Partial Activation of Muscle Phosphorylase by Replacement of Serine 14 with Acidic Residues at the Site of Regulatory Phosphorylation†

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ABSTRACT: Phosphorylation of glycogen phosphorylase at residue Ser14 triggers a conformational transition that activates the enzyme. The N-terminus of the protein, in response to phosphorylation, folds into a 3_{10} helix and moves from its location near a cluster of acidic residues on the protein surface to a site at the dimer interface where a pair of arginine residues form charged hydrogen bonds with the phosphoserine. Site-directed mutagenesis was used to replace Ser14 with Asp and Glu residues, analogs of the phosphoserine, that might be expected to participate in ionic interactions with the arginine side chains at the dimer interface. Kinetic analysis of the mutants indicates that substitution of an acidic residue in place of Ser14 at the site of regulatory phosphorylation partially activates the enzyme. The S14D mutant shows a 1.6-fold increase in V_{max}, a 10-fold decrease in the apparent dissociation constant for AMP, and a 3-fold decrease in the $S_{0.5}$ for glucose 1-phosphate. The S14E mutant behaves similarly, showing a 2.2-fold increase in V_{max}, a 6-fold decrease in the apparent dissociation constant for AMP, and a 2-fold decrease in the $S_{0.5}$ for glucose 1-phosphate. The ability of the mutations to enhance binding of AMP and glucose 1-phosphate and to raise catalytic activity suggests that the introduction of a carboxylate side chain at position 14 promotes docking of the N-terminus at the subunit interface and concomitant stabilization of the activated conformation of the enzyme. Like the native enzyme, both mutants show significant activity only in the presence of the activator, AMP. Full activation, analogous to that provided by covalent phosphorylation of the enzyme, likely is not achieved because of differences in the charge and the geometry of ionic interactions at the phosphorylation site.

Protein phosphorylation is a common mechanism for coupling the regulation of enzymatic activity to extracellular signals from hormones and intracellular second messengers (Johnson, 1994; Parsadanian, 1994; Cohen, 1989). The control of gene transcription, cell division, and metabolism all depend on the covalent phosphorylation of proteins. The first enzyme found to be regulated by phosphorylation was glycogen phosphorylase (Fischer & Krebs, 1955). The allosteric properties of glycogen phosphorylase are altered by phosphorylation at residue Ser14, which is catalyzed by phosphorylase kinase (Fischer & Krebs, 1955; Fischer et al., 1957, 1959). The activity of phosphorylase kinase is in turn controlled by neuronal and hormonal signals linked to a cyclic AMP second messenger system. The unphosphorylated form of phosphorylase, phosphorylase *b*, is inactive in the absence of the metabolite activator, AMP, and substrates and effectors bind to the enzyme cooperatively. The phosphorylated form of the enzyme, phosphorylase *a*, is nearly fully active in the absence of AMP, and phosphorylation enhances the affinities of substrates and activators (Graves & Wang, 1972; Madsen et al., 1976; Madsen, 1986).

The structural basis for the activating effects of phosphorylation on phosphorylase has been investigated by X-ray crystallography (Sprang et al., 1988; Johnson, 1992; Barford et al., 1991). A comparison of structures of phosphorylated and unphosphorylated forms of phosphorylase shows that phosphorylation induces structural changes at the N-terminus of the enzyme that result in an altered subunit arrangement and manifold changes in structure throughout the monomer (see Figure 1). In structures of phosphorylase *b*, residues 5−16 of the N-terminus are generally disordered in the absence of inhibitors. In the complex of the enzyme with the inhibitor glucose, the N-terminus is located on the surface of the enzyme near a cluster of acidic residues (Martin et al., 1990). Phosphorylation at Ser14 initiates a refolding of residues of the N-terminus into a 3_{10} helix, which moves into a cleft at the subunit interface where the phosphoserine forms salt-bridge interactions with Arg43 from the cap (residues 40−45) of one subunit and Arg69 from the α_2 helix of the adjacent subunit (Sprang et al., 1988). The repositioning of residues of the N-terminus displaces residues 838−841 of the C-terminus from the subunit interface. These combined structural changes draw the two monomers closer together and trigger rotations of the subunits in opposite directions by 5°. Changes in tertiary structure have been described for the active site and the allosteric effector sites for glucose, purines, glycogen, and AMP (Browner & Fletterick, 1992; Johnson, 1989).

In numerous enzymes, phosphorylase included, arginine residues form the recognition sites for phosphate groups of substrates, effectors, or cofactors (Riordan et al., 1977). The geometry of the planar, branched guanidinium group enables arginine to form multiple hydrogen bonds with a phosphate group, and the charged interaction is an order of magnitude stronger than that between neutral groups. Arginine is also commonly found in the enzymatic binding sites for carboxylate groups and is capable of forming two hydrogen bonds

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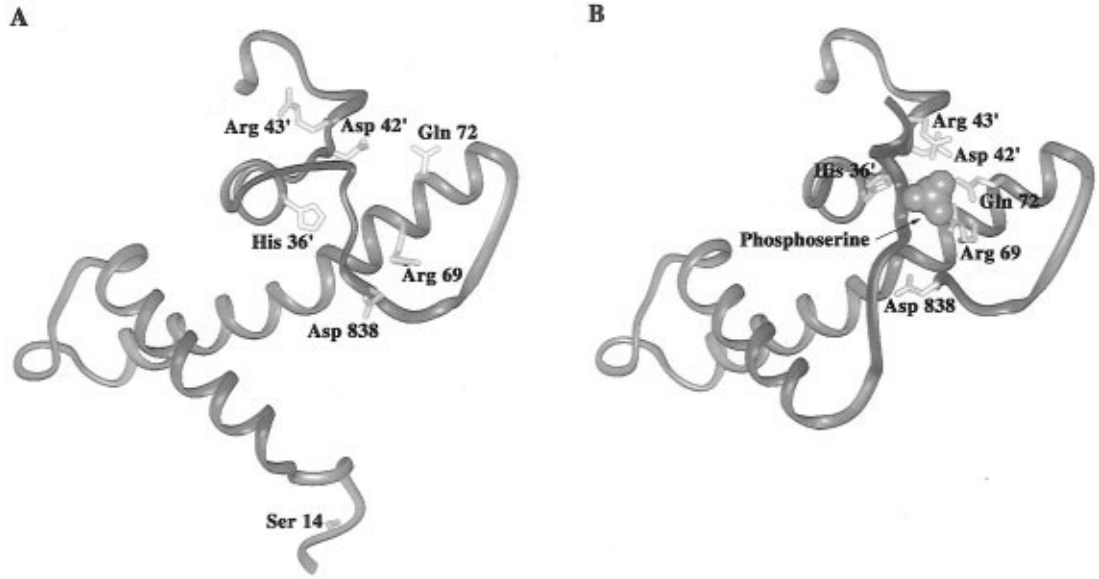

FIGURE 1: Structural changes at the dimer interface of glycogen phosphorylase induced by phosphorylation at Ser14 (Sprang et al., 1988). The main chain of the protein is drawn as a ribbon, and selected side chains are shown in a ball and stick representation. (A) View of phosphorylase *b*. The N-terminus extends away from the dimer interface while the C-terminus forms van der Waals contacts and hydrogen-bonding interactions with residues from both subunits. His36 forms an intersubunit hydrogen bond with Asp838 (residues beyond 838 are disordered), and Arg43 forms an intrasubunit hydrogen bond with Leu115. These interactions are disrupted in phosphorylase *a*. (B) View of the phosphoserine site in phosphorylase *a*. The N-terminus moves to the dimer interface causing the C-terminus to move away. Salt-bridge interactions form between the phosphoserine and Arg69 of the same subunit and Arg43 of the opposite subunit.

with carboxylate oxygens (Riordan et al., 1977; Mitchell et al., 1992). Acidic residues have been substituted at the sites of regulatory phosphorylation in several enzymes and found to mimic, albeit inexactly, the effects of phosphorylation (Alessandrini et al., 1996; Mansour et al., 1996; Huang et al., 1995; Orr & Newton, 1994; Hurley et al., 1990b). In the present study, site-directed mutagenesis was used to replace Ser14 with Asp and Glu residues to test whether an acidic residue would promote the docking of the N-terminus of phosphorylase at the subunit interface and activate the enzyme. Kinetic analysis of the mutants shows that substitution of an acidic residue in place of Ser14 results in partial activation.

MATERIALS AND METHODS

Oyster glycogen type II and phosphorylase kinase were purchased from Sigma. *Escherichia coli* strain 25A6 was supplied by Genentech Inc. (South San Francisco, CA).

Mutagenesis. The S14D and S14E mutations were made in phosphorylase by oligonucleotide-directed mutagenesis according to the procedure of Kunkel (1985) with some modifications as described by Browner et al. (1991). The cDNA of rabbit muscle phosphorylase, which was cloned into the *E. coli* plasmid pHSe5 (Browner et al., 1991), was used for mutagenesis and protein expression. An Applied Biosystems PCRmate synthesizer was used for synthesis of the oligonucleotides needed for site-directed mutagenesis and DNA sequencing. The oligonucleotides were 25 bases in length and contained base substitutions for introduction of the mutation sites as well as conservative substitutions which introduced a (*Kpn*I) restriction site next to the Ser14 mutations and enabled restriction digests to be used for screening the mutants. In order to determine that the correct mutations had been incorporated in the DNA, the cDNAs were sequenced in the regions of the mutation sites and within 300 base pairs on either side of the mutations.

Protein Expression and Purification. Wild-type phosphorylase and the S14D and S14E mutants were expressed in cultures of *E. coli* strain 25A6 (W3110; *ton*A, *lon*Δ, *qal*E, *htp*P^{ts}) at 22 °C under conditions that have been described previously (Browner et al., 1991). About 5−10 mg of the mutants was obtained from 1 L of culture. The enzyme variants were purified by chromatography on fast-flow metal chelating Sepharose and fast-flow DEAE-Sepharose as described previously (Luong et al., 1992). Wild-type phosphorylase *a* was prepared from purified phosphorylase *b* by phosphorylating the enzyme with phosphorylase kinase. Phosphorylase kinase, 1 mg, was added to a reaction mixture containing 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, 6 mM NaF, 0.2 mM ATP, and 5 mg of phosphorylase *b* in a total volume of 10 mL and allowed to incubate for 6 h. The phosphorylase *a* was then purified by chromatography on fast-flow DEAE-Sepharose using a 150 mL linear gradient of 0−250 mM KCl and 25 mM glycerophosphate, pH 7.0. The phosphorylated enzyme elutes at about 160 mM KCl, well separated from phosphorylase *b*, which elutes at the beginning of the gradient.

Kinetic Analysis of Phosphorylase Variants. The enzyme variants were desalted by chromatography on Sephadex G25 before use in assays. The extinction coefficient of the wild-type enzyme of 1.32 cm^2/mg (Buc et al., 1971) was used to determine the protein concentrations of the mutants. Glycogen was purified as described previously to remove contaminating phosphate (Buchbinder et al., 1995). Initial velocities were determined at 30 °C by measuring the rate of phosphate produced from the reaction of glucose-1-phosphate and glycogen (Carney et al., 1978). The assays contained 50 mM BES,[1] pH 6.8, 1 mM EDTA, 1 mM DTT, and 1% glycogen in a final volume of 0.5 mL. The enzyme was preincubated with glycogen and AMP for 5 min at 30

[1] Abbreviations: BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; Gp *b*, phosphorylase *b*; Gp *a*, phosphorylase *a*.

Table 1: AMP Activation of Wild-Type Phosphorylase and Mutant Enzymes[a]

enzyme	V_{max} (μmol min^{-1} mg^{-1})	n_H	K_{AMP}[b] (μM)
wild-type Gp *b*	29.4 ± 0.3	1.57 ± 0.04	16.5 ± 0.4
wild-type Gp *a*	94 ± 5	1.0[c]	1.4[d]
S14D	53.7 ± 0.8	1.50 ± 0.05	1.66 ± 0.05
S14E	52 ± 1	1.8 ± 0.1	2.7 ± 0.1

[a] Enzymatic activities were measured at 30 °C at pH 6.8. The concentration of glucose 1-phosphate was held constant at 10 mM. The kinetic parameters were determined from a nonlinear, least squares fit to the Hill equation. [b] K_{AMP}, AMP concentration at half the maximal observed specific activity. [c] n_H for AMP reported by Helmreich et al. (1967). [d] K_d for AMP determined by equilibrium dialysis in the presence of 1% glycogen and 50 mM glucose 1-phosphate; reported by Helmreich et al. (1967).

Table 2: Kinetic Parameters for Wild-Type and Mutant Forms of Phosphorylase[a]

enzyme	V_{max}[b] (μmol min^{-1} mg^{-1})	n_H	$S_{0.5[G-1-P]}$[c] (mM)
wild-type Gp *b*	28.5 ± 0.3	1.40 ± 0.02	1.19 ± 0.02
wild-type Gp *a*	95 ± 6	1.1 ± 0.1	0.17 ± 0.02
S14D	46 ± 1	1.27 ± 0.04	0.32 ± 0.02
S14E	64 ± 2	1.36 ± 0.03	0.50 ± 0.02

[a] Enzymatic activities were measured at 30 °C at pH 6.8. The concentration of AMP was held constant at 100 μM. The kinetic parameters were determined from a nonlinear, least squares fit to the Hill equation. [b] Maximal observed specific activity. [c] $S_{0.5(G-1-P)}$, glucose 1-phosphate concentration at half the maximal observed specific activity.

°C before initiating the reaction with glucose-1-phosphate. Trichloroacetic acid (10%) was used to stop the reactions. The apparent dissociation constant, K_{AMP}, and Hill coefficient, n_H, of AMP were determined by varying AMP from 0 to 10 μM while glucose 1-phosphate was held constant at 10 mM. The substrate concentration at half-maximal velocity, $S_{0.5}$, and n_H of glucose 1-phosphate were determined by varying the concentration of glucose 1-phosphate from 0 to 3 mM while AMP was held constant at 100 μM. The assays were done in triplicate, and the kinetic data were fit to the Hill equation by nonlinear regression with the program JMP (SAS Institute Inc., 1989). Kinetic parameters are reported with their standard errors determined from the curve-fitting programs.

Molecular Modeling. The program Chain (Sack, 1988) was used to display the structure of phosphorylase *a* with different rotamers of aspartate and glutamate modeled at residue 14. Structures were displayed on a Silicon Graphics system, and the potential interactions of the carboxylate groups in the mutants were compared to those of the phosphoserine in the wild-type enzyme.

Calculation of Electrostatic Surface Potential. The program GRASP (Nicholls et al., 1991) was used to calculate the electrostatic surface potentials for the wild-type phosphorylase *a* and the model of the S14E mutant in the absence of ligands and in the complex with AMP. Calculations were performed using a solvent dielectric constant of 80 and a protein dielectric constant of 2, and the accessible surface was determined with a probe radius of 1.4 Å.

RESULTS AND DISCUSSION

The introduction of an acidic residue at the position of Ser14 might be expected to alter the interaction of the N-terminus with the enzyme and affect regulatory and catalytic behavior. The effects of the mutations on maximal velocities and the apparent affinities for AMP and the substrate glucose 1-phosphate were analyzed kinetically. Kinetic parameters determined from AMP saturation curves for the wild-type and mutant enzymes are shown in Table 1. In comparison to wild-type phosphorylase *b*, the S14D mutant shows a 1.6-fold increase in V_{max}, a 10-fold decrease in K_{AMP}, but little change in cooperativity for AMP. Substitution of a Glu for Ser14 similarly causes a 2.2-fold increase in V_{max}, a 6-fold decrease in K_{AMP}, and a somewhat elevated cooperativity for the activator. Kinetic parameters determined from glucose 1-phosphate saturation curves are shown in Table 2. The S14D mutant shows a 3-fold decrease in the $S_{0.5}$ for glucose 1-phosphate and a slight reduction in cooperativity for the substrate in comparison to wild-type phosphorylase *b*. The S14E mutant shows a 2-fold decrease in the $S_{0.5}$ for glucose 1-phosphate and cooperativity for the substrate that is comparable to wild-type phosphorylase *b*.

The combined results indicate that the mutants are partially activated by the substitutions. Maximal velocities are nearly double that observed for phosphorylase *b*, and the apparent dissociation constants for AMP and the $S_{0.5}$'s for glucose 1-phosphate approach the values observed for phosphorylase *a*. Unlike the phosphorylated wild-type enzyme, no significant activity is observed for the mutants in the absence of AMP. Wild-type phosphorylase *a* exhibits a maximal velocity in the absence of AMP that is about 90% of that observed in the presence of saturating concentrations of the activator (Engers et al., 1970). Another dissimilarity from wild-type phosphorylase *a* is that the mutants show a higher level of substrate cooperativity, indicated by n_H values more typical of phosphorylase *b*. The lack of cooperativity for glucose 1-phosphate in phosphorylase *a* reflects the nearly complete conversion of the wild-type enzyme to the active state as a result of phosphorylation. In the mutants, a carboxylate group in place of the phosphate is apparently unable to shift the equilibria between inactive and active conformational states, perhaps because of an inability of the N-terminus to bind at the subunit interface in proper register to induce the rotation of the subunits that is required for activation. The mutants are still fully dependent upon the binding of AMP to trigger the allosteric transition; however, the substitutions allow the mutants to achieve a more activated conformational state in the presence of AMP than is typical of phosphorylase *b*.

The partial activation of the mutants suggests that in the presence of AMP, which induces the activated quaternary association of the subunits, the mutations allow the N-terminus to dock at the dimer interface and trigger changes in tertiary structure that further activate the enzyme. Replacement of Ser14 with an acidic residue may allow the N-terminus of the enzyme to assume a 3_{10} conformation, and 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. In native phosphorylase *b*, unfavorable electrostatic interactions among cationic residues Lys9, Arg10, Lys11, Arg16, Arg69, and Arg43, clustered within 8 Å of Ser14, presumably prevent the N-terminus from adopting a 3_{10} conformation and moving to the dimer interface. The substitution of a negatively charged residue for Ser14 may partially neutralize

the constellation of positive charge and allow the refolding of the N-terminus to resemble its conformation in the phosphorylated enzyme.

Differences between carboxylate and phosphate groups in charge and geometry may prevent full activation. The phosphate most likely exists partially as a dianion at pH 6.8 (Vogel & Bridger, 1983) and has tetrahedral geometry whereas the carboxylate group is a monoanion and has trigonal geometry. Although it is impossible to accurately determine the energetic contributions of particular charged interactions to activation, a salt bridge between an arginine and a carboxylate group is estimated to provide about 2−3 kcal/mol (Fersht et al., 1985; Wells et al., 1987; Cunningham & Wells, 1993) and represent a somewhat weaker linkage than that between arginine and phosphoserine, estimated to provide about 4−5 kcal/mol (Vogel & Bridger, 1983). Calculations of the energy required for activation of the enzyme are also imprecise, but assuming a two-state concerted model for the allosteric transition, phosphorylation is estimated to lower the energy required for activation by about 3.5 kcal/mol (Madsen, 1986). These estimates indicate that a salt bridge from a carboxylate group could contribute substantially to activation. The singly charged Glu or Asp residues, however, likely do not provide adequate charge compensation for surrounding positively charged side chains, which as a result may become solvated. The weaker interactions of a carboxylate group with surrounding charged residues together with structural perturbations in the vicinity of the phosphorylation site may be responsible for the observed suboptimal activation in the mutants.

The electrostatic effects of replacing a dianionic phosphate with a monoanionic carboxylate group were analyzed by calculation of the electrostatic surface potential in the vicinity of the phosphorylation site (Figure 2). In wild-type phosphorylase *a*, the side chains of Arg43′ (the prime indicates a residue from the opposing subunit) and Arg69 lie in regions of negative potential. In the mutants, the presence of a carboxylate group at the position of the serine phosphate, in contrast, provides insufficient negative charge to neutralize the surrounding cationic residues, and the electrostatic potential appears to be positive at the side chains of Arg43′ and Arg69. The strong negative potential at the phosphoserine in the wild-type enzyme may be required to attract the arginines into the correct position for activation. AMP binding approximately 10 Å away from the phosphorylation site brings additional negative potential into the vicinity of the phosphoserine and helps to compensate for the deficiency of negative charge in the mutants (Figure 2C). Electrostatic calculations for the S14E mutant show that the side chain of Arg69 lies in a region of negative potential in the complex with AMP; however, electrostatic potential surrounding the side chain of Arg43′ remains positive, albeit reduced from that in the absence of AMP. The ability of AMP to partially neutralize the excess positive charge at the arginines may account for the activation of the mutants in the presence of AMP to levels above that normally observed for wild-type phosphorylase *b*.

The limited activation of the S14D and S14E mutants may be the result of differences in the separation of the subunits and the precise positioning of side chains that normally interact with the phosphoserine. Molecular modeling was used to examine the hypothetical interactions of the Asp and Glu residues that were substituted at Ser14. The preferred

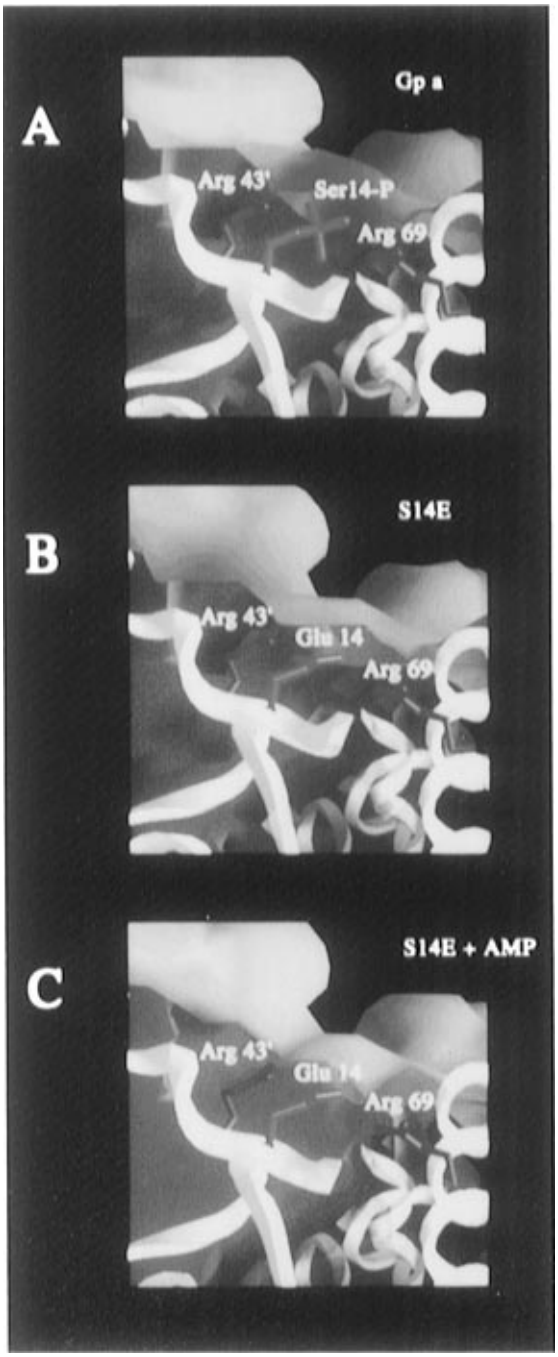

FIGURE 2: Representation of the electrostatic surface in the vicinity of residue 14 in (A) wild-type phosphorylase *a*, (B) the model of the S14E mutant, and (C) the model of the S14E mutant in complex with AMP. Negative potential is colored red and positive potential is in blue. The protein secondary structure is drawn as a ribbon diagram. The figure was prepared using the program GRASP (Nicholls et al., 1991).

rotamers of Asp and Glu that most closely approximated the position of the phosphoserine in the native enzyme were modeled into the crystallographic structure of phosphorylase *a*. The phosphoserine in the native enzyme forms salt-bridge interactions with Arg43 from the cap of one subunit and Arg69 from the α_2 helix of the adjacent subunit (see Figure 1). Molecular modeling indicates that these interactions are not precisely simulated in the mutants (see Figure 3). For the S14D mutant, if the preferred side chain rotamer of Asp that places the carboxylate group closest to the phosphoserine is modeled into the structure, the guanidinium groups of Arg43′ and Arg69 and the carboxylate oxygens of the

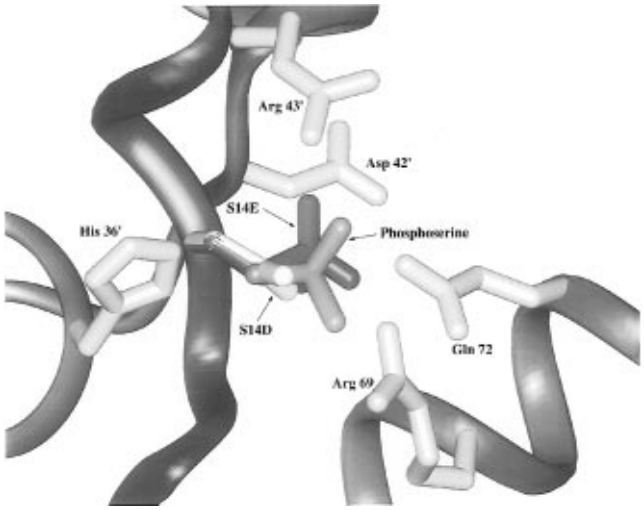

FIGURE 3: Interactions of the phosphoserine in native phosphorylase *a* (Sprang et al., 1988) and a comparison with mutants based on molecular modeling. Aspartate and glutamate residues were modeled at residue 14 with the preferred side chain rotamers that most closely approximate the position of the phosphoserine. The main chain of the protein is represented as a ribbon. The phosphoserine is shown in medium gray, the carboxylate side chain of the S14D mutant is in light gray, and the S14E mutant is in dark gray.

aspartate side chain are too far apart to form salt-bridge interactions. The aspartate side chain, if torsion angles are allowed to deviate from the preferred rotamer position, can be moved to place the carboxylate oxygens within hydrogen-bonding distance of either Arg43′ or Arg69, but not within hydrogen-bonding range of both arginines simultaneously. Further allowing adjustment of the arginine side chains still does not enable the carboxylate group to span the distance required to form hydrogen bonds with both arginines. Some local shifting of backbone atoms would be required in this mutant to accommodate the carboxylate group. In the S14E mutant, the longer side chain enables the placement of the carboxylate group closer to the position of the phosphoserine. In the preferred rotamer position of Glu14 that most closely matches the position of the phosphoserine in the wild-type enzyme, the side chain carboxylate $O_{\epsilon 2}$ of the glutamate and the guanidinium NH_1 of Arg69 lie within hydrogen-bonding distance of each other. The carboxylate $O_{\epsilon 1}$ of Glu14 and the guanidinium NH_1 of Arg43′ are, however, only 1.9 Å apart and approach too closely. A relatively small adjustment in side chain torsion angles would place the carboxylate group of Glu14 within appropriate hydrogen-bonding distance of both arginines.

Several lines of evidence suggest that activation of phosphorylase depends on the placement of the cap and α_2 helix and their particular interactions at the subunit interface (Sprang et al., 1988; Browner et al., 1994). The enzyme can be partially activated by binding of divalent cations at engineered metal binding sites located between the cap and α_2 helix (Browner et al., 1994). The binding of transition metal ions was shown by X-ray crystallography to simulate some of the structural changes, such as the drawing together of the monomers and the contraction of the α_2 helix, normally induced by activating ligands. Natural metabolite effectors including the activator, AMP, and the inhibitor, glucose 6-phosphate, also bind between the cap and α_2 helix about 10 Å away from the site of phosphorylation and induce changes in quaternary and tertiary structure that affect ligand affinities and catalytic activity (Sprang et al., 1987, 1988; Barford et al., 1991; Lorek et al., 1984). Studies with AMP analogs indicate that the dianionic and not the monoanionic form of AMP is responsible for activation (Withers & Madsen, 1980; Murray & Atkinson, 1968) and that 2′-AMP and 3′-AMP are unable to activate phosphorylase, most likely because of mispositioning of the phosphate relative to enzymatic residues at the effector site (Okazaki et al., 1968; Mott & Bieber, 1970). Protein phosphorylation and metabolite effectors have in common the ability to affect the disposition of the cap and the α_2 helix in glycogen phosphorylase through their particular atomic interactions. The precise ligand interactions apparently determine whether an effector activates or inhibits the enzyme. Given the sensitivity of the allosteric response to the particular interactions at the subunit interface, it is perhaps not surprising that an acidic residue cannot entirely mimic a phosphoserine. In general, Glu or Asp side chains cannot be expected to fully replace phosphothreonine or phosphoserine residues in proteins regulated by phosphorylation where precise charge balance and phosphopeptide registration are primary features of the activation mechanism.

Crystallographic structures are now available for several proteins that are regulated by phosphorylation and have provided insights into the mechanisms by which the covalent modification may exert its effects. Isocitrate dehydrogenase, for example, is phosphorylated at a position near its active site where the phosphate group inhibits the enzyme by electrostatically and sterically interfering with substrate binding (Hurley et al., 1990a). In protein kinases, cAMP-dependent protein kinase, MAP kinase, and cyclin-dependent kinase 2, phosphorylation sites are located in loops at the entrances of their active sites (Knighton et al., 1991a,b; Zhang et al., 1994; De Bondt et al., 1993; Russo et al., 1996). Phosphorylation of the T loop of cyclin-dependent kinase causes movement of the loop away from the catalytic cleft and affects packing interactions between the kinase and cyclin A. Structures are not yet available for both phosphorylated and unphosphorylated forms of the other protein kinases; however, phosphorylation is believed to cause activation of these enzymes by affecting either substrate binding, the positioning of catalytic residues, or interactions with regulatory subunits. Although the mechanisms differ, covalent phosphorylation in each of these enzymes provides a regulatory switch which, in response to extracellular signals and intracellular second messengers, alters enzymatic structure and catalytic function.

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