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INVITED PAPER, SPECIAL SECTION IN HONOR OF MAX PERUTZ

Electrostatic effects in the control of glycogen phosphorylase by phosphorylation

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Abstract

Electrostatic effects are important in the initial activation mechanism of glycogen phosphorylase by phosphorylation. Analysis of the electrostatic surface potential of glycogen phosphorylase with the program GRASP shows that in the unphosphorylated state, the N-terminal 20 residues, which include a number of basic amino acids, are located close to a position on the surface of the molecule that is highly acidic. Upon phosphorylation by phosphorylase kinase at Ser 14, the N-terminal residues change their position and conformation so that the Ser-P is directed away from the acidic patch and to an intersubunit site where 2 arginines bind the phosphate. This recognition site is created through tertiary and quaternary structural changes that accompany the activation mechanism.

Keywords: activation; electrostatic effects; glycogen phosphorylase; phosphorylation; protein kinases; surface potential

Control by reversible phosphorylation is a ubiquitous mechanism by which intracellular events are linked to extracellular signals. Phosphorylation/dephosphorylation pathways inside the cell are initiated in response to growth factor, hormonal, or neuronal signals on the outside of the cell that lead to modifications of enzymes, linker molecules, and transcription factors; these have profound effects on diverse processes such as metabolism, growth, differentiation, motility, transport, learning, and memory. The responses can be elicited by a variety of molecular mechanisms. Phosphorylation can result in activation (as in glycogen phosphorylase) or inhibition (as in glycogen synthase). It can promote conformational changes that may be local or remote from the site of phosphorylation. It can act by steric blocking (as in isocitrate dehydrogenase) or by relief of steric blocking by the protein (as in the cyclin-dependent protein kinase Cdk2) or relief of autoinhibition by a pseudosubstrate domain in protein kinases (e.g., calmodulin-dependent protein kinase II). It can alter the surface properties of a protein that may affect self association or association of the protein with other molecules. Some of these structural mechanisms have been reviewed (Roach, 1990; Soderling, 1990; Stroud, 1991; Cohen, 1993; Johnson & Barford, 1993; Taylor & Radzio-Andzelm, 1994).

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How can these diverse effects be achieved by the covalent attachment of a phosphate group? The specificity of the kinases ensures that the site of attachment of a phosphate group is governed by the local amino acid sequence surrounding the phosphorylated residue and, in some systems, by the conformation of both the local peptide and the whole protein. The rather broader specificity of the phosphatases allows for different regulatory controls in the reverse reaction. A phosphate group has the property of conferring a double negative charge localized at a specific site. The dianionic character is a unique property that cannot be conferred by the naturally occurring amino acids. The phosphate group also has the properties of hydrogen bonding potential and steric bulk, but these properties are available from the amino acids. The results on the structural changes observed in glycogen phosphorylase upon phosphorylation suggest that the electrostatic properties of the phosphate group are of critical importance in the initial events of activation, although local changes in tertiary structure combined with larger changes in quaternary structure are also of importance for transmission of the activation signal. In this report we document these effects with the aid of the computer graphics program GRASP (Nicholls & Honig, 1991).

Glycogen phosphorylase is activated by phosphorylation on a single serine residue, Ser 14, by the action of phosphorylase kinase, a highly specific enzyme whose only established physiological substrate is glycogen phosphorylase but which will also phosphorylate other proteins such as glycogen synthase, the α

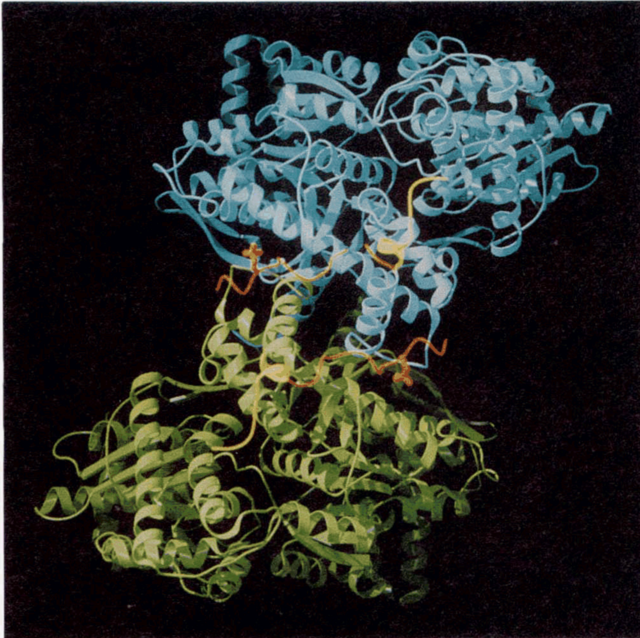


Fig. 1. Schematic representation of the phosphorylase *a* (GPa) dimer viewed down the 2-fold axis. The two subunits are colored green and blue. The N-terminal residues 14–25 for the nonphosphorylated phosphorylase *b* (GPb) are shown in yellow. The N-terminal phosphorylated residues 10–24 of GPa are shown in orange with the Ser 14(P) side chain displayed. Drawing by MOLSCRIPT (Kraulis, 1991).

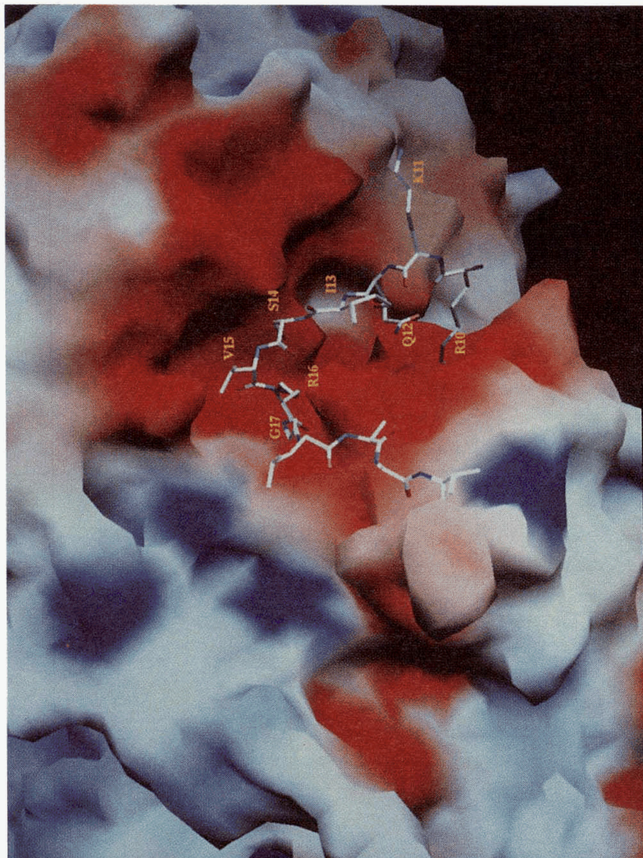
and β subunits of phosphorylase kinase itself, and troponin I in vitro. The structural changes upon conversion of inactive glycogen phosphorylase *b* (GPb) to active glycogen phosphorylase *a* (GPa) have been described (Sprang et al., 1988; Barford & Johnson, 1989; Barford et al., 1991) and reviewed (Johnson, 1992; Johnson & Barford, 1993). In brief, to a first approximation the enzyme can be viewed according to the Monod, Wyman, and Changeux model in which the enzyme exists in 2 states: a T state, which is less active and has low affinity for substrate, and an active R state, which has high affinity for substrate. In the inactive T state of GPb, N-terminal residues 10–20 (residues 1–10 are not located) are some of the least well-defined regions of the structure; their positions were first established in the complex of the enzyme with glucose, a T state inhibitor (Martin et al., 1990). These residues make intrasubunit contacts (Fig. 1). On activation to GPa there is a conformational change involving changes in the dihedral angles of the main chain in the region of residues 22 and 23 so that the N-terminal residues are directed through 120° and reach up to make intersubunit contacts (Fig. 1). The Ser-P docks between 2 arginine residues: Arg 69 from its own subunit and Arg 43′ from a symmetry-related subunit. Both of these arginines shift to make contact with the Ser-P, and these shifts together with other local conformational changes and a quaternary conformational change lead to a tightening of the subunit interface of the dimer. In GPb, C-terminal residues 836–842 are located in this intersubunit region and there is an intersubunit ion pair between Asp 838 and His 36′. Upon phosphorylation and localization of the N-terminal tail at this site, the C-terminal region is displaced. Thus, phosphorylation leads to ordering of the N-terminal tail and disordering of the C-terminal tail and concomitant other changes in structure.

The site of phosphorylation, Ser 14, is located in a sequence of residues, 10–16 (R.K.Q.I.S.V.R), that contains 3 basic and no acidic residues. This characteristic of the N-terminal tail was noted in 1959 by Fischer et al., who speculated that it may have some importance in the control mechanism. Figure 2A shows the surface of the GPb molecule without N-terminal residues 10–20 with the electrostatic potential displayed by the program GRASP; potentials corresponding to -7 kT are colored deep red and potentials of $+7$ kT are colored deep blue. It is apparent that there is a strong acidic patch on the protein surface caused by the proximity of a number of groups, including Glu 105, Asp 109, Glu 110, Glu 120, Glu 501, Glu 505, and Glu 509. The N-terminal tail is associated with this region (Fig. 2B). Ser 14 is directed inward and hydrogen bonds to Glu 501, and Arg 16 is directed to either Glu 105 or Asp 109 (Acharya et al., 1991). In order for these residues to become accessible to phosphorylase kinase, the N-terminal region would need to be displaced from the surface; indeed, those ligands that promote changes from the T state to the R state also make phosphorylase a better substrate for its kinase (Harris & Graves, 1990, and references therein).

In the R state, the surface charge potential is changed slightly by the tertiary and quaternary changes. The surface of the R state GPa without residues 10–20 is shown in Figure 2C. There is a basic patch at the subunit interface caused by displacement of the C-terminal tail and the movement of the 2 arginines to create the Ser-P recognition site. The location of the N-terminal peptide is shown in Figure 2D. The Ser-P forms ionic links with the 2 arginines. The adjacent nonpolar residues Ile 13 and Val 15 dock into nonpolar sites on the protein. Arg 16 is exposed to solvent. Arg 10 contacts the C-terminal end of an α-helix (Sprang et al., 1988; Barford et al., 1991).

Although the electrostatic interactions had been noted previously, the display of the electrostatic potential with GRASP provides an easily appreciated representation. The acidic environment encountered by the N-terminal tail in GPb makes a favorable location for the basic sequence of residues but an inhospitable environment for the phosphorylated residue. A conformational change is therefore obligatory, but it is only when the enzyme clicks to the R conformation that the basic recognition site for the Ser-P is created.

The importance of electrostatic effects in the activation mechanism was anticipated by the observation that sulfate ions at high concentration can activate GPb and produce concomitant R state properties such as enhanced affinity for AMP, reversal of glucose-6-P inhibition, and induction of enzyme tetramerization (Engers & Madsen, 1968; Sotiroudis et al., 1978; Leonidas et al., 1991). The crystal structure of GPb obtained from crystals grown in the presence of 1 M ammonium sulfate showed that sulfate ions could occupy the Ser-phosphate recognition site and induce ordering of the N-terminal tail (Barford & Johnson, 1989). Phosphorylase *b*′ (GPb′), the proteolytic fragment obtained from limited digestion with subtilisin that lacks the first 16 amino acids, cannot be activated by sulfate (Leonidas et al., 1991). These results indicate the importance of the N-terminal residues in the activation process, because GPb′ still contains all the basic residues for the dianionic recognition site (i.e., Arg 69 and Arg 43′). The amino acid sequence surrounding the phosphorylation site confers specificity of activation on the Ser-P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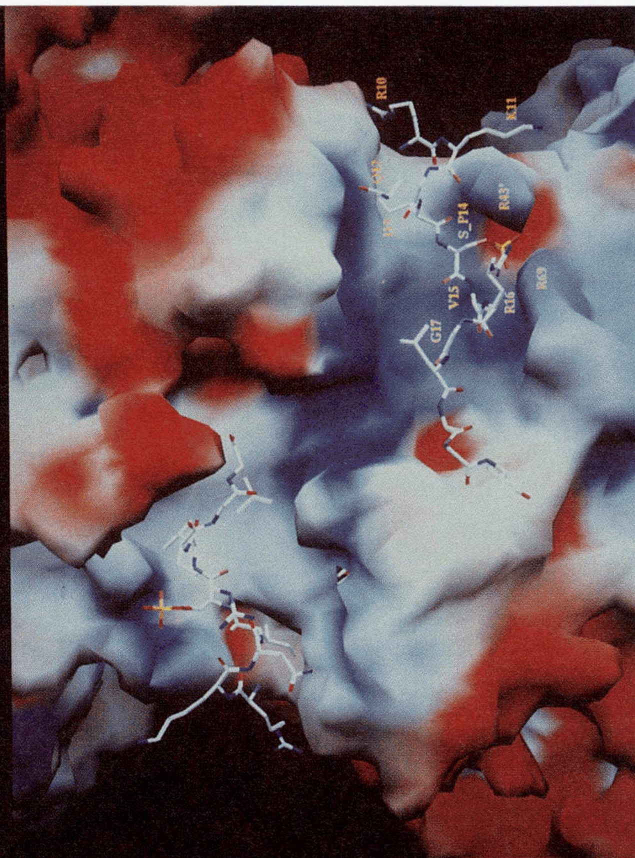
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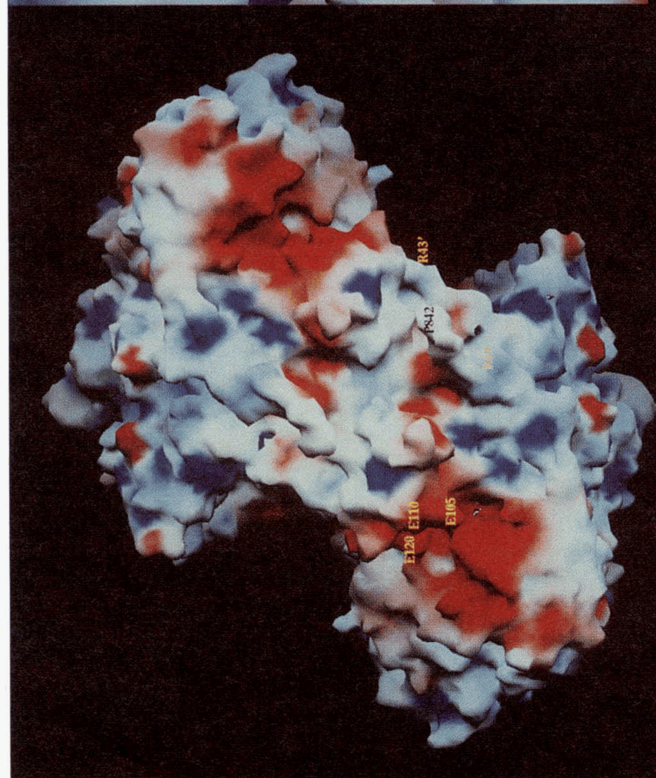
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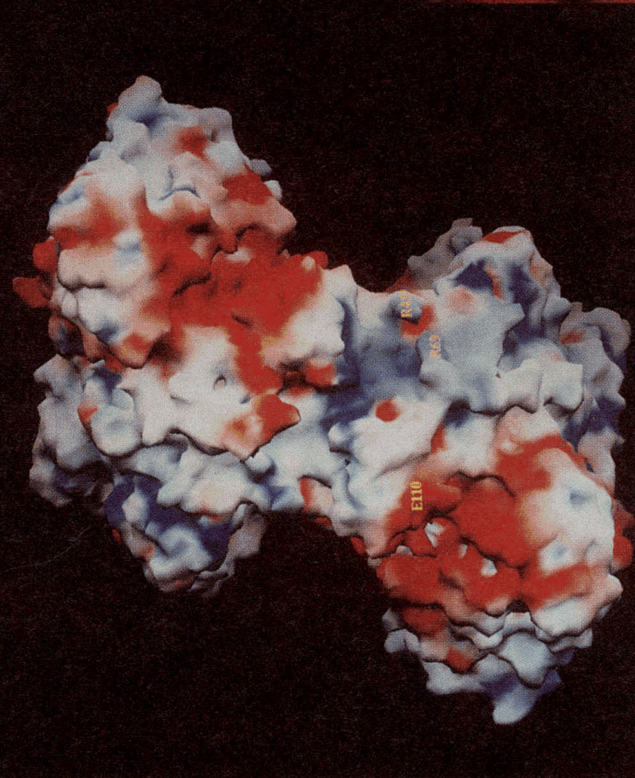
C
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residue. The development of bacterial expression systems for mammalian phosphorylases have allowed site-directed mutagenesis experiments to probe the roles of individual residues (Browner et al., 1991, 1994; Coats et al., 1991). Interestingly, the mutation Ser 14 → Asp does not result in activation (M.F. Browner & R.J. Fletterick, pers. comm.), a result that supports the notion that only a dianionic group is able to promote the conformational changes that lead to activation, although structural data are needed to test this. The Ser-P side chain is partially buried, suggesting that, similar to the requirement for phosphorylation, a displacement is required to allow access for dephosphorylation by the protein phosphatase.

How important are electrostatic effects likely to be in other mechanisms controlled by phosphorylation? The other enzyme system for which the structures are known in both the phosphorylated and nonphosphorylated state is isocitrate dehydrogenase. In this enzyme, phosphorylation acts by an electrostatic steric blocking mechanism that inhibits substrate binding with only small local conformational changes in the enzyme (Hurley et al., 1990; Stroud, 1991). The different contributions of the steric and electrostatic effects have been addressed by site-directed mutagenesis studies, which showed that electrostatic effects contributed between 2×10^5 and 7.5×10^5 to the total reduction of V_{max}/K_m of 10^9-fold (Dean & Koshland, 1990). Here, electrostatic effects are important but can be partially mimicked by substitution of a monoanion such as a glutamate residue. Additional inhibition is achieved by steric blocking.

A fascinating but tantalizing picture of the role of phosphorylation in control of protein kinase activity is emerging from the structural studies on 4 kinases. The structure of the basic archetype, cyclic AMP-dependent protein kinase, is known in the activated phosphorylated state (Knighton et al., 1991a, 1991b; Bossmeyer et al., 1993); the structures of 2 others, Cdk2 (De Bondt et al., 1993) and MAP kinase (Zhang et al., 1994), are known in the inactive nonphosphorylated states; and a fourth structure, twitchin kinase (Hu et al., 1994), does not require activation by phosphorylation but is inhibited by a pseudosubstrate intrasteric blocking mechanism. In the cyclic AMP-dependent protein kinase the phosphorylation of Thr 197 appears to contribute several effects in the activation of the kinase: it confers stability on the enzyme, it creates a recognition site for the regulatory subunit, it stabilizes interdomain contacts, and probably it helps to locate part of the substrate binding pocket in the correct position (Taylor & Radzio-Andzelm, 1994). In Cdk2 the loop that carries the phosphorylatable threonine (Thr 160) blocks the substrate binding site in the inactive conformation, and it has been surmised that phosphorylation will relieve this blocking. In contrast, in MAP kinase the equivalent loop is displaced significantly away from the catalytic site and the 2-domain structure is in an inactive open conformation. It is suggested that phosphorylation (at Thr 183 and Tyr 185) may promote closure of the domains and the correct location of the substrate recognition loop. In vivo control of these kinases by phosphorylation of residue(s) in the activation loop is a carefully regulated process, but other kinases (e.g., twitchin kinase and phosphorylase kinase) do not require this particular post-translational modification for activation although they also exhibit strict control by other mechanisms. Thus, the correct conformation of the activation loop can be achieved by the packing of the naturally occurring amino acids.

As first shown with hemoglobin (Perutz, 1970a, 1970b), electrostatic interactions between ionizable groups can play significant roles in the stabilization of different tertiary and quaternary conformational states and thereby in the regulation of macromolecules. Phosphate groups have the potential to provide enhanced electrostatic interactions not available to the naturally occurring amino acids. As shown with glycogen phosphorylase and isocitrate dehydrogenase, these are of critical importance in the regulatory mechanism but are not the only factors involved. The importance of electrostatic interactions for other phosphorylation control processes remains to be elucidated.

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Fig. 2 (*facing page*). Electrostatic surface potential on the phosphorylase dimer displayed with the program GRASP (Nicholls & Honig, 1991). The view is similar to Figure 1. Negative potentials (−7 kT) are displayed in deep red, and positive potentials (+7 kT) are displayed in deep blue. The positions of some side chains are indicated with the single-letter code. **A:** The GPb dimer without residues 10–20. The negative region created by Glu 105, Glu 110, Glu 120, and other residues is apparent. The C-terminal region represented by Pro 842 blocks the intersubunit site. Arg 43′ and Arg 69 are exposed. **B:** Close-up of the negative region of the upper subunit of GPb with the atomic positions for residues 10–20 superimposed. Ser 14 and Arg 16 are directed into a pocket on the enzyme surface. **C:** The GPa dimer without residues 10–20. The 2 arginines, Arg 43′ and Arg 69, have shifted to create a phosphate recognition site, and there are further changes in tertiary and quaternary structure from GPb. The C-terminal tail has been displaced. The view is slightly displaced from the view down the 2-fold axis. **D:** Close-up of the Ser 14(P) recognition site in GPa, with the atomic positions for residues 10–20 superimposed. The phosphate is sandwiched between the 2 arginines.

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