Mechanism of Regulation in Yeast Glycogen Phosphorylase*

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The mechanism of yeast glycogen phosphorylase activation by covalent phosphorylation involves structural elements distinct from the mammalian homologs. To understand the role of the amino-terminal 39-residue extension in the phosphorylation control mechanism, mutants with 22 and 42 amino-terminal residues removed were expressed in Escherichia coli, and their properties were compared with the wild-type (WT) enzyme. The unphosphorylated WT enzyme had a specific activity of 0.1 unit/mg and was not activated significantly by the substrate, glucose 1-phosphate. Phosphorylation by protein kinase resulted in a 1300-fold activation. Glucose 6-phosphate inhibited the unphosphorylated enzyme more effectively than the phosphorylated form, and inhibition of the latter was cooperative. Glucose was a more effective inhibitor for both the unphosphorylated and phosphorylated WT enzyme with $K_i$ > 300 mM. The rate of phosphorylation by protein kinase depended on substrates and interactions of the amino terminus. Maltoheptaose increased the rate of phosphorylation of the WT enzyme by yeast phosphorylase kinase 5-fold. The 22-residue deletion mutant (Nd22) had overall kinetic properties similar to the WT enzyme, except that Nd22 was a better substrate for the protein kinase and the rate of phosphorylation was unaffected by maltoseptaose. The 42-residue deletion mutant (Nd42), which lacks the phosphorylation site, was measurably active, although much less active than phosphorylated WT. Sedimentation equilibrium analysis indicated that the WT, Nd22, and Nd42 exist as tetramer, partially dissociated tetramer, and dimer, respectively. Phosphorylation of the WT and Nd22 converted both to dimer. The results indicated that the amino terminus affects quaternary structure and mediates activity regulation through conformational transition.

Glycogen phosphorylase (EC 2.4.1.1) catalyzes the mobilization of glucose 1-phosphate (Glc-1-P)$^3$ from glycogen and hence provides readily usable energy when needed. Present among diverse tissues and organisms, the enzyme has evolved with different control features to regulate activity. Among such features, only feedback inhibition by the metabolite Glc-6-P is conserved within the enzyme family and may represent the most ancient control feature (1). The mechanism of activation by phosphorylation appears to occur only in eukaryotes.

Among eukaryotic glycogen phosphorylases studied, the mammalian muscle isozyme shows the most elaborate regulation. Biochemical and structural characterization has defined the mechanisms by which activators and inhibitors regulate enzyme activity (2–4). The unphosphorylated enzyme is inactive even in the presence of high concentrations of substrates, Glc-1-P and glycogen. Activation requires either phosphorylation of Ser$^{14}$ by phosphorylase kinase or AMP binding. AMP and phosphoserine bind nonoverlapping sites at the subunit interface of the functional dimer, some 35 Å distant from the active site (5, 6). Both stabilize the active conformation by causing the subunits to be drawn together at the interface and triggering the active site to open. The inhibitors Glc-6-P and ADP also bind at the AMP site, but their binding stabilizes the inactive conformation by denying AMP or phosphoserine from this site (7). Glucose is an even more potent inhibitor of the muscle enzyme, abrogating activation by phosphorylation. Glucose stabilizes the inactive conformation by binding in the active site cleft between the amino- and carboxyl-terminal domains (8). Glucose inhibition promotes dephosphorylation of the muscle phosphorylase by protein phosphatase 1 as a result of freeing the phosphorylated amino termini from their binding sites (9).

Several properties distinguish the yeast and muscle enzymes. Yeast phosphorylase is neither activated by AMP nor inhibited by glucose at physiological concentration (10, 11). Although phosphorylation activates the yeast enzyme, the phosphorylation site and the kinase involved are different from the muscle enzyme (12–14). A threonine residue at the amino terminus is phosphorylated by either yeast phosphorylase kinase or cAMP-dependent protein kinase, neither of which recognizes the muscle enzyme.

Sequence alignment of the yeast and muscle enzymes showed that the former has an unique 39-residue amino-terminal extension (numbered as −1 to −39 toward the amino terminus) relative to residue 1 of the muscle enzyme (13, 15). After amino acid 80, the sequences exhibit 49% identity at alignable positions. While some biochemical differences between the two enzymes can be rationalized on the basis of nonconservation, others cannot. For example, the lack of activation by AMP in yeast enzyme can be attributed to poor conservation of residues involved in binding the base moiety of AMP. However, it is not understood why glucose is a poor inhibitor for the yeast enzyme because all residues involved in binding glucose in the muscle enzyme are conserved. Also, the structural mechanism by which phosphorylation activates muscle enzyme cannot apply to the yeast enzyme because of differing structural contexts; phosphorylation occurs on Ser$^{14}$ in muscle enzyme and on Thr$^{10}$ in yeast enzyme. In addition, the residues involved in binding the phosphoserine in the muscle enzyme, Arg$^{13}$ and Arg$^{60}$, are not conserved.

Previous characterization of yeast phosphorylase suggests that the amino-terminal extension functions to stabilize the enzyme in an inactive conformation. Becker et al. (16) have
Regulation of Yeast Phosphorylase

observed that a proteolytic product of the yeast phosphorylase lacking the amino-terminal 39 residues elutes in the active fractions during gel filtration. Furthermore, the crystal structure of the unphosphorylated yeast enzyme has revealed that the amino-terminal extension binds near the catalytic site of the neighboring subunit (Fig. 1), suggesting that it prevents the substrate from entering the active site (17). Both observations are consistent with the notion that phosphorylation activates the yeast enzyme by displacing the amino terminus from the active site. An observation that the unphosphorylated enzyme forms a tetramer and that phosphorylation converts the enzyme to a dimer has not been linked to regulation (18).

In this study, the role of the amino-terminal extension and dimer-tetramer transition in activity regulation were investigated. Kinetic characterization of the Escherichia coli-expressed wild-type and amino-terminal deletion mutant enzymes, both unphosphorylated and phosphorylated, were carried out. The oligomerization states of the wild-type and mutant enzymes in the presence of different activators and inhibitors were determined by sedimentation equilibrium to establish the role of the dimer-tetramer equilibrium and the amino terminus in the regulatory mechanism of yeast phosphorylase.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were purchased from New England Biolabs. Ampiltaq polymerase was from Perkin Elmer Cetus. Isopropyl-1-thio-β-D-galactopyranosidase, Glc-6-P, glucose, ATP, BisTris, and bovine heart kinase catalytic subunit were from Sigma. Glc-1-P was from ICN. Sodium succinate was from Fisher. [γ-32P]ATP was from Amersham Corp. Oligosaccharides were purchased from Aldrich. DEAE-Sepharose was from Pharmacia Biotech Inc. Oyster glycogen from Sigma was repurified by repeated ethanol precipitations, and then followed by ion-exchange chromatography using resin AG501-X8. Yeast phosphorylase kinase was provided by Dr. Arnold C. Schwartz at the Biochemical Institute, University of Texas, Austin, Texas.

Construction of Expression Plasmid and Generation of Deletion Mutants—From the cloned yeast phosphorylase gene described previously (19), a DNA fragment containing the entire protein coding region was inserted into the pTACTAC expression vector at the unique BamHI site downstream of the TACTAC promoter. To facilitate the cloning procedures, BamHI sites were introduced into the 5′- and 3′-noncoding regions immediately adjacent to the protein coding region. This was accomplished using polymerase chain reaction with oligonucleotide primers 5′-GGGGGATCCCA TATGGCGGCA GCTGACTA GTAC-3′ for the forward primer and 5′-GGGGATCCT GAGAATGTT CACTGTTCA ACCTGTTT-3′ as a reverse complement primer. The polymerase chain reaction products were cut with BamHI prior to subcloning into pTACTAC and verification by restriction digestion. Deletion mutants were also generated by polymerase chain reaction-directed mutagenesis using forward oligonucleotides introducing new initiation codons and a reverse complement oligonucleotide. The Nd22 and Nd42 mutants were generated using the forward primers 5′-CCTACTGCTT CACCATGGC ACC-3′ and 5′-CAAGTCAA TGTTCCCAA GTACCAC-3′, respectively. The common reverse primer encodes a unique XbaI site, 5′-GCAAATCGTT TTTTCTAGA TCGGATC-3′, about 1 kilobase downstream of the forward primer. The polymerase chain reaction products were cut with NdeI and XbaI restriction enzymes and subcloned into the pTACTAC expression vector. Mutants were identified by restriction digestion patterns.

Expression of Yeast Phosphorylase in E. coli—E. coli 25A6 cells (W3110; tonA, lonA, gelE, htpP) containing yeast phosphorylase cDNAs in the pTACTAC vector were grown overnight in LB/amp (Luria broth plus 100 mg/ml ampicillin). The cells were used to inoculate (1:200 dilution) an enriched media containing 2% Bacto-tryptone, 1.5% yeast extract, 0.8% NaCl, 0.2% Na2HPO4, 0.1% KH2PO4, 0.2% glucose, 3 mM MnCl2, 0.5 mM pyridoxine, and 100 mg/ml ampicillin. The culture was grown at 37°C until the A595 nm reached 0.5. Isopropyl-1-thio-β-D-galactopyranosidase was added to final concentration of 0.1 mg/ml, and the culture was grown for an additional 40 h. Cells were then cooled on ice, pelleted, and resuspended in ice cold buffer A containing 20 mg/ml BisTris (pH 6), 2 mg/ml EDTA, 2 mg/ml DTT, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, 1 mg/ml phenylmethylsulfonyl fluoride, and 0.01% (w/v) benzamidine-HCl. The resuspended cells were quickly frozen in liquid nitrogen and stored at −70°C.

Purification of Wild-type and Mutant Phosphorylases—All enzyme forms were purified by similar overall procedures. Frozen cells were thawed out slowly in a 4°C water bath. All procedures were performed at 4°C unless otherwise stated. Extracts were prepared by sonication, followed by centrifugation at 16,000 × g for 1 h. Polyethylene glycol 8000 was added to the extract to a final concentration of 18% (w/v) with constant stirring for 20 min. The pellet was obtained by centrifugation at 8000 × g for 15 min and was resuspended in buffer A so that the final protein concentration is about 20 mg/ml. The sample was then loaded on a DEAE-Sepharose column equilibrated with buffer A, and the column was washed with 5 column volumes of buffer A. For the wild-type and the Nd42 mutant enzymes, a salt gradient of buffer B to buffer A plus 300 mM NaCl was applied. For the Nd22 mutant, a gradient of buffer A to buffer B plus 600 mM NaCl was applied. The fractions containing phosphorylase activity were pooled and dialyzed against buffer B containing 100 mM potassium phosphate (pH 6.0), 2 mM EDTA, 2 mM DTT, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 5 μg/ml pepstatin A, 1 mg/ml phenylmethylsulfonyl fluoride, and 0.01% (w/v) benzamidine-HCl. The precipitate formed during dialysis was spun down, and the clear sample was loaded on a DEAE-Sepharose column equilibrated with buffer B. The column was washed with 5 column volumes of buffer B and then eluted with a gradient of buffer B to buffer B plus 700 mM potassium phosphate (pH 6.0). The fractions containing purified phosphorylase were pooled, concentrated by Amicon ultrafiltration to 40 mg/ml, and dialyzed against a storage buffer containing 10 mM BisTris (pH 5.8), 100 mM NaCl, 2 mM DTT, 0.2 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride, and 0.02% sodium azide. Enzyme purity was assessed by SDS-PAGE (20).

Conversion of Phosphorylase to the Phosphorylated Form and Its Purification—Phosphorylation was catalyzed by bovine cAMP-depend-
ent protein kinase catalytic subunit from Sigma. The reaction contained 1:200 molar ratio of kinase to phosphorylase in the presence of 1 mM ATP, 5 mM MgCl₂, and 2 mM DTT in BiTris buffer at pH 6.0. The reaction mixture was incubated at 25 °C for 5 h and then dialyzed against buffer C containing 0.2 mM EDTA, 2 mM DTT, 1 μM leupeptin, 1 μM pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride, and 100 mM sodium succinate at pH 5.8. The sample was loaded onto a 1 × 100-cm DEAE-Sepharose column equilibrated with buffer C, after which the column was eluted with a gradient of buffer C plus 25 mM sodium succinate (400 ml) to buffer C plus 45 mM sodium succinate (400 ml). The phosphorylated enzyme eluted at about 135 mM sodium succinate. Enzyme purity was assessed by SDS-PAGE (20). The phosphorylated enzyme was concentrated to 20 mg/ml and dialyzed against the storage buffer.

Slot-Blot for Phosphorylase Activity—Phosphorylase activity was followed during purification by a rapid slot blot assay. Approximately 50 μl of column fractions were slot blotted onto a nitrocellulose filter pre-wet in water. The filter was then incubated for 30 min at room temperature in 100 mM sodium succinate (pH 5.8), 1% (w/v) glycogen, and 75 mM Glc-1-P. Slots on the filter containing phosphorylase stained purple upon soaking in an iodine solution containing 0.4% KI and 0.2% iodine.

Steady-state Kinetic Analysis—A modification of the procedures of Carney et al. (21) was used to carry out steady-state kinetic studies on yeast glycogen phosphorylase in the direction of glycogen synthesis. Detailed reaction components are provided under "Results." All reactions were performed at 30 °C at pH 5.8 using 100 mM sodium succinate as buffer. Glc-1-P and Glc-6-P were titrated to pH 5.8 before adding to reaction mixtures. In all experiments, reactions were stopped within the linear velocity range, and less than 5% of the substrates were converted to products. The colorimetric detection of phosphate was used to measure the amount of phosphate generated by the conversion of Glc-1-P into glycogen (21). Aliquots from the stopped reactions were added to the color reagent and were incubated exactly 6 min at room temperature before measurement of 316 nm absorbance. Kinetic data were analyzed using the nonlinear regression calculation of the KinetAsst II program (29). Kinetic equations applied for data analysis are shown in the figure or table legends.

Monitoring the Time Course of Phosphorylation Catalyzed by Protein Kinase—[γ-32P]-Labeled ATP was used to monitor the time course of covalent phosphorylation on yeast glycogen phosphorylase. The amount of enzyme-labeled 32P-specific activity was used to quantitate the incorporation. The kinase reaction was started by adding yeast phosphorylase kinase to a mixture containing [γ-32P]-ATP and yeast phosphorylase (and oligosaccharides in some experiments). The control experiment, which was run in parallel with the reaction, contained bovine serum albumin instead of phosphorylase. At each time point, 10 μl of the reaction mixture and control were removed and spotted on two pieces of Whatman P-81 phosphocellulose paper, which were then washed in 5 mM phosphoric acid 3 times to remove noncovalently bound phosphate. The paper was then transferred to a vial containing scintillant, mixed, and counted.

Determination of Phosphorylase-bound Phosphate—The phosphate content of yeast glycogen phosphorylase was determined using the ashing procedure of Ames (22) and the phosphate assay of Itaya and Ui (23) essentially as described by Stull and Buss (24). Various amounts of yeast glycogen phosphorylase (0.5–4 nM) in storage buffer were mixed with 30 μl of 10% MgNO₃-4H₂O in 95% ethanol and gently heated to dryness. The samples were then ashed over intense flame. After dissolving the residues in 300 μl of 1.2 N HCl, 100 μl of filtered phosphate reagent (containing 1 volume of 10% [NH₄]₂MoO₄-D₂O in 4 N HCl to 3 volumes of 0.2% malachite green) was added, and the developed color was measured at 660 nm after 5 min. The standards (0.2–5 nM KH₂PO₄) were also ashed and assayed by the same procedure.

Molecular Weight Determination by Sedimentation Equilibrium—Sedimentation was performed using the Beckman Optima XL-A ultra-centrifuge. Protein samples were prepared in the presence of various ligands, with the final protein concentration about 0.5 mg/ml. For each sample, a reference was prepared with the same composition as the sample protein, but without protein. In each run, nine samples and nine reference scans were spun at 5000 rpm at 25 °C. The 280 nm absorbance was scanned at 0.01-mm steps across the cell (average of five measurements) every 4 h until equilibrium was reached (usually 30 h). The difference spectrum between scans was used as a criteria for judging whether equilibrium was reached. The data (Aₚ – Aₙ) (reading versus radius r from the center of rotor) were least squares fitted to the equation for a single ideal solute, Cᵢ = Cᵢ[exp(r²/wM)(1 – r²/rt²)] – Cᵢ, where Cᵢ is the solute concentration at radius r, Cᵢ is the solute concentration at a reference distance rᵢ, w is the angular velocity, r is the gas constant, T is the temperature in Kelvin, ρ is the density of the solution, ν is the partial specific volume of the protein, and Mᵢ is the molecular weight of the protein. All molecular weights were determined using a value of 1 g/cm³ for ρ and 0.76 mol/g for ν. The ρ values for N22 and N42 mutants are within 0.2% difference from the WT value when calculated using nᵢ, the number of residues per mol of the ith amino acid in the protein, ρᵢ is the residue molecular weight (the molecular weight of the amino acid minus the weight of 1 mol of water), and νᵢ is the partial specific volume of the i-th residue.

RESULTS

Expression and Purification of WT, N22, and N42 Yeast Glycogen Phosphorylases—The optimized expression and purification procedures (see "Experimental Procedures") allowed isolation of approximately 30–50 mg of highly purified WT and mutant enzymes from 1 liter of bacterial culture. All enzyme forms purified by this procedure appeared to be homogeneous as judged by SDS-PAGE (Fig. 2a). The comparison between the rabbit muscle enzyme and the various yeast phosphorylase constructs is shown in Fig. 2b.

Glc-1-P-dependent Velocities of Unphosphorylated WT and Mutant Enzymes—The Glc-1-P-dependent velocity plots of the WT, N22, and N42 enzymes are shown in Fig. 3. Only the N42 mutant, lacking 42 amino-terminal amino acids, showed significant activity. At 50 mM Glc-1-P, the specific activity for WT and N22 was about 0.1 unit/mg, while that for N42 was about 0.7 units/mg. The initial velocity of the N42 mutant increased exponentially with increasing Glc-1-P concentration, characteristic of cooperative substrate binding, and reached an

![Image](314x435 to 556x732)

**Fig. 2.** a, SDS-PAGE of the WT, N22, and N42 yeast glycogen phosphorylases showing the decrease in apparent molecular weight for the two deletion mutants. The two outer lanes contain high molecular weight markers (HMW). The molecular weight of protein standards are indicated on the right-hand side, b, schematic representation of the amino acid sequences of the muscle and yeast glycogen phosphorylases. The regions shaded light gray are about 50% identical. The amino-terminal region, from residue 1 to 80, are about 20% identical. The yeast enzyme has an extended amino terminus, residue 1 to 39, which is not present in the muscle enzyme. The phosphorylation sites are marked with triangles (Thr in yeast enzyme, Ser in muscle enzyme). The N42 mutant lost the phosphorylation site as a result of the deletion.
yet had different properties. Specific activities of the earlier
indistinguishable when analyzed by SDS-PAGE (not shown),
separation of two distinct phosphorylase peaks in the elution
first peak had a phosphate content of 1.95 \( \times 10^{-3} \) mol/mol of
subunit, determined by \( ^{32} \text{P} \) assay, was incorporated for
2

The curve for Nd42 was obtained by fitting the
data to the Hill equation, \( v = V_{\max }S/(S_{0.5} + S) \). The resultant fit has
a Hill coefficient of 1.5 \( \pm 0.03 \), and \( V_{\max } \) and \( S_{0.5} \) values of 110 \( \pm 15 \)
units/mg and 40 \( \pm 6 \) mM, respectively. The glycogen concentration in the
assays is 1%.

Phosphorylation and Activation by Protein Kinase—The time
course of phosphate incorporation into the WT, Nd22, and
Nd42 enzymes, catalyzed by yeast phosphorylase kinase, is
shown in Fig. 4. As expected, there was no detectable phos-
phorylation site, Thr\( ^{10} \), was removed from this mutant (Fig.
2b). A steady-state level of about 0.9 mol of phosphatemol of
enzyme subunit, determined by \( ^{32} \text{P} \) assay, was incorporated for
both WT and Nd22 mutant under the experimental conditions.
However, the rate of phosphorylation for the Nd22 mutant was
about 5 times faster than the WT. The difference in rate sug-
gests that Nd22 mutant is a better substrate for the yeast
phosphorylase kinase than the WT enzyme. Phosphorylation
activated both enzymes. A steady-state activity level of about
115 \( \pm 5 \) units/mg, which is 1000-fold more activity than found
in the unphosphorylated forms, was reached by phosphorylated
WT and Nd22 enzymes.

The oligosaccharide, maltotetraose, increased the rate of
phosphorylation of the WT enzyme by yeast phosphorylase
kinase (Fig. 5). In the presence of 20 mM maltotetraose, the
rate of phosphorylation of the WT enzyme mimicked that of the
Nd22 mutant. Shorter oligosaccharide had less effect at the
same concentration, with maltotriose showing no noticeable ef-
fect. Addition of oligosaccharides to the Nd22 mutant, however,
did not change the rate of phosphorylation (data not shown).

Purification of the Phosphorylated WT and Nd22—The phos-
phorylated form of the enzyme can be separated from the
unphosphorylated form employing ion-exchange chromatogra-
phy as described under “Experimental Procedures.” The shal-
low 125–145 mM sodium succinate gradient achieved a clean
separation of two distinct phosphorylase peaks in the elution
profile (not shown). Phosphorylases from the two peaks were
indistinguishable when analyzed by SDS-PAGE (not shown),
yet had different properties. Specific activities of the earlier
eluted peak 1 and later eluted peak 2 were 125 \( \pm 5 \) units/mg
and 2 \( \pm 0.2 \) units/mg, respectively. Phosphorylase from the
first peak had a phosphate content of 1.95 \( \pm 0.10 \) mol/mol of
subunit, twice as much as compared with phosphorylase from
the second peak, 0.97 \( \pm 0.08 \) mol/mol of subunit. The unphos-
phorylated and phosphorylated enzymes contain one pyridoxal
phosphate/subunit but would expectedly differ by the one ad-
ditional phosphate/subunit from the kinase reaction. Hence,
these results indicated that peaks 1 and 2 represent phosphory-
lated and unphosphorylated enzyme, respectively. Furthermore,
sedimentation equilibrium experiments determined that peak 1
enzyme was dimeric while the peak 2 enzyme was tetrameric.

Glc-1-P-dependent Velocity of the Phosphorylated WT and
Nd22—The velocity versus Glc-1-P concentration plots for the
phosphorylated WT and Nd22 mutant are shown in Fig. 6. Both
enzymes exhibited hyperbolic velocity change with respect to
varied Glc-1-P concentration, typical of Michaelis-Menten ki-
netics. The apparent \( K_m \) values of Glc-1-P for the WT and Nd22
mutant are 0.86 \( \pm 0.02 \) and 0.65 \( \pm 0.04 \) mM, respectively. The
\( V_{\max } \) for the WT and Nd22 mutant are 142 \( \pm 2 \) units/mg and
137 \( \pm 3 \) units/mg, respectively. It should be noted for compar-
ison that \( V_{\max } \) for the Nd42 mutant, which exhibited homo-
tronic Glc-1-P cooperativity, is about 110 units/mg, and its
estimated \( S_{0.5} \) is about 40 mM. The comparison indicates that
although removing the amino-terminal 42 residues activates the enzyme, the mutant lacks a stabilized high affinity Glc-1-P binding site established by phosphorylation.

Glc-6-P and Glucose Inhibition in Yeast Phosphorylase—Glc-6-P and glucose are effective inhibitors for the rabbit muscle phosphorylase with $K_i$ values in the low millimolar range (25). For the yeast enzyme, in spite of the conservation of the residues involved in binding Glc-6-P and glucose, only Glc-6-P appears to be the physiological relevant inhibitor (10). To investigate whether the extended amino terminus of the yeast enzyme plays a role in influencing Glc-6-P or glucose inhibition, inhibition studies were carried out on the WT and the deletion mutants.

The results are summarized in Table I. The apparent $K_i$ for Glc-6-P of the unphosphorylated WT and Nd22 was $0.29 \pm 0.01$ and $0.21 \pm 0.01$ mM, respectively. The apparent $K_i$ for Glc-6-P of Nd42 was $12.5 \pm 0.5$ mM. Interestingly, in contrast to the noncooperative inhibition pattern of the unphosphorylated enzymes, as reflected by their Hill coefficients, inhibition of the phosphorylated WT and Nd22 by Glc-6-P were cooperative, with Hill coefficients of 1.36 and 1.37, respectively. The cooperative character of Glc-6-P inhibition for the phosphorylated enzymes is also indicated by Dixon plot in Fig. 7. Glucose is a poor inhibitor of all enzyme forms, with apparent $K_i > 300$ mM.

Effects of Amino Terminus, Phosphorylation, and Effectors on the Oligomerization State of Yeast Phosphorylase—In the absence of effector, the phosphorylated yeast enzyme exists as a dimer, and the unphosphorylated enzyme forms a tetramer (18). One issue is whether the tetramer-dimer equilibrium correlates with the enzyme’s activation state or with its state of phosphorylation. Another question is whether the functional differences between the WT and mutant enzymes and the effect of maltoheptaose in accelerating phosphorylation of the WT enzyme by protein kinase are related to changes in quaternary structure. In order to investigate these possibilities, the apparent molecular weights for the WT, Nd22 and Nd42 were determined by sedimentation equilibrium in the absence and presence of inhibitors and/or substrates.

Table II summarizes the results of sedimentation analysis. In the absence of effector, the tetramer to dimer transition of the WT enzyme upon phosphorylation was confirmed (from 393 to 198 kDa). The Nd42 mutant behaved as a dimer under all conditions (ranging from 194 to 220 kDa). The unphosphorylated Nd22 mutant exhibited an apparent molecular mass of 322 kDa, indicating a mixture of dimer and tetramer; phosphorylation of Nd22 stabilizes the dimeric state (200 kDa). Interestingly, addition of 50 mM Glc-6-P to both the phosphorylated WT and Nd22 reverted the oligomerization state to that of the unphosphorylated form (from 198 to 370 kDa for the WT, and 200 to 320 kDa for the Nd22). Since 50 mM Glc-6-P would completely inhibit the phosphorylated enzyme, one could argue that the dimer-tetramer equilibrium correlates with the activation state of the enzyme, with dimer and tetramer being active and inactive, respectively. Somewhat contrary to this interpretation, Nd22 mutant remained dimeric in the presence of 50 mM Glc-6-P, which would also inhibit the enzyme based on kinetic studies.

Maltoheptaose destabilized the tetrameric state of the unphosphorylated enzyme. Addition of 20 mM maltoheptaose to the unphosphorylated WT enzyme resulted in a change of apparent molecular mass from 393 to 203 kDa. The tetramer to dimer transition might account for the effect of maltoheptaose in increasing the rate of phosphorylation of the WT enzyme by protein kinase (Fig. 5). This interpretation is consistent with the observation that Nd22 served as a better substrate for the protein kinase than the WT enzyme (Fig. 4) and that Nd22 exists as a mixture of dimer and tetramer. In addition, maltoheptaose overcame the effect of Glc-6-P to promote tetramer formation of the phosphorylated enzyme. In the presence of 20 mM maltoheptaose, phosphorylated WT and Nd22 remained dimeric (236 kDa) even upon addition of 50 mM Glc-6-P.

Studies on muscle enzyme showed that oligosaccharides bind preferentially at the high affinity glycogen storage site (26, 27). The high sequence homology between the muscle and yeast enzyme at this site implies functional homology. The crystal structure of the unphosphorylated yeast phosphorylase tetramer revealed that the glycogen storage site forms part of the dimer-dimer contact. For maltoheptaose to bind, the dimer-dimer contact must be disturbed. Maltotriose did not affect the oligomerization state of any enzyme forms, presumably because it could not provide enough binding energy to stabilize

![Fig. 6. The Glc-1-P concentration-dependent velocity of the phosphorylated WT (filled square) and Nd22 (filled triangle) yeast phosphorylases.](image-url)

**TABLE II**

Inhibition of WT, Nd22 and Nd42 by glucose 6-phosphate and glucose

|          | WT          | Nd22        | Nd42        |
|----------|-------------|-------------|-------------|
|          | Unphosphorylated | Phosphorylated | Unphosphorylated | Phosphorylated | Unphosphorylated | Phosphorylated |
| $K_i$ (Glc-6-P) | $0.29 \pm 0.01$ | $4.8 \pm 0.4$ | $0.21 \pm 0.01$ | $0.52 \pm 0.05$ | $12.5 \pm 0.5$ |
| (Hill coefficient n) | 0.97 | 1.36 | 1.01 | 1.37 | 1.05 |
| $K_i$ (glucose) | $>300$ | $>300$ | $>300$ | $>300$ | $>300$ |

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the dimer. A consistent observation is that maltotriose did not enhance the rate of phosphorylation of the WT by protein kinase (Fig. 5).

**DISCUSSION**

An extraordinary dimension of eukaryotic glycogen phosphorylases is the tailoring of isozymes for specific cell and tissue environments through evolution of regulatory features. Yeast phosphorylase represents an intermediate in the development of the intricate control features in mammalian phosphorylases as it shares a number of structural attributes of the mammalian regulatory apparatus (conservation of Glc-6-P site, glucose binding residues, glycogen storage site). However, its covalent phosphoryregulatory apparatus is distinct. An important focus of yeast glycogen phosphorylase studies is to elucidate novel mechanistic roles of protein phosphorylation in regulation.

In rabbit muscle glycogen phosphorylase, phosphorylation results in greater than 200-fold enzyme activation. Phosphorylation of the yeast enzyme was previously reported to result in only 5-fold activation, from about 25 to 135 units/mg (18), whereas we report a 1000-fold activation, from 0.1 to 115 units/mg. The discrepancy arises because the specific activity for the E. coli-expressed enzyme is 50–250-fold lower than values for the enzyme purified from yeast (15, 18). The latter values are likely due to contamination by the phosphorylated enzyme form as a consequence of purifying from yeast, where the endogenous protein kinases activate a fraction of the enzyme.

The previously determined crystal structure of unphosphorylated yeast phosphorylase showed that the amino terminus of each subunit binds near the catalytic site of the neighboring subunit in the homodimer and suggested that inactivation could be a consequence of active site occlusion by the amino-terminal peptide (17). However, the lack of activity in the Nd22 mutant, where 22 amino-terminal residues are removed, indicates otherwise. That Nd22 also requires phosphorylation for activation argues that phosphorylation of the WT enzyme functions other than to displace a steric hindrance to substrates. Although there is indication that the amino terminus serves to stabilize the inactive conformation of the enzyme, from the observation that eliminating the amino-terminal 42 residues leads to an appreciable activity, the Nd42 mutant falls short of a fully activated WT enzyme and, structurally, may resemble more an inactive conformer. Whether or not the phosphopeptide region binds as a ligand to stabilize the active conformation, as observed for rabbit muscle phosphorylase, awaits the determination of the crystal structure of the phosphorylated enzyme.

The amino terminus is also involved in stabilizing the tetrameric form of the enzyme. While the unphosphorylated WT enzyme forms a stable tetramer, the Nd42 mutant remains dimeric under all conditions, and the Nd22 mutant exists as a mixture of dimer and tetramer in rapid equilibrium, indicating weakened dimer/dimer interaction. Results also indicate that the active conformation of the enzyme is incapable of forming stable tetrameric structure. The activated phosphorylated WT enzyme is dimeric, but the Glc-6-P-inhibited phosphorylated WT enzyme is tetrameric.

The crystal structure of unphosphorylated yeast phosphorylase reveals that the tetrameric state of the enzyme is inactive because the two dimers interact on opposing catalytic faces, thereby limiting active site access to glycogen. Is tetramerization the mechanism adopted by the yeast enzyme to switch off activity? Could the enzyme be inhibited in the dimeric state? Two of our observations indicate that the yeast enzyme could be inhibited as dimer. First, Glc-6-P inhibits the Nd42 mutant with apparent $K_i$ about 12.5 mM, while at 50 mM Glc-6-P, this mutant remains dimeric. Second, Glc-6-P inhibits the phosphorylated WT enzyme cooperatively with a $K_i$ of 5 mM, yet the enzyme is dimeric at 50 mM Glc-6-P when 20 mM maltotetraose is present. Based on the assumption that maltotetraose mimics glycogen and binds to the glycogen storage site, the second observation suggests that the dimer is the physiological rele-

![Figure 7](image_url)

**FIG. 7.** The cooperative effect of Glc-6-P inhibition. The figure shows 1/velocity versus Glc-6-P concentration plots on phosphorylated WT (a) and phosphorylated Nd22 (b) yeast phosphorylases. The Glc-1-P concentration used was 5 mM, and glycogen concentration was 1%.

**TABLE II**

| Molecular weights of WT, Nd22, and Nd42 measured by sedimentation equilibrium |
|------------------|------------------|------------------|
|                  | WT               | Nd22             | Nd42             |
|                  | Unphosphorylated | Phosphorylated   | Unphosphorylated | Phosphorylated | Unphosphorylated | Phosphorylated |
|                  | kDa              | kDa              | kDa              | kDa            | kDa              |
| Enzyme alone     | 393 ± 3.1 (4)    | 198 ± 2.2 (2)    | 322 ± 2.9 (2)    | 200 ± 5.8 (2)  | 194 ± 1.2 (2)    |
| Enzyme + Glc-6-P | 370 ± 4.1 (4)    | 193 ± 2.5 (2)    | 307 ± 4.1 (2)    | 190 ± 4.7 (2)  | 192 ± 1.9 (2)    |
| Enzyme + maltotetraose | 203 ± 3.7 (2) | 229 ± 3.9 (2) | 175 ± 3.0 (2) | 288 ± 3.8 (2) | 208 ± 3.0 (2) |
| Enzyme + Glc-6-P + maltotetraose | 379 ± 4.2 (4) | 288 ± 3.8 (2) | 236 ± 2.2 (2) | 206 ± 2.0 (2) | 188 ± 3.6 (2) |
| Enzyme + maltotriose | 379 ± 4.2 (4) | 288 ± 3.8 (2) | 236 ± 2.2 (2) | 206 ± 2.0 (2) | 188 ± 3.6 (2) |
| Enzyme + glucose  | 204 ± 2.5 (2)    | 229 ± 3.9 (2)    | 236 ± 2.2 (2)    | 206 ± 2.0 (2)  | 188 ± 3.6 (2)    |
| Enzyme + Glc-1-P  | 288 ± 3.8 (2)    | 236 ± 2.2 (2)    | 206 ± 2.0 (2)    | 188 ± 3.6 (2)  | 188 ± 3.6 (2)    |
vant inhibited form, as phosphorylation in action has to bind glycogen. It is also worth noting that the cooperative effect of Glc-6-P inhibition on the phosphorylated enzyme cannot be attributed to dimer to tetramer transition, since glycogen in the assays assures the dimeric state of the enzyme. The cooperative effect is more likely due to interaction of the Glc-6-P binding sites within the dimer. It is possible that tetramerization, which occurs only when the enzyme is not bound to the glycogen particles, plays a role in stabilizing phosphorylase at rest. It is interesting that tetramer formation in the absence of glycogen also occurs for the muscle enzyme but only when it is phosphorylated.

The dimeric and tetrameric forms of yeast phosphorylase have distinct biochemical properties. The separation of the phosphorylated (peak 1) and the unphosphorylated (peak 2) forms by ion-exchange chromatography is likely due to different surface charge of the dimer and tetramer. The introduction of the phosphate group cannot alone account for the separation, as one would expect the phosphorylated enzyme to elute later than the unphosphorylated form on an anion exchanger. The two oligomerization states differ further in that the dimeric state is a better substrate for the protein kinase. This was demonstrated by the faster rate of phosphorylation of the Nδ22 mutant, and the enhanced rate of phosphorylation of the WT enzyme in the presence of maltosephatase. Hence, another possible role for tetramerization is allowing the enzyme population bound on the glycogen particles to be phosphorylated preferentially when energy supply is needed.

The results from this study indicate that yeast glycogen phosphorylase is an allosteric enzyme and can be partly described using the two-state model of Monod et al. (28). The unphosphorylated form is in the low activity T state. Phosphorylation stabilizes the high activity R state, which exhibits a hyperbolic velocity curve with respect to substrate, Glc-1-P. Glc-6-P is an allosteric inhibitor. It inhibits the unphosphorylated enzyme noncooperatively by stabilizing the T state, and it inhibits the phosphorylated enzyme cooperatively, causing an R to T state transition. A model describing the allosteric transitions between glycogen bound and free forms is shown in Fig. 8. The unphosphorylated T state enzyme can exist as either dimer (glycogen bound) or tetramer (glycogen free). The dimeric T state enzyme is a better substrate for protein kinase than the tetrameric T state enzyme. Covalent phosphorylation results in the formation of dimeric R state, which can be converted, upon Glc-6-P binding, to either tetrameric T state (glycogen free) or dimeric T state (glycogen bound). It is intriguing that despite the conservation of the glucose-binding residues between the rabbit muscle and yeast enzymes, glucose is a poor inhibitor for the yeast enzyme. One speculation is that protein structure surrounding the site could influence these residues to bind glucose effectively. Understanding this observation requires a structural determination of the glucose bound yeast phosphorylase. Diffraction quality crystals of the phosphorylated yeast phosphorylase in complex with glucose have recently been obtained in this laboratory, and the structure determination is underway.

Acknowledgments—We thank Dr. Arnold C. Schwartz for providing yeast phosphorylase kinase.

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