STRUCTURAL RELATIONSHIPS AMONG REGULATED AND UNREGULATED PHOSPHORYLASES

Jenny L. Buchbinder¹, Virginia L. Rath², and Robert J. Fletterick¹

¹Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143; e-mail: buchbinder@incyte.com; flett@msg.ucsf.edu
²Exploratory Medicinal Sciences, Central Research, Pfizer Inc., Eastern Point Road, Groton, Connecticut 06340; e-mail: rath@pfizer.com

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Abstract Species and tissue-specific isozymes of phosphorylase display differences in regulatory properties consistent with their distinct roles in particular organisms and tissues. In this review, we compare crystallographic structures of regulated and unregulated phosphorylases, including maltodextrin phosphorylase (MalP) from Escherichia coli, glycolgen phosphorylase from yeast, and mammalian isozymes from muscle and liver tissues. Mutagenesis and functional studies supplement the structural work and provide insights into the structural basis for allosteric control mechanisms. MalP, a simple, unregulated enzyme, is contrasted with the more complicated yeast and mammalian phosphorylases that have evolved regulatory sites onto the basic catalytic architecture. The human liver and muscle isozymes show differences structurally in their means of invoking allosteric activation. Phosphorylation, though common to both the yeast and mammalian enzymes, occurs at different sites and activates the enzymes by surprisingly different mechanisms.

CONTENTS

INTRODUCTION ................................................ 192
E. coli MALTODEXTRIN PHOSPHORYLASE (MalP) ............... 193
    An Unregulated Catalytic Enzyme ................................. 193
    Domain Structure—Preserved in Bacterial and Human Enzymes .............. 193
    Oligosaccharide Binding ........................................ 194
YEAST PHOSPHORYLASE, AN EVOLUTIONARY ADVANCE IN REGULATION ................................................ 194
    Regulation at Its Simplest ........................................ 194
    Effectors at the Dimer Interface—A Phosphopeptide Competes with Glc-6-P ..... 197
MUSCLE PHOSPHORYLASE—THE MOST ELABORATELY REGULATED .... 197
    A Competition by Activators and Inhibitors .......................... 197
    Structure ............................................................ 198
INTRODUCTION

In the early biotic world, the ability to maintain carbohydrate reserves must have granted selective advantage to those organisms with even primitive management of glycogen. In human beings, glucose is stored as glycogen in high concentrations in skeletal muscle and liver tissues, where glycogen degradation is exquisitely regulated by the control of the activity of glycogen phosphorylase. Phosphorylase responds to metabolites within the cell, including AMP, ATP, glucose 6-phosphate (Glc-6-P), and glucose, and also to hormonal signals from epinephrine at the cell surface. Regulation is achieved primarily by covalent phosphorylation and by binding of metabolite effectors, which mediate conformational changes between inactive and active forms of the enzyme. Isozymes with specialized regulatory properties are expressed in different cell types and provide tissue-specific control of glycogen degradation. Three isozymes of phosphorylase are found in human beings and are named after the tissues where they are predominantly expressed: brain, liver, and muscle. They share ~80% sequence identity. All are activated by phosphorylation at Ser-14 but differ primarily in their responses to allosteric effectors. In this review, we examine phosphorylases from the primitive versions in *Escherichia coli* and yeast to the elaborate sensory molecules found in human tissues. We compare the most simple unregulated enzyme with the more complicated species and tissue-specific isozymes that have evolved distinct mechanisms of allosteric control.

Biochemical and physiological studies of phosphorylase began in the late 1930s and continue to this day (23, 44, 45). Phosphorylases from all species are active as homodimers with molecular weights of ~200,000. All have highly conserved active sites and catalyze phosphorolysis of glycogen by the same catalytic mechanism. The product, Glc-1-P, is converted to Glc-6-P for entry into the glycolytic pathway to provide energy to the cell. Nearly three decades of crystallographic studies of phosphorylases from different species provide structures of phosphorylated and unphosphorylated forms of the enzyme in assorted complexes with activators, substrates, and inhibitors (6, 20, 30, 32). Regulated and unregulated phosphorylases adopt a range of conformational states, which differ substantially in structure and subunit association. Mutagenesis and functional studies have helped define the mechanisms linking changes in structure to changes in function. We have organized this review from an evolutionary perspective, primarily to achieve focus and simplicity, though at the expense of certain details. We start with a brief...
description of the structure of the \textit{E. coli} homologue, the simplest, unregulated phosphorylase, and then proceed to discuss the regulated enzymes from yeast and mammals. We focus on the mammalian isozymes from muscle and liver tissues. The brain isozyme is omitted from the review because no crystallographic structures are available for it. We address the questions: How are the regulated enzymes inhibited and activated by effectors binding at sites distant from the active site? What is the structural basis for allosteric control? How do structural elements link the allosteric sites with one another and with the active site? How are effector sites related, from an evolutionary perspective, among the phosphorylases?

\textbf{\textit{E. coli} MALTODEXTRAN PHOSPHORYLASE (MalP)}

\textbf{An Unregulated Catalytic Enzyme}

MalP is a homologue of glycogen phosphorylase in \textit{E. coli} that catalyzes the phosphorolysis of $\alpha$ (23, 30, 44, 45) glucosyl linkages from linear rather than from branched oligosaccharides. It has less than 1\% activity with branched glycogen (60). Unlike regulated phosphorylases, the enzyme is not inhibited by Glc-6-P nor activated by AMP or phosphorylation. Enzyme levels are controlled instead by transcriptional regulation (12). MalP represents a basic catalytic phosphorylase with no allosteric controls.

\textbf{Domain Structure—Preserved in Bacterial and Human Enzymes}

Crystallographic studies show that the enzyme has an $\alpha/\beta$ fold consisting of two domains (Figure 1; 68). A distinct surface structure within the N-terminal domain contains a site for binding maltodextrase. Muscle phosphorylase (mGP) attaches to glycogen particles at this position. The C-terminal domain has the familiar nucleotide binding fold similar in structure to lactate dehydrogenase, but with a coating of additional $\alpha$-helices. The PLP cofactor is covalently linked to the side chain of Lys-680 within the nucleotide binding fold adjacent to the substrate. The active site of the enzyme is located in a crevice between the N-terminal and C-terminal domains.

The N-terminal’s hundred or so amino acids are especially important for allosteric control in regulated phosphorylases. Two simple structural elements, the “cap” loop and the $\alpha$2 helix, which directly follows the cap, form approximately half the intersubunit contacts in the phosphorylase dimer. The phosphoserine and AMP binding sites in mGP nestle close to each other between the twofold related cap and the $\alpha$2 helix. These regulatory sites lie about 40 Å away from the active site. MalP lacks the phosphoserine site of mGP because the first 17 residues of mGP, which form the first $\alpha$ helix of the structure, are missing from its sequence. The binding site for AMP is also absent in MalP because of sequence differences in residues from the cap and $\alpha$2 helix. Arg-309 and Arg-310, which bind the
phosphate of AMP in mGP, have their side chains pointing in opposite directions in the *E. coli* enzyme. The intersubunit contacts in MalP are unrelated to those in structures of phosphorylases from other species. Regulatory sites apparently developed at the dimer interface by the incorporation of side chains that were appropriately positioned to bind effectors.

**Oligosaccharide Binding**

The structure of MalP, cocrystallized with maltohexaose, provides the only phosphorylase structure featuring an oligosaccharide bound at the catalytic site (51). Comparison to the structure of the enzyme alone indicates that an induced-fit mechanism closes the N- and C-terminal domains over the oligosaccharide substrate. An active site element, the 380’s loop moves by about 7 Å in response to oligosaccharide binding to close the domain interface. Domain closure is accompanied by formation of ionic and van der Waals interactions that stabilize the loop in this position. Although the 380’s loop residues form no direct interactions with the oligosaccharide, site-directed mutagenesis studies show that the substitution E382A reduces the affinity for oligosaccharide and suggest the importance of these interactions for proper positioning of active site residues (16).

Perhaps correlated with the structural reorganization, oligosaccharides are known to bind weakly to the active sites of phosphorylases. Although MalP was crystallized with maltohexaose, the structure shows only two glucose units bound at the active site (Figure 2). All hydroxyls of the sugar form hydrogen bonds to residues at the active site. The side chain of the universally conserved Tyr-280 points into the active site, where it forms a stacking interaction with the hydrophobic face of the sugar. Site-directed mutagenesis studies confirm the importance of many of these residues for oligosaccharide recognition and catalytic activity (16). A Y280A substitution increases the $K_m$ for maltoheptaose 200-fold and reduces $k_{cat}/K_m$ by $10^4$ (51).

The constitutive activity of MalP contrasts with regulated phosphorylases and may be accounted for in part by differences in the position of a movable gate, the 280’s loop, which controls access to the active site (51, 68). The 280’s loop in MalP is located away from the active site. The 280’s loop, in structures of inactive mGP, enters the active site, blocks substrate binding, and furthermore binds inhibitors (33, 34, 64). In structures of inhibited mGP, Arg-569, which binds the phosphate and oligosaccharide substrates in MalP, is not positioned correctly to bind either substrate (4, 22, 64).

**YEAST PHOSPHORYLASE, AN EVOLUTIONARY ADVANCE IN REGULATION**

**Regulation at Its Simplest**

Regulation of yeast phosphorylase (yGP) is relatively simple compared to the more complicated mammalian isozymes. It is activated solely by phosphorylation and
inhibited only by binding of Glc-6-P (5, 21). A yeast cAMP-dependent protein kinase phosphorylates Thr\(^{-10}\) to activate yeast yGP. Thr\(^{-10}\) is located in an N-terminal extension (residues \(-1\) to \(-39\), numbered backwards from residue 1 of the mGP sequence) not found in either MalP or mGP. Structures of unphosphorylated and phosphorylated forms of yGP provide a glimpse of the allosteric transition between inhibited and activated states (39, 40, 57, 58).

Phosphorylation control mechanisms are known for only a handful of enzymes, and a similarity between yeast and higher eukaryotes is often assumed. It comes as a surprise that the mechanism by which phosphorylation activates yGP is utterly unrelated to that of mGP. Sequence differences at the N-terminus of the yeast enzyme eliminate the phosphorylation site found in mGP (28). Yeast GP has an Asn in place of Ser-14, the site of regulatory phosphorylation in mGP, and in the region that corresponds to the N-terminus of mGP, the two enzymes have only one identical residue. It is surprising that the structure of yeast GP \(b\) (unphosphorylated phosphorylase) shows that this region adopts a conformation and position more similar to that of the phosphorylated than to that of the unphosphorylated muscle enzyme (57, 63). This observation implies that an ancient precursor enzyme was active with its N-terminus in a helical conformation bound between the subunits. The mammalian enzyme became inactive when its N-terminus became extended and no longer bound at the dimer interface. Phosphorylation is required in the muscle enzyme to initiate the refolding of its N-terminus into a helix that binds between the subunits. Electrostatic repulsion between the phosphate and surrounding acidic residues may be required in the muscle enzyme to drive the movement of the N-terminus to the subunit interface (11, 31). In the yeast enzyme, differences in the sequence allow this region to adopt a helical conformation and dock at the subunit interface in the absence of phosphorylation. Moreover, phosphorylation of the yeast enzyme has little effect on the structure of this region (40, 57). yGP is inactive though because the subunit contacts have evolved to stabilize the inactive form.

Ionic interactions that form in mGP between the negatively charged serine-phosphate and Arg-43 and Arg-69 are not conserved in the yeast enzyme. Another difference between the yeast and muscle enzymes is that the C-terminus of yGP is shorter than in the muscle enzyme. C-terminal residues in mGP compete with the phosphorylated N-terminus for binding at the subunit interface (63). In yGP, the corresponding C-terminal residues that impede binding of the N-terminus in mGP are absent.

The location of the N-terminal extension of yGP depends on the phosphorylation of Thr\(^{-10}\) (Figure 3). In unphosphorylated yGP, residues \(-27\) to \(-38\) of the N-terminal extension bind between the N- and C-terminal domains to block the entrance to the active site (57). The position of the N-terminus indicates that it regulates activity by controlling access of substrates to the active site. Both hydrophobic and ionic interactions fuel the conformational change. In response to phosphorylation of the enzyme, the N-terminus refolds and moves away from the active site to the subunit interface (40). Residues from 5 to \(-7\) assume an
α-helical conformation, and residues from −8 to −10 form a hairpin structure that binds between the subunits to build a hydrophobic cluster. The phosphorylated Thr−10 forms ionic interactions with the side chains of Arg-309 and Arg-310 at a site that corresponds to the AMP binding site in mGP (40). In striking mimicry of mammalian AMP activation, the phosphopeptide competes with Glc-6-P for binding the pair of arginines. Ionic interactions with the phosphopeptide serve to properly position the N-terminal peptide at the dimer interface. Phosphorylation is accompanied by a small increase in the separation of the two subunits. The movement of the subunits apart, as a result of phosphorylation, contrasts with mammalian activation in which phosphorylation or AMP binding draw the subunits closer together.

The structural basis for allosteric control extends to the active site through specific linkages. In the unphosphorylated, inhibited state, the 380’s loop forms contacts with the N-terminus near the entrance to the active site (57). The N-terminus and the 380’s loop together block binding of substrates to the enzyme in the inactive state. These contacts are disrupted during phosphorylation by the refolding and movement of the N-terminus, and as a result, the 380’s loop moves away from the active site (40). The two active sites are linked by the two tower helices and active site gates. The change in position of the tower helices as a result of phosphorylation brings together a cluster of conserved hydrophobic residues Phe-252, Leu-254, Phe-257, Tyr-262, Tyr-163′, Val-278′, Tyr-280′, Pro-281′, Phe-285′, and Leu-291′ near the active site, which stabilize the activated enzyme. The conservation of these residues in all phosphorylases suggests that the condensation of hydrophobic side chains is a common feature in all active phosphorylases (39). Figure 3 shows the hydrophobic residues, which form a cluster in the phosphorylated form of the enzyme. The aromatic ring of Tyr-280 flips 180° during activation to point into the active site. Although no structure has ever been determined of yGP with oligosaccharide bound at the active site, the structure of MalP with maltohexaose shows that Tyr-280 is a key residue involved in oligosaccharide recognition (51).

In both the unphosphorylated and phosphorylated forms of yGP, the separation between the N-terminal and C-terminal domains is greater than in either the complex of MalP with oligosaccharide or the complex of mGP inhibited with glucose (40, 51, 57, 64). That is, the domains are rotated apart such that the active site cleft would be open for substrate binding in the unphosphorylated enzyme if not for the blockage by the N-terminus and the 380’s loop. The active site gate of yGP a is 10 Å away from its position in structures of inhibited mGP, and it is in the position identical to that in MalP. The movement of the N-terminus and the 380’s loop thus appears to promote substrate binding and link the two active sites. In structures of both unphosphorylated and phosphorylated forms of yGP, the gate is held in a position where it does not block the active site (40, 58). The side chain of Arg-569 in both unphosphorylated and phosphorylated forms of yGP occupies the same position observed for activated mGP and is in the proper position to bind the substrate phosphate.
Mutagenesis studies further support a role for the N-terminal extension in regulation of activity (38). An enzyme variant that has the first 42 residues of yGP deleted displays significant activity, though it is incapable of being phosphorylated. Removal of the N-terminus results in partial activation of the enzyme, presumably because substrates can bind at the unblocked active site. Without the N-terminal phosphopeptide at the dimer interface to induce further conformational changes, however, the mutant does not achieve full activation.

Effectors at the Dimer Interface—A Phosphopeptide

Glc-6-P inhibits yGP by displacing the phosphorylated N-terminus from the effector site (40). Residues that bind Glc-6-P in yGP are mostly the same as in mGP, though the mode of binding of Glc-6-P differs in the two enzymes (58). Muscle GP binds the α anomer of Glc-6-P, whereas yGP binds the α anomer. The precise positioning of the inhibitor and the specific contacts it makes with the enzyme differ accordingly in the two structures. Notably, the hexose rings of Glc-6-P are oriented differently, by 90°, in the two enzymes. In yGP, the position of Trp-67 matches that of the activated muscle enzyme, which necessitates the change in position of the hexose ring of Glc-6-P. Like the muscle enzyme, Arg-309 and Arg-310 form ionic interactions with the phosphate group of the inhibitor. The phosphate group of Glc-6-P forms ionic interactions with the same two arginine residues as the Thr-10-phosphate of yGP. Since interactions of the phosphate groups of activators and inhibitors at this site are similar, other interactions with the effectors must differentiate the allosteric response. At the subunit interface, interactions with hydrophobic residues of the N-terminus differ from those with Glc-6-P and provide the discrimination. Hydrophobic interactions of the phosphopeptide inserted between the subunits lead to the separation of the subunits that triggers activation. Unlike mGP, AMP does not activate yGP because it lacks the residues in mGP that interact with the adenine ring of AMP (28).

MUSCLE PHOSPHORYLASE—THE MOST ELABORATELY REGULATED

A Competition by Activators and Inhibitors

In skeletal muscle, phosphorylase activity is activated during muscle contraction when glycogenolysis is required to produce ATP for energy. The enzyme is regulated by metabolites of glycolysis, including ATP and glucose-6-P, which act as feedback inhibitors, and AMP, which serves as an allosteric activator (14, 15, 35, 50, 54). In addition, the enzyme is activated during stress by covalent phosphorylation at residue Ser-14. Phosphorylase is interconverted between unphosphorylated and phosphorylated states that have different allosteric properties by phosphorylase
kinase and protein phosphatase PP-1, whose activities in turn are controlled by a complex multienzyme cascade responsive to hormonal regulation, nerve stimulation, and intracellular Ca²⁺ concentration (13, 17–19, 29, 37).

Structure

The overall structure of the enzyme is similar to other phosphorylases; however, the subunits associate in different orientations than in *E. coli*, MalP, or yGP, and mGP has regulatory sites not present in the other enzymes (20, 32). The site of covalent phosphorylation and the binding site for AMP in mGP reside in an activation subdomain consisting of the first six N-terminal helices. Inhibitors ATP and Glc-6-P bind competitively with AMP at overlapping regulatory sites in this region (46, 50, 54, 67). Activation of the enzyme by phosphorylation at Ser-14 is associated with a structural rearrangement of the N-terminus and movement of the N-terminus over a distance of 35 Å to the subunit interface (Figure 4; 63). In phosphorylase b, N-terminal residues 5–16 are disordered, and residues 10–22, which have an extended conformation, form intrasubunit contacts (47, 69). Phosphorylation causes the N-terminus to refold into a 3₁₀ helix, and upon relocation to the subunit interface, the phosphoserine forms ionic interactions with Arg-43 of the α₂ helix within the activation subdomain.

AMP binds to the subunit interface between the cap and the adjacent α₂ helix at a site approximately 10 Å away from the phosphoserine. The phosphate group of AMP forms ionic interactions with Arg-309 and Arg-310, which were also shown to have a role in binding Glc-6-P in yGP. The purine ring of AMP forms an aromatic stacking interaction with the aromatic ring of Tyr-75 in the third helix of the activation subdomain, and the ribose group forms a hydrogen bonding interaction with the side chain of Asp-42′ from the cap. The formation of intersubunit contacts mediated by AMP or the phosphoserine draws the cap and α₂ helix closer together (3, 62, 63, 66). This movement of secondary structural elements triggers a transition in quaternary structure. The subunits rotate in opposite directions by 5° about two axes approximately perpendicular to the molecular twofold (3, 4, 22, 66, 65). Further tertiary structural changes propagate allosteric effects to the active site.

At the opposite end of the subunit interface, the tower helices (helix α₇, residues 264–277) form intersubunit contacts that link the two active sites. These tower helices are named for their obvious extension away from the body of the monomer. Intersubunit contacts between the helices are altered by the rotation of the subunits (4, 22). In the inactive state, the tower helices assume an approximately antiparallel configuration. Rotation of the subunits changes the geometry of the helices such that the angle formed by the helices crossing each other is approximately 80°. The reorientation of the tower helices is accompanied by the movement of a loop of amino acids (residues 280–288 are the active site gate), which directly follows each tower helix. The role of the active site gate is similar to the N-terminal peptide of yGP that controls access of substrates to the active site. The gate blocks the active
site when the enzyme is in an inactive conformation and moves away from the active site when the enzyme is in an activated conformation (4, 22, 70). Mimicry as seen at the Glc-6-P site in yGP is also apparent here. Residue Asp-283 mimics the substrate phosphate at the active site (4, 22). In the inactive conformation, the carboxylate side chain occupies the phosphate subsite of the active site and electrostatically and sterically interferes with substrate binding. The carboxylate group of Asp-283 forms charged hydrogen bonds with the side chains of His-571 and Asn-284. These interdomain contacts contribute to the stabilization of the inactive conformation of the enzyme in which the N- and C-terminal domains are rotated toward each other to close the active site.

The two residues following Asp-283, Asn-284 and Phe-285, of the active site gate loop participate in binding allosteric inhibitors. Asn-284 forms a hydrogen bond with the C1 hydroxyl group of glucose, which binds at the active site at the position of the substrate, glucose 1-phosphate (Glc-1-P) (64). The aromatic ring of Phe-285 forms aromatic stacking interactions with purine inhibitors that bind just outside the active site (61). The identity of the physiological inhibitor that may bind at this conserved purine site in vertebrate phosphorylases remains a mystery. Binding of glucose and purine inhibitors stabilizes the inactive conformation of the active site gate loop and hinders substrate binding. yGP is not inhibited by glucose even though all of the residues that are involved in binding glucose in mGP are conserved, perhaps because the active site gate does not assume the correct position to bind the inhibitor.

During activation of mGP by either AMP or phosphorylation, the N- and C-terminal domains rotate apart by 5°, the active site gate moves away from the active site, and Arg-569 moves into the correct position to bind the substrate, phosphate (3, 4, 22, 63, 66). The opening of the domains and the movement of the active site gate make the active site accessible to oligosaccharide and phosphate. Further structural changes may be required for activation; however, the crystallographic structures of mGP, either phosphorylated or with AMP bound, do not depict full activation since the enzyme crystallized as a tetramer and not as a functional dimer.

Glc-6-P inhibits phosphorylase by competing with AMP (67). The binding site for the inhibitor overlaps that of the activator. The phosphate group of Glc-6-P, similarly to AMP, forms ionic interactions with the side chains of Arg-309 and Arg-310, and in addition, Arg-242 contributes an ionic interaction. The position of the glucopyranose ring differs from that of the ribose ring of AMP. Phosphorylase senses the state of phosphorylation of the nucleotide in the activator site so that only AMP activates. ATP and ADP, like Glc-6-P, inhibit the enzyme by competing with AMP for binding at the subunit interface. Discrimination among nucleotides is based on the charged interactions of the phosphate groups and different placements of their adenine rings. For ATP, the α- and β-phosphate groups form ionic interactions with Arg-309 and Arg-310 (62). Additional ionic interactions are formed between the γ-phosphate of ATP and Lys-41′ and the β-phosphate and Arg-242. The ribose ring of ATP forms one hydrogen bonding interaction with the enzyme. The adenine ring of ATP is shifted from the position observed for AMP because of
the longer triphosphate cluster. The adenine ring extends out into the solvent and forms no productive interactions with the enzyme. ATP and Glc-6-P form a different set of interactions than the activator AMP and similarly inhibit the enzyme by mediating intersubunit contacts that stabilize the inactive quaternary association of the subunits. Unlike AMP, these inhibitors do not cause the appropriate movement of the cap and \( \alpha_2 \) helix closer together than is necessary for triggering activation. It is only the correct condensation of the three elements of structure, the cap, the \( \alpha_2 \) helix, and the arginine cluster, that activates and only AMP can perform the condensation.

**Mutagenesis Studies**

Allosteric activation of mGP can be mimicked by the binding of divalent transition state metals between the cap and \( \alpha_2 \) helix (7). Site-directed mutagenesis was used to replace Val-45 of the cap and Tyr-75 of the helix with histidine residues to create a metal binding site at the subunit interface. As metal ions bind to residues from both monomers, the cap and \( \alpha_2 \) helix move closer together, simulating the effects of AMP binding or phosphorylation. Activation levels of up to 10% of that for AMP have been achieved by the binding of metal ions at engineered divalent cation sites. mGP can also be partially activated by acidic substitutions at Ser-14, the site of regulatory phosphorylation (11). An S14D mutant exhibits a 1.6-fold increase in \( V_{\text{max}} \), a tenfold decrease in the apparent dissociation constant for AMP, and a threefold decrease in the \( S_0.5 \) for Glc-1-P. An S14E mutant shows a 2.2-fold increase in \( V_{\text{max}} \), a sixfold decrease in the apparent dissociation constant for AMP, and a twofold decrease in the \( S_0.5 \) for Glc-1-P. Both mutants still require AMP for activity and show cooperativity for Glc-1-P in contrast to the phosphorylated enzyme. Native GP\( \alpha \) (phosphorylated phosphorylase) neither displays substrate cooperativity nor requires AMP for activation because phosphorylation converts the enzyme almost entirely to the active state. An acidic substitution at the N-terminus apparently does not induce the quaternary transition associated with allosteric activation on its own. In the presence of AMP, however, which triggers the subunit rotation, the mutants are activated to a greater extent than native mGP\( \beta \).

The mutations may, therefore, enable the N-terminus to refold and dock at the subunit interface when the monomers are oriented in their activated configuration. Molecular modeling indicates that a carboxylate side chain at the position of the serine phosphate may participate in salt-bridge interactions with the arginines that normally interact with the phosphoserine. Differences in the charge and in the geometry of carboxylate versus phosphate groups and the weaker ionic interactions with charged residues presumably prevent full activation.

The crystallographic studies suggest the importance of the tower helix and active site gate in coupling structural changes induced by allosteric effectors binding at the subunit interface to changes at the active site. The tower helices undergo a large change in tertiary structure during the allosteric transition, though the movement of the tower helices during activation could simply be the result of the subunit rotation.
triggered by AMP or phosphorylation. Mutagenesis studies designed to test the functional significance of particular tower helix, gate interactions, and structural changes in these regions show that mutations in the tower helix and active site gate do affect allosteric regulation (9, 10). The entire tower helix of mGP was replaced with the tower helix from yGP to test the importance of specific intersubunit interactions on allosteric activation and catalytic activity. Only 33% of the amino acids in the helix of yGP are identical to those of mGP, and the helix replacement changes helix-helix intersubunit interactions. Kinetic studies of this mutant show that the replacement with the yGP tower helix results in an eightfold reduction in $V_{\text{max}}$, a loss in cooperativity for both AMP and Glc-1-P, but little change in the $K_a$ for AMP, and the $S_{0.5}$ for the Glc-1-P is reduced twofold. The ability of this mutant to be activated by AMP shows that the helix replacement does not destroy the coupling between the AMP and active sites. The relatively modest loss in catalytic activity shows that the specific interactions of the helices are not essential for allosteric activation or catalytic activity, though they do determine the precise response to allosteric effectors. The helices have been tuned during evolution to provide a calibrated response at the active site to regulatory signals from the opposite face of the dimer.

Some amino acids in the active site have multiple functions. The importance of active site gate residue Asp-283 for catalytic activity and allosteric regulation was tested by site-directed mutagenesis (9). Replacement with either Asn or Ala results in a tenfold stronger association of Glc-1-P with retention of full substrate cooperativity. The increased apparent affinity for Glc-1-P suggests that the substrate binds to the inactive conformer in these mutants because of the loss of competition between the substrate phosphate and the negatively charged carboxylate side chain of Asp-283. The 15-fold reduced levels of catalytic activity in the D283A and D283N mutants suggest that Asp-283 plays more than an inhibitory role at the active site. Although no structure is available for mGP with oligosaccharide bound at the active site, the structure of MalP with maltohexaose suggests that residues of the active site gate loop participate in oligosaccharide recognition. In MalP, a D283A substitution results in a sevenfold increase in the $K_m$ for maltoheptaose and a threefold decrease in $k_{\text{cat}}$. The structure of MalP shows that Asp forms no direct interactions with the oligosaccharide but may contribute secondary interactions that affect the positioning of residues at the active site.

HUMAN LIVER PHOSPHORYLASE

Regulation in the Unselfish Organ

The liver and muscle isozymes fulfill different physiological requirements and accordingly respond differently to allosteric effectors. In muscle, where the role of phosphorylase is to mobilize carbohydrate for intracellular use, the enzyme displays a greater sensitivity to ligands, such as AMP and ATP, which signal the energy state of the cell. The unphosphorylated form of mGP is potently activated...
by AMP and is sensitive to ATP or Glc-6-P, which competitively inhibit AMP binding (8, 25, 26). In contrast, the primary role of the liver enzyme is to break down glycogen for release as free glucose into the bloodstream to ensure a constant supply of this substrate to other tissues. The liver isozyme is consequently less sensitive to intracellular ligands. The unphosphorylated form of liver glycogen phosphorylase (lGP) is inactive, shows only weak activity in the presence of AMP (10%–20%), and is insensitive to inhibition by ATP or Glc-6-P (36, 42, 43). The enzyme, instead, depends strongly on phosphorylation to achieve activity. The phosphorylation state of the enzyme is subject to hormonal control; thus, lGP activity is regulated primarily extracellularly in keeping with the function of this isozyme in providing glucose outside of the cell. It is uncertain from an analysis of sequence differences between the isozymes what accounts for their differential responses to ligands. The liver and muscle enzymes are about 80% identical and, with one exception, all of the residues that bind AMP are conserved.

Crystallographic Structures Show Changing Interfaces

The first structures of human lGP in both active and inhibited conformations have recently been determined (56). The phosphorylated enzyme was crystallized in an active conformation with AMP bound and in an inactive conformation complexed with a potent but nonphysiological glucose analog, N-acetyl-β-D-glucopyranosylamine (71). Overall, the structures of both the active and inactive conformations are similar to their counterparts in mGP. A comparison of the inactive and active conformations of lGP and mGP shows that in both isozymes, the solvent accessible surface area of the subunit is reduced when the enzyme is activated. Hydrogen bonds specific for the inactive conformation are replaced by a greater number of hydrogen bonds tailored to stabilize the active conformation. Activation of both isozymes involves a rotation of the subunits about a conserved axis accompanied by domain closure, and these changes are similar in magnitude in both isozymes.

Despite these overall similarities, there are significant differences between mGP and lGP in both the active and inactive conformations. The crystal structures revealed that mGP and lGP do not share the same subunit interface in either conformation. This is unexpected because all but three of the residues involved in the subunit interface are conserved between the two enzymes (59). Repacking of the subunit interface is the result of changes in the relative orientation of the hydrophobic cores of the subunits and in hydrogen bonds and van der Waals contacts between the subunits. Half of the hydrogen bonds exclusive to either the active or inactive conformation are isozyme specific; the remainder are found in both mGP and lGP. Repacking of the two subunits creates a lGP dimer, which is distinct from the mGP dimer and could not have been predicted by sequence comparison.

The crystal structure of the dimeric, activated complex of lGP/AMP also reveals how more than 40 residues of the catalytic site work together to activate the enzyme (Figure 5). On activation of lGP a, residues 250–290 undergo order or disorder
transitions, changes in secondary structure, and/or repacking, resulting in a larger subunit interface with new hydrogen bonds and van der Waals contacts. In the activated IGP structure, the tower helix is shorter by two turns and does not form the hydrogen bonds to its mate in the other subunit, which are seen in inactive IGP α. The gate is well ordered and is moved up and away from the entrance to the active site such that substrate has direct access to the catalytic residues. In its open position, the gate residues make van der Waals contacts with the 250’s loop of the other subunit, and the length of the gate is shortened by extending the helix that follows it (helix 8). This shorter gate can no longer contact the 380’s loop, which is less well defined in the electron density. Thus, when open, the gate contacts the 250’s loop of the second subunit, and as a consequence, the 380’s loop of its own subunit becomes more mobile, probably to permit the interaction with substrate observed in the MalP complex with maltoheptaose.

These 40 residues include most of the cluster of conserved hydrophobic residues, which stabilize the active enzyme in yGP. The structure of IGP/AMP shows that the hydrophobic cluster is preserved structurally in the liver enzyme (although yGP and mGP share only 49% sequence identity) and suggests that the more closely related mammalian phosphorylases (80% sequence identity between human liver and human mGP) are also stabilized in the same way. This result cannot be confirmed in mGP because we lack a crystal structure of the fully activated, dimeric mGP enzyme. In all the mGP structures solved to date, the 250’s loop, a key part of the hydrophobic condensation, is disordered, and the enzyme is either inhibited in some way or forms a tetramer in which the catalytic site is blocked.

The IGP structures also suggest why AMP is not a strong activator of IGP. The loss of AMP cooperative binding and weak enzyme activation in the liver isozyme results from changes at the subunit interface, where the AMP site is located, notably in the conformation of the adenine loop (residues 315–325). Despite the conservation of binding site residues, many of the interactions with AMP seen in mGP are absent in the complex of the liver enzyme with AMP. The loss of contacts to binding site residues is primarily the result of a rigid body rotation between the two subunits and secondarily of alterations in side chain positions. The rotation of the subunits with respect to each other increases the distance between Cα carbons of the cap and the α2 helix by 0.5 Å (on average), with the loss of five hydrogen bonds seen in the mGP complex. The fact that AMP forms fewer hydrogen bonds to residues of the α2 helix partly explains the loss of cooperative binding in IGP because the signal that AMP is bound to one site is propagated through the α2 helix to the other subunit.

In mGP, the adenine N6 amino group of AMP is recognized by the main chain carbonyls of residues within the adenine loop (66). These contacts are absent in the IGP complex because the loop adopts a different conformation that moves it away from the AMP site. Hydrogen bonds, which stabilize the conformation of the adenine loop in mGP, are absent in IGP as a result of sequence changes. The adenine loop connects the AMP site to the active site gate through helix α8. Without the contacts between AMP and the adenine loop, local structural changes
induced by AMP binding may be poorly coupled to the active site and may account for weaker AMP activation (36).

Inhibitors of Liver Phosphorylase for Treatment of Non-Insulin-Dependent Diabetes

Recently, there has been a growing interest in using inhibitors of IGP in treating noninsulin-dependent or Type 2 diabetes. Type 2 diabetes mellitus is a disease characterized by high levels of glucose in the plasma and leads to complications such as nerve and kidney damage, blindness, premature atherosclerosis, and heart disease. The molecular basis of the disease is not well understood but is characterized by peripheral insulin resistance and pancreatic defects in insulin secretion. Intensive control of blood glucose levels prevents or delays the onset of diabetic complications (1) but is rarely achieved with oral antidiabetic agents. In diabetic subjects, breakdown of glycogen by IGP remains an important contributor to hepatic glucose output even when blood glucose levels are high, establishing a potential role for phosphorylase inhibitors in diabetes therapy.

Inhibitors of phosphorylase include glucose analogs (active site) (24, 48), an AMP site inhibitor (71), and hydroxylated piperidines and pyrrolidines (2). Recently, two series of indole-2-carboxamide inhibitors were reported (49, 27). The indole-2-carboxamides inhibit synergistically with glucose, a desirable property that could minimize the risk of hypoglycemia, a potentially severe side effect of many antidiabetic agents. Preliminary results have shown that the indole-2-carboxamide inhibitors show both cellular and oral activity in reducing blood glucose levels in ob/ob mice (49, 27).

To identify the binding site for these compounds on the enzyme, the crystal structure of IGP \( a \) complexed with one of these inhibitors was determined at 2.3 Å resolution (Figure 6; 55). The electron density of the bound inhibitor was located in a difference map of a crystal of IGP \( a \) grown in the presence of excess compound and a glucose analog, GlcNAc (63). The compound binds to a new, highly specific, allosteric site on the enzyme. This site is distant from the catalytic site and has not been reported previously. The compound is bound within the solvent cavity, which forms part of the dimer interface, very close to the axis of the molecular twofold symmetry operator. The binding site for the inhibitor is made up of residues from both subunits and consists of a primarily hydrophobic half and a mixed hydrophobic and polar half. The secondary structural elements that form this novel binding site are conserved in the known crystal structures of yGP, mGP, and IGP, and they are expected to be present in the brain isozyme as well.

The discovery of this series suggests that screening for allosteric inhibitors (or, potentially, activators) may be generally useful in identifying new classes of drugs. The indole-2-carboxamide series was identified by screening the Pfizer sample bank for compounds that would inhibit enzyme activity in the presence of physiological concentrations of glucose. Because glucose itself is an active site inhibitor, the screen amounted to a search for compounds that would inhibit
phosphorylase activity by allosteric mechanisms. Phosphorylase has multiple effector sites, each of which could be the target of a separate screen. The discovery of a new, highly specific, allosteric inhibitor site on such a well-studied enzyme reveals the remarkable complexity and plasticity of phosphorylase.

CONCLUSIONS: Evolution Provides Regulatory Diversions

In conclusion, allosteric control mechanisms appear to have evolved independently in the yeast and mammalian enzymes. Mammalian and yeast enzymes differ in their subunit interactions and in their mode of binding phosphopeptides and Glc-6-P. Their phosphorylation sites differ and though N-terminal phosphorylation activates both types of enzymes, the mechanisms of their activation differ. The structural changes triggered at the interface by effectors are distinct. During activation of the mammalian enzyme, the subunits draw closer together; in yGP, the subunits separate. Both mammalian and yeast enzymes are inhibited by Glc-6-P; however, they display different anomer preferences and bind the inhibitor in different orientations. The enzymes have in common a binding pocket at the subunit interface that recognizes the phosphate groups of either activators or inhibitors. Regulation is achieved by the competition of effectors for binding at this site. In yGP, this site binds either the phosphate of the phosphorylated N-terminus or Glc-6-P. In mammalian phosphorylases, this site binds either the phosphate of AMP or Glc-6-P or the triphosphate of ATP. The ancestral phosphorylase was presumably a simple constitutively active catalytic enzyme similar to MalP that over time, in response to environmental conditions, evolved distinct sets of binding amino acids for the pertinent effectors. Because all phosphorylases must come to the same final configuration of active site amino acids around the substrates, it is the family of inactive states that varies among species. The binding of a competitive activator that displaces inhibitors to drive the stabilization of an active conformation represents a common feature of the regulatory mechanisms of phosphorylases. Recent evidence suggests that in the yeast and mammalian phosphorylases, stabilization of the active state involves the condensation of 12 conserved hydrophobic side chains into a hydrophobic cluster near the active site. In the inactive states, these side chains are found in different positions in the yeast and mammalian enzymes.

Crystallographic studies indicate that regulated phosphorylases adopt at least three conformational states. The unphosphorylated enzyme assumes an inactive conformation in which the active site is inaccessible to substrates. In yGP, the N-terminus and 380’s loop block the entrance to the active site. In mammalian phosphorylases, the N- and C-terminal domains are rotated together to close the active site cleft, and the active site gate occupies the phosphate subsite of the active site. Allosteric inhibitors, including Glc-6-P, glucose, and purines, stabilize the enzyme in an inactive conformation. Phosphorylation and/or AMP binding promote a transition to an activated conformation: The N-terminus in yGP and the active site gate in mGP move away from the active site to relieve inhibition. The
N- and C-terminal domains rotate apart to open the active site to substrate binding, and key residues move into positions to increase affinities for phosphate and sugar substrates. Finally, substrates bind at the active site, and the enzyme undergoes an oligosaccharide-induced domain closure involving movements of the 280s and 380s loops to create the catalytically competent ternary complex.

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Figure 1  Ribbon diagram of MalP dimer. The binding site for maltodextrin is shown in magenta. The PLP cofactor (yellow) is covalently linked to the side chain of Lys-680 within the nucleotide binding fold motif of the C-terminal domain. The active site of the enzyme is located in a crevice between the N-terminal and C-terminal domains and is shown with bound maltose (purple), glycerol (purple), and sulfate (pink).
Figure 2  View of the active site of MalP shows PLP cofactor (yellow) and bound maltose (dark blue), glycerol (dark blue), and sulfate (pink). Hydrogen bonding interactions are indicated with dashed lines (RCSB Protein Data Bank entry 1AHP).
Figure 3  Comparison of (A) phosphorylated (RCSB Protein Data Bank entry 1YGP) and (B) unphosphorylated yeast phosphorylase (22). The phosphorylase dimer is depicted as a Connolly surface with one monomer colored blue and the other purple. N-terminal residues 1-22, corresponding to the N-terminus of muscle phosphorylase, are shown as a ribbon in white. The unique N-terminal extension of yeast phosphorylase (residues -1 through -39) is drawn as a ribbon in pink. The structure of yGP a is of a truncated form of the enzyme, which contained a deletion of the first 22 N-terminal residues; therefore, only residues -1 through -14 of the N-terminal extension are shown. The structure of yGP b is of the full-length enzyme; however, residues -12 through -22 of yGP b were disordered and are not shown. In the unphosphorylated enzyme, the N-terminal extension blocks the entrance to the active site. Phosphorylation results in the movement of the N-terminal extension to an
allosteric site at the subunit interface where it displaces the inhibitor, glucose 6-phosphate (orange). Thr-10, the site of regulatory phosphorylation, is colored pink. The position of the active site is indicated by the PLP cofactor (yellow) and in yGP a, by the additional presence of a bound phosphate (pink). Hydrophobic residues (Phe-252, Leu-254, Phe-257, Tyr-262, Tyr-163', Val-278', Tyr-280', Pro-281', Phe-285' and Leu-291'), shown in coral, condense to form a hydrophobic cluster near the active site in yGP a.
Figure 4  Comparison of (A) phosphorylated (27) and (B) unphosphorylated muscle phosphorylase (RCSB Protein Data Bank entry 2GPB). The phosphorylase dimer is depicted as a Connolly surface with one monomer colored blue and the other purple. N-terminal residues (5-22 in mGP a and 12-22 in mGP b) are drawn as a ribbon in white. Residue Ser14 is shown in dark pink and the PLP cofactor is shown in yellow. AMP (light pink) is bound at the allosteric site of mGP a. The inhibitor glucose (orange) is bound at the active site of mGP b. In response to phosphorylation, the N-terminus of mGP refolds, assuming a helical conformation, and moves to the dimer interface. This structural change triggers the allosteric transition and results in the rotation of the subunits and further tertiary structural changes that lead to activation.
Figure 5  Refolding and repacking of the protein core. Cartoon showing the structural changes that occur on the catalytic face of the enzyme in the inactive and active conformations of IGP α. The subunits of the functional dimer are shown as circles, with the N-terminal and C-terminal domains indicated. In the inactive structure, hydrogen bonds between the tower helices are shown as orange-dotted lines; regions of the 250’s loop that are disordered are shown as dashed lines; the tower helix is two turns longer (shown as a solid orange cylinder). In the active structure, the subunit rotation axis is shown in green; helix 8 is extended by 1 turn (shown as a solid purple cylinder).
Figure 6 The new allosteric inhibitor site. Human liver glycogen phosphorylase $\alpha$ is depicted as a ribbon diagram, one subunit in purple (helices) and pink (sheets) and the other in green (helices) and blue (sheets). AMP (gray); Ser-14-P (pink, red phosphates); PLP (pyridoxal phosphate, the essential cofactor in red); GlcNAc, the glucose analog (purple, marks the glucose binding site); and CP-403,700 (carbon, pink; nitrogen, blue; oxygen, red) are shown in CPK. The twofold symmetry operator relating the subunits is located between the two molecules of CP-403,700, orthogonal to the plane of the page. The binding sites for Ser-14-P and AMP are located close to each other but do not overlap. To show all the binding sites in one image, a composite of two crystal structures was made; AMP and residues 5-22 (including Ser-14-P) from the crystal structure of HLGP $\alpha$ complexed with AMP (62) and the rest from the complex of HLGP $\alpha$ complexed with GlcNAc and CP-403,700 (71).