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# The N-Terminus of Glycogen Phosphorylase *b* Is Not Required for Activation by Adenosine 5′-Monophosphate[†]

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ABSTRACT: The, so far unsuccessful, search for selective effective inhibitors of glycogen phosphorylase for the treatment of type II diabetes has made phosphorylase an active target of research for the past 20 years. Many crystallographic structures of phosphorylase are currently available to aid in this research. However, those structures have been interpreted, at least in part, on the basis of work conducted with a proteolytically derived form of phosphorylase that lacked the N-terminus (phosphorylase *b*′). It has been reported that phosphorylase *b*′ shows no allostery, neither homotropic nor heterotropic. The original report on phosphorylase *b*′ examined the allosteric characteristics over very narrow ranges of effector and substrate concentrations and reported the presence of proteolytic cleavages in addition to the removal of the N-terminus. We have applied molecular biological techniques to generate a truncate lacking the N-terminus with know primary structure, and we have established conditions for fully quantifying the allosteric effect of AMP on glycogen phosphorylase *b*. We report here for the first time the full thermodynamic effect of AMP on phosphorylase *b*. Our findings with a truncate lacking the N-terminus show that the effect of AMP binding does not depend on the N-terminus.

Glycogen phosphorylase was one of the first enzymes recognized as being allosterically regulated, as well as the first case of regulation by phosphorylation (*1*−*3*). The phosphorylation of phosphorylase *b* at serine 14 converts the enzyme to the more active phosphorylase *a*. Activation by phosphorylation and allosteric activation by AMP[1] are well-established. However, over the past 20 years, muscle phosphorylase has continued to be studied as a model for the regulation of liver glycogen phosphorylase, which is linked to type II diabetes (*4*−*7*). Unfortunately to date, this approach has yielded no therapeutics that are able to effectively exploit phosphorylase for the treatment of diabetes.

The recognition that muscle phosphorylase was activated by phosphorylation at serine 14 led to much early interest in the role of the N-terminus in the activation of phosphorylase (*8*, *9*). The work done to establish the role of the N-terminus was published in 1968 and led to the formation of phosphorylase *b*′, a form of the enzyme that had the first 16 residues removed via proteolysis (*8*). The reported results implied that all of the allosteric responsiveness in phosphorylase was lost in the absence of the N-terminus. Specifically, it was found that AMP did not affect the affinity for phosphate and phosphate did not affect the affinity for AMP. These findings led to the conclusion that the N-terminus was responsible for transmitting the allosteric signal in phosphorylase *b*. The conclusions drawn from phosphorylase *b*′ were and have remained the basis for interpreting the crystallographic structures of glycogen phosphorylase (*10*−*12*). It must be noted, however, that phosphorylase *b*′ was derived by limited proteolysis of the enzyme purified from rabbit muscle and that additional effects on the primary structure of the enzyme were present as confirmed by polyacrylamide gel electrophoresis that yielded a diffuse band when stained for either protein or phosphorylase activity (*8*). Given that modern experimental techniques, particularly in molecular biology and data analysis, have advanced the ability to specifically address the role of the N-terminus of glycogen phosphorylase, we have undertaken this study to evaluate the significance of the N-terminus of glycogen phosphorylase *b* in the activation by AMP. We report here, for the first time, the full magnitude of the effect of AMP on glycogen phosphorylase *b*. We also show that the N-terminal truncate, Δ2−17, of rabbit muscle glycogen phosphorylase retains all of the heterotropic and homotropic cooperativity observed in wild-type phosphorylase. However, Δ2−17 has a reduced affinity for both AMP and phosphate. In addition, we were able to test the proteolytically derived phosphorylase *b*′ under our experimental conditions. These data yield results that are comparable to the original findings with phosphorylase *b*′.

## MATERIALS AND METHODS

Phosphorylase *b* was obtained from bacterial expression of the recombinant rabbit muscle glycogen phosphorylase gene in the pTACTAC plasmid as previously described (*13*). Purified proteins were stored at 4 °C and generally used within 1 week.

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[1]Abbreviations: AMP, adenosine 5′-monophosphate; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; NADP, nicotinamide adenine dinucleotide phosphate.

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β-Glycerophosphate was from Sigma-Aldrich (St. Louis, MO) or USB (Cleveland, OH). Restriction enzymes were from New England Biolabs (Beverly, MA). Ion exchange chromatography resins were from Amersham Biosciences (Piscataway, NJ). Size exclusion resin, glycogen phosphorylase kinase, trypsin, and soybean trypsin inhibitor were from Sigma-Aldrich. Glucose-6-phosphate dehydrogenase was from Roche Applied Sciences (Indianapolis, IN). Phosphoglucomutase was from Roche Applied Sciences or Sigma-Aldrich. Rabbit liver glycogen used in this study was purchased from Sigma-Aldrich and was used as purchased. Glycogen was assayed for AMP prior to use with a coupled enzyme assay, and no detectable contamination was observed. Other chemicals were from Sigma-Aldrich.

*Mutagenesis and Molecular Biology*. The pTACTAC plasmid with the gene for wild-type glycogen phosphorylase from rabbit muscle inserted between NdeI and HindIII sites as previously described was used as the starting template for mutagenesis (*13*). The truncate Δ2−17 was created by using the Quik Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) to introduce a second NdeI cut site positioning the starting ATG at codon 17. The resulting plasmid was digested to completion with NdeI and gel purified to remove the DNA sequence between codons 1 and 17. The plasmid was then religated to produce the plasmid with a phosphorylase gene missing the DNA residues encoding amino acids 2−17. The nucleotide sequences over the region of interest were verified via DNA sequencing at the Gene Technology Laboratory at Texas A&M University.

*Protein Expression and Purification*. Wild-type and mutant phosphorylase were expressed from plasmid pTACTAC as previously described (*13*) with the exception that the growth was conducted in *Escherichia coli* strain DF1020 [*pro-82, glnV44*(AS), Δ*pfkB201, recA56, endA1,* Δ(*rhaD-pfkA*)*200, thi-1, hsdR17*] (*14*, *15*) for 48 h at 27 °C in 1.5 L cultures that were inoculated with 40 mL of culture grown to saturation at 37 °C in the absence of inducer. Cell pellets from expression were stored at −80 °C prior to protein purification. Protein purification was conducted with modifications of previously described methods (*13*). Briefly for the wild type, cell pellets from 1.5−3 L of culture were thawed and resuspended in 75 mL of resuspension buffer [50 mM β-glycerophosphate (pH 7.0), 30 mM β-mercaptoethanol, 1 mM EDTA, 0.2 mM PMSF, 0.7 μg/mL pepstatin A, 0.5 μg/mL leupeptin, and 0.01% benzamidine]. Cells were lysed by sonication until the $OD_{600}$ was approximately $^1/_{10}$ of the starting value. Debris was pelleted by centrifugation at 14000*g* for 45 min. The supernatant was brought to 0.5% polyethylenimine by addition of a 10% solution (40 mM β-glycerophosphate and 10% polyethylenimine) and stirred on ice for 30 min. The precipitate was removed by centrifugation at 14000*g* for 45 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to 50% saturation, and the mixture was stirred on ice for 20 min. The precipitated protein was recovered by centrifugation at 14000*g* for 30 min. The pellet was resuspended in 25 mL of column buffer [25 mM β-glycerophosphate (pH 7.0), 1 mM β-mercaptoethanol, 1 mM EDTA, 0.2 mM PMSF, and 0.01% benzamidine] and dialyzed against three changes of the same buffer. Protein was then run over DEAE-Sepharose fast flow and SP-Sepharose Fast Flow columns run in tandem. Fractions from the load and wash showing activity were pooled and used for subsequent kinetic and fluorescence experiments.

For Δ2−17, the purification procedure was the same as that described above until the chromatography step. Δ2−17 was loaded on a Q-Sepharose Fast Flow column and eluted with a 0 to 300 mM KCl gradient in column buffer. The cleanest fractions (as assessed by 10% SDS−polyacrylamide gel electrophoresis) with activity were pooled, dialyzed to remove salt, and loaded on a DEAE-Sepharose Fast Flow column. Elution was conducted with a 0 to 300 mM KCl gradient in column buffer. The cleanest fractions with activity were pooled and dialyzed to remove salt prior to being used in kinetic and fluorescence studies. Protein was quantified using the Pierce BCA protein assay.

*Phosphorylase a and Phosphorylase b′*. Phosphorylase *a* was produced and isolated utilizing purified phosphorylase *b* and commercially available phosphorylase kinase as previously described (*16*). Phosphorylase *b′* was made from phosphorylase *a* following the protocol of Graves (*8*).

*Kinetic Measurements*. Glycogen phosphorylase activity was followed in the direction of glycogen degradation at 25 °C utilizing phosphoglucomutase and glucose-6-phosphate dehydrogenase in a coupled enzyme assay system to link the degradation of glycogen to the production of NADPH, which was followed at 340 nm on a Beckman 600 series UV−vis spectrophotometer. Assays were conducted in a 600 μL reaction volume containing 50 mM PIPES (pH 6.8), 100 μM EDTA, 5 mg/mL rabbit liver glycogen, 0−5 mM AMP, 0−300 mM potassium phosphate, 360 μM NADP, 4 μM glucose 1,6-bisphosphate, 10 mM $MgCl_2$, 6.7 units/mL phosphoglucomutase, and 3 units/mL glucose-6-phosphate dehydrogenase. Changes in ionic strength due to varying phosphate concentrations were compensated by the addition of appropriate amounts of KCl to maintain an ionic strength of approximately 0.69 M. The temperature was maintained within 1 °C by a circulating water bath. The assay mixes were preincubated at 25 °C, and the reaction was initiated by addition of appropriately diluted glycogen phosphorylase.

*Steady State Fluorescence*. Spectral measurements were recorded on an SLM-4800 instrument with a Phoenix upgrade package from ISS (Champaign, IL). Spectra were recorded with a 295 nm excitation wavelength at 25 °C in a 1 cm × 1 cm cuvette. Excitation slits were set to 2 nm, and emission slits were set to 8 nm. All measurements were collected with protein in column buffer. The same buffer without protein was used as a blank to correct the spectra. For experiments with AMP, 2 mM AMP was added to the blank as well as the sample.

*Data Analysis*. Initial velocity data were plotted as titrations for both phosphate and AMP, and individual titration curves were fit to the Hill equation to determine $K_{1/2}$ and the Hill number (*17*). The measured Hill numbers for AMP binding at low concentrations of phosphate ( < 40 mM for the wild type and < 20 mM for Δ2−17 and phosphorylase *b′*) from multiple experiments were averaged, and the average values were used to estimate the homotropic coupling between AMP sites in the absence of phosphate per eq 1 (*18*).

$$Q_{AMP} = \left(\frac{n_H}{2 - n_H}\right)^2 \quad (1)$$

where $Q_{AMP}$ is the homotropic coupling quotient of AMP binding in the absence of phosphate and $n_H$ is the average of multiple determinations of the Hill number for AMP binding at limiting low concentrations of phosphate (see Table 1). Analogous approaches were used to quantify the homotropic coupling quotient for AMP binding in the saturating presence of phosphate ($Q_{AMP/P_i}$), as well as the homotropic coupling quotient for

Table 1: Kinetic Constants and Coupling Free Energies for the Wild Type (WT *b*), Δ2−17, and Phosphorylase *b*′ (*b*′)

| | WT *b* | Δ2−17 | *b*′ |
|---|---|---|---|
| $K_{P_i}^{o}$ (mM)[a] | 259 ± 3 | 848 ± 29 | 129 ± 10 |
| $K_{AMP}^{o}$ (mM)[a] | 0.43 ± 0.01 | 5.8 ± 0.4 | 0.033 ± 0.005 |
| $n_H(P_i)$[b] | 1.95 ± 0.05 (8) | 1.81 ± 0.04 (5) | 1.00 ± 0.03 (11) |
| $n_H$(AMP)[c] | 1.52 ± 0.03 (20) | 1.79 ± 0.02 (9) | 0.56 ± 0.03 (10) |
| $\Delta G_{AMPP_i}$ (kJ/mol)[d] | −7.5 ± 0.4 | −10 ± 2 | −6 ± 1 |
| $\Delta G_{P_i}$ (kJ/mol)[e] | −18 ± 4 | −11 ± 2 | 0.0 |
| $\Delta G_{P_i/AMP}$ (kJ/mol)[e] | −1.4 ± 0.2 | −0.2 ± 0.1 | 0.0 |
| $\Delta G_{AMP}$ (kJ/mol)[f] | −5.9 ± 4 | −11.5 ± 0.6 | 4.6 ± 0.8 |
| $\Delta G_{AMP/P_i}$ (kJ/mol)[f] | −0.9 ± 0.4 | −7 ± 2 | −0.39 ± 0.07 |

[a]Dissociation constants and heterotropic coupling free energy determined from replots of $K_{1/2}$ for phosphate as a function of AMP concentration. [b]Average value of the Hill number for phosphate binding at low AMP concentrations. Reported as the mean ± standard error (*n*), where *n* is the number of independent experimental values averaged. [c]Average value of the Hill number for AMP binding at low phosphate concentrations. Reported as the mean ± standard error (*n*), where *n* is the number of independent experimental values averaged. [d]$\Delta G_{AMPP_i}$ is coupling free energy between AMP and phosphate, which is calculated from the heterotropic coupling quotient. [e]$\Delta G_{P_i}$ and $\Delta G_{P_i/AMP}$ are the homotropic coupling free energies in the absence and saturating presence of AMP, respectively. [f]$\Delta G_{AMP}$ and $\Delta G_{AMP/P_i}$ are the homotropic coupling free energies for AMP in the absence and saturating presence of phosphate, respectively. Homotropic free energies calculated from the average Hill numbers at high and low concentrtions of another ligand.

phosphate binding at low (< 3 μM) and saturating concentrations of AMP ($Q_{P_i}$ and $Q_{AMP/P_i}$, respectively).

Quantification of the heterotropic coupling between AMP and phosphate was conducted by fitting $K_{1/2}$ for phosphate as a function of AMP concentration to eq 2 (*18*). The values for homotropic coupling quotients for AMP binding, $Q_{AMP}$ and $Q_{AMP/P_i}$, appearing in eq 2 were fixed to the values established utilizing eq 1 as just described.

$$K_{1/2} = K_{P_i}^{o}\{(K_{AMP}^{o\,2} + 2K_{AMP}^{o}[AMP] + Q_{AMP}[AMP]^2)/ [K_{AMP}^{o\,2} + 2K_{AMP}^{o}Q_{AMPP_i}(Q_{AMP}/Q_{AMP/P_i})^{1/2}[AMP] + Q_{AMPP_i}^{2}Q_{AMP}[AMP]^2]\}^{1/2} \quad (2)$$

where $K_{P_i}^{o}$ is the dissociation constant for phosphate in the absence of AMP, $K_{AMP}^{o}$ is the dissociation constant for the first equivalent of AMP in the absence of phosphate, and $Q_{AMPP_i}$ is the apparent heterotropic coupling quotient between AMP and phosphate.[2]

The measured coupling quotients were converted to the free energy of coupling by the relationship between free energy and the coupling quotient given in eq 3.

$$\Delta G_{coupling} = -RT \ln Q \quad (3)$$

where $\Delta G_{coupling}$ is the free energy of coupling, *R* is the gas constant, *T* is the temperature, and *Q* is the coupling quotient of interest.

*Activity Gels*. Equal units of activity for wild-type and Δ2−17 glycogen phosphorylase *b* were applied to a 7.5% nondenaturing polyacrylamide gel and electrophoresed at 100 V for 2 h on ice. As previously described, gels were incubated with 1% glycogen, 20 mM glucose 1-phosphate, and 10 mM AMP in 50 mM PIPES buffer (pH 6.8) for AMP activation or 1% glycogen, 30 mM glucose 1-phosphate, and 0.6 M $(NH_4)_2SO_4$ in 50 mM PIPES (pH 6.8) for sulfate activation (*19*). Incubation was conducted for 4 h at room temperature. Following incubation, gels were stained with a saturated solution of $I_2$ and photographed.

[2]The apparent value of $Q_{AMPP_i}$ includes terms that reflect the homotropic coupling between the two substrate ($P_i$) binding sites in the absence and presence of bound AMP (*18*).

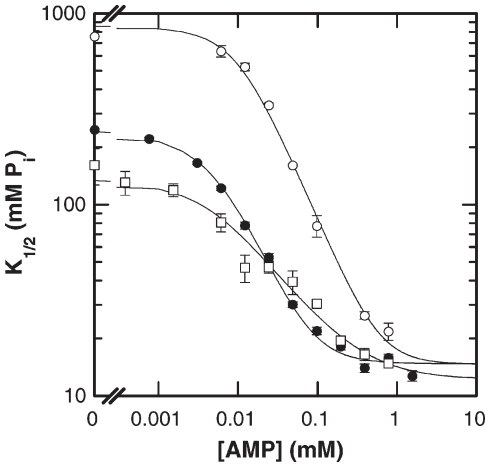


FIGURE 1: Replot of $K_{1/2}$ for phosphate as a function of AMP concentration for the wild type and N-terminal truncates. Data for the wild type (●), Δ2−17 (○), and phosphorylase *b*′ (□) are shown with standard errors. $K_{1/2}$ values were determined from a phosphate titration fit with the Hill equation. Lines represent the best fits to eq 2, with values for homotropic coupling quotients for AMP fixed to the values determined from the Hill numbers observed in AMP titrations at high and low phosphate concentrations. All experiments were conducted with a constant ionic strength at 25 °C with 5 mg/mL glycogen.

## RESULTS

*Wild-Type Expression and Kinetics*. Glycogen phosphorylase when expressed in *E. coli* is in the pure *b* form because of the lack of phosphorylase kinase. The wild type was successfully expressed in *E. coli* DF1020 with yields similar to those previously reported (*13*). Titrations were conducted from 0 to 330 mM phosphate and from 0 to 5 mM AMP. There is little to no effect on $V_{max}$ for the enzyme upon addition of AMP, while there is a dramatic effect on the affinity for phosphate. The replots of Hill number versus phosphate or AMP concentration allow the quantification of the homotropic cooperativity (Figure 3 and Table 1). Once the values for homotropic effects in AMP binding are established, the replot of $K_{1/2}$ for phosphate versus AMP concentration can be fit to eq 2 and the coupling between AMP and phosphate can be quantified (Figure 1 and Table 1). This experimental approach allows for the measurement of activity even in the absence of AMP.

*Δ2−17 Expression and Kinetics*. Mutation of the gene for rabbit muscle glycogen phosphorylase allows for the creation of a modified protein that lacks the N-terminus (Δ2−17). The modified protein was successfully created, expressed, and purified. When the kinetics of the Δ2−17 mutant are measured, it displays a reduced affinity for AMP and phosphate which can be observed by the curve being shifted up and to the right in Figure 1 (also see Table 1). A clear dependence of the affinity for phosphate on the presence of AMP is observed, and conversely, there is a clear dependence of the affinity for AMP on the presence of phosphate (Figures 1 and 2). Comparing the free energies of coupling in Table 1, one can see that there is slightly more interaction observed between AMP and phosphate binding with the Δ2−17 mutant than with the wild type. The absence of

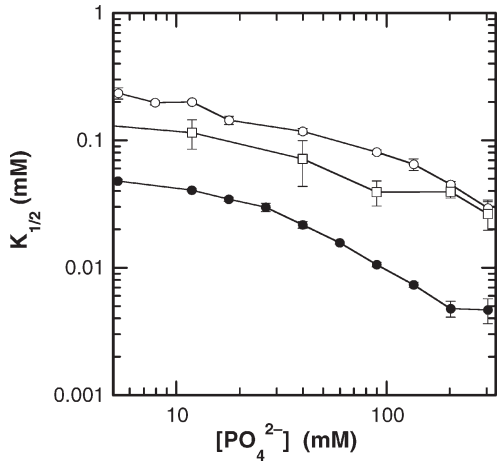


FIGURE 2: Line plot of $K_{1/2}$ for AMP as a function of phosphate concentration. Data for the wild type (●), Δ2−17 (○), and phosphorylase *b*′ (□) are shown with standard errors. Experiments were conducted at a constant ionic strength at 25 °C with 5 mg/mL glycogen.

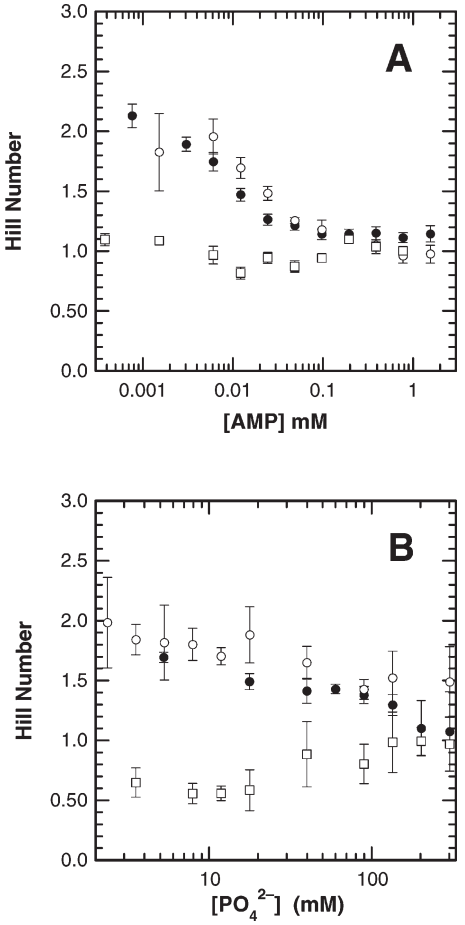


FIGURE 3: Replots of the Hill number vs the other ligand for the wild type and N-terminal truncates. (A) Replot of the Hill number for phosphate vs AMP. (B) Replot of the Hill number for AMP vs phosphate. Data for the wild type (●), Δ2−17 (○), and phosphorylase *b*′ (□) are shown.

the N-terminus clearly did not alleviate the heterotropic cooperativity in phosphorylase; on the contrary, it is actually significantly enhanced relative to that of the wild type (Figure 1). The Δ2−17 enzyme also retains homotropic cooperativity with respect to both AMP and phosphate as reported in Table 1. To rule out the

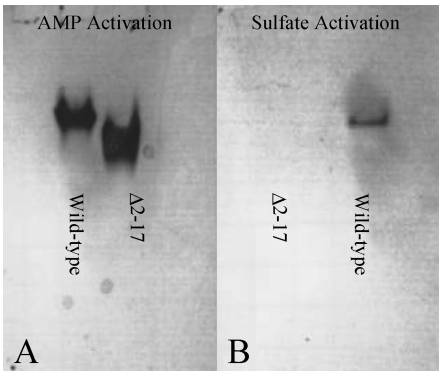


FIGURE 4: Activity gels with wild-type and Δ2−17 glycogen phosphorylase *b*. Activity shown by a 4 h incubation of roughly equal units of activity of the wild type and Δ2−17 in (A) 1% glycogen, 20 mM glucose 1-phosphate, and 10 mM AMP or (B) 1% glycogen, 20 mM glucose 1-phosphate, and 1 M $(NH_4)_2SO_4$. Stain developed by incubation with iodine.

possibility of significantly altered secondary or tertiary structure in the Δ2−17 mutant, the intrinsic tryptophan fluorescence emission spectrum was recorded with excitation at 295 nm. The spectrum of Δ2−17 matched the spectrum of the wild type for both apo and 2 mM AMP (data not shown). To test the ability of sulfate to activate Δ2−17, the wild type and Δ2−17 were electrophoresed on 7.5% nondenaturing polyacrylamide gels. After reaction in the direction of glycogen synthesis by incubation with glycogen and glucose 1-phosphate, the gels were stained with iodine. Although AMP activates both the wild type and Δ2−17, Δ2−17 is not activated by sulfate (Figure 4).

*Phosphorylase b*′. To assess the differences between our findings with Δ2−17 and the originally published results in the absence of the N-terminus, we created phosphorylase *b*′ from WT phosphorylase *a* according to the original protocols (*8*). As previously reported, phosphorylase *b*′ had a higher affinity for phosphate and AMP (Figure 1 and Table 1). However, there is still a substantial dependence of the affinity for phosphate on the presence of AMP. Interestingly, the slope of the transition region is much reduced with respect to those of both WT and Δ2−17, which might suggest why this activation was previously overlooked given the relatively low concentration range of AMP used (*8*). In agreement with the previous work (*8*), there is no evidence of cooperativity in binding of phosphate at any concentration of AMP. At low phosphate concentrations, the Hill number for AMP is approximately 0.56 ± 0.03, indicating significant negative homotropic cooperativity in AMP binding (Figure 3B). The Hill number returns to unity at higher phosphate concentrations, indicating that this is a real case of negative homotropic cooperativity.

## DISCUSSION

*Wild-Type Phosphorylase*. Previously, phosphorylase *b* has been assumed to be inactive without AMP present (*1*, *2*, *10*), but under the experimental conditions used in this study, activity can be measured and the affinity for phosphate determined even in the absence of AMP. These data, when analyzed with linkage analysis rather than simply using a model that assumes an equilibrium between active and inactive forms of the enzyme, allow the complete quantification of the allostery in glycogen phosphorylase *b* with respect to AMP and phosphate. Many previous reports have shown the activation by AMP, but none have quantified the extent of coupling (*8*, *10*, *13*). This report

provides a complete analysis of the extent of heterotropic coupling between phosphate and AMP in glycogen phosphorylase *b*.

*Δ2−17 and Phosphorylase b′*. The results with the truncate Δ2−17 are in clear disagreement with the previously reported loss of cooperativity in phosphorylase *b′* (*8*). The loss of affinity for both substrate and effector is evident, but there is no loss, rather an enhancement, in the apparent heterotropic coupling (Figure 1). Similarly, it is clear that substantial homotropic cooperativity with both AMP and phosphate has been retained in the absence of the N-terminus. In light of these findings, the long-standing proposal that the N-terminus transmits the allosteric signal across the subunit interface becomes highly improbable. The possibility that these differences are due to the trivial cause of different experimental conditions has been ruled out by examination of phosphorylase *b′*. When phosphorylase *b′* was tested under the same conditions as Δ2−17, results similar to those originally reported were found, confirming that there are different heterotropic and homotropic cooperativities in Δ2−17 and phosphorylase *b′*. The likely source of these differences is the use of proteolysis to generate phosphorylase *b′*. The original publication describing phosphorylase *b′* reported the presence of multiple cleavages that were apparent on SDS−PAGE in addition to the removal of the N-terminus (*8*). These additional cleavages were assumed to be inconsequential at the time. However, the finding that Δ2−17, which has a known primary structure, retains both homotropic and heterotropic cooperativity indicates that the additional cleavage sites in phosphorylase *b′* have significant effects. Irrespective of these differences, examining phosphorylase *b′* over a much larger concentration range of AMP shows that there is a great deal of heterotropic activation retained nonetheless. The two variants lacking the N-terminus, therefore, both show that allosteric activation by AMP is present in the absence of the N-terminus, though it is much reduced with phosphorylase *b′* (Figure 1). The conclusion that the N-terminus is not required for the activation by AMP is therefore unavoidable. It appears that the N-terminus probably does have a role in establishing the binding characteristics of apophosphorylase *b*, since the affinities for phosphate and AMP are greatly altered in its absence. However, that role is independent of any role in the allosteric coupling in the enzyme, which is not greatly disturbed.

It has been a long-standing theory that the activation of phosphorylase proceeds via the same mechanism regardless of the activation (*1*, *10*). The N-terminus is clearly implicated in activation by phosphorylation and by high concentrations of sulfate (*20*, *21*). Of course, phosphorylase cannot be activated by phosphorylation in the absence of the N-terminus, but activation by sulfate is also dependent on the N-terminus (Figure 4) (*21*). While the high concentrations of sulfate required for activation make quantitative comparison to effects of AMP difficult due to the potential complications of nonspecific ionic strength effects, previous structural studies have shown that the activation by sulfate proceeds via an interaction with both the N-terminus and the phosphoserine recognition site (*21*). The lack of activation by sulfate in the absence of the N-terminus (Figure 4) provides a strong indication that the mechanism of activation with phosphorylation may in fact be distinct from that of the activation by AMP.

## CONCLUSIONS

Our understanding of the functional characteristics of allosteric activation of glycogen phosphorylase has to date been largely based on investigations with a proteolytically derived enzyme of unknown primary structure looking at relatively narrow concentration ranges of substrates and AMP (*8*). The new findings reported here indicate that, contrary to the conclusions obtained from those investigations, heterotropic AMP activation of phosphate binding is if anything somewhat enhanced when the N-terminus is cleanly removed through mutagenesis. The positive homotropic cooperativities in the binding of AMP and phosphate are also retained. The interpretations of the structural changes that give rise to the allosteric activation of glycogen phosphorylase should be reevaluated in light of these new findings.

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