Divalent Cations but Not Other Activators Enhance Phosphorylase Kinase's Affinity for Glycogen Phosphorylase†

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ABSTRACT: To better understand the physical interaction between glycogen phosphorylase-*b* (P-*b*) and its only known kinase, phosphorylase kinase (PbK), and the relationship of this interaction to the activation of PbK, direct binding studies are necessary. By utilizing an enzyme-linked immunosorbent assay, a method was developed for measuring the binding of PbK to immobilized P-*b* under a variety of experimental conditions. A monoclonal antibody specific for the α subunit of PbK that had no effect on the phosphorylation of P-*b* by PbK or on the interaction of PbK with its known effectors was used to detect PbK bound to plated P-*b*. Hyperbolic binding curves were obtained regardless of whether the concentration of PbK or P-*b* was varied, and the assay detected changes in relative affinity caused by certain effectors of the kinase. The allosteric effector ADP, alkaline pH, and phosphorylation by cAMP-dependent protein kinase, all activators of PbK, did not cause significant changes in its relative affinity for P-*b*; however, Ca^{2+} and Mg^{2+} ions, which also stimulate PbK, increased its affinity for P-*b*, with Mg^{2+} being more effective. Mn^{2+}, which inhibits the P-*b* conversion activity of PbK, was found to be the most potent enhancer of its affinity for P-*b*. These results indicate that activation of PbK is not necessarily caused by an increase in its affinity for P-*b*, although divalent cations may enhance binding. Inclusion of ATP analogs in the binding assay with Ca^{2+} and Mg^{2+} to simulate catalytic assay conditions did not further affect the apparent affinity for P-*b*, which is consistent with the previously reported rapid equilibrium random bi-bi kinetic mechanism for P-*b* conversion.

Glycogen phosphorylase (EC 2.4.1.1) catalyzes the phosphorolysis of glycogen, the first step in glycogenolysis. The enzyme from skeletal muscle is a homodimer (97.4 kDa per monomer) and is subject to both allosteric control and regulation by phosphorylation−dephosphorylation [for a review, see Acharya et al. (1991)]. The only kinase known to phosphorylate glycogen phosphorylase-*b* (P-*b*),[1] thus generating an active form independent of AMP, is PbK (EC 2.7.1.38) [for reviews, see Pickett-Gies and Walsh (1986) and Heilmeyer (1991)]. Skeletal muscle PbK is a hexadecamer with a mass of 1.3×10^6 Da that is composed of four different subunits, $(\alpha\beta\gamma\delta)_4$. The γ subunit is catalytic (Skuster et al., 1980; Reimann et al., 1984), and the other three subunits are regulatory, with δ being an intrinsic molecule of calmodulin (Cohen et al., 1978). At neutral pH, the activity of the nonactivated PbK holoenzyme is very low and shows a pronounced lag in the rate of product formation (Krebs et al., 1964). Neural (Ca^{2+}; Brostrom et al., 1971), hormonal (Ca^{2+} and phosphorylation by PK-A; Yeaman & Cohen, 1975), and metabolic (ADP; Cheng et al., 1985) signals stimulate PbK's activity. Its activity can also be enhanced *in vitro* by alkaline pH, by partial proteolysis of its α and β subunits, and by preincubation with Ca^{2+} plus Mg^{2+} ions (King & Carlson, 1981).

Based on early studies comparing the kinetic parameters for P-*b* conversion by activated *versus* nonactivated PbK, it became widely accepted that activation by various means is a result of an increase in PbK's affinity for P-*b* [i.e., a decrease in K_m; reviewed in Pickett-Gies and Walsh (1986)]. However, two recent reports have concluded that the mechanism of PbK's activation may be more associated with an increase in its general catalytic efficiency than in its binding affinity for P-*b*. For instance, the enzyme's ATPase activity, which occurs independently of any protein cosubstrate, was found to be stimulated completely in parallel with its P-*b* conversion activity in response to various modes of activation (Paudel & Carlson, 1991). Moreover, Newsholme and Walsh (1992) have reinvestigated the kinetic parameters of the P-*b* conversion reaction catalyzed by activated and nonactivated PbK and concluded that activation can be fully accounted for by an increased V_{max}. In none of these previous studies, however, was an attempt made to directly measure the relative affinities of activated *versus* nonactivated PbK for P-*b*, which is the subject of this report. A preliminary account of this work has been presented (Xu et al., 1993).

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[1] Abbreviations: AMP-PNP, 5′-adenylyl imidodiphosphate; AP, alkaline phosphatase; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Fab′, monovalent antigen-binding fragment; F(ab)′$_2$, divalent antigen-binding fragment; IgG, immunoglobulin G; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; P-*b*, phosphorylase-*b*; PbK, phosphorylase-*b* kinase; PK-A, cAMP-dependent protein kinase.

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EXPERIMENTAL PROCEDURES

Reagents. Protein A−agarose was from Bio-Rad. AMP-PNP was obtained from Boehringer Mannheim. $Mg(CH_3CO_2)_2$ was from Fisher Scientific and $CaCl_2$ from Mallinckrodt. Other compounds were from Sigma, with glycogen (G-8751) being further purified by several cycles of alternating precipitation with trichloroacetic acid (15%) and ethanol (increasing from 40% to 60%), followed by a batch charcoal treatment.

Proteins and Enzymes. The mAb157 was generated as described (Wilkinson et al., 1994) and used in ELISAs directly from cell culture supernatant without purification. Goat anti-mouse IgG−AP conjugate was from Southern Biotechnology, Inc. The catalytic subunit of PK-A was from Promega.

P-*b* was isolated from rabbit skeletal muscle as described (Fischer & Krebs, 1958), and residual AMP was removed with activated charcoal (Sigma, C-4386). For use in binding assays, crystallized P-*b* was dissolved in 25 mM Hepes (pH 6.8)/0.1 mM EDTA/1 mM dithiothreitol, and treated with charcoal.

PbK was purified from the back skeletal muscle of New Zealand White rabbits through the DEAE-cellulose chromatography step (Cohen, 1973) and stored frozen in 50 mM Hepes (pH 6.8)/0.2 mM EDTA/10% sucrose. Phosphorylation of PbK by the catalytic subunit of PK-A was by the method of Pickett-Gies and Walsh (1985). The final concentrations in the phosphorylation mixture were 50 mM β-glycerophosphate (pH 6.8), 0.5 mM EGTA, 1.5 mM $Mg(CH_3CO_2)_2$, 0.25 mM ATP, 0.1 mM dithiothreitol, 50 mM NaF, 0.5 mg/mL PbK, and 1.5 μg/mL catalytic subunit of PK-A. The reaction was allowed to proceed for 10 min at 30 °C. After precipitation with 50% ammonium sulfate, the resuspended PbK was desalted over a Sephadex G-25 column equilibrated with 50 mM Hepes (pH 6.8)/0.2 mM EDTA/10% sucrose and stored in small aliquots at −80 °C. The extent of ^{32}P incorporation was 1.1 mol of phosphate per mole of $\alpha\beta\gamma\delta$ protomer as determined by the phosphocellulose paper assay (Roskoski, 1983), which corresponded to 0.5 mol of phosphate per mole of α and β subunit, as determined following excision of the individual subunit bands from SDS−PAGE gels (Laemmli, 1970). The pH 6.8/pH 8.2 activity ratio of phosphorylated and purified PbK was determined to be 0.21, compared to 0.04 for its nonactivated predecessor.

The concentrations of PbK and P-*b* were spectrophotometrically determined using the appropriate absorbance indices (Cohen, 1973; Kastenschmidt et al., 1968), and that of phosphorylated PbK was matched by the Bio-Rad protein assay against its nonphosphorylated counterpart. The molecular weights used in calculations were 1.3×10^6 for PbK and 1.95×10^5 for P-*b*. The concentrations of mAb157 and its Fab′ and F(ab)′$_2$ fragments were determined by the Bio-Rad assay with BSA as the standard.

Generation and Purification of Fab′157. The mAb157 was purified from culture supernatant by protein A−agarose affinity chromatography. After precipitation with 50% ammonium sulfate, the pellet was dissolved in the loading buffer (10 mM sodium borate, pH 9.0/3.3 M NaCl) and applied to a preequilibrated protein A column (1-mL bed volume). The elution was effected using Affi-MAPS II elution buffer (Bio-Rad), with the pH adjusted to 4.5. The mAb fractions, located by the absorbance at 280 nm, were pooled and exchanged into 50 mM Hepes (pH 6.8)/0.5 mM EGTA (buffer A), while being concentrated by Centricon-30 (Amicon, Inc.). F(ab)′$_2$ of mAb157 was generated from pepsin digestion in 0.45 M sodium citrate (pH 4.5)/25 mM Hepes/0.25 mM EGTA at 37 °C for 2 h with a 7.5:1 (w/w) ratio of mAb157 to pepsin. The digestion was stopped by first raising the pH of the solution to approximately pH 8 with 3 M NaOH, followed by a 25-fold dilution with cold loading buffer containing 1 μg/mL BSA. Undigested IgG molecules were removed by passage over a protein A−agarose column. The F(ab)′$_2$ was further purified over a Sephadex G-200 gel filtration column equilibrated with buffer A. The Fab′157 was obtained by reducing the F(ab)′$_2$ with 100 mM dithiothreitol at 4 °C overnight.

Phosphorylation Assays. For determination of the pH 6.8/pH 8.2 activity ratio of PbK phosphorylated by the catalytic subunit of PK-A, the P-*b* conversion activity of the kinase holoenzyme was measured at 30 °C, with product formation monitored by a phosphocellulose paper assay (Roskoski, 1983). The final concentrations in the assays were 50 mM Tris, 50 mM β-glycerophosphate (pH 6.8 or pH 8.2), 3.1 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 (ICN), and 1 μg/mL or 0.1 μg/mL PbK at pH 6.8 or pH 8.2, respectively.

To test the influence of Fab′157 on the P-*b* conversion activity of PbK, the kinase holoenzyme (0.81 μg/mL) was preincubated at room temperature for 30 min with an excess amount of Fab′157 (0.125 mg/mL, which is 1000 times the molar concentration of the α subunit) plus or minus 1 mg/mL BSA. The inclusion of BSA was to compensate for possible kinase stablization by the large excess of protein (i.e., Fab′) in the preincubation mixture. The activity assay was then initiated by a 10-fold dilution of the kinase/Fab′ solution into the reaction mixture at 30 °C. The final concentrations of components in the assay were 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 (ICN), 0.081 μg/mL PbK, and 12.5 μg/mL Fab′, plus or minus 0.1 mg/mL BSA.

Direct ELISA. A summary of the direct ELISA procedures for measuring PbK and P-*b* binding using either varied PbK or varied P-*b* concentrations, with and without effectors, is outlined in Figure 1. The fixed concentrations for PbK and P-*b* were 15 μg/mL and 5 μg/mL, respectively, with their varied concentrations generally as shown in Figure 3. All ELISAs were performed at room temperature using Immulon I plates (Dynatech). The volumes per well were 100 μL for addition of samples and 230 μL for rinsing after each binding step. The amount of PbK bound to immobilized P-*b* was detected with a saturating amount of mAb157 (1:50 dilution of the crude culture supernatant), which was in turn detected by IgG−AP (1 μg/mL). Control experiments with all appropriate permutations of the various ELISA proteins showed no significant nonspecific binding of PbK or mAb157 to BSA or of mAb 157 to P-*b*. The hydrolysis of the AP substrate *p*-nitrophenyl phosphate (1 mg/mL) in the substrate buffer (0.9 M diethanolamine, pH 9.8/15 mM $MgCl_2$) was monitored by the absorbance at 405 nm. It should be noted that the rate of color development varied between experiments (but always within the linear range of absorbance); so, only the concentration of the varied protein

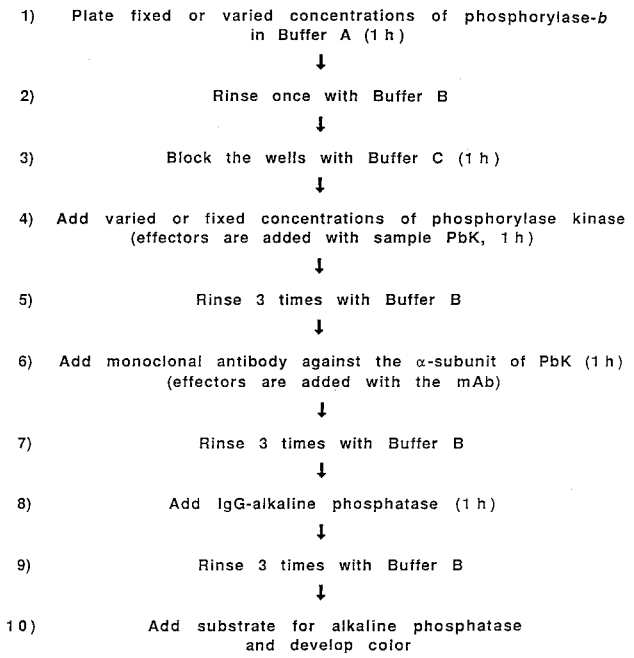

FIGURE 1: Outline of the direct ELISA method for measuring the binding of PbK to P-*b*. This ELISA procedure is further described under Experimental Procedures. Buffers used are buffer A (50 mM Hepes, 0.5 mM EGTA, pH 6.8 or 7.5); buffer B (50 mM Hepes, 0.5 mM EGTA, 1% BSA, pH 6.8 or 7.5); and buffer C (50 mM Hepes, 0.5 mM EGTA, 5% BSA, pH 6.8 or 7.5).

that gave 50% saturation of the other was meaningful. This concentration, which we term $K_{d'}$, is analogous to an apparent dissociation constant, but with one of the two interacting molecules immobilized. When effectors of PbK were tested for their influence on the binding of P-*b*, they were included during incubation with PbK (step 4) and were present from step 6 throughout. This corrected for their possible influence on the detection system in both control sets and sets with effectors, which were performed simultaneously on the same plate. Half of the wells on the 96-well plate were used to generate the control binding curve, and the remaining half were for the binding curve in the presence of effector. Consequently, each concentration of P-*b* or PbK used in generating a binding curve represented the average value of four to six replicate wells from the same plate. A minimum of four independent binding curves were generated for each effector, using at least two different preparations of PbK and P-*b*. When $MnCl_2$ was tested, it was present as described above, except that it was not included in steps 9 and 10 due to its precipitation at high pH.

For determination of the K_a values for the stimulation by Mg^{2+} and Mn^{2+} of PbK's binding of P-*b*, fixed concentrations of P-*b* (5 μg/mL) and PbK (25 μg/mL) were employed with varied concentrations of Mg^{2+} or Mn^{2+}, which were only present in the PbK binding step and not included in any other steps. The performance of all other steps was the same as in the ELISA procedure described above. At least four independent measurements were made with Mg^{2+} and Mn^{2+}.

Each binding curve was analyzed by nonlinear regression using the Enzfitter program (Elsevier-Biosoft, 1987) for determination of the apparent $K_{d'}$ values of PbK/P-*b* binding and the K_a values for Mg^{2+} and Mn^{2+} stimulation. Similar results were obtained when the data were analyzed using a Scatchard plot. To evaluate whether there was a statistical difference between the apparent $K_{d'}$ values obtained in the presence and absence of effectors, paired *t*-test analyses of the binding data were performed as described by Miller and Miller (1986). Mean values that were different at $P < 0.01$ were considered to be significantly different.

Competition ELISA. All competition ELISAs were performed at room temperature using Immulon I plates (Dynatech). A fixed concentration of P-*b* (20 μg/mL) in 50 mM Hepes (pH 6.8)/0.5 mM EGTA/0.1 mM DTT (100 μL) was plated for 1 h. After washing once with buffer A (pH 6.8) (230 μL/well), the remaining adsorption sites of the wells were blocked with buffer B (pH 6.8) (230 μL) for 1.5 h. A fixed concentration of PbK (0.5 μg/mL) was incubated for 40 min with, or without, various concentrations of P-*b*. Aliquots of 100 μL from these mixtures were then added to the blocked wells, and the remaining unbound PbK in the PbK/P-*b* preincubation mixture was allowed to bind to the plated P-*b* for 20 min. When effectors of PbK were tested for their influence on the binding of PbK to P-*b*, they were included only in the preincubation and the subsequent binding of PbK to the plated P-*b*. A control set without added effectors was develped at the same time. Detection of the amount of PbK bound was as in the direct ELISA, except that when PbK's effectors were tested, they were not present in steps 5−9 (Figure 1).

The percentage decrease in absorbance in the presence of increasing amounts of solubilized P-*b*, compared to that of the control developed in the absence of P-*b* solution, was calculated and plotted against the concentrations of the P-*b* solutions. The plots were analyzed as described above for determination of the apparent $K_{d'}$ values of PbK binding to P-*b* in solution.

RESULTS

Development of a Binding Assay. Because of the lack of a suitable analytical technique for quantifying the interaction completely in solution between two proteins as large as PbK and P-*b* (Xu, 1994), we developed an ELISA binding assay utilizing a mAb that had been previously generated and characterized in our laboratory (Wilkinson et al., 1994). This mAb (mAb157) binds to a C-terminal region of the α subunit of PbK with subnanomolar affinity without affecting the interaction of the kinase with its known effectors, nor do effectors influence the interaction of PbK with the mAb. The effect of mAb157 on P-*b* conversion by PbK was previously evaluated in preliminary experiments and concluded to be insignificant (Wilkinson et al., 1994). However, because the validity of employing mAb157 in the binding assay described herein absolutely requires that the mAb have no influence on the interaction between PbK and P-*b*, we further evaluated its effect on the phosphorylation of P-*b* by PbK using a large excess of the mAb (in its Fab′ form) and a low concentration of P-*b* (approximately one-tenth the K_m value of PbK for P-*b* determined under the same conditions that the effect of the Fab′ was evaluated). That the mAb has little, if any, effect on PbK's interaction with P-*b*, even under these stringent conditions, is shown in Figure 2, in which PbK was preincubated with a 1000-fold molar excess (per $\alpha\beta\gamma\delta$ protomer) of the Fab′ fragment of mAb157 and then tested for its ability to phosphorylate P-*b*. The use of the Fab′ fragment instead of the whole IgG molecule of mAb157 was to avoid cross-linking of the tetravalent PbK by the divalent

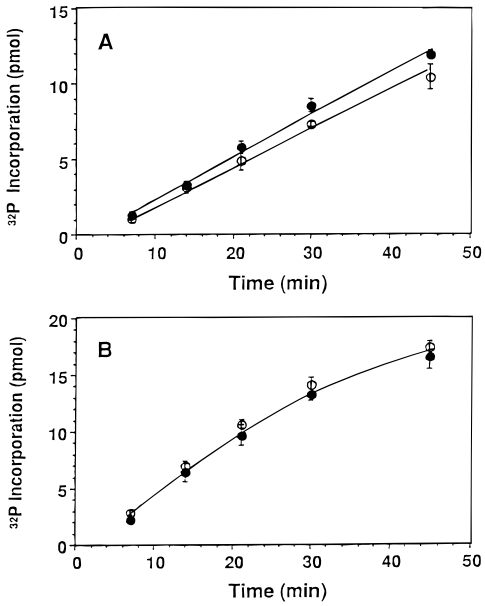

FIGURE 2: Effect of the Fab′157 fragment on P-*b* conversion by PbK. The ^{32}P incorporation into P-*b* at 30 °C and pH 8.2 catalyzed by PbK in the absence (○) or presence (●) of a 1000-fold molar excess of Fab′157 was measured as described under Experimental Procedures. Aliquots were removed at the indicated time points. (A) Without BSA; (B) with BSA. The data points are the average of triplicates, with the error bars representing standard deviations.

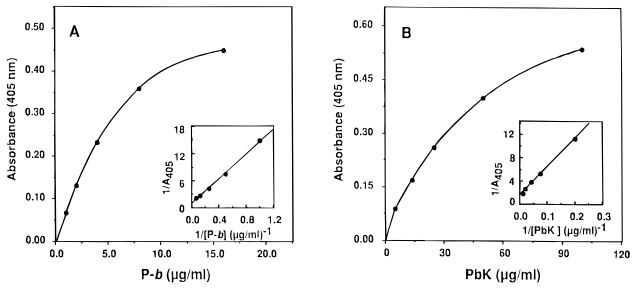

FIGURE 3: Representative binding curves obtained with ELISAs. The measurement of PbK binding to P-*b* at pH 7.5 by ELISAs was performed as described under Experimental Procedures, with varied concentrations of either P-*b* (A) or PbK (B). (A) P-*b* concentrations were varied at 1, 2, 4, 8, and 16 μg/mL, with the concentration of PbK fixed at 15 μg/mL. (B) PbK concentrations were varied at 5, 13.7, 25, 50, and 100 μg/mL, with P-*b* fixed at 5 μg/mL. Insets: Double-reciprocal replots of the data giving $K_{d'}$ values of 39.1 nM (A) and 40.1 nM (B).

IgG. When BSA was included in the preincubation mixtures (Figure 2B), the slight apparent stimulation of activity by the Fab′ observed at later time points in Figure 2A disappeared. This suggests that the slight stimulation in Figure 2A is likely due to a general protein stablization effect caused by the high concentration of Fab′157, which was absent in the control set. As determined by ELISAs, mAb157 did not cross-react with either P-*b* or BSA. Thus, it was established from these experiments that mAb157 is an acceptable probe for detecting and quantifying PbK bound to P-*b* in ELISAs.

The scheme for the direct ELISA utilized in this study is illustrated in Figure 1, with details described under Experimental Procedures. It is demonstrated in Figure 3 that hyperbolic binding curves and similar apparent $K_{d'}$ values [39.4 ± 0.1 nM (Figure 3A) and 40.1 ± 0.1 nM (Figure 3B)] were obtained regardless of whether the concentration of PbK or P-*b* was varied. Although the apparent $K_{d'}$ values derived from this binding assay with immobilized P-*b* do not necessarily represent those that would be found if both PbK and P-*b* were in solution, this assay nevertheless does provide the first method for measuring the relative affinities between PbK and P-*b* under various conditions known to activate the kinase. Because its activation is most apparent when PbK is assayed at pH 6.8, we chose this pH to test the effects on P-*b* binding of activation by ADP, glycogen, and phosphorylation by PK-A. To allow comparison with previous literature data, the effects of divalent metal ions (Mg^{2+}, Mn^{2+}, and Ca^{2+}) and ATP analogs were tested at pH 7.5.

Effect of Activation of PbK on Its Relative Affinity for P-b. Phosphorylation and the resultant activation of PbK by PK-A are important *in vivo* in the cascade activation of glycogenolysis initiated by certain hormones. If activation of PbK is associated with an increase in its affinity for P-*b*, we reasoned that we should be able to detect such a change in relative affinity using the ELISA binding method. Therefore, we prepared PbK that was activated 5-fold (as measured after purification) via phosphorylation by the catalytic subunit of PK-A under conditions where autophosphorylation was minimized. After the concentrations of the activated and nonactivated forms of PbK were carefully matched by the Bio-Rad protein assay, using the nonphosphorylated (nonactivated) form as standard, the relative affinity of P-*b* for the two forms of PbK was compared by ELISAs and found to be essentially the same (Table 1). These results indicate that although the kinase had been activated, its affinity for P-*b* was not affected significantly, which is consistent with recent conclusions that a decrease in the K_m for P-*b* does not account for PbK's activation by phosphorylation (Paudel & Carlson, 1991; Newsholme & Walsh, 1992).

Because PbK has a much higher specific activity at pH 8.2 than at pH 6.8, we compared the relative affinity of PbK for P-*b* at these two pH values. To take into account the possible influence of pH on the detection system, ELISA controls were performed simultaneously at both pH 6.8 and pH 8.2, with all steps after PbK binding being carried out at both pH values. As with phosphorylation, we found that pH 8.2 had little effect on the apparent $K_{d'}$ of PbK for P-*b* (Table 1).

ADP stimulates both the P-*b* conversion and autophosphorylation activities of the kinase through a high-affinity allosteric site, probably on the β subunit (Cheng et al., 1985, 1988). The effect of ADP on the PbK/P-*b* interaction was evaluated at a concentration 10 times its reported K_d value for the kinase (see Experimental Procedures for the measures taken with all effectors to assess their potential influence on the detection system). The results showed that ADP had little effect on the affinity of PbK toward P-*b* (Table 1); GDP, another allosteric activator (Cheng & Carlson, 1988; Cheng et al., 1988), was likewise found to have little effect (data not shown).

Glycogen has been reported to stimulate P-*b* conversion by PbK and to lower the K_m for P-*b* (Krebs et al., 1964). When tested for its effect on the relative affinity of PbK for P-*b* in the binding assay, glycogen (5.5 mg/mL) did cause an approximately 50% increase in the apparent affinity at pH 6.8 (Table 1); however, because glycogen binds to both PbK (Steiner & Marshall, 1982) and P-*b*, this effect cannot yet be attributed to a specific influence on just one of the two proteins.

Table 1: Comparison of $K_{d'}$ Values under Different Conditions[a]

condition	apparent $K_{d'}$ (nM)	condition	app $K_{d'}$ (nM)
(1) control	39.6 ± 7.9 (26)	(7) Mg^{2+}	2.9 ± 0.5** (6)
(2) phosphorylation	34.7 ± 5.8 (4)	(8) Mg^{2+}/Ca^{2+}	5.2 ± 0.7** (4)
(3) pH 8.2	44.6 ± 8.0 (4)	(9) Mg^{2+}/ADP	3.8 ± 0.7 (8)
(4) ADP	42.1 ± 1.8 (4)	(10) Mg^{2+}/ADP/Na_2HPO_4	5.9 ± 1.7 (4)
(5) glycogen	16.7 ± 2.9** (4)	(11) Mg^{2+}/ADP/$NaNO_3$	1.9 ± 0.1 (4)
(6) Ca^{2+}	18.0 ± 3.8** (4)	(12) Mg^{2+}/Ca^{2+}/AMP-PNP	3.6 ± 0.6 (4)

[a] The apparent $K_{d'}$ values under various conditions were measured with either fixed PbK or fixed P-*b* as described under Experimental Procedures, where the appropriate pH is also given. The concentrations of effectors were: Mg^{2+} (10 mM), Ca^{2+} (0.7 mM total), glycogen (5.5 mg/mL), ADP (0.5 mM), $NaNO_3$ and Na_2HPO_4 (5 mM), and AMP-PNP (0.8 mM). The values for the apparent $K_{d'}$ are averages with standard deviations of independent experiments (the number of experiments performed under each condition is given in parentheses). Two different PbK preparations were used under each condition. For the paired *t*-test analyses, condition 1 was the control for conditions 2−7, condition 7 was the control for conditions 8−11, and condition 8 was the control for condition 12. Where there is a significant difference ($P < 0.01$) between the control and test conditions, the apparent $K_{d'}$ values are denoted with double asterisks ($K_{d'}$**).

Effects of Divalent Metal Ions. The activity of PbK has been previously shown to be influenced by several divalent metal ions, among them Mg^{2+}, Ca^{2+}, and Mn^{2+}. To gain a greater understanding of their mechanisms of action, we compared the effects of these three divalent cations on the PbK/P-*b* interaction. It was found that Mg^{2+} greatly enhanced the apparent affinity of the kinase for P-*b*, as indicated by a large decrease in the apparent $K_{d'}$ value to about $^1/_{13}$th that of the control (Table 1). Similar results were obtained regardless of whether the concentration of P-*b* was fixed or varied. This is the first effector of PbK found to substantially alter its affinity for P-*b*; this finding also demonstrates that the ELISA binding method is sensitive to changes in the relative affinity of PbK for P-*b* resulting from the influence of effectors.

The physiologically important regulatory cation Ca^{2+}, which has been reported to decrease the K_m of PbK for P-*b* by a factor of 13 (Heilmeyer et al., 1970), also stimulated P-*b* binding, but to a considerably lesser extent than Mg^{2+}. The decrease in the apparent $K_{d'}$ was by a factor of about 2 (Table 1), similar to the effect of glycogen. When Mg^{2+} and Ca^{2+} were present together, there was no further decrease in the apparent $K_{d'}$ value compared to that observed with Mg^{2+} alone (Table 1), indicating that Mg^{2+} accounts for most of the increase in affinity.

Mn^{2+} was even more potent than Mg^{2+} in enhancing the interaction between PbK and P-*b*; 10 mM Mn^{2+} caused a decrease in the apparent $K_{d'}$ by a factor of 99. The concentrations of Mg^{2+} and Mn^{2+} required to cause half-maximal stimulation of P-*b* binding were determined by ELISA to be 1.42 ± 0.33 mM and 1.11 ± 0.12 mM, respectively. To further compare the effectiveness of Mg^{2+} and Mn^{2+} in enhancing the binding of P-*b*, we determined the apparent $K_{d'}$ values for P-*b* at equivalent extents of saturation with the metal ions. At 7 times their respective K_a values, Mn^{2+} was about 10 times more effective than Mg^{2+} in increasing the affinity for P-*b*. At twice their respective K_a values, however, Mn^{2+} showed only a 2-fold greater stimulatory effect than Mg^{2+} (data not shown).

Effects of ATP Analogs. The kinetic mechanism of both the PbK holoenzyme and its isolated γ subunit has been reported to be most consistent with rapid equilibrium random bi-bi (Tabatabai & Graves, 1978; Farrar & Carlson, 1991). For this mechanism, ATP and P-*b* would not be expected to affect each others binding affinity, especially inasmuch as the double-reciprocal plots of the initial velocity data at varied concentrations of substrates intersect on, or near, the abscissa. To evaluate the effect of ATP on the binding of P-*b*, we used the analog AMP-PNP, so as to avoid phosphorylation of P-*b* and autophosphorylation of PbK during the binding assays. In addition, Mg^{2+} and Ca^{2+} ions were included to simulate catalytic reaction conditions. Inclusion of AMP-PNP with the cations had little effect on PbK's affinity for P-*b* (Table 1). Similar results were obtained with ADP plus either NO_3^- or P_i to roughly approximate the structure of ATP. Together, these results indicate that nucleotide does not affect the binding of P-*b*, which is consistent with the previous conclusions of a random kinetic mechanism.

Use of a Competition ELISA To Confirm Results Obtained with the Direct ELISA. Because of the concern regarding potential effects of immobilization of P-*b* in the direct ELISA, a competition ELISA (described under Experimental Procedures) was developed to verify results obtained with the direct ELISA. An advantage of the competition ELISA technique is that it allows PbK and P-*b* to interact in solution, although immobilized P-*b* must still be utilized in the assay. Without added effector, the apparent $K_{d'}$ value of PbK for P-*b* was determined by competition ELISAs to be 29.8 ± 1.3 nM, which is similar to the value obtained by direct ELISAs. Not only was the control binding affinity measured with these two different assays similar, so were the relative effects of Mg^{2+}, Ca^{2+}, and glycogen in enhancing the binding of P-*b* (data not shown). The competition ELISA was also used to confirm that the observed affinity enhancement by Mg^{2+} was actually due to the Mg^{2+} cation, as opposed to its acetate counterion or to an increase in ionic strength. In this case, 10 mM $Mg(CH_3CO_2)_2$ enhanced the measured affinity by approximately 17-fold, whereas 20 mM $NaCH_3CO_2$ was without effect.

DISCUSSION

Quantification of the interaction between proteins as massive as PbK (1.3×10^6) and P-*b* (1.95×10^5) presents a special set of problems, which are undoubtedly exacerbated by the multivalent nature of the two proteins (tetravalent and bivalent, respectively). The first attempt to directly study the interaction between these proteins relied on detection of complex formation by frontal gel filtration chromatography, but the complex eluted with an apparent mass of only half the theoretical value (Gergely et al., 1975). Fluorescence polarization utilizing derivatized P-*b* has also been evaluated (Dimitrov, 1976; Xu, 1994), but there are a number of problems in using this technique with this particular protein pair. Among the more serious of these are the alteration of

Table 2: Reported Half-Maximal Values of Mg^{2+}-Induced Effects on PbK

half-max Mg^{2+} concn (mM)	pH	enzyme form	function stimulated	ref
0.6	8.2	holoenzyme	P-*b* conversion	Clerch & Huijing (1972)
4	6.8	holoenzyme	synergistic activation with Ca^{2+}	King & Carlson (1981a)
4	6.8	holoenzyme	EGTA-insensitive P-*b* conversion	King & Carlson (1981b)
4	7.0	holoenzyme	formation of PbK−glycogen complex	Steiner & Marshall (1982)
3.1	8.2	holoenzyme	autophosphorylation	Hallenbeck & Walsh (1983)
1.1	8.0	γ subunit	peptide phosphorylation	Farrar & Carlson (1991)
2.7	8.0	$\gamma-\delta$ complex	peptide phosphorylation	Farrar & Carlson (1991)
1.7	6.8	holoenzyme	binding of anti-β and anti-γ mAbs	Wilkinson (1993)
1.4	7.5	holoenzyme	binding of P-*b*	this report

the enzymatic properties and stability of P-*b* by derivatization, the availability of a fluorescent probe of sufficiently long lifetime, and photobleaching (Xu, 1994). Affinity electrophoresis and Hummel−Dreyer gel filtration−high-performance liquid chromatography have also been evaluated, but were eliminated for technical reasons (Xu, 1994). Thus, the ELISA approach described in this report represents the first method that allows convenient measurement of the interaction between these two proteins under a variety of conditions. In turn, the ability to detect changes in its relative affinity for P-*b* brought about by substrates and effectors of PbK has allowed further evaluation of the kinetic and activation mechanisms of this kinase. Because the ELISA method does require immobilization of one of the two interacting proteins, the measured binding affinities do not necessarily represent the K_d values for the same protein pair interacting in solution. Based upon its kinetic mechanism and reported K_m value for P-*b* of 20 μM (Tabatabai & Graves, 1978), the K_d value for the interaction of PbK and P-*b* in solution may be considerably weaker than that measured by the ELISA method. However, until an independent method to directly measure the binding of P-*b* to PbK in solution can be developed, the ELISA method is useful for detecting relative changes in affinity induced by different conditions and effectors.

A variety of approaches have indicated that activation, by phosphorylation for instance, of the PbK holoenzyme results from deinhibition associated with conformational changes, most apparent in the regulatory β subunit, that release constraints on the activity of the catalytic γ subunit imposed by the quaternary structure of the nonactivated enzyme (Fitzgerald & Carlson, 1984; Trempe & Carlson, 1987; Paudel & Carlson, 1987; Cheng et al., 1988). However, the precise mechanistic basis underlying the enhancement of catalytic ability upon activation remains unresolved. Although the studies of Paudel and Carlson (1991) and Newsholme and Walsh (1992), described in the introduction, agreed that a change in the affinity for P-*b* is unlikely to be the cause of PbK's activation, they did not directly address the question of whether a change in affinity nevertheless occurs concomitantly with activation. The results of the present study indicate, however, that regardless of whether PbK is activated by phosphorylation, alkaline pH, or the allosteric effector ADP, there is not a significant change in its affinity for P-*b*, as measured by ELISAs. Divalent cations, on the other hand, do induce a large increase in the apparent affinity for P-*b*, which may indicate that they bring about activation through a different mechanism. Although it is clear that activation of the catalytic subunit of the PbK holoenzyme can be initiated by changes in its regulatory subunits [reviewed in Pickett-Gies and Walsh (1986) and Heilmeyer (1991)], it is possible that the activation by Mg^{2+}, and in some instances by Mn^{2+}, may be initiated partially, or perhaps wholly, by changes in the catalytic subunit itself, as is discussed below.

Inclusion of Mg^{2+} ions in excess of ATP causes a relatively large stimulation of the P-*b* conversion activity of PbK. Stimulation by this second Mg^{2+} ion, the first being that necessary for formation of a Mg^{2+}−nucleotide complex, occurs with all forms of PbK: holoenzyme (Chelala & Torres, 1968; Villar-Palasi & Wei, 1970; Clerch & Huijing, 1972), $\alpha\gamma\delta$ and $\gamma\delta$ complexes (Chan & Graves, 1982), isolated full-length γ subunit (Kee & Graves, 1987; Farrar & Carlson, 1991), and several engineered truncated forms of the γ subunit (Cox & Johnson, 1992; Huang et al., 1993). Singh et al. (1982) reported that the K_m of the nonactivated PbK holoenzyme for P-*b* was decreased by a factor of 5 when the concentration of Mg^{2+} greatly exceeded that of ATP, which is consistent with the increased affinity for P-*b* induced by Mg^{2+} in this study (Table 1). Because activation by excess Mg^{2+} is observed with the free catalytic γ subunit, which by definition interacts with P-*b*, that subunit must supply a binding site through which the second Mg^{2+} ion exerts its stimulatory effect on P-*b* conversion and, presumably, on P-*b* binding. The recently solved crystal structure of the truncated γ subunit complexed with MgADP does, in fact, show two Mg^{2+} ions bound; however, both appear to be involved in multiple interactions with the phosphates of the nucleotide, in addition to their interactions with the γ subunit (Owen et al., 1995). One might ask then whether all effects of Mg^{2+} on the PbK holoenzyme can be attributed to occupancy of these two sites on the γ subunit. This question becomes more intriguing when considering that some effects of Mg^{2+}, such as in this study, occur in the complete absence of other ligands, including the nucleotide substrate. The similarity of the reported values for the half-maximal concentration of Mg^{2+} required to stimulate various functions of PbK (Table 2) is consistent with a common mechanism, or binding site(s), being responsible for the influence of Mg^{2+} on these diverse functions. If in fact all of these observed effects of Mg^{2+} on the holoenzyme are initiated through the binding sites on the γ subunit, then in response to this ion the γ subunit would be influencing the conformation of the regulatory subunits, just as the regulatory subunits control the conformation of γ in response to other effectors. As potential examples of this, Mg^{2+} is known to protect the regulatory β subunit from proteolysis (Trempe & Carlson, 1987) and to enhance its interaction with a subunit-specific mAb (Wilkinson, 1993). In the latter case, there is a parallel increase in the binding of two different

mAbs specific for the β and γ subunits in response to progressively increasing concentrations of Mg^{2+}, with a K_a for the two of 1.7 mM.

Although crystallography indicates that Mg^{2+} and Mn^{2+} ions bind to the γ subunit in a structurally equivalent manner (Owen et al., 1995), these two metal ions have remarkably different effects on PbK's activities. In comparison to Mg^{2+}, excess Mn^{2+} causes a large inhibition of the P-*b* conversion activity of all forms of the kinase (Clerch & Huijing, 1972; Chan & Graves, 1982; Kee & Graves, 1987; Cox & Johnson, 1992; Huang et al., 1993). In contrast, the autophosphorylation, ATPase, and tyrosine phosphorylation activities of PbK, which occur at much slower rates than P-*b* conversion (by a factor of 2−3 orders of magnitude; Paudel & Carlson, 1991; Yuan et al., 1993), all utilize Mn^{2+} relatively efficiently. In the case of autophosphorylation, MnATP is as effective as MgATP in supporting the initial reaction, and an excess of either cation over nucleotide is stimulatory, although the pattern of subunit phosphorylation is different for the two cations (Hallenbeck & Walsh, 1983). The ATPase activity of the truncated γ subunit is also supported equally well by MnATP and MgATP; however, unlike Mg^{2+}, excess Mn^{2+} causes no further rate enhancement (Huang et al., 1993). In further contrast to P-*b* conversion, the tyrosine phosphorylation activity of PbK is supported by MnATP, but not by MgATP (Yuan et al., 1993). Thus, Mn^{2+} can substitute for, or improve upon, Mg^{2+} in the three slow activities of PbK (autophosphorylation, ATPase, and tyrosine phosphorylation), but not in its rapid activity (P-*b* conversion). Apparently, the rate-limiting steps for the fast and slow activities have different metal ion sensitivities. In comparison to Mg^{2+}, Mn^{2+} decreases the K_m for P-*b* of the γ subunit (Yuan et al., 1993), and greatly enhances the affinity for P-*b* of the holoenzyme (this study). It is possible that the enhancement of P-*b* binding and inhibition of P-*b* conversion by Mn^{2+}, in comparison to Mg^{2+}, are different manifestations of a more fundamental feature of PbK that is affected differently by the two cations, because Mg^{2+} and Mn^{2+} have considerably different effects on PbK's structure. For example, autophosphorylation in the presence of one ion *versus* the other results in a large difference in the extent of phosphate incorporated into the β subunit (Hallenbeck & Walsh, 1983). Moreover, Mn^{2+}, in contrast to Mg^{2+}, provides very little protection against trypsinolysis of the β subunit (Trempe & Carlson, 1987). In addition, simultaneous changes in the binding of certain subunit-specific mAbs to the β and γ subunits induced by Mg^{2+} are not observed with Mn^{2+} (Russell et al., 1992). To further understand the reasons for the different effects of these two cations on activity and structure, it would be of great value to know whether all of their binding sites on PbK are the same, whether relevant sites exist apart from the two on the γ subunit that are known (Owen et al., 1995), and whether a differential interaction with P-*b* contributes to their different effects on its conversion (Hallenbeck & Walsh, 1983).

Ca^{2+} ions stimulate PbK's activity measured in the presence of excess Mg^{2+}, and their stimulatory effect is presumed to be mediated entirely through the enzyme's intrinsic calmodulin subunit, δ. As with Mg^{2+} and Mn^{2+}, Ca^{2+} has also been reported to decrease PbK's K_m for P-*b* (Heilmeyer et al., 1970); however, this effect of Ca^{2+} has recently been questioned (Newsholme & Walsh, 1992). Regardless of whether Ca^{2+} ions lower the K_m, they do increase the affinity of PbK for P-*b* as measured by the ELISA method (Table 1). This result is in agreement with an earlier observation by Gergely et al. (1975), who found that Ca^{2+} ions were required for formation of a PbK/P-*b* complex (the effect of Mg^{2+} was not evaluated in that study). Ca^{2+} was much less effective than Mg^{2+} in enhancing PbK's binding of P-*b* (Table 1), which is consistent with the primary locus of action of the two cations being on different subunits. In fact, it is possible that the primary subunit through which any ligand or event acts to stimulate the P-*b* conversion activity of the PbK holoenzyme may be reflected by the enzyme's relative affinity for P-*b*. For example, those ligands or events whose actions are mediated through the regulatory α and/or β subunits (phosphorylation and probably ADP and alkaline pH) had no influence on the affinity for P-*b*. Ca^{2+}, on the other hand, acts through the regulatory δ subunit, which interacts strongly with the γ subunit, and caused a modest enhancement of affinity. Finally, Mg^{2+} and Mn^{2+}, which presumably act, at least in part, directly through the catalytic γ subunit itself, caused the greatest increase in the affinity for P-*b*.

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