THE FAMILY OF GLYCOGEN PHOSPHORYLASES: STRUCTURE AND FUNCTION

Authors: **Christopher B. Newgard**
Department of Biochemistry and the Center for Diabetes Research
University of Texas Southwestern Medical Center
Dallas, Texas

Peter K. Hwang
Robert J. Fletterick
Department of Biochemistry and Biophysics
University of California at San Francisco
San Francisco, California

Referee: Neil B. Madsen
Department of Biochemistry
University of Alberta
Edmonton, Alberta, Canada

I. INTRODUCTION

Glycogen phosphorylase (1,4 α-D-glucan: orthophosphate α-D-glucosyltransferase, E.C. 2.4.1.1) isozymes play a central role in the mobilization of carbohydrate reserves in a wide variety of organisms and tissues. By far, the best-studied phosphorylase isozyme is that from rabbit skeletal muscle. This enzyme was discovered in 1936 and shown to be dependent on AMP for activity in 1938.[1,2] Demonstration of a covalent mechanism of interconversion of active and inactive forms of phosphorylase was demonstrated as early as 1943,[3,4] but the role of phosphorylase *b* kinase and phosphorylase phosphatase in covalent phosphorylation/dephosphorylation was first established in 1955.[5,6] The 6-Å X-ray crystal structure of the inactive phosphorylase *b* enzyme was published in 1974,[7] followed by the 3-Å structure of phosphorylase *a* in 1976.[8] The atomic resolution structures for the *a* and *b* enzymes are now complete, and a detailed comparison of the two forms has revealed the role of covalent phosphorylation.[9] The complete primary sequence of the enzyme, deduced entirely by protein sequencing techniques, was first reported in 1977.[10] Finally, the entire nucleotide sequences of the rabbit muscle phosphorylase cDNA and the human muscle phosphorylase gene have been completed within the last 2 years.[11-13] In between these landmark achievements, work on phosphorylase has continued unabated, making this enzyme one of the most carefully studied proteins in history.

A deficiency in the otherwise excellent extant review articles on glycogen phosphorylase is that they have focused almost exclusively on the structure and function of the rabbit muscle isozyme.[14-20] This is true, despite the fact that phosphorylases from a large number of organisms, including bacteria, fungi, yeast, plants, insects, and animals, as well as from mammalian tissues other than skeletal muscle such as liver and brain, have been identified and characterized. In most cases, these other isozymes exhibit diverse regulatory properties and physiological roles when compared with the muscle enzyme.

Recently, the use of recombinant DNA techniques has led to the determination of primary sequences for a wide variety of phosphorylase isozymes.[11-13,21-25] Concurrently, fresh insight

has been gained into the structural basis for allosteric regulation of the muscle enzyme by effectors such as AMP and maltoheptaose.[26,27] Therefore, it seems timely to review the new structural information in the context of the insight it provides into the function and evolution of the family of phosphorylases. We begin by briefly reviewing the functional properties of the "reference" enzyme, rabbit skeletal muscle phosphorylase, and then compare the functional properties of phosphorylases from other tissues and organisms to those of the muscle isozyme. Important new information about the tissue and organism-specific expression patterns and the chromosomal localizations of phosphorylase genes gleaned from recombinant approaches is described. We then focus in depth on relating the differences in functional properties among phosphorylases to the new structural information. Finally, the insights into evolutionary relationships afforded by the new sequence information are discussed, and our views of future directions for study of this highly complex and important enzyme are summarized.

II. PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF PHOSPHORYLASE ISOZYMES

A. Rabbit Skeletal Muscle Phosphorylase

All of the glycogen phosphorylases catalyze the same reversible reaction:

$$(\alpha\text{-}1,4\text{-glucoside})_n + P_i \rightarrow (\alpha\text{-}1,4\text{-glucoside})_{n-1} + \alpha\text{-D-glucose-1-P}$$

Although the equilibrium constant of this reaction at pH 6.8 is 0.28, strongly favoring glycogen synthesis, phosphorylase works in the glycogenolytic direction *in vivo* because the ratio of P_i to glucose-1-P concentration greatly exceeds the equilibrium constant. A high ratio of P_i to glucose-1-P is maintained in large part by the enzyme phosphoglucomutase, which rapidly converts glucose-1-P to glucose-6-P in a reaction whose equilibrium is strongly in the direction of glucose-6-P *in vivo*. All glycogen phosphorylases contain pyridoxal phosphate (PLP) as cofactor,[14,16,20,28] covalently bound via a Schiff base to an active site lysine (lys 680 in the rabbit muscle phosphorylase sequence). Experiments involving the removal of PLP from the apoenzyme and reconstitution with either PLP, pyridoxal, or PLP analogs have demonstrated the absolute requirement of the phosphate-containing cofactor for catalytic activity.[14,29] Controversy remains with respect to the exact catalytic mechanism.[14,20,29-36] One model implicates the 5′-phosphate of PLP acting in tandem with orthophosphate as proton donor and acceptor (general acid-base catalysis). In this scenario, orthophosphate donates a proton to O-4 of the glycogen chain and acquires a proton, possibly from PLP. The carbonium ion intermediate formed in this step reacts with P_i to form glucose-1-P. Other groups suggest that the glucosyl carbonium ion intermediate cannot exist long enough to react with inorganic phosphate, invoking instead interaction with protein functional groups for stabilization of the intermediate and as the source of the proton. Even the recent elegant studies of Hajdu et al., using fast crystallographic data collection with a Synchrotron Radiation source to allow direct observation of the phosphorylase-catalyzed reaction in phosphorylase *b* crystals, showed a clear interaction of the substrate and cofactor phosphates, but could not discriminate between inorganic phosphate and protein functional groups as the source of proton.[36]

The most active configuration assumed by rabbit muscle glycogen phosphorylase is a dimer composed of two identical monomers; the complete sequence analysis of the subunit assigned it a mol wt of 97,500.[10,12] Earlier studies suggesting that conversion of phosphorylase *b* to phosphorylase *a* was accompanied by a conversion of the molecule from a dimer to a tetramer[37,38] have been largely supplanted by work showing that the most active form of phosphorylase *a* in the presence of glycogen is a dimer.[39] The current consensus is that

phosphorylase exists as a dimer under normal physiological conditions and in most *in vitro* assays, but that phosphorylase *a* can form tetramers in concentrated solutions that still retain some activity.[15,16,20] The monomer is best described as consisting of two domains, each composed of a β-sheet core surrounded by layers of α-helical segments. The N-terminal domain is also referred to as the "regulatory domain", since it contains the covalent modification site Ser 14, the binding sites for most of the allosteric effectors (see below), and the residues that provide the intersubunit contacts in the dimer that are required for propagating allosteric effects. The C-terminal domain, also called the "catalytic domain", contains the Lys 680 that covalently binds the cofactor pyridoxal phosphate[28,40] and, with residues contributed by the N-terminal domain, forms the catalytic site as a deep crevice between the domains of the protein. The active site entrance has a moveable gate (residues 280-285) that partly shields substrates from the aqueous environment, thus favoring phosphorolysis over hydrolysis of the glycosidic bond in glycogen.

The physiological role of muscle phosphorylase is to provide the fuel for the energy production required for muscular contraction. The enzyme is interconverted between the active "R" and inactive "T" states by the integration of covalent and allosteric regulatory mechanisms. The functioning of both these mechanisms allows regulation of muscle phosphorylase activity by external stimuli, such as circulating catecholamines, as well as by intracellular signals that reflect the energy balance of the tissue. Extracellular factors generally exert their effect indirectly by interacting either with the adenylate cyclase system (the "glycogenolytic cascade") or by affecting Ca^+ flux, which in turn affect the activity of phosphorylase kinase.[41-43] Both of these pathways affect phosphorylase activity by promoting either phosphorylation of phosphorylase *b* or dephosphorylation of phosphorylase *a*. Phosphorylation occurs at the specific Ser 14 residue in rabbit muscle phosphorylase[44,45] resulting in the binding of the helical N-terminus to intra- and inter-subunit regions of the molecule, thus promoting the R state.[46] In contrast, compounds reflecting intracellular conditions exert their affects directly by acting as allosteric ligands that bind to discrete, well-defined sites in the muscle phosphorylase structure and either activate or inactivate the enzyme by inducing the R or T conformations. It is important to emphasize that these mechanisms are not mutually exclusive, since covalent modification is ultimately under the control of allosteric ligands. Thus, for example, the negative allosteric effector glucose binds to phosphorylase *a* and promotes the inactive T state, which is not only intrinsically less active, but is also a better substrate for phosphorylase phosphatase.[47,48]

The observation by Cori and Cori that AMP is required to demonstrate catalytic activity of muscle phosphorylase *b* represents the first example of enzyme regulation by a ligand that is not a substrate.[1,2] In subsequent years a number of other naturally occurring compounds have been shown to have allosteric effects on muscle phosphorylase activity, as summarized in Table 1. These compounds exert their activating and inhibitory effects by binding to discrete sites within the phosphorylase dimer that are at varying distances from the active site. All of the sites are described individually below.

Some allosteric ligands (glucose, glucose-1-P, and P_i) bind at the active site, each using a specific set of side chains. As mentioned above, glucose promotes the T state and is thus an allosteric inhibitor, while glucose-1-P and P_i are at once substrates and allosteric activators, since binding of these compounds to the active site promotes the R conformation. Glucose is a less prominent physiological inhibitor of the muscle enzyme than of the liver enzyme, since glucose rapidly equilibrates across the liver plasma membrane by an insulin-independent facilitated diffusion mechanism, in contrast to the muscle, where the process is insulin-dependent.[48,49] Thus, intracellular concentrations of glucose more closely approximate circulating glucose concentrations in liver than in muscle.

The "purine inhibitor" or "I" site is located close to the active site (approximately 10 Å away). It exists as a slot formed by parallel "stacking" of the hydrophobic side chains

Table 1
ALLOSTERIC EFFECTOR LIGANDS AND THEIR BINDING SITES ON RABBIT MUSCLE GLYCOGEN PHOSPHORYLASES *a* AND *b*

Ligand	Function	*a* or *b*	Binding locus	Conformer stabilized
Glucose	Inhibition	Both	Active site	T
Purine	Inhibition	Both	Near active site	T
glc-6-P	Inhibition	*b*	Nucleotide	T
ATP	Inhibition	*b*	Nucleotide	T
Glycogen	Activation	Both	Storage	R-like
glc-1-P	Activation	Both	Active	R
Pi	Activation	Both	Active	R
frc-1-P[a]	Activation	Both	Active	R
UDPG[a]	Activation	Both	Active	R
AMP	Activation	*b*	Nucleotide	R
N-term Ser 14-Pi	Activation	*b*	Intersubunit	R

Note: Some ligands are only effective on the dephosphorylated enzyme, phosphorylase *b*.

[a] These ligands are inhibitors that bind at the active site. The binding stabilizes the R conformer but blocks the active site to substrates.

of Phe 285 and Tyr 613.[50-53] A number of purine analogs or related heterocyclic ring compounds such as adenosine, caffeine, theophylline, FAD or FMN, and even the allosteric activator AMP (at superphysiological concentrations) bind to this site and inhibit enzyme activity.[50,51] Caffeine and glucose inhibit phosphorylase *a* in a synergistic manner, with each of these compounds promoting the binding of the other.[51-53] Interestingly, glucose or caffeine alone are very poor inhibitors of phosphorylase *a* in the presence of AMP, but significantly reduce activity when they are present together. It is currently not known whether a physiological ligand exists that interacts with the purine inhibitor site. An intriguing possibility is that the inhibitory effect of insulin on phosphorylase *a* activity in cells[54,55] may be mediated by an as yet unidentified compound interacting with the purine inhibitor site.[51]

Glycogen is another example of a phosphorylase substrate that is also an allosteric affector. Unlike glucose-1-P or P_i, however, glycogen does not exert its allosteric activating effect by binding at the active site. Activation is instead achieved by glycogen binding to a "glycogen storage site" comprised of a pair of helices located on the globular surface of the molecule some 30 Å distant from the active site.[56] Maltoheptaose, a seven-residue oligomer of α-D-glucose, also binds to this site; use of this analog allowed the measurement of increased binding of the substrates glucose-1-P and P_i at the active site without interference by the bulky glycogen glucosyl chain.[56,57] Remarkably, maltoheptaose, and presumably glycogen bind to the glycogen storage site at least 20-fold more tightly than to the active site. Thus, measurements of glycogen binding to purified phosphorylase should largely reflect binding at the glycogen storage site.[56]

The AMP allosteric site is in many ways the most complex. In addition to binding the allosteric activator AMP, portions of the site are also used to bind the allosteric inhibitors ATP and glucose-6-P. The site is located some 40 Å from the active site, making it the most distant of the known ligand effector sites. It is the only site that is formed by chain segments contributed by both monomers of the active dimer. The site can be thought of as three subsites, one each for the phosphoryl, sugar, and base moieties of AMP. Thus, glucose-6-P binds to only the sugar and phosphoryl subsites of the AMP site. Recent crystallographic analysis has revealed that whereas AMP interacts with all three subsites of the nucleotide binding site, only the triphosphate group and one hydroxyl of the ribose of ATP make

significant contact with the enzyme.[26] These data underscore the complexity of the nucleotide binding site by suggesting that it can function as an inhibitor or activation site, depending on which of its subsites are bound to a ligand. AMP is a potent activator of rabbit muscle phosphorylase *b* capable of activating the enzyme to 80% of its fully active state (phosphorylase *a*) at physiological concentrations of AMP.[1,2] Binding of this ligand to phosphorylase *a* results in a slight additional activation and also makes the enzyme refractory to glucose inhibition.[58,59] The conformational changes occurring upon binding of AMP to crystals of phosphorylase *a* prepared in the presence of the inhibitor glucose have been described recently at high resolution.[26] AMP binds to the muscle phosphorylase *b* dimer in a cooperative fashion. Binding of AMP to one subunit of the dimer both promotes the binding of AMP to the second subunit[60-64] and activates the dimer for substrate binding and catalysis.

The site of covalent phosphorylation, Ser 14, is 10 Å from the AMP locus. The activation by covalent phosphorylation is, in a sense, also a "ligand" binding event, since on phosphorylation the N-terminal 17 amino acids bind to the main body of the protein.[9,18,19,46] This binding occurs in a shallow crevice formed in part by Arg 69 from the same subunit as the phosphorylated serine and Arg 43 from the opposite subunit. The phosphate of Ser 14 interacts with the guanidinium groups of the two Arg residues, and there are also hydrophobic interactions between several residues of the N-terminus and the main body of the protein including the methylene portions of the two arginines.

B. *E. coli* Phosphorylases

E. coli contains both a true glycogen phosphorylase and a maltodextrin phosphorylase. These organisms also accumulate the specific substrates for the two enzymes, glycogen and maltodextrin.[65-67] Under certain conditions, notably nitrogen limitation, coliforms may store glycogen to 15 to 40% of the cell dry weight. Glycogen phosphorylase appears to be constitutively expressed in bacteria and is present at low specific activity, consistent with a role for the enzyme in the slow degradation of glycogen during extended periods of substrate deprivation.[68,69] Bacteria also synthesize short-chained unbranched α-1,4 polyglucoses (maltodextrins). Maltodextrin phosphorylase catalyzes the phosphorolysis of maltodextrins, but is poorly active against the more complex, branched glycogen molecule.[70] This enzyme is encoded by the malP gene of the maltose regulon and is inducible by maltose.[71-74] Chen and Segel[68,69] were able to separate glycogen and maltodextrin phosphorylases by ammonium sulfate precipitation or by DEAE-cellulose chromatography and evaluate their respective functional properties. Both enzymes contain pyridoxal phosphate as cofactor, but neither are regulated by covalent phosphorylation/dephosphorylation.[66,68,69,75] Both enzymes are slightly activated by AMP, but unlike muscle phosphorylase the effect is on V_{max} rather than on the K_m for the substrate glucose-1-P. Like liver and muscle phosphorylase, bacterial glycogen phosphorylase is activated by high concentrations of NaF and Na_2SO_4.

C. Potato Phosphorylases

Like *E. coli*, potato tubers contain two types of phosphorylase that can be distinguished on the basis of their preferred substrates and molecular sizes.[76-78] The type I enzyme, which is also found in sweet corn and in spinach leaf chloroplasts, has a high affinity for amylopectin, amylose, and maltodextrin, but an extremely low affinity for glycogen. Type I enzyme has a subunit mol wt of about 110,000. The type II enzyme is smaller (approximate mol wt of 90,000) and has an equally high affinity for glycogen as for amylopectin or amylose. This form is found as a minor component in potato tubers, but constitutes the major activity in spinach leaf nonchloroplast fractions. As is the case for muscle phosphorylase, both plant enzymes contain 1 mol of pyridoxal phosphate per monomer. Thorough kinetic studies have only been done for the type I enzyme. This phosphorylase exists only in an active form that is independent of covalent phosphorylation and is generally unaffected

by allosteric regulators, including AMP. An exception to this generalization is the strong competitive inhibition of type I potato phosphorylase activity by 6 to 8 residue cyclodextrins.[79]

D. Yeast Glycogen Phosphorylase

Yeast phosphorylase represents a functional intermediate between the essentially unregulated prokaryotic and plant phosphorylases and the highly regulated mammalian phosphorylases. The enzyme contains 1 mol of pyridoxal phosphate per subunit and is most active as a dimer.[80-82] Unlike bacterial or plant phosphorylases, yeast phosphorylase is regulated by reversible covalent phosphorylation.[80-82] The enzyme differs from mammalian phosphorylases in that phosphorylation occurs at a specific Thr 19 instead of at Ser 14.[23,81,82] Phosphorylation of yeast phosphorylase is catalyzed by a specific phosphorylase kinase and perhaps additionally by a yeast cAMP-dependent protein kinase, an enzyme with broad specificity; yeast phosphorylase is not a substrate for muscle phosphorylase kinase.[82] Covalent regulation of yeast phosphorylase is similar to the mammalian system in that dephosphorylation is catalyzed by a protein phosphatase with broad specificity, although the exact range of substrates for the yeast and mammalian enzymes is not known. Yeast phosphorylase *b* (unphosphorylated) is potently inhibited by the allosteric effector glucose-6-P with a K_i of 1 m*M*. The phosphorylated enzyme is somewhat less sensitive to this inhibitor, with a K_i of 11 m*M*. Glucose-6-P inhibition is noncompetitive with respect to glucose-1-P binding, indicating that glucose-6-P exerts its effect by binding to a site other than the active site.[83] Fosset et al. found no effect of AMP on phosphorylase activity from 0.05 to 5 m*M*.[80] Tanabe et al., however, have shown that 10-m*M* AMP competes with the substrate, glucose-1-P, to produce about 55% inhibition.[84] No clear evidence exists for any regulatory effects of other possible ligands, such as glucose, glycogen, or purines.[83,156]

In addition to allosteric controls, yeast phosphorylase is subject to transcriptional control. Immunological evidence has indicated that enzyme production occurs at the late exponential growth phase;[85] intracellular levels of the enzyme appear highest in yeast cultures grown to stationary. Recent hybridization analysis of mRNA isolated from yeast at various stages of growth confirms that gene transcription is induced during the late exponential stage of growth.[86] It has also been shown that intracellular glycogen, scarce in exponentially growing yeast, accumulates dramatically in the approach to stationary phase.[87,88] Taken together, these observations are supportive of a primary physiological role for yeast phosphorylase of utilizing a reserve energy source during periods of substrate depletion or starvation. The regulated expression of the yeast phosphorylase gene product raises questions about the physiological role of allosteric regulation of this enzyme by reversible phosphorylation and glucose-6-P inhibition.

Yeast phosphorylase activity may also be regulated through partial proteolytic cleavage. Becker et al. have observed two slightly different sizes of the enzyme subunit.[81] The specific yeast phosphorylase kinase activates the larger subunit but not the smaller subunit; however, at least some proportion of the smaller subunits are activated by the cAMP-dependent protein kinase.[89] From comparison of partial peptide sequences for these subunits to the determined primary structure of yeast phosphorylase, it is clear that the smaller subunits are derived from the larger, intact subunit by removal of the N-terminal residues. Hwang and Fletterick have noted that the sequence at the phosphorylation site in yeast phosphorylase matches a consensus for substrates of the cAMP-dependent kinase.[23] Thus, an interesting functional role of the N-terminus may be to impede recognition and binding by phosphorylase kinase. That the smaller subunit may be an artifact of purification cannot yet be ruled out; whether N-terminal proteolysis has physiological significance in regulating phosphorylase remains to be established.

While further study is required to define the role of allosteric regulation in yeast phos-

Table 2
THE PHYSIOLOGICAL ROLES AND REGULATORY PROPERTIES OF THE ISOZYMES OF GLYCOGEN PHOSPHORYLASE

Isozyme	Physiological role	Allosteric control
Muscle	Rapid mobilization of metabolizable carbohydrate provides the energy for muscular function	Covalent phosphorylation Ser; cooperative AMP activation; glucose inhibition; purine inhibition; glycogen activation; Glc-6-P inhibition
Liver	Provides free glucose for extrahepatic tissues, regulates blood glucose	Covalent phosphorylation Ser; poor AMP activation (noncooperative); weak Glc-6-P inhibition; glucose inhibition; specific purine inhibition
Brain	Provides glucose for short anoxic or hypoglycemic episodes	Same as muscle, except potent AMP activation (noncooperative); weak Glc-6-P inhibition

Note: The isozymes are named after the tissues in which they are preferentially expressed in adult mammals. Tissues other than muscle, liver, and brain generally contain a mixture of phosphorylase isozymes that varies in proportions according to the species.

phorylase, current information provokes some reasonable speculations. We have already mentioned that glycogen and phosphorylase appear at the late exponential stage of growth. It seems likely that as cells approach stationary phase, phosphorylase predominates in the unphosphorylated *b* form. Although relatively inactive when compared to the *a* enzyme, this form has sufficient enzymatic activity to meet the energy requirements of dormant yeast. Stationary yeast remain viable for months at room temperature, with a gradual diminution of glycogen content.[87] Degradation of glycogen by phosphorylase *b* would be effectively modulated by intracellular levels of glucose-6-P, a more effective inhibitor of phosphorylase *b* than of phosphorylase *a*. In this scenario, activation of phosphorylase by covalent phosphorylation, involving activation of the accessory kinase, would only need to be invoked when stationary yeast are switched into rich growth conditions. Under these conditions, glycogen levels are known to be rapidly reduced, with concommittant increases in cAMP levels and cAMP-dependent kinase activity in yeast.[90-93]

E. Other Mammalian Phosphorylase Isozymes (Liver and Brain)

1. Tissue Distribution of Phosphorylase Isozymes and Isozyme-Specific mRNAs

At least three isozymes of glycogen phosphorylase are found in mammalian tissues that are named after the tissues in which they are preferentially expressed: liver, muscle, and brain.[94-102] Protein gel electrophoresis has demonstrated that glycogen phosphorylase isozyme expression is regulated in a developmental and tissue-specific manner.[97-109] Although all of these isozymes are interconverted between phosphorylase *b* and phosphorylase *a* forms by covalent phosphorylation/dephosphorylation, they are differentially regulated by allosteric effectors in a fashion that reflects their differing physiological roles as summarized in Table 2. In fetal rat tissues, the predominant isozyme comigrates with the brain type.[101,102,106] At birth, the fetal isozyme is replaced by species comigrating with the liver and muscle forms in liver and muscle tissues, respectively.[101-103,107,108] Other adult tissues express a mixture of all three types of phosphorylase in proportions that vary depending on the species.[97,99,101,102] The predominant isozyme expressed in tissues other than brain, liver, and skeletal muscle in the rat comigrates with the brain type;[101,102] in humans, a form identical with the liver type is most prevalent in other tissues.[99] Separation of the isozymes on the basis of size is complicated by the fact that liver and muscle phosphorylases have nearly identical molecular weights. Better separation and identification can be achieved on native gels not containing SDS; tissue-specific phosphorylases have different net charge, and enzyme activity can be assayed in such gels under conditions that favor the activation of a particular isozyme.[99,102,106]

A number of other molecular species with mobilities distinct from the three major forms are variously described as hybrid dimers consisting of mixtures of subunit types,[97] oligomeric species,[100] degradation products,[102] or possibly other forms of mammalian phosphorylase that have not been definitively characterized.[104,109]

There is evidence to suggest that phosphorylase levels are regulated by physiological conditions in both liver and muscle. Thus, total muscle phosphorylase activity decreases in athletes or rats subjected to a long training period and then increases to above-normal values if these individuals are "detrained".[110,111] Phosphorylase activity in liver has been described as decreased in rats with chemically induced[112-114] or spontaneous diabetes,[115] but has been shown to increase in genetically diabetic (*db/db*) mice.[116] Only the latter study included immunological measurements of phosphorylase protein, which was shown to increase in parallel with enzyme activity. In all experiments, the changes were quantitatively modest, in the range of 40 to 60% of the normal values, in contrast to the many-fold induction that occurs for other metabolic enzymes, such as glucokinase or fatty acid synthase in response to changing dietary conditions.[117,118] Bahnak and Gold[113] used a double labeling technique[119] to measure turnover of glycogen phosphorylase in rat liver and demonstrated both increased degradation and, to a lesser extent, increased synthesis of the protein in chemically induced diabetes. The net decrease in phosphorylase activity could be largely overcome by administration of insulin, implying a regulatory role for this hormone in phosphorylase biosynthesis. In contrast, Miller et al. showed no effect of insulin on total phosphorylase activity in hepatocytes isolated from either diabetic or normal rats.[114] In sum, although there are undoubtably alterations in total phosphorylase activity and enzyme protein associated with long-term physiological perturbations, rapid changes in phosphorylase activity that occur in response to acute fluctuations in circulating hormone or substrate levels are entirely mediated by covalent phosphorylation/dephosphorylation and allosteric regulation acting in concert.[48]

In the past 3 years, the cDNAs encoding the entire coding sequence of rabbit muscle phosphorylase,[11,12] human liver phosphorylase,[22] human brain phosphorylase,[25] and portions of rat and human muscle,[11,24] and rat liver phosphorylases[24] have been cloned and sequenced. These achievements have allowed experiments assessing phosphorylase mRNA expression profiles to be carried out. The human liver cDNA hybridizes preferentially to a mRNA in human adult and fetal liver and in adult rabbit liver that is approximately 3.2 kB in size. The rabbit or human muscle phosphorylase cDNAs hybridize strongly to a slightly larger mRNA in human or rabbit muscle of 3.4 kB that is weakly recognized by the liver or brain phosphorylase cDNAs. Finally, the recently cloned brain phosphorylase cDNA hybridizes strongly to a 4.2-kB message in the temporal and frontal lobes of human brain and to a similarly sized but less abundant message in human muscle, liver, and fetal liver samples;[25] this large message is also seen in rabbit brain mRNA when probing with the liver or muscle cDNA probes at low stringency.[22] Thus, we have learned that human liver, muscle, and brain tissues generally contain more than one type of phosphorylase mRNA,[25] similar findings have also been described in the rat.[102] In particular, the temporal lobe of human brain expresses significant amounts of liver phosphorylase mRNA, in contrast to the rabbit, where no liver phosphorylase mRNA is detectable in whole brain extracts.[22,25] In addition, human liver and muscle contain low levels of brain phosphorylase mRNA. The reasons for having more than one phosphorylase mRNA expressed in a given tissue are unclear, especially since the Northern blot data do not correlate well with earlier protein electrophoresis studies that showed no liver phosphorylase activity in human brain extracts and no brain phosphorylase activity in liver or muscle tissues.[99,102]

2. *Gene Mapping Studies*

The presence of multiple forms of phosphorylase mRNA in a given tissue does not reflect alternative splicing from a single phosphorylase gene. The human transcripts are clearly

derived from separate genes, as demonstrated by the fact that the three phosphorylase cDNAs map to different chromosomes. The muscle and liver phosphorylase genes were mapped to chromosomes 11 and 14, respectively, by hybridizing the DNA probes to laser-sorted human chromosomal fractions spotted onto nitrocellulose.[120,121] Similar analysis with the human brain cDNA probe resulted in hybridization to the spot containing chromosome 20 and also to the spot containing a mixture of chromosomes 9, 10, 11, and 12.[25] Newgard et al.[25] carried out further mapping studies with the brain phosphorylase cDNA using genomic DNA purified from hamster/human hybrid cell lines. The lines were chosen so as to be able to unequivocably distinguish between chromosomes 9, 10, 11, and 12. A line containing chromosome 20 was also included. To further clarify the issue, the hybridization was performed using a cDNA fragment encompassing the 3′ untranslated region of the brain-specific cDNA, since this region has no homolog in the muscle- or liver-specific phosphorylase cDNAs.[25] The results confirmed the strong hybridiztion to a gene found on chromosome 20, but also revealed the presence of a somewhat less homologous fragment on chromosome 10. In sum, these studies indicate that the human phosphorylase genes are unlinked, and are in fact dispersed throughout the genome. They also show that two nearly identical copies of the brain phosphorylase gene exist that map to separate chromosomes. We have begun studies aimed at determining which of these genes is transcribed to yield the large brain-specific transcript.

3. Liver Phosphorylase Physiology and Biochemistry

The physiological role of liver phosphorylase is to ensure a constant supply of glucose for extrahepatic tissues, especially the central nervous system, which relies on glucose as its major source of fuel.[48,122] This function is in contrast to the muscle phosphorylase isozyme, which functions primarily to supply energy for the sole use of the tissue in which it is found. The funneling of glucose carbon into metabolic pathways in muscle is ensured by the lack of glucose-6-phosphatase, an enzyme that converts glucose-6-P (derived from glucose-1-P, the product of the phosphorylase reaction *in vivo*) to free glucose. Glucose-6-phosphatase is present in liver at high activity, allowing rapid release of free glucose into the bloodstream in response to glycogenolytic stimuli.[123] The contrasting physiological roles of the liver and muscle isozymes of phosphorylase are reflected in the differential response of these enzymes to certain allosteric effectors. As stated earlier, muscle phosphorylase activity is controlled by an integration of the whole complement of regulatory sites, since this enzyme must be able to respond to external nervous and hormonal signals, as well as to changes in the concentration of intracellular ligands that are indicative of energy balance. The liver enzyme, in contrast, is essentially inactive in the absence of the covalent activation mechanism, which is under the control of extracellular factors. This statement follows from the observation that animals deficient in liver phosphorylase kinase are unable to break down liver glycogen, resulting in glycogen storage disorders,[124,125] while animals with muscle phosphorylase kinase deficiency are essentially asymptomatic.[126,127]

In vitro experiments have shown that the liver enzyme is poorly activated by AMP. Whereas muscle phosphorylase *b* can be activated to 80% of its full activity by this effector, AMP only activates liver phosphorylase *b* to 10 to 20% of liver phosphorylase *a* activity.[128-135] In addition, AMP binding to liver phosphorylase is noncooperative. AMP appears to be a poor activator of mammalian liver phosphorylase regardless of species; it has been suggested, however, that the effect of AMP on the human enzyme is largely on V_{max} rather than on K_m for glucose-1-P, as in other species.[132] Activation by AMP can be potentiated to 50 to 60% of the *a* form by the addition of the salts Na_2SO_4 or NaF.[61,131] Elegant studies by Graves and co-workers with an AMP analog, N^6-(6-aminohexyl) AMP, which is specific for the activator site and does not bind to the purine inhibitor site (as AMP does) revealed that the poor activation is not due to a preferential binding of AMP to the inhibitor site in

liver phosphorylase.[135] These investigators also demonstrated that the primary defect in AMP activation in the liver enzyme is in the allosteric linkage between the activator site and the active site; binding of AMP to liver phosphorylase lowers the K_m of the enzyme for its substrate glucose-1-P, but the effect is tenfold less than observed for the muscle enzyme. Finally, the liver enzyme binds AMP and its analogs with somewhat lower affinity (two- to sevenfold) than does the muscle enzyme, implying that the conformation of the activator site may be altered in liver phosphorylase. Glucose-6-P, which is known to bind at the AMP activation site in muscle phosphorylase, binds to muscle phosphorylase with approximately eightfold greater affinity than it does to the liver enzyme, providing further support for the latter scenario.[135] The K_a of the liver and muscle enzymes for AMP, or the concentration required to achieve half-maximal AMP activation, is nearly the same and within the physiological range (60 to 75 μM);[64,135] the maximal AMP-inducible activity of the liver enzyme, however, is significantly less than that for the muscle enzyme.

The other known allosteric effectors of muscle phosphorylase generally have similar effects on liver phosphorylase activity. Glycogen binds to liver phosphorylase with nearly identical affinity as it does to the muscle enzyme and decreases the K_m for P_i from 11 to 0.8 mM and of glucose-1-phosphate from 1.3 to 0.28 mM.[136] Since binding to the muscle enzyme primarily reflects binding to the glycogen storage (activation) site, it seems reasonable to assume that glycogen binds to this site in the liver enzyme as well. Glucose is a potent allosteric inhibitor of both the muscle and liver forms of phosphorylase *in vitro*, but has more pronounced effects on the liver enzyme *in vivo* due to rapid equilibration of this hexose across the liver plasma membrane. In studies on phosphorylase *a*, Kasvinsky and co-workers demonstrated that the rabbit liver enzyme does have a functional purine inhibitor site that binds caffeine synergistically with glucose to completely inhibit phosphorylase *a* activity.[51,53] The apparent K_is for caffeine, theophylline, and allopurinol, however, all fused heterocyclic ring compounds that bind to the purine inhibitor site in muscle phosphorylase, are two- to fourfold higher in the liver enzyme compared to the muscle enzyme. In addition, the liver enzyme is not inhibited by other compounds that affect muscle phosphorylase activity, such as FMN and folic acid. These authors also demonstrated that AMP could partially overcome the inhibitory effect of glucose + caffeine in both enzymes. A possible explanation for the AMP effect on the liver enzyme is that AMP competes with caffeine for binding at the purine inhibitor site, since caffeine and AMP were added to the assay at nearly equal concentrations (1 and 0.4 mM, respectively). The muscle enzyme is both more potently activated by AMP binding to the AMP activator site and more sensitive to AMP binding to the purine inhibitor site. Thus, in contrast to the liver enzyme, removal of glucose + caffeine inhibition in the muscle enzyme by AMP (0.3 mM) is more likely due to the potent effect of AMP on promoting the R state in muscle phosphorylase.

Another possibility is that a lesser conformational change is required for AMP to affect glucose binding than is needed for this ligand to influence binding of the substrate glucose-1-P. In this scenario, AMP can exert enough of a conformational effect to diminish the role of inhibitors in both enzymes, but can only significantly activate muscle phosphorylase *b*. Such a model, if correct, appears to have only limited physiological relevance, since the modest increments in glucose induced by eating a normal meal are sufficient to cause rapid glucose-mediated inactivation of liver phosphorylase.[48] Also, under these normal physiological conditions, there is little variation in hepatic levels of AMP.[137]

4. Brain Phosphorylase Physiology and Biochemistry

Although kinetic studies have been carried out on phosphorylase from mammalian brain, they have been less extensive than those for the liver and muscle isozymes. The physiological role of this enzyme is also poorly understood, especially given that the brain stores far less glycogen than either liver or muscle, but it is generally thought to supply an emergency

glucose supply during stressful periods.[138-143] Brain glycogen levels have been shown to decrease in response to ischemia, anoxia, or convulsions with concomittant increases in phosphorylase *a* activity, followed by increases in glycogen content during the recovery period. Repeated convulsive seizures induced by pharmacological agents ultimately result in self-generating seizures, suggesting that the brain has a limited capacity to adjust to increased energy demands.[141]

As in the case for liver and muscle phosphorylase, the active form of brain phosphorylase is a dimer. Like muscle phosphorylase, but different from the liver isozyme, the enzyme is potently activated by AMP. Buc and co-workers have suggested that the affinity of the brain type enzyme for this ligand is greater than seen for the muscle enzyme, based on the fact that the brain enzyme exhibits a sevenfold lower K_a (concentration of AMP required to produce half the activity in the presence of saturating AMP).[64] In addition, the brain and liver enzymes share the property of noncooperative AMP binding (Hill coefficient = 1.0), whereas the muscle enzyme binds AMP with a high degree of cooperativity (Hill coefficient = 1.9). The authors speculate that the brain enzyme is "set" in a conformation that has a high affinity for AMP, analagous to the conformation assumed by muscle phosphorylase when 1 mol of AMP is bound per enzyme dimer (i.e., 1 of 2 AMP binding sites occupied). The brain isozyme also differs from the liver and muscle types in that the phosphorylated *a* form of the enzyme is dependent on AMP for activity when glycogen concentrations are low.[62,102] Interestingly, a hybrid enzyme consisting of muscle and brain subunits that occurs naturally in heart tissue has AMP binding and activation properties intermediate between the the muscle and brain dimers.[97]

Buc and co-workers also demonstrated that glucose-6-P had no effect on the conversion of brain phosphorylase *b* to *a*. The conversion of muscle phosphorylase, in contrast, was inhibited 80% by glucose-6-P,[64] a finding consistent with the notion that the brain enzyme is in a conformation that favors binding of activators over inhibitors at the AMP activation site. To our knowledge, no direct inhibitory effect of glucose-6-P has been demonstrated on brain phosphorylase. It is also not known whether brain phosphorylase is inhibited by purines or activated by glycogen.

Studies performed on tranformed astrocytes in culture indicate a potent inhibitory effect of glucose on brain phosphorylase *a* activity,[142,143] even in the presence of β-adrenergic agents such as norephinephrine that dramatically increase intracellular cAMP levels and cause covalent activation of phosphorylase. The applicability of these data to the brain isozyme of phosphorylase per se have been called into question by the recent finding that transformed human astrocytes contain significant amounts of a phosphorylase mRNA that hybridizes specifically to the liver phosphorylase cDNA.[25]

III. THE NEW STRUCTURAL DATA

In 1977, Titani and co-workers reported the amino acid sequence of the entire 841-residue rabbit muscle phosphorylase, the first phosphorylase for which the complete primary sequence was deduced. In the 10 years since the report of this monumental achievement, and particularly in the past 5 years, the number of known phosphorylase sequences has increased dramatically. This has occurred largely as a result of the advent of recombinant DNA technology, which allows rapid and accurate sequence information to be obtained from cDNA and gene sequences. Only the sequence of potato phosphorylase among the new sequences was obtained by direct protein sequencing.[144,145] Cloning of a partial cDNA for rabbit muscle phosphorylase[11] led to the cloning and sequencing of cDNAs containing the entire coding region of rabbit muscle,[12] human liver,[22] and human brain phosphorylase,[25] as well as to partial cDNA clones encoding the rat muscle[11,24] and liver[24] and human muscle proteins.[11] The rabbit muscle cDNA was also used to clone the entire structural gene for

human muscle phosphorylase.[13] Sequence analysis of the rabbit muscle phosphorylase cDNA coupled with the resolution of the X-ray crystal structure of this enzyme at 2.1 Å[9,12] revealed a few mistakes in the original protein sequence,[10] most notably the omission of an amino acid at position 314. Thus, rabbit muscle phosphorylase actually contains 842 amino acids; the residue numbers cited in this review reflect this change and are thus different from previous papers. The gene sequence for *E. coli* maltodextrin phosphorylase was deduced from gene fragments comprising the malP locus of the maltose operon, itself cloned by complementation of genetic mutants.[21,73] The sequence of the *E. coli* glycogen phosphorylase gene has also been determined.[157] Finally, the gene-encoding yeast phosphorylase was cloned[23] by preparing a mixed oligonucleotide probe comprised of all the possible codons corresponding to the previously sequenced peptide fragment surrounding and containing the pyridoxal phosphate binding site of yeast phosphorylase.[145]

An interesting finding that emerged from the sequence analysis of rabbit muscle and human muscle, liver, and brain isozymes is that despite a high level of amino acid identity, the liver phosphorylase gene utilizes a distinct codon usage pattern compared with those encoding the brain and muscle enzymes.[22,25] Specifically, the muscle and brain phosphorylase DNA sequences contain a higher number of deoxycytosine (C) and deoxyguanosine (G) residues compared to the liver sequence, and in particular contain a much higher percentage of G + C at the third codon position. Analysis of other liver and muscle coding sequences revealed that the tissue-specific codon usage pattern first observed for phosphorylases reflects a general bias in these two tissues (brain sequences were not analyzed for codon usage preference, due to an insufficient number of available sequences).

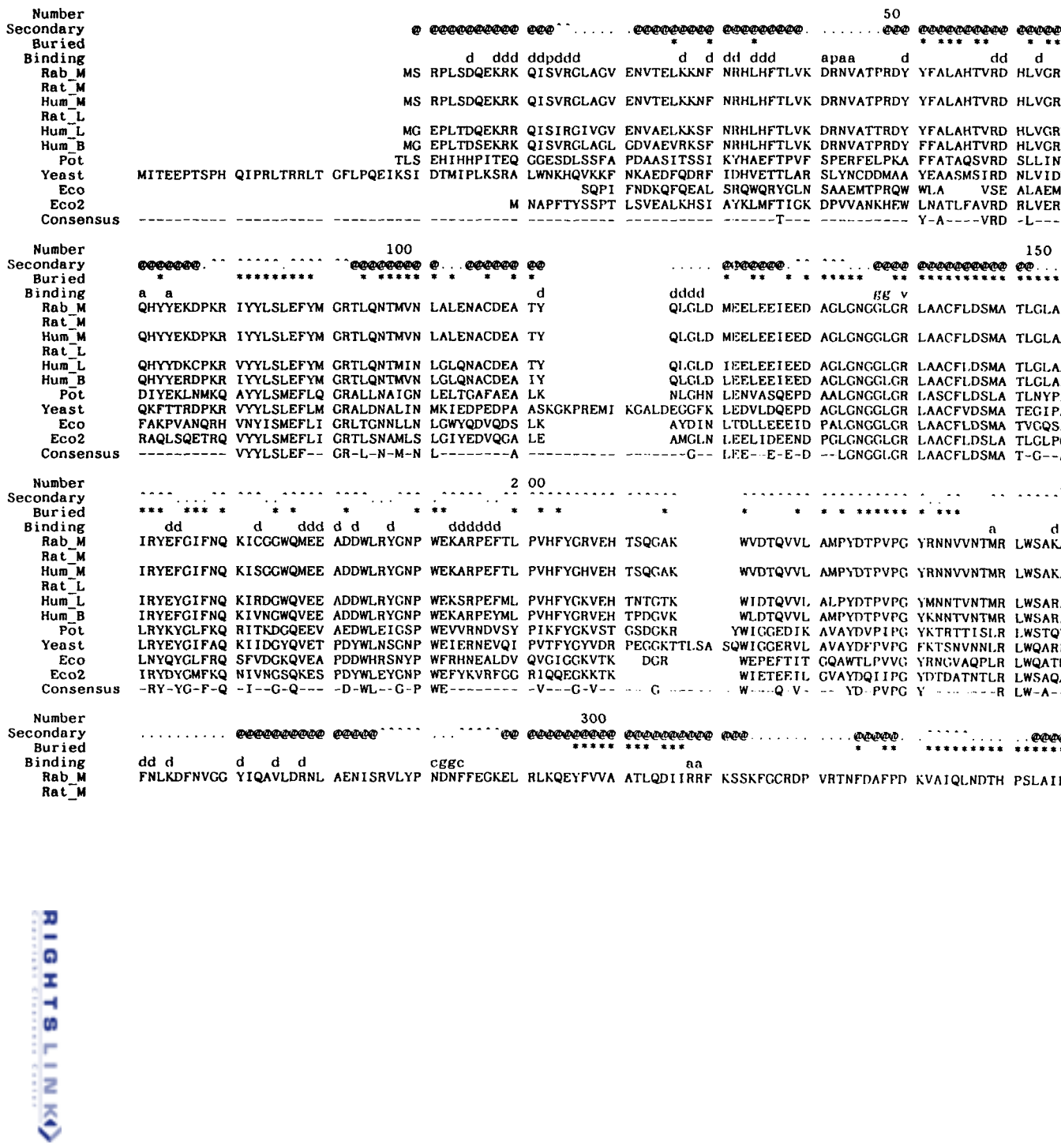
The amino acid sequences of all the above-mentioned phosphorylase isozymes are presented in Figure 1. The sequences have been aligned to best fit the muscle sequence, requiring several insertions and deletions in the potato, yeast, and *E. coli* enzymes. Based on this alignment, the percent identities of the amino acid sequences have been determined and are presented in Table 3. These data demonstrate a remarkable conservation of primary sequence in phosphorylase isozymes, even when comparing lower life forms to mammals, and an especially high conservation among the mammalian isozymes. Below, we summarize recent efforts at correlating the new structural information with the different functional characteristics exhibited by the various organism- and tissue-specific forms of glycogen phosphorylase.

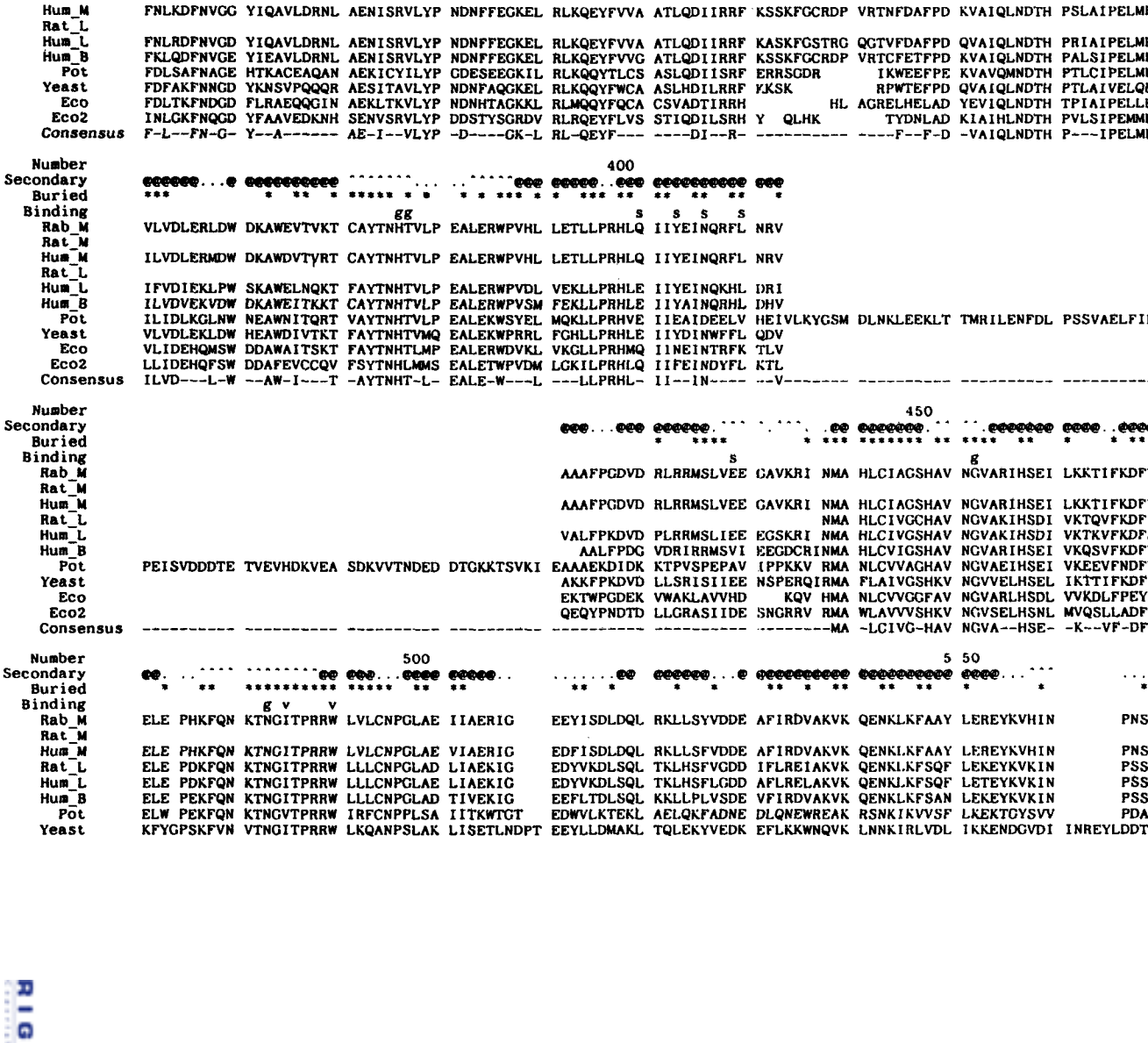
IV. STRUCTURE/FUNCTION RELATIONSHIPS IN THE FAMILY OF GLYCOGEN PHOSPHORYLASES

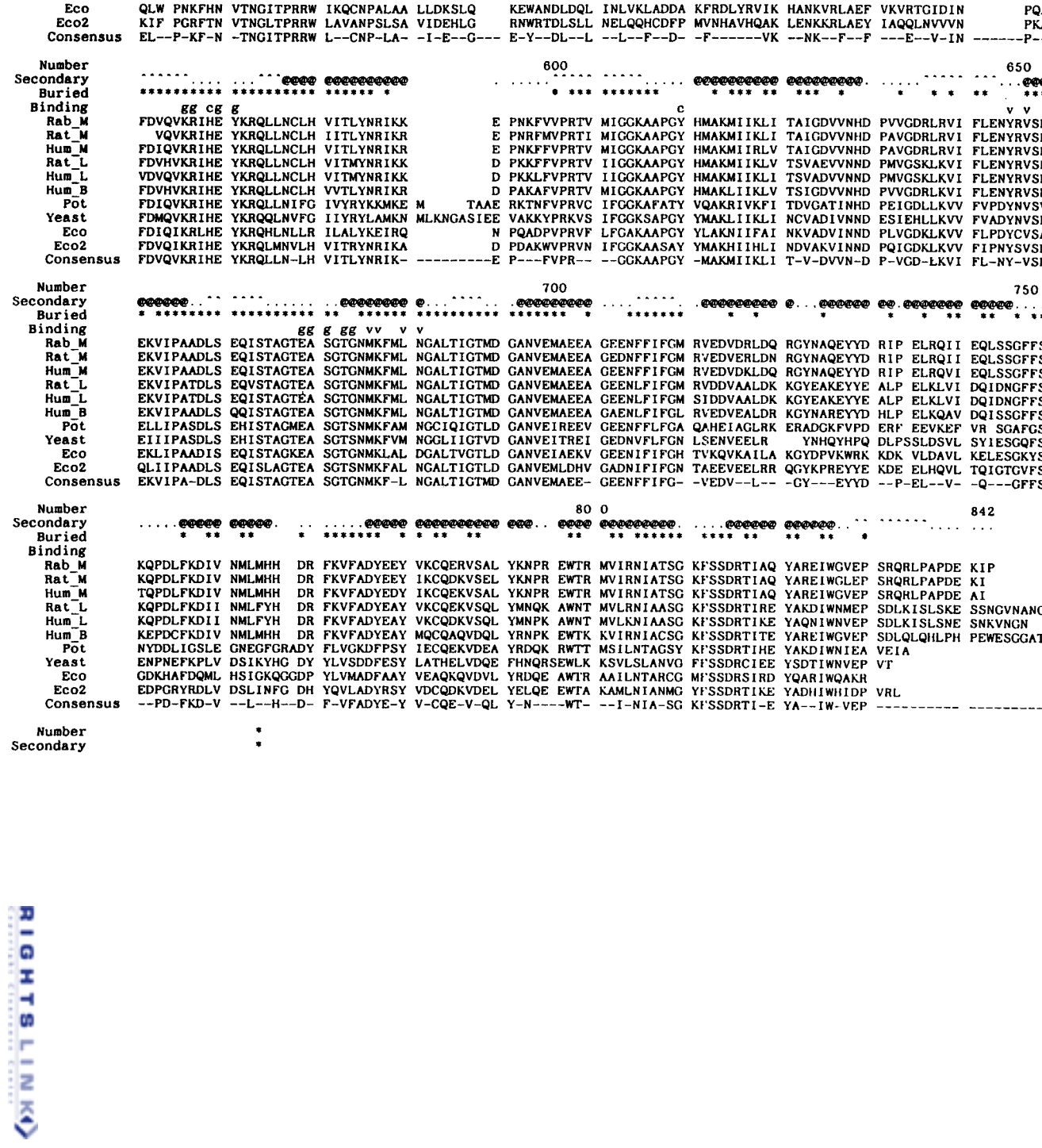
Despite the recent advances in primary sequence information, rabbit muscle phosphorylase remains the only phosphorylase isozyme for which the three-dimensional structure is known. The structural basis for differences in function among isozymes must therefore be interpreted by modeling amino acid substitutions that occur in the various sequences onto the rabbit muscle structure. Obviously, such an approach is most valid for the more homologous mammalian sequences, but valuable information can be derived from this type of analysis on more primitive phosphorylases as well. In the following section we interpret the structural bases for differences in function among phosphorylase isozymes.

A. *E. coli* Phosphorylases

The sequences of both the bacterial maltodextrin phosphorylase and the true glycogen phosphorylase have been determined. The former protein is 796 amino acids in length, as compared to 842 amino acids for the rabbit muscle enzyme.[21] The latter contains 809 amino acids.[157] The *E. coli* maltodextrin enzyme lacks the 17 N-terminal amino acids of muscle phosphorylase that contains the covalent phosphorylation site, consistent with the lack of a covalent regulatory mechanism for the bacterial enzyme. Palm and co-workers introduced







```
Buried                                *
Binding                               *
Rab_M                                 *
Rat_M                                 *
Hum_M                                 *
Rat_L                                 *
Hum_L                                 *
Hum_B       WAPPELCTHL AMY*
Pot                                   *
Yeast                                 *
Eco                                   *
Eco2                                  *
Consensus   ---------- ----
```

FIGURE 1. Alignment of the amino acid sequences of glycogen phosphorylases. The consensus amino sequence is given when six or more sequences have the identical amino acid at a particular position. The row labeled "Number" gives the amino acid number of rabbit muscle phosphorylase beginning with the Ser residue that immediately follows the initiator methionine. The secondary structure of rabbit muscle phosphorylase is indicated by symbols over the sequence in the row marked "Secondary" as follows: @, α-helix; ., turn; ^, β-strand. If an amino acid is not accessible to water in the three-dimensional structure of rabbit muscle phosphorylase, it is considered buried and is marked with an *. Amino acid side chains that participate in a subunit contact or ligand binding are labeled as follows; d, dimer contact; a, nucleotide activator site; p, Ser 14 phosphorylation site; g, active site resides; s, glycogen storage site; c, purine inhibitor site; v, pyridoxal phosphate binding residues. Amino acids are marked only if they fall within 4.0 Å of the ligand as determined by X-ray crystallography. Phosphorylase sequences from the following sources are included: Rab_M, rabbit muscle; Rat_M, rat muscle; Hum_M, human muscle; Rat_L, rat liver; Hum_L, human liver; Hum_B, human brain; Pot, potato; Yeast; Eco, *E. coli* maltodextrin phosphorylase; Eco2, *E. coli* glycogen phosphorylase.

Table 3
PERCENT IDENTITIES OF THE AMINO ACID SEQUENCES OF PHOSPHORYLASES FROM VARIOUS SOURCES[a]

	RMus	HMus	HLiv	HBr	Pot	Yeast	EC 1	EC 2
RMus	—	97	80	82	44	49	45	47
HMus	97	—	80	83	44	47	43	48
HLiv	80	80	—	80	46	50	43	49
HBr	82	83	80	—	47	50	43	49
Pot	44	44	46	47	—	46	40	42
Yeast	49	47	50	50	46	—	42	46
EC1	45	43	43	43	40	42	—	45
EC2	47	48	49	49	42	46	45	—

[a] Sources: RMus, rabbit muscle; HMus, human muscle; HLiv, human liver; HBr, human brain; Pot, potato; yeast; EC1, *E. coli* maltodextrin phosphorylase; EC2, *E. coli* glycogen phosphorylase. Note that only the full-length sequences, aligned as shown in Figure 2, were used in compiling this table. Comparisons were made over the aligned regions only; the number of mismatches between two sequences was determined (insertions were not counted), subtracted from the number of amino acids in the shorter of the two sequences being compared, and then divided by that number to yield the percent identity.

four small gaps, totaling 17 amino acid residues, in order to induce the best-fit alignment of the *E. coli* and rabbit muscle phosphorylase sequences.[21] With this alignment the C-terminal residue of the bacterial maltodextrin phosphorylase is found to terminate at position 830. The portion of the bacterial sequence that aligns with the C-terminal or catalytic domain of rabbit muscle phosphorylase (residues 482 to 842) is much more conserved than the N-terminal or regulatory domain (54 vs. 38% identity, respectively). Residues comprising the catalytic site and cofactor binding sites are exactly conserved, except for Glu 645, which is changed to a proline in the bacterial enzyme.

The substrate preference of *E. coli* maltodextrin phosphorylase for unbranched maltodextrins over larger and highly branched glycogen molecules may be due in part to the poor conservation of the glycogen storage site. Only three of the eight residues thought to directly interact with glycogen and only 42% of the residues that comprise the entire helical glycogen binding domain (residues 380 to 440) on the external surface of the muscle phosphorylase monomer[27,56,158] are conserved in the bacterial enzyme. It has been suggested that the glycogen storage site of muscle phosphorylase might function to position the terminus of the highly branched glycogen molecule in the active site crevice, thus facilitating phosphorolysis.[56,145] In this context, the absence of correct positioning due to a nonfunctional glycogen storage site in the bacterial enzyme might explain the low glycogen phosphorylase activity. An alternative explanation is that maltodextrin has greater access to the active site in bacterial maltodextrin phosphorylase due to cumulative conformational effects of amino acid replacements throughout the molecule, resulting in a smaller opening to the active site crevice.

Recent crystallographic analysis by Sprang and co-workers has defined the specific amino acid residues interacting with the ribose, purine, and phosphate moieties of AMP and ATP in rabbit muscle phosphorylase,[26] making it possible to interpret the poor activation of *E. coli* maltodextrin phosphorylase by AMP in a structural context. None of the residues that interact with either the ribose or purine portions of AMP are conserved in the bacterial enzyme; in fact, the N-terminal 120 amino acids are in general quite dissimilar. In contrast, Arg 309 and Arg 310, which form charge-charge interactions with the phosphate group of AMP, and Arg 242, which interacts with phosphate in ATP, are conserved. This suggests that the slight stimulatory effect of AMP on maltodextrin phosphorylase might arise from weak binding to a primitive AMP binding site, consisting of a well-formed phosphate binding site and an undeveloped ribose and purine binding region. Interestingly, the *E. coli* enzyme

contains Tyr 613, but not Phe 285, which forms the mammalian purine inhibitor binding site; the lack of a functional inhibitor site might be a factor that allows a relatively weak, nonspecific activation by AMP in the bacterial enzyme.

The true glycogen phosphorylase of *E. coli* starts at residue 12 of the rabbit muscle numbering system and also has no phosphorylation site. The *E. coli* enzyme is shorter in the N and C-termini and has deletions at positions 214 and 317. There is a single residue insertion at position 768. The deletions and insertions are all at surface positions and presumably do not represent functional changes. Remarkably, *E. coli* glycogen phosphorylase shares greater sequence identity with mammlian phosphorylases (47 to 49%) than it does with *E. coli* maltodextrin phosphorylase (45%)(see Table 3).

Significant amino acid sequence identity between the rabbit muscle and bacterial glycogen phosphorylases starts at amino acid 25, but only two of the six residues implicated in AMP binding are conserved in the N-terminal 80 amino acids. Two of the three phosphoryl-binding Arg residues are conserved. Thus, like the maltodextrin bacterial enzyme, any activation of the *E. coli* glycogen phosphorylase by AMP must occur by a mechanism distinct from that seen for the muscle enzyme. In contrast, all of the side chains involved in binding glycogen are conserved, with the exception of Tyr 404, which is a Phe in the bacterial glycogen phosphorylase. This finding likely explains the preference of this enzyme for glycogen as substrate. The purine-binding residue Phe 285 is a Thr, suggesting that this inhibitor site does not function. Interestingly, Tyr 613 (the other purine-binding residue) is conserved in all mammalian enzymes, the yeast, and both *E. coli* enzymes. As this side chain stands strategically at the gate of the active site, a role in binding glycogen is likely.

B. Potato Phosphorylase

The primary sequence of type I potato tuber phosphorylase contains 916 amino acids.[144,145] Nakano and Fukui showed that the protein is two residues longer at the NH_2 terminus and, similar to the bacterial enzymes, nine residues shorter at the COOH terminus than the muscle enzyme when the two proteins are aligned for maximum identity. Such an alignment must accomodate a 78-residue insertion in the potato sequence that occurs between residues 414 and 415 in the rabbit muscle sequence, as well as a few small deletions or insertions at other positions. The N-terminus of potato phosphorylase is not lost, as it is in the bacterial enzyme, but is entirely unrelated to that of the muscle enzyme over the first 80 amino acids. This includes the lack of a site in the potato enzyme resembling the covalent phosphorylation site of mammalian phosphorylase, in keeping with the inability of the former enzyme to be regulated by this mechanism. Furthermore, the AMP binding site is essentially nonexistent in the potato enzyme, although interestingly the phosphate-binding arginine pair found at positions 309 and 310 in the muscle enzyme and in an analagous position in the bacterial enzyme are present three residues away in the potato enzyme. Also, as in the bacterial enzyme, Arg 242 is conserved. Finally, as in the bacterial enzyme, Tyr 613, but not Phe 285, of the purine inhibitor site is conserved. It is unclear why bacterial phosphorylase responds weakly to AMP and not at all to potato phosphorylase, especially in light of the fact that the intersubunit contact that are critical for propagating the allosteric effects of this ligand in muscle phosphorylase are poorly conserved in both the potato and bacterial enzymes.[21,145]

The lack of a glycogen allosteric effect and the poor enzymatic activity of the enzyme with glycogen as substrate are more easily understood. Fukui and co-workers showed by kinetic and binding analyses that the potato enzyme does not contain a glycogen storage site, and that smaller glucans bind directly to the active site.[76,77,147] The 78-residue insertion (see Figure 1) occurs in the middle of the helical subdomain (residues 380 to 440) that binds glycogen in the muscle enzyme. The insertion is itself highly charged and predicated to be helical in character. This segment may block the entry of highly branched glucans into the

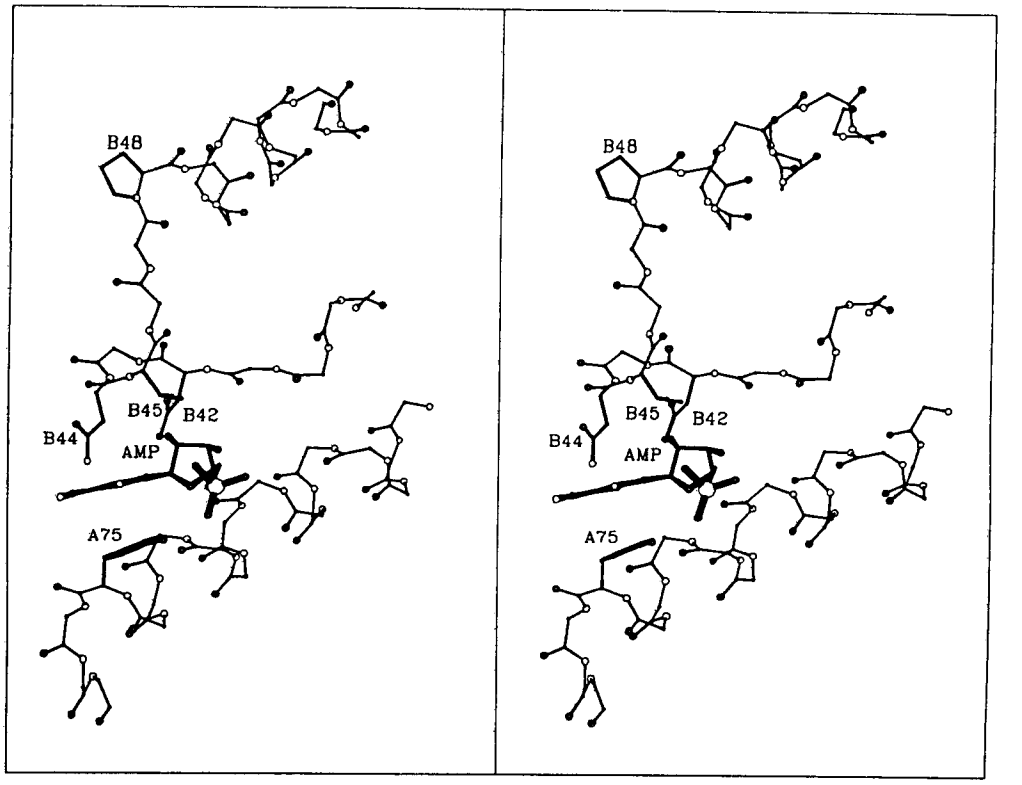


FIGURE 2. Stereoscopic view of main chain segments forming part of the AMP binding site. Carbon atoms are shown as small open circles, nitrogen atoms as large open circles, and oxygen atoms as larger filled circles. AMP and the side chains of residues that interact with it are shown with darkened bonds. Residues are labeled according to whether they belong to the A or B monomer of the active phosphorylase dimer. Parts of each of the symmetry-related C-helices (residues 48 to 80) are illustrated. The roof of the AMP binding site (residues 42 to 45) is connected to the C-helix by a two-amino-acid linker composed of residues 46 and 47. The C-helix begins at proline 48. The loss of AMP activation and/or cooperativity in liver phosphorylase may be due to the substitution of threonine for proline 48. One possible effect of this substitution is that changes in the backbone dihedral angles at this position may alter the orientation of the linker and consequently that of the roof of the AMP site.

active site, while allowing access to the smaller linear polysaccharides. Supportive of this idea is the finding that a second form of phosphorylase occurs in plants that have a similar size and affinity for glycogen as muscle phosphorylase, presumably because it lacks the 78-residue insertion. Elucidation of the exact structure of the insertion and its role in substrate specificity must await the resolution of the three-dimensional structure of the type 1 potato enzyme, a project that is underway and beginning to show promise.[148]

C. Yeast Glycogen Phosphorylase

The primary sequence of yeast phosphorylase, like potato phosphorylase, is longer than the muscle enzyme, consisting of 890 amino acids.[23] Hwang and Fletterick[23] aligned the yeast and rabbit muscle phosphorylase sequences, requiring a total of 14 insertions and deletions, as shown in Figure 2. This configuration results in a yeast phosphorylase protein that is 28 residues longer than muscle phosphorylase at the N-terminus and, like the bacterial and plant enzymes, 11 residues shorter at the C-terminus. Despite the large number of alignment gaps, it is likely that yeast and muscle phosphorylases have a similar tertiary structure, at least in the hydrophobic core, since all of the gaps map to the hydrophilic surface of the rabbit muscle three-dimensional structure. In addition, most of the noncon-

servative amino acid substitutions between the two sequences involve surface residues of the muscle enzyme. The yeast enzyme contains pyridoxal phosphate and has a similar catalytic mechanism as the muscle enzyme; in accordance with this observation, the pyridoxal phosphate attachment site (Lys 680 in the muscle structure) and substrate-binding residues of the catalytic site are exactly conserved.

Although yeast phosphorylase is interconverted between active and inactive states by covalent phosphorylation, there is no homology between the yeast N-terminal region and corresponding regions of any of the other eukaryotic or prokaryotic phosphorylases. It has been shown previously that the phosphorylated peptide of yeast phosphorylase does not match the peptide in vertebrate phosphorylases and that yeast phosphorylase cannot be phosphorylated by mammalian phosphorylase kinase.[80] The yeast phosphorylase N-terminus does not contain any region of homology with the mammalian phosphorylation site, but does contain the consensus sequence (Arg-Arg-Leu-Thr) found in substrates of cyclic AMP-dependent protein kinase.[23]

Yeast phosphorylase retains some but not all of the intricate regulatory properties of the mammalian phosphorylases. In keeping with its functional properties, the yeast enzyme exhibits significantly greater structural identity with the muscle enzyme at most of the allosteric sites. Yeast phosphorylase effectively catalyzes the phosphorolysis of the branched polysaccharide glycogen, in contrast to the *E. coli* maltodextrin phosphorylase and the potato type 1 enzyme. The affinity of the yeast enzyme for glycogen is probably conferred in part by a functional glycogen storage site, since seven out of eight residues known to bind glycogen at this site in muscle phosphorylase are either exactly conserved (five residues) or are conservative replacements (two residues). The yeast enzyme also differs from the bacterial and plant forms in that both the glucose and purine inhibitor binding sites are exactly conserved with the muscle enzyme, although these ligands have no effect on yeast phosphorylase activity.[84,86]

Yeast phosphorylase is not activated by AMP, but is potently inhibited by glucose-6-P. As in the prokaryotic and plant phosphorylases, the positively charged Arg 242, Arg 309, and Arg 310 residues that interact with the phosphate moiety of AMP, ATP, and glucose-6-P are conserved. Although yeast phosphorylase is like bacterial and plant phosphorylases in that it is poorly homologous to muscle phosphorylase in the N-terminal 80 amino acids, one of the two residues (Gln 71) thought to form the sugar subsite of the AMP activator site in muscle phosphorylase is only conserved in the yeast enzyme. All other residues known to interact with AMP are lacking in the yeast N-terminal region. These observations suggest that conservation of the phosphate site and partial conservation of the sugar site are sufficient to retain glucose-6-phosphate binding and allosteric regulation (recall that glucose-6-phosphate does not compete with glycogen or glucose-1-P for binding, meaning that glucose-6-P effects must be explained by binding to a site other than the active site). AMP allostery, on the other hand, is lost due to the lack of any recognition site for the base moiety of the nucleotide. An interesting point is that allosteric regulation is conserved in the yeast enzyme, despite substantial disruption of the intersubunit contacts found in the muscle enzyme. These changes involve not only point mutations, but include two substantial length variations (insertions) near the dimer interface. Thus, it would appear that the propagation of allosteric effects in the yeast enzyme involves structural components and conformational changes that are distinct from its muscle counterpart.

D. Other Mammalian Phosphorylases (Liver and Brain)

As shown in Figure 1 and Table 3, glycogen phosphorylases that are derived from the same tissue in different species have significantly more sequence identity than do the tissue-specific isozymes from one species. In particular, full-length skeletal muscle phosphorylase sequences are available from human and rabbit that show an overall sequence identity of

Table 4
AMINO ACIDS DIFFERING BETWEEN HUMAN LIVER AND RABBIT MUSCLE PHOSPHORYLASES

	Total	Nonconservative
Number of substitutions	171	86
Number buried	41	13
Involving subunit contacts	2	0
Involving domain contacts	2	1
Active site	0	0
Nucleotide activation site	0	0
Phosphorylation site	0	0
Glycogen activation site	3	1
Purine inhibitor site	0	0

97% and no structural variation in any catalytic or allosteric sites[10,12,13] in keeping with the conserved regulatory properties of the enzyme from these two sources. Thus, in this section we disregard interspecies differences and instead focus on the comparison of the muscle, liver, and brain sequences in the context of the differences in their regulatory properties.

The proteins predicted by the human liver and brain cDNA sequences are 846 and 862 amino acids long, respectively. The increased size of both proteins relative to the human or rabbit muscle form (841 and 842 amino acids, respectively) is entirely due to insertion of amino acids at the C-terminus; the three human phosphorylase isozymes can be aligned without insertions or deletions so that amino acids 1 to 830 match[25] (Figure 1). The liver protein sequence is 80% identical to either the brain or muscle sequence, although there are unique substitutions in each pair. The brain and muscle forms are 83% identical. Thus, the sequences of the human muscle and brain phosphorylase isozymes are more closely related than either are to the liver isozyme.

Fletterick and co-workers have performed the most detailed phosphorylase modeling study to date by superimposing the primary sequence of human liver phosphorylase on the rabbit muscle phosphorylase three-dimensional structure.[149-151] Such an analysis is more rigorously valid among mammalian isozymes than between mammalian and more distant species because of the higher degree of sequence identity and the lack of insertions and deletions in the alignment of the former. In support of this premise, it has been found recently that rat anionic and bovine cationic trypsin, isozymes with a similar degree of relatedness (80%) for which both crystal structures are known, show a strong conservation of tertiary structure.[152] Furthermore, 75% of the phosphorylase residues that differ in the liver isozyme are found on the surface of the rabbit muscle structure, where they would be least likely to alter the structure or function of the liver enzyme. As shown in Table 4, the entire hydrophobic core of the protein is highly conserved, including exact conservation of the catalytic site and the buried allosteric effector sites.

Like muscle phophorylase, liver phosphorylase is interconverted between active and inactive states by covalent phosphorylation/dephosphorylation. Phosphorylation of liver phosphorylase can be catalyzed by purified muscle phosphorylase kinase. The site of phosphorylation (Ser 14) is unchanged in liver phosphorylase, and although there are three substitutions nearby (Ser 5 to Thr, Lys 11 to Arg, and Val 15 to Ile), the conservative changes involved are unlikely to disrupt the helix formed by residues 7 to 13 in the muscle structure. In addition, the cluster of 11 basic side chains found within a 15-Å radius of Ser 14 in the muscle structure that may be involved in recognition or binding of the interconverting enzymes[153] are identical in the two isozymes.

Remarkably, the binding sites for the various allosteric effectors have been exactly con-

served, with the exception of the glycogen storage site, where three substitutions have occurred. Conservation of the glucose inhibitory site is not unexpected, given that glucose binds at the active site. Slightly more surprising, in light of the reported difference in substrate specificity between liver and muscle phosphorylases, is that the purine site and all residues surrounding it are also exactly conserved. As stated earlier, it is not known whether glycogen activates liver phosphorylase, but the enzyme binds this substrate with an affinity equal to the muscle enzyme. Two maltoheptaose binding sites per muscle phosphorylase monomer that comprise the glycogen storage site are termed the major and minor site on the basis of occupancy and temperature factors.[27,57,158] Two of the substitutions within the glycogen storage site are found at the minor site (Gln 401 to Glu and Arg 409 to Lys) and one is at the major site (Val 431 to Ile). The substitutions at the minor site predict a slight increase in the affinity of oligosaccharide binding in favor of the liver enzyme and the disruption of a weak salt bridge that is seen in the muscle structure. The effect of the major site substitution is that it may alter van der Waals contacts with the oligosaccharide that are present in the muscle structure.[151] Overall, these substitutions are unlikely to compromise the function of the glycogen storage site in liver phosphorylase.

The biggest surprise held by the liver phosphorylase sequence was that all of the residues that have been shown to interact with AMP in the muscle enzyme are exactly identical. Thus, the profound difference in response to AMP in the liver and muscle enzymes is the result of a more subtle difference between the two structures. One possible explanation might be that intersubunit contacts are disrupted in the liver enzyme, resulting in the loss of cooperative binding of AMP and reduced allosteric transmission from the binding site to the catalytic site. The work of Rath et al. has demonstrated that this is not the case, since only 2 of the 22 amino acids that form hydrogen bonds between the monomers have been substituted in the liver sequence.[151] Both of these substitutions (Lys 11 to Arg and Lys 247 to Arg) are conservative and appear to allow the retention of the hydrogen bonds with no steric hinderance. Another explanation is that the difference in response is due to the cumulative effects of amino acid replacements throughout the liver phosphorylase molecule. Not only is this hypothesis virtually impossible to test at either the molecular modeling or biochemical level, but it lacks credence, since both liver and muscle phosphorylase are activated upon covalent phosphorylaton, suggesting that the T to R transition and the pathways along which conformational changes take place are intact in the liver enzyme.

Thus, Rath and co-workers[151] focused on yet another explanation, namely, that the differences in AMP activation are due to a few critical amino acid substitutions occuring in the vicinity of the AMP activator site. By comparing the sequences of human muscle, human brain, rabbit muscle, and human liver phosphorylase, they were able to identify a set of 57 residues unique to the liver sequence, 40 of which are either on the surface of the protein or solvent accessible. Only one of the unique residues is in close proximity to the AMP binding site, the substitution of a Thr in the liver sequence for Pro in the other three sequences at position 48. The two AMP binding sites in the muscle phosphorylase dimer are formed by the meeting of two identical α-helices, one from each submit, as shown in Figure 2. These helices are composed of residues 48 to 78 and are oriented head to tail with respect to each other. Residues 42 to 45 form the roof of the AMP binding site and residues 42, 44, and 45 are in direct contact with the ligand. At the other end of the helix, Tyr 75 forms the floor of the site in the opposite monomer and interacts with the adenine ring of AMP. The roof of the AMP site is connected to the intersubunit helix by a two-amino-acid linker (residues 46 and 47). Thus, the effect of the PRO 48 to Thr substitution in liver phosphorylase might be an alteration of backbone torsional angles at residue 48. This can affect several physical parameters, including the length of the intersubunit helix, the position of the roof of the AMP binding site, or the dynamic structure of the binding locus. Any of these effects could alter the distances between AMP and its binding residues in the AMP allosteric site,

resulting in weakened binding of the ligand and less effective allosteric communication between the AMP site and the active site.

Another potentially significant substitution is Thr 197 to Met. Unlike the change at 48, this substitution is not unique to the liver enzyme, as it is found in the brain phosphorylase sequence as well. This residue may function to stabilize a turn formed by residues 192 to 195. The muscle structure contains intersubunit interactions between Glu 195 and Lys 41 (salt bridge) and Arg 193 with Leu 39 and Val 40 (hydrogen bonds). These contacts require that the 192 to 195 turn be held in precise conformation and could be critical in forming the roof of the AMP binding site. Stability of the turn in muscle phosphorylase may be conferred by a hydrogen bond between the side chain oxygen of Thr 197 and the main chain carbonyl of Pro 194. Such an interaction is lost in liver and brain phosphorylases, both of which contain Met at position 197. The fact that brain phosphorylase is activated by AMP in a noncooperative fashion suggests that the substitution at position 197 may have a greater effect on cooperativity than on activation per se. One possibility is that both the liver and brain AMP sites are locked in an "open" position, allowing free access to AMP at both sites on the dimer, thereby explaining the lack of cooperativity. In this scenario, ligand binding to the brain or muscle enzymes results in a "clamping down" of the site around the lignad, thereby initiating conformational changes that ultimately reach the catalytic site. Due to the change at residue 48 in the liver enzyme, the "clamping" action is weakened, in turn attenuatating AMP activation. Experiments are currently underway to directly test the role of the residues implicated in AMP effects, as described below.

V. EVOLUTION OF PHOSPHORYLASES

The appearance of each new phosphorylase sequence in the literature has been accompanied by speculation on its evolutionary relationship to the rabbit muscle sequence. The common theme in all such discussions is that the catalytic or C-terminal domain is always highly conserved, while the regulatory or N-terminal domain is variable. This finding suggests that the primordial phosphorylase gene encoded at least the C-terminal portion of the protein and that it has been conserved by selective pressure to maintain a particular catalytic functon. The N-terminal or regulatory domains of different phosphorylases, in contrast, appear to have evolved by independent mechanisms. Palm et al. suggest that freedom from the selective pressures placed on the catalytic domain have allowed rapid changes in the N-terminal regions in organisms for which the latter domain has no discernable function.[21] Thus, outside of a few packets of conserved residues involved in the formation of the active site, the N-terminal domain of bacterial phosphorylase may have evolved at a constant rate, constrained only by maintenance of the conformation of the catalytic site.

Based on the sequence of potato phosphorylase, Nakano et al. suggested that the ancestral phosphorylase gene was generated by the fusion of two exons of nearly equal molecular weight, since the large 78-residue insertion found in this sequence is positioned near the N-terminal/C-terminal domain junction.[145] In this scenario, the amylopectin preferring type I plant phosphorylase would have evolved from a primordial phosphorylase with little or no preference for a particular glycan as substrate (the type 2 enzyme) by the insertion of the 78-residue fragment.

Hwang and Fletterick have proposed that allosteric regulation in yeast and mammalian enzymes evolved by distinct but converging paths. The N-termini of these enzymes are not homologous, yet they have similar functional roles; they contain the sites of phosphorylation as well as surface regions involved in the intersubunit contact. The lack of sequence homology in this region between either yeast or mammalian enzymes and the unregulated enzyme forms (bacteria, plants) suggests that the yeast and mammalian phosphorylation sites were acquired through separate gene fusions to the N-terminus of the primordial sequence.[23] The

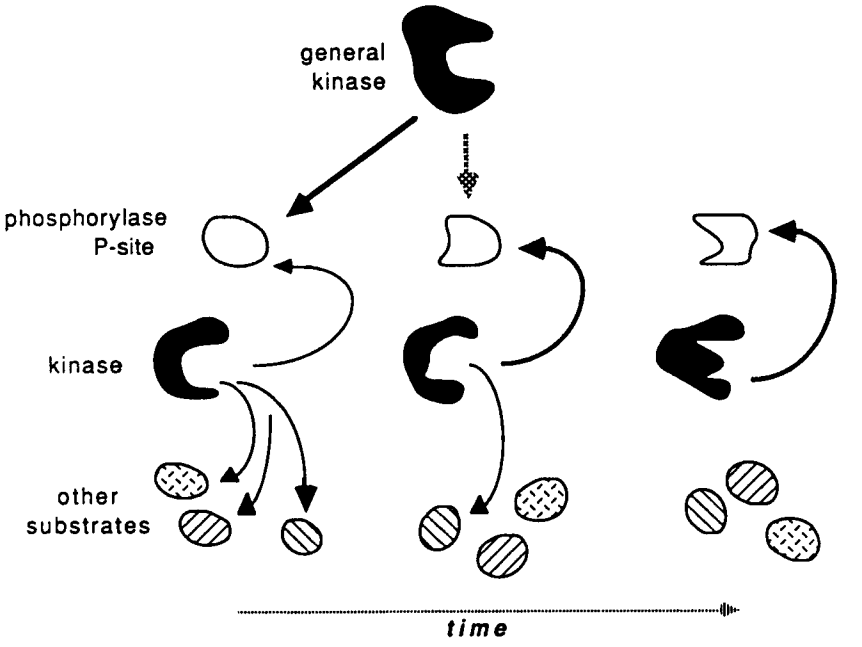


FIGURE 3. Schematic of a possible mechanism for the evolution of a specific phosphorylase kinase regulating eukaryotic phosphorylases. In this model, the ancestral protein contains a phosphorylation site recognized by a general kinase; divergence of this site leads to loss of recognition by the general kinase. Concurrently, other nonspecific kinases gradually restrict their range of substrates, ultimately giving rise to a specific phosphorylase kinase.

acquisition of phosphorylation sites alone was not sufficient to confer allosteric regulation on the enzymes, but must have been accompanied or followed by the evolution of appropriate intersubunit contacts that would allow communication between the regulatory site and the active site. The mechanisms of allosteric regulation in the yeast and mammalian enzymes are related by convergent evolution, since the subunit contacts found in muscle phosphorylase and in the yeast enzyme are clearly dissimilar and thus effect different pathways of intersubunit communication. Hwang and Fletterick further proposed a model for the evolution of a regulatory kinase cascade that may account for differences in kinase specificities for the yeast and mammalian phosphorylases. According to this model, as shown in Figure 3, a phosphorylation site recognized by the general protein kinase was originally spliced onto the primordial enzyme; divergence of this site led to poorer recognition and eventually loss of recognition by the general kinase. Coevolution of other nonspecific kinases gradually restricted their range of substrates, ultimately giving rise to a specific phosphorylase kinase.

From comparison of phosphorylase sequences also emerges a chronology of the evolution of the AMP site in mammalian phosphorylases. Conservation of residues involved in binding the phosphate group of AMP is found even in the bacterial enzyme and suggests that a phosphate binding site existed in the ancestral enzyme.[21] In the eukaryotic lineage, next there evolved a sugar-binding subsite; yeast and vertebrate sequences, but not those from bacteria or plants, have conserved residues required for binding of the ribose moiety of AMP. After separation from the yeast lineage, the mammalian enzyme developed the final component for functional AMP binding, namely, the adenine subsite, which in the yeast lineage became the functional site of glucose-6-P inhibition. Effective AMP activation was then presumably lost from the liver enzyme as a safeguard against inappropriate hepatic glycogen depletion, thus ensuring a glucose supply for extrahepatic tissues, especially the

central nervous system, under conditions of short-term starvation. The ability of AMP to increase the Km for glucose of both liver and muscle phosphorylase, coupled with the exact conservation of the residues that contact AMP in the two proteins, suggests the possibility that AMP may play an as yet undefined regulatory role distinct from allosteric activation in the liver under certain physiological conditions.

Remarkably, human muscle, brain, and liver phosphorylase sequences are more conserved in the N-terminal domain than in the C-terminal domain.[13,22,25] Newgard et al. further suggested that the N-terminal 80 amino acids of mammalian phosphorylase represents a unique segment that was fused on to the ancestral gene.[22] Evidence for this mechanism includes the almost complete lack of homology between mammalian and more primitive phosphorylases within this region, a sudden change in "liver-like" codon usage pattern to a "muscle-like" pattern beginning at the codon for amino acid 80 in the liver phosphorylase sequence,[22] and the finding by Burke et al. that there is an intron/exon junction in the muscle phosphorylase gene at the codon for amino acid 80.[13] The fact that conservation of sequence between mammalian and more primitive phosphorylases begins at or near amino acid 80 may reflect the fact that residue 80 marks the beginning of the first β-strand of the hydrophobic core of the protein.

VI. FUTURE DIRECTIONS

Crystallographic and recombinant approaches have led to a much broader understanding of structure/function relationships in the family of glycogen phosphorylases. Still, a true understanding of functional properties at the structural level for these proteins ultimately must include crystallographic analysis of phosphorylases from sources other than skeletal muscle. Ever-improving DNA-sequencing technology has skewed the advances strongly in the favor of primary sequence information. With the advent of new techniques for X-ray data collection and processing in structural biology, such as area detectors coupled with recombinant approaches for expression of large quantities of the cloned phosphorylase of interest, we are in a position to narrow this gap. In the meantime, predictions advanced from structural modeling can be tested by site-directed mutagenesis. To this end, Hwang et al. have recently developed an expression system for eukaryotic phosphorylases in yeast.[154] To facilitate easy assay of the expressed phosphorylase, a strain of yeast deficient in endogenous phosphorylase activity was constructed by transfecting with a plasmid containing a disrupted yeast phosphorylase gene. This procedure resulted in the deletion of the intact functional gene in some progeny strains via homologous recombination. An expression vector was also constructed by creating a restriction site via site-directed mutagenesis at the 5′ and 3′ junctions of the yeast structural gene and its flanking regions that allows insertion of the mammalian cDNAs. To date, this system has been used to express muscle, liver, and yeast phosphorylase in quantities sufficient for assay. Site-directed mutagenesis studies of residue 48 in liver and muscle phosphorylases are underway, allowing a direct test of the predictions advanced by structural modeling. Palm and co-workers also recently reported preliminary experiments where site-directed mutagenesis is being used to probe the catalytic site and the role of the C-terminus in bacterial phosphorylase; the native and mutant bacterial proteins were overexpressed under control of the malP, Q operator.[155] Given the size and complexity of glycogen phosphorylase, these experiments represent only the early stages of what promises to be a rich period to come. Phosphorylase deserves the attention, not only because of its central role in carbohydrate metabolism, but also because the unraveling of its regulatory mechanisms has broad implications for understanding allosteric regulaton in general.

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