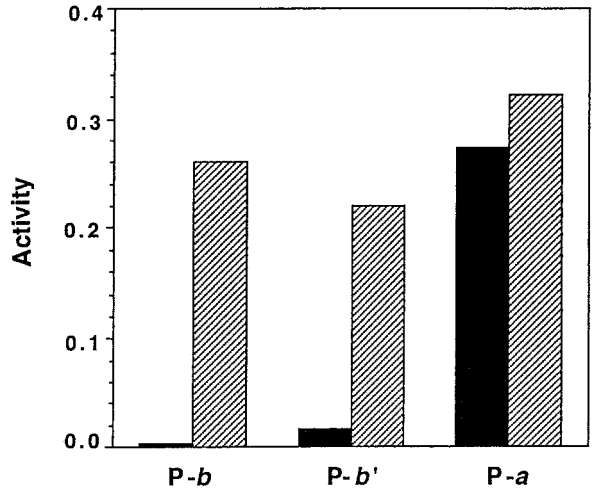

FIGURE 1: Comparison of the activities of P-*b*, P-*b*′, and P-*a*. The activities of the three forms of phosphorylase were measured in the absence (black bars) and presence (hatched bars) of AMP as described in Experimental Procedures. The ordinate values denote the absorbance at 760 nm of the chromophoric complex of the phosphate product of the reaction.

the same plate. When the binding of γ_{300} to P-*b* was tested, it was plated instead of PhK at a fixed concentration. When P-*b*′ was measured for its extent of binding to PhK, it was added instead of P-*b* at varied concentrations. Other steps were as described above for the binding of P-*b* to plated PhK.

For the competition ELISA, the concentration of plated PhK was 10 μg/mL. A fixed amount of P-*b* (8 μg/mL) was incubated in solution for 40 min with or without various concentrations of PhK. Aliquots of 100 μL from these mixtures were then added to the coated wells, and the P-*b* remaining unbound in the preincubation mixture was allowed to bind to the plated PhK for 20 min. Detection of the amount of P-*b* bound was performed as in the direct ELISA described above.

RESULTS

Validation of P-a and P-b′ *for Use in Studies on Their Interaction with PhK.* The P-*a* prepared for this study had no detectable PhK activity and was incapable of incorporating ^{32}P in the PhK-catalyzed reaction (data not shown), indicating the absence of residual P-*b*. In the absence of AMP, the P-*a* had high activity that was increased about 20% in its presence (Figure 1), which is characteristic of P-*a*.

P-*b*′ was generated from purified P-*a* by limited trypsin digestion using an improved procedure described in Experimental Procedures. Analysis by SDS−PAGE of the purified P-*b*′ sample revealed a single predominant band that comigrated with P-*b*. Eight cycles of N-terminal sequence analysis of purified P-*b*′ gave a single sequence (GLAGVENV) at a confidence level above 96%, confirming that trypsin cleaved the N-terminal tail of P-*b* between Arg-16 and Gly-17 (*22*), with Ser-14 being the sole site phosphorylated by PhK. No PhK activity or intact P-*b* molecules could be detected in the purified P-*b*′ (data not shown). The P-*b*′ exhibited high activity only in the presence of AMP (Figure 1), indicating that little, if any, undigested P-*a* was present and that the P-*b*′ was catalytically functional. The lack of residual P-*a* is also consistent with the results of pilot experiments in which P-*b*′ prepared from ^{32}P-labeled P-*a* had no remaining radioactivity (data not shown). Thus, P-*b*′ and P-*a* prepared as described above are suitable for use in experiments for evaluating their interactions with PhK.

Table 1: Summary of $K_{d'}$ Values and Effects of Mg^{2+} (10 mM) and Ca^{2+} (0.2 mM) on the Affinities of P-*b*, P-*b*′, and P-*a* for PhK As Measured by Direct ELISAs Using mAb 157 and Ab-R13[a]

enzyme form	$K_{d'}$ (nM) using mAb 157			$K_{d'}$ (nM) using Ab R13
	control	with Ca^{2+}	with Mg^{2+}	
P-*b*	40.9 ± 3.9	18.6 ± 1.5	3.0 ± 0.3	54.6 ± 8.8
P-*b*′	64.9 ± 3.9	40.6 ± 3.1	6.6 ± 0.7	114.8 ± 10.6
P-*a*	70.5 ± 3.1	47.0 ± 3.9	7.0 ± 1.2	136.5 ± 12.5

[a] The apparent $K_{d'}$ values of P-b, P-b′, and P-a for PhK were measured by direct ELISAs using either mAb157 or Ab-R13 as described in Experimental Procedures and represent relative values describing the interaction in which one member of the interacting protein pair is immobilized. The values for the apparent $K_{d'}$ are averages with standard deviations of at least four independent experiments. Two different preparations of these enzyme forms and PhK were used in these experiments.

Interaction of P-b′ *and P-a with PhK Measured by Direct and Competition ELISAs Utilizing mAb157.* The interactions of P-*b*′ and P-*a* with PhK were first studied using the previously developed direct and competition ELISAs, as described in Experimental Procedures, employing a mAb against the α subunit of PhK, mAb157 (*17*, *18*). As a control, the extent of binding of P-*b* to PhK was measured simultaneously on the same plates under the same conditions. In the direct ELISA, both P-*b*′ and P-*a* were found to bind to PhK, with apparent $K_{d'}$ values that were only 59% and 72% greater, respectively, than that of P-*b* for PhK (Table 1). This is the first time that P-*b*′ has been shown to be able to bind to PhK. Binding of P-*b*′ and P-*a* to PhK was also observed using competition ELISAs, and moreover, binding affinities similar to those determined using the direct ELISA were obtained (data not shown).

The interaction of P-*b* with PhK was previously shown to be stimulated by Ca^{2+} and Mg^{2+} ions, both activators of PhK (*17*; repeated in Table 1). The influence of Ca^{2+} and Mg^{2+} ions on the binding affinities of P-*b*′ and P-*a* for PhK was also investigated in this study using the direct ELISA. It was found that the binding of both P-*b*′ and P-*a* to PhK was enhanced nearly 2-fold by Ca^{2+} and approximately 10-fold by Mg^{2+} (Table 1). This stimulation in binding affinity by Ca^{2+} and Mg^{2+} was similar to the extent of stimulation by these metal ions on the binding of P-*b* to PhK.

The fact that P-*b*′ lacks the phosphorylatable Ser-14 residue allowed the extent of binding of P-*b*′ to PhK to be measured in the presence of ATP, Mg^{2+}, and Ca^{2+}, conditions under which P-*b* would be phosphorylated. ATP (1 mM) affected little, if any, the apparent $K_{d'}$ value of P-*b*′ for PhK, which is similar to results previously observed when the interaction between P-*b* and PhK was assessed in the presence of the ATP analogue AMP-PNP with Mg^{2+} and Ca^{2+} ions (*17*). On the basis of an early study (*26*), it is likely that significant amounts of autophosphorylation of PhK occurred during the binding assays in the presence of ATP, Mg^{2+}, and Ca^{2+}, suggesting that the binding of autophosphorylated, and thus activated, PhK to P-*b*′ may be similar to that of nonphosphorylated, nonactivated PhK. This conclusion is consistent

with our previous observations that phosphorylation of PhK prior to binding assays has little influence on its interaction with P-*b* (*17*).

Development of an Antibody against P-b and Its Use in Binding Assays Utilizing ELISAs. Although the use of mAb157 against PhK allowed direct measurement of the extent of binding of P-*b*′ and P-*a* to PhK for comparison with that of P-*b*, the assay required immobilization of phosphorylase, which could potentially compromise the extent of differences in the interactions of the three forms of the substrate enzyme with PhK. Moreover, without a probe (such as an antibody) capable of sensitively and directly detecting phosphorylase, the interaction of phosphorylase with the γ subunit of PhK could not be evaluated, nor could the influence of effectors of phosphorylase on its binding to PhK. Because the crystal structure of P-*b* has been determined (*27*−*29*), P-*b* peptides with defined location and known structural and functional roles can be selected for induction of desirable polyclonal antibodies against the P-*b* holoenzyme. The peptide chosen for the generation in rabbits of anti-phosphorylase antibodies corresponds to residues 727−748 of phosphorylase, a region having 59% surface exposure located at the opposite side of the regulatory face of P-*b* to which PhK must bind. Our premise was that an antibody against this region would least influence, or be influenced by, phosphorylase−PhK interactions.

One polyclonal antibody, termed Ab-R13, was found to recognize and bind well to the P-*b* holoenzyme as screened by ELISAs. The Ab-R13 also recognized P-*b* that was bound to plated PhK, indicating that the epitope for Ab-R13 is, as designed, away from where PhK and P-*b* interact. To employ Ab-R13 in binding assays absolutely requires that the antibody has no influence on the interaction between P-*b* and PhK. To test this and to avoid cross-linking of the homodimeric P-*b* by the divalent IgG, monovalent Fab-R13 was utilized. A large excess of purified Fab-R13 (30-fold molar excess over P-*b*) was evaluated for its effect on the phosphorylation by PhK of a sub-K_M concentration of P-*b* (approximately $^1/_{10}$ of the K_M value of PhK for P-*b* determined under the same conditions under which the effect of the Fab was evaluated). The Fab-R13 had little, if any, effect on P-*b* conversion by PhK, even under these stringent conditions (data not shown). In addition and as expected, Ab-R13 was found to bind to P-*b*′ as well as to P-*b* (data not shown). Therefore, Ab-R13 was deemed suitable for use as a probe for all three forms of phosphorylase in binding assays.

Both direct and competition ELISAs using Ab-R13 were developed to measure the extent of binding of phosphorylase to plated PhK. Representative binding curves for P-*b* measured by direct ELISAs are shown in Figure 2; the apparent $K_{d'}$ values that were obtained were not only in the same concentration range with both direct and competition ELISAs but also similar to those obtained using mAb157 (Table 1). Using Ab-R13, P-*b*′ was again tested for its binding to PhK. The results confirmed that P-*b*′ binds to PhK; however, its apparent $K_{d'}$ value using this assay was slightly more than 2 times greater than that of P-*b* (Table 1), a larger difference than the 59% increase determined in the direct ELISAs using mAb157. A similar difference in the two assays was observed for the binding of P-*a* (Table 1).

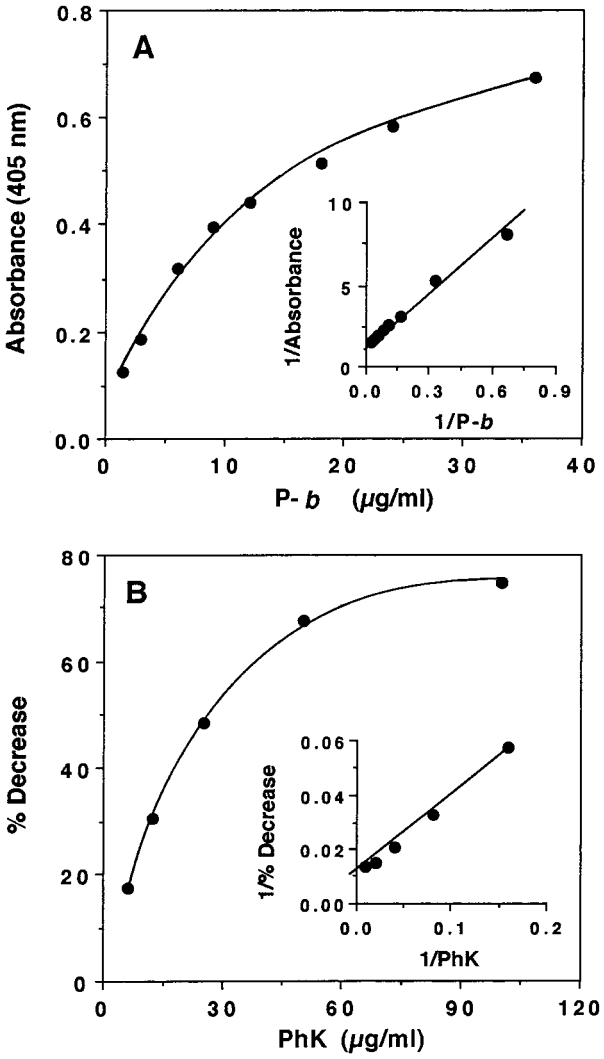

FIGURE 2: Representative binding curves obtained with ELISAs using Ab-R13. The measurement of P-*b* binding to PhK at pH 6.8 by direct (A) and competition (B) ELISAs was performed as described in Experimental Procedures. (A) P-*b* concentrations were varied at 2, 4, 6, 8, 12, 18, 24, and 36 μg/mL, with the concentration of PhK fixed at 3 μg/mL. (B) PhK concentrations were varied at 6.25, 12.5, 25, 50, and 100 μg/mL, with the concentration of P-*b* fixed at 8 μg/mL. (Insets) Double-reciprocal replots of the data giving $K_{d'}$ values of 43.4 nM (A) and 31.7 nM (B).

Use of a Truncated PhK γ Subunit in Binding Assays Using Ab-R13. The generation of the anti-phosphorylase Ab-R13 allowed us to compare the binding of phosphorylase to the isolated γ subunit of PhK versus the $(\alpha\beta\gamma\delta)_4$ PhK holoenzyme used to this point. Using the direct ELISA described above, an engineered, truncated, high-activity form of the γ subunit (γ_{300}) was plated to test its ability to bind phosphorylase, with PhK plated simultaneously on the same plate for comparison. The γ_{300} did bind P-*b*, although only at concentrations significantly higher than required to measure binding by PhK; to obtain similar readings in ELISAs, it was necessary to plate 116 nM γ_{300} versus 9 nM PhK $\alpha\beta\gamma\delta$ protomer (still 23% higher than the former in terms of absorbance at 405 nm). Under this condition, an apparent $K_{d'}$ of 90.3 ± 3.4 nM was obtained for the binding of P-*b* to γ_{300} (Figure 3), but because of the different amounts of γ plated, this value cannot be directly compared to those in Table 1. Addition of Mg^{2+} ions, which greatly stimulate the binding of P-*b* to PhK (*17*), resulted in a 1.6-fold

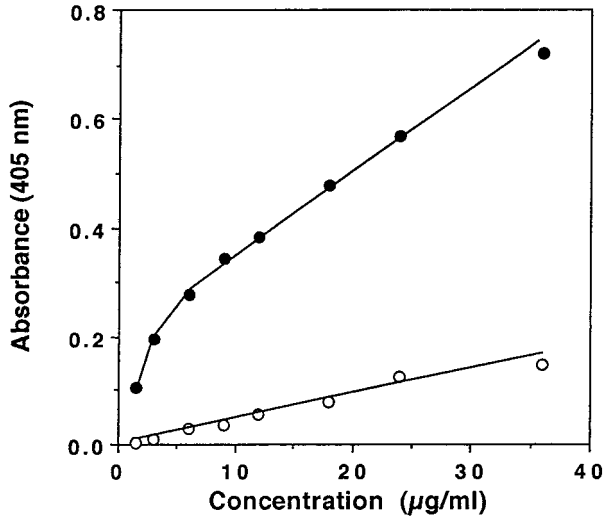

FIGURE 3: Interaction of γ_{300} with P-*b* and P-*b*′ measured by direct ELISA. The direct ELISA using Ab-R13 was performed at pH 6.8 as described in Experimental Procedures, with the concentrations of P-*b* (●) and P-*b*′ (○) varied at 1.5, 3, 6, 9, 12, 18, 24, and 36 μg/mL. The concentration of plated γ_{300} was 4 μg/mL.

enhancement in the binding of P-*b* to γ_{300}. Ca^{2+}, on the other hand, had little, if any, effect on the interaction of P-*b* with γ_{300}. The isolated γ_{300} was also tested to see whether it could bind to P-*b*′. Although weak binding was observed at very high concentrations of P-*b*′, the extent of binding was insufficient to allow determination of an apparent $K_{d'}$.

Influence of AMP on the Binding of P-b to PhK. Besides being phosphorylated and activated by PhK, P-*b* is also activated by the allosteric activator AMP, which binds at an activation site within its N-terminal domain and promotes the active R conformation (*30*). However, the question of whether the direct binding of P-*b* to PhK is affected by the conformational change induced by AMP has not been previously studied. In activity studies, 1 mM AMP has been reported to cause some inhibition of PhK (*31*); however, it is unclear whether that effect was on the substrate P-*b* or on the kinase. To answer this question, we employed the tetradecapeptide alternative substrate, which we term the S-peptide, that corresponds to the N-terminal convertible region of P-*b* and has been used previously to determine whether the influence of effectors on activity in this system is enzyme-directed or substrate-directed (*31*). Of a number of effectors of phosphorylase that were tested (see below), AMP was the only one that influenced P-*b* conversion (40% inhibition by 1 mM AMP) without also affecting S-peptide phosphorylation and/or PhK's autophosphorylation, indicating that at this concentration it exerted its inhibitory effect solely on P-*b* (*25*).

The effect of the same concentration of AMP (1 mM), an amount approximately 10 times greater than its reported K_d value for P-*b* (*4*), on the binding of P-*b* to PhK was evaluated using anti-P-*b* Ab-R13 and both direct and competition ELISAs. The control set (i.e., no AMP present) was performed each time simultaneously on the same plate, allowing the $K_{d'}$ in the presence of effector versus the corresponding control $K_{d'}$ to be compared and presented as percent change. In the direct ELISA, AMP was found to increase the apparent $K_{d'}$ by 38% compared to its control. In the competition ELISAs, which measure the extent of binding of the two proteins in solution, the $K_{d'}$ in the presence of AMP was 31% higher than that of the control. Thus, AMP causes parallel and similar inhibitory effects on the binding and phosphorylation of P-*b* by PhK.

Other effectors of P-*b* (UDP-glucose, caffeine, glucose, and glucose 6-phosphate) were similarly tested (*25*). UDP-glucose, like AMP, promotes the R conformation of P-*b*, but through binding to its catalytic site; however, in contrast to AMP, UDP-glucose increased the binding affinity of P-*b* for PhK by 50−60% in both ELISAs. Caffeine, glucose, and glucose 6-phosphate all preferentially stabilize the T conformation of P-*b*, but through binding to different sites on the enzyme (caffeine at the inhibitor site, glucose at the catalytic site, and glucose 6-phosphate at the AMP binding site). In competition ELISAs, caffeine and glucose increased the binding affinity of P-*b* for PhK by less than 10%, while glucose 6-phosphate decreased it by 17%. Because all of these effectors of P-*b* were also found to inhibit the ability of PhK to phosphorylate the S-peptide and/or itself (*25*), it is difficult to assess whether these particular effectors influence the interactions between P-*b* and PhK by binding to the former, the latter, or both.

DISCUSSION

Despite extensive studies on the activity, regulation, and structure of both glycogen phosphorylase and PhK, few studies have been undertaken to investigate the direct interaction between these two enzymes. Most available data are related to the primary structure specificity determinants surrounding the convertible Ser-14 of P-*b*, namely, how amino acid substitutions in this region influence its phosphorylation by PhK. The use of synthetic peptide analogues has shown specific residues to be important phosphorylation determinants, particularly those in the stretch from positions 11 to 16 (*5*, *15*, *32*); it should be noted in this regard that P-*b*′ begins at residue 17. Other than these primary structure determinants surrounding Ser-14 that affect phosphorylation, little is known concerning other structural factors contributing to the binding specificity between PhK and P-*b*. Because the short synthetic peptide substrates do not retain the specificity toward PhK that P-*b* does and because their K_M values for PhK are considerably greater than that of P-*b*, it has long been speculated that higher orders of structure in P-*b* and/or regions outside of its phosphorylation site may represent additional important determinants in its recognition and binding by PhK (*5*, *15*, *16*). However, direct binding studies have not been previously performed to evaluate these ideas, or the potential contribution to the binding of P-*b* by regions of PhK outside of its catalytic site.

The N-terminal region of phosphorylase is the primary locus for controlling its catalytic activity in response to several effectors, which affect its conformation (*30*, *33*), and through the action of its kinase and phosphatase, which act on Ser-14. The change in structure at the N-terminal tail in P-*b* induced by phosphorylation is indirectly indicated by the ability to proteolytically generate P-*b*′ from P-*a*, but not from P-*b*. Because of the conformational changes at the N-terminal tail after phosphorylation, tryptic digestion of P-*a* can be made selective for Arg-16, leaving the remainder of the molecule relatively intact (*34*). The resultant P-*b*′, lacking the first 16 N-terminal residues, retains up to 90% of the catalytic activity of its P-*b* precursor and can still be activated

by AMP; therefore, it is an excellent tool for evaluating whether regions outside of the N-terminal phosphorylation site of P-*b* contribute to its interactions with PhK.

With the generation of the anti-P-*b* Ab-R13, the extent of binding of soluble P-*b*, P-*a*, and P-*b*′ to plated PhK was measured using both the direct and competition ELISAs developed in this study. It is noteworthy that with two different ELISAs and two different antibodies (anti-PhK mAb157 and anti-P-*b* Ab-R13), $K_{d'}$ values in a similar concentration range were obtained for the binding of P-*b* to PhK (ref *17* and this study), supporting the validity of the binding assays. However, as has been previously emphasized (*17*), because these ELISAs require immobilization of one of the two interacting proteins, the measured binding affinities do not necessarily represent the K_d values that would be measured for the same protein pair interacting in solution. Nevertheless, the $K_{d'}$ values obtained in this study do allow the comparison of relative binding affinities under different conditions and of different conformers or forms of phosphorylase.

Because P-*a* is a product of the P-*b* conversion reaction catalyzed by PhK, it is not surprising that it interacts with the kinase; however, it is notable that its affinity for PhK is only 42% lower than that of P-*b*. Comparison of the crystal structures of P-*b* and P-*a* reveals that the N-terminal tail of P-*b* is disordered and mobile, since it is not observed in the electron density map, and that there is not much interaction between the tail and the remainder of the protein. Upon phosphorylation, the N-terminal tail becomes ordered, rotates, and binds across the interface to a region of the adjacent subunit (*33*), resulting in an increased level of interactions (*27*, *28*, *35*, *36*). Thus, it is clear from these structural studies that the N-terminal phosphorylatable region in P-*b* is significantly different from that in P-*a*. It might reasonably be expected then that the interactions of P-*b* and P-*a* with PhK would be quite different. The fact that P-*a* binds to PhK with an apparent affinity of more than half of that of P-*b* suggests that the conformation of the N-terminal region of phosphorylase does not dictate its affinity for PhK as measured by ELISAs or, alternatively, that this region of phosphorylase becomes deformed in the process of binding to PhK. The data are also consistent with the possibility that regions outside of the phosphorylation site of P-*b* may significantly contribute to its interactions with PhK. The validity of this last possibility is strongly suggested by the relatively tight binding of P-*b*′, which lacks the first 16 residues, including the phosphorylatable Ser-14 and the important basic residues surrounding it. A similar conclusion was reached by Dasgupta and Blumenthal (*37*), who found that two distinct CaM-binding peptides corresponding to noncontiguous regions of the C-terminus of the γ subunit synergistically inhibited the Ca^{2+}-activated PhK holoenzyme. Because the sequence of one of those peptides is similar to a region of phosphorylase approximately 50 residues C-terminal to Ser-14, they suggested that PhK has at least two separate binding sites for phosphorylase, one of them distinct from its phosphorylation site. It is noteworthy in this regard that Mg^{2+} and Ca^{2+} ions, which act directly on PhK and have been previously reported to enhance its binding of P-*b* (*17*), had the same effect on the binding of P-*a* and P-*b*′ (Table 1), suggesting a common mechanism for the enhanced binding by these cations that involves regions of phosphorylase distinct from its phosphorylatable Ser-14 and surrounding residues.

Evaluation of the potential contribution to the binding of the different forms of phosphorylase by regions distinct from the catalytic domain of the PhK holoenzyme was allowed by the availability of γ_{300} and of the anti-phosphorylase Ab-R13 specific for a region of phosphorylase apparently not involved in its interactions with PhK. Although high concentrations of plated γ_{300} were required, P-*b* did bind specifically to this catalytic domain of PhK, as would be expected given that γ_{300} phosphorylates P-*b*. Further, Mg^{2+} ions, which alter the conformation of the isolated γ subunit somewhere within the stretch of residues 100−240 (*38*), enhanced the binding of P-*b* to γ_{300}, as was discussed above with respect to the PhK holoenzyme. In contrast to their effect with the holoenzyme, Ca^{2+} ions did not stimulate the binding of P-*b* by γ_{300}, which is expected given that the effects of Ca^{2+} are mediated by PhK's δ subunit, obviously missing in γ_{300}. Compared to P-*b*, P-*b*′ bound very poorly to γ_{300}, suggesting that the 16 N-terminal residues of P-*b* play a key role in its interaction with the catalytic domain of PhK, an idea that is consistent with the previously discussed results from studies with synthetic peptides. However, the poor binding of P-*b*′ to γ_{300} is in direct contrast to its relatively tight binding to the PhK holoenzyme, which is in turn weaker than that of P-*b* to PhK. These findings would seem to suggest that aside from the 16 N-terminal residues that interact with the catalytic domain of PhK, other regions of P-*b* also interact with the kinase, presumably with its large α and/or β subunits. When it is considered that together these two subunits have a mass nearly 6 times greater than that of the catalytic subunit and that both PhK and P-*b* are oligomers, it would not be surprising if there were extensive contacts between them in addition to those between the catalytic subunit and the N-terminal phosphorylation site. Our findings that the binding of P-*b* to PhK has the highest affinity, while those of P-*b*′ to PhK and P-*b* to γ_{300} are intermediate, and that of P-*b*′ to γ_{300} is the lowest, support the idea that structural features outside the catalytic domain of PhK and outside the phosphorylatable region of P-*b* are both necessary for the maximal interaction of these two proteins. An unavoidable caveat here is that within the PhK holoenzyme the regulatory subunits could potentially influence the structure of the catalytic domain so as to facilitate its interaction with P-*b*; if this were the case, then conclusions based on the interaction of γ_{300} with P-*b* and P-*b*′ would not necessarily apply to the holoenzyme.

The conformation of P-*b* is influenced by phosphorylation and by the binding of allosteric effectors, and it has been clearly demonstrated that dephosphorylation of P-*a* by phosphoprotein phosphatase is influenced by these effectors. For example, the allosteric activator AMP inhibits dephosphorylation, while the allosteric inhibitor glucose 6-phosphate stimulates dephosphorylation. The action of these effectors is considered to be substrate-directed, i.e., acting through binding to P-*a*, because they do not affect the dephosphorylation of an alternative phosphopeptide substrate (*39*, *40*). In contrast, there have been few reports concerning the possible influence of the conformation of P-*b* on its phosphorylation by PhK (*31*, *41*). Harris and Graves (*42*) compared the kinetic parameters for the phosphorylation of the purified P-*b* dimer against those of the P-*a*−P-*b* dimeric

hybrid and found that the hybrid had a lower K_M, suggesting that conformational changes resulting from the phosphorylation of one subunit make the other subunit a better substrate for PhK. They also found that the $\gamma\delta$ complex from PhK was less sensitive to this conformational difference than the PhK holoenzyme, which is consistent with the findings in this study suggesting that the regulatory subunits of PhK may interact with phosphorylase. With the exception of AMP, all effectors of P-*b* that were tested were found to also directly influence PhK (*25*); therefore, it is difficult to ascertain whether an influence of these effectors on the binding of P-*b* to PhK is actually due to an effector-induced conformational change in P-*b*. This complication of the simultaneous targeting of PhK by many effectors of P-*b* does raise, however, the intriguing possibility of an additional layer of regulation on this already complex system of glycogenolysis.

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BI9901836