Distinct phosphorylation signals converge at the catalytic center in glycogen phosphorylases

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Background: Glycogen phosphorylases (GPs) catalyze the conversion of the storage form of carbohydrate (glycogen) to the readily usable form (glucose-1-phosphate) to provide cellular energy. Members of this enzyme family have evolved diverse regulatory mechanisms that control a conserved catalytic function. The mammalian and yeast GPs are expressed as inactive forms requiring phosphorylation for activation. Phosphorylation of yeast GP occurs at a distinct site from that of mammalian GP. This work addresses the structural basis by which distinct activation signals relay to the conserved catalytic site in yeast and mammalian GPs. Such knowledge may help understand the principles by which diverse biological regulation evolves.

Results: We have compared the crystal structures of the unphosphorylated and phosphorylated forms of yeast GP and propose a relay which links phosphorylation to enzyme activation. Structural components along the activation relay becomes more conserved within the GP family downstream along the relay, towards the catalytic center. Despite distinct upstream activation signals, a response element downstream of the relay leading to the catalytic center is conserved in all GPs. The response element consists of ten hydrophobic residues dispersed over two subunits of the homodimer. Phosphorylation induces hydrophobic condensation of these residues via structural rearrangement, which triggers conformation change of the active site GATE loop, leading to enzyme activation.

Conclusions: Members of the GP family with diverse activation mechanisms have evolved from a constitutively active ancestral enzyme which has the TOWER hydrophobic response element in the active position. Diverse regulation evolved as a result of evolutionary constraint on the downstream response element in the active state, coupled with flexibility and variability in elements of the upstream relays.

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Introduction

Reversible protein phosphorylation is the fundamental mechanism mediating cellular signal transduction. The hallmark of the mechanism is the ability to utilize a simple chemistry to perform a versatile regulatory function on target proteins. Lack of detailed structural and mechanistic knowledge, however, limits our understanding on how this mechanism works and how it has evolved to transduce signals. The mammalian and yeast glycogen phosphorylases (GPs) are two out of only four proteins for which the phosphorylation control mechanisms have been described through functional studies and determination of the unphosphorylated and phosphorylated structures of the same molecule. The other two examples are *Escherichia coli* isocitrate dehydrogenase [1,2] and cyclin-dependent protein kinase [3]. The pair of GP homologs provide a unique opportunity to examine the evolution and functional principles of reversible protein phosphorylation. Although both enzymes are activated by single-site phosphorylation, the targeted protein segments and the kinases involved are distinct. Mammalian GP is phosphorylated by a specific phosphorylase kinase at Ser14 [4]. Yeast GP, with a 39-residue extended N terminus relative to the mammalian homolog, is phosphorylated by a cyclic adenosine monophosphate-dependent protein kinase at a threonine located 24 residues towards the N terminus from the site in mammalian GP. Understanding how these distinct phosphorylation switches regulate a conserved catalytic site, and the principles by which they have evolved to do so, can serve as a paradigm for deciphering other protein phosphorylation control systems. One example is the family of protein kinases for which a sub-group requires phosphorylation of residue(s) within the activation loop for activity. Although the sequence and relative position of the phosphorylation site vary, phosphorylation invariably results in the correct alignment of the conserved catalytic residues [5,6].

GP catalyzes the conversion of glycogen to glucose-1-phosphate (Glc-1-P) to provide energy. In *Saccharomyces*

cerevisiae, the enzyme is simply regulated by two events: inhibition by the allosteric inhibitor glucose-6-phosphate (Glc-6-P) and activation by protein kinase catalyzed phosphorylation [7–9]. Phosphorylation of a threonine residue near the N terminus results in a local protein refolding event that activates the enzyme by promoting a global conformational change. Strikingly, the phosphorylated N-terminal segment refolds and, through its covalently linked phosphate, anchors at the allosteric Glc-6-P site within the dimer interface [10]. Thus, Glc-6-P inhibits the phosphorylated form of the enzyme by competing with the phosphorylated residue for binding at the allosteric site.

The structural basis by which phosphorylation initiates mammalian GP activation is distinct from that of yeast. Activation is initiated through binding of a phosphorylated serine residue (Ser14) within the dimer interface, 14 Å away from the allosteric site [11,12]. The allosteric site of mammalian GP has evolved to become a bifunctional switch, operated through the competitive binding of two mutually exclusive molecules. Binding of Glc-6-P at the allosteric site, as in the yeast enzyme, results in enzyme inhibition [13]. Alternatively, the same site can accommodate adenosine monophosphate (AMP), a feature unique to mammalian GPs, which acts either independently or synergistically with the phosphorylation of Ser14 to activate the enzyme [11,12,14,15]. The structural change upon enzyme activation involves subunit and domain movements and extensive exchange of interactions at the subunit and domain interfaces. The structural changes have been described in great detail, but the signaling pathway remains elusive. A complication in the analysis of the mammalian GP activation mechanism arises in the absence of glycogen, which allows activated mammalian GP dimers to form tetrameric structures, and as a result access to the catalytic site is obstructed [16–18]. All activated mammalian GP crystal structures determined so far are tetrameric, and do not define the active dimeric form.

An approach to assist identification of important components on the activation pathway is to examine the activation mechanisms of evolutionarily related GPs. Yeast GP has an overall 49% amino acid sequence identity with the mammalian enzyme and a unique N-terminal extension where the phosphorylation site resides. In contrast to the mammalian enzyme, activated yeast GP exists as a dimer even without glycogen [8,9]. Presumably, pathways of distinct activation signals of yeast and mammalian GPs converge at common features prior to turning on the conserved catalytic machinery.

We have previously compared the phosphorylation switch of the yeast GP with the phosphorylation and AMP control switches of the mammalian GP [10]. In this work, we analyze the structural relay linking the upstream phosphorylation switch to the downstream enzyme catalytic site of yeast GP, and compare it with the corresponding mechanism in the mammalian enzyme. Sequence comparison between the structural components of the relay and the corresponding regions of thirteen other GPs revealed that there is increasing conservation downstream of the relay. This observation provides insight into the evolution of the GP regulatory mechanism and how phosphoregulatory mechanism evolved to operate in general.

Results

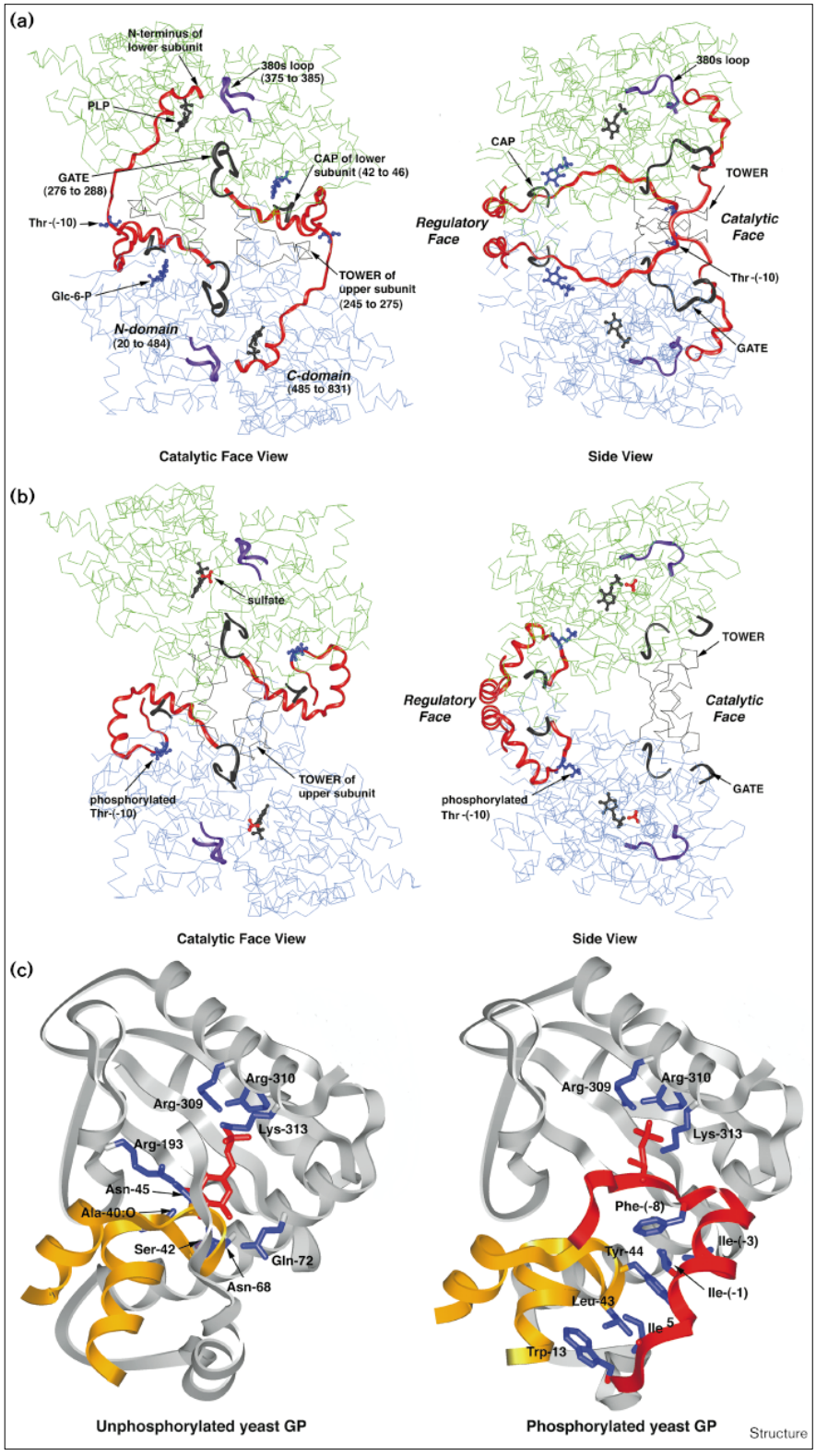
Enzyme allosteric site as an ON/OFF phosphorylation switch

The structures of the unphosphorylated (inactive) and phosphorylated (active) forms of yeast GP dimer are shown in Figures 1a and b, respectively. The overall domain and subunit architecture of the yeast GP is similar to that of the mammalian GP with the exception of the unique N-terminal 39-residue extension where the phosphorylation site, Thr(–10), resides (residues are numbered –1 to –39 towards the N terminus relative to the mammalian GP N terminus). The catalytic center is sandwiched in between the N-terminal and C-terminal domains of each subunit, with the catalytically essential cofactor pyridoxal phosphate (PLP) covalently linked to a lysine residue at the bottom of the catalytic cleft. Structural components which are important in the activation process are also highlighted in Figure 1. Other structures used for analysis and discussion are the sulfate activated mammalian GP [14] and glucose-6-P inhibited mammalian GP [13].

In the unphosphorylated structure, the subunits of yeast GP are restrained from movement by the N-terminal extension which wraps around from the regulatory face of one subunit to the catalytic face of the other subunit (Figure 1a), making numerous non-polar contacts along its path [19]. Phosphorylation of Thr(–10) induces withdrawal of the N terminus towards the regulatory face, which disrupts the contacts of the N terminus with three key elements that participate in linking the subunits or domains: the 380s loop, TOWER and CAP (defined in Figures 1a and b). Presumably, these N-terminal contacts are responsible for stabilizing the enzyme in an inactive conformation. Consistent with this hypothesis, a mutant GP with the N-terminal 42 residues deleted showed significant catalytic activity at high substrate concentration not observed in the native enzyme, suggesting a shift in conformational equilibrium towards the active state [9]. As will be described, structural components involved in these contacts need to adopt a different conformation for activation. The phosphorylated Thr(–10) moves 36 Å in space and becomes buried in the enzyme's allosteric site (Figure 1b), substituting Glc-6-P in the unphosphorylated state.

The comparison of the interactions at the allosteric site between the unphosphorylated (with Glc-6-P bound)

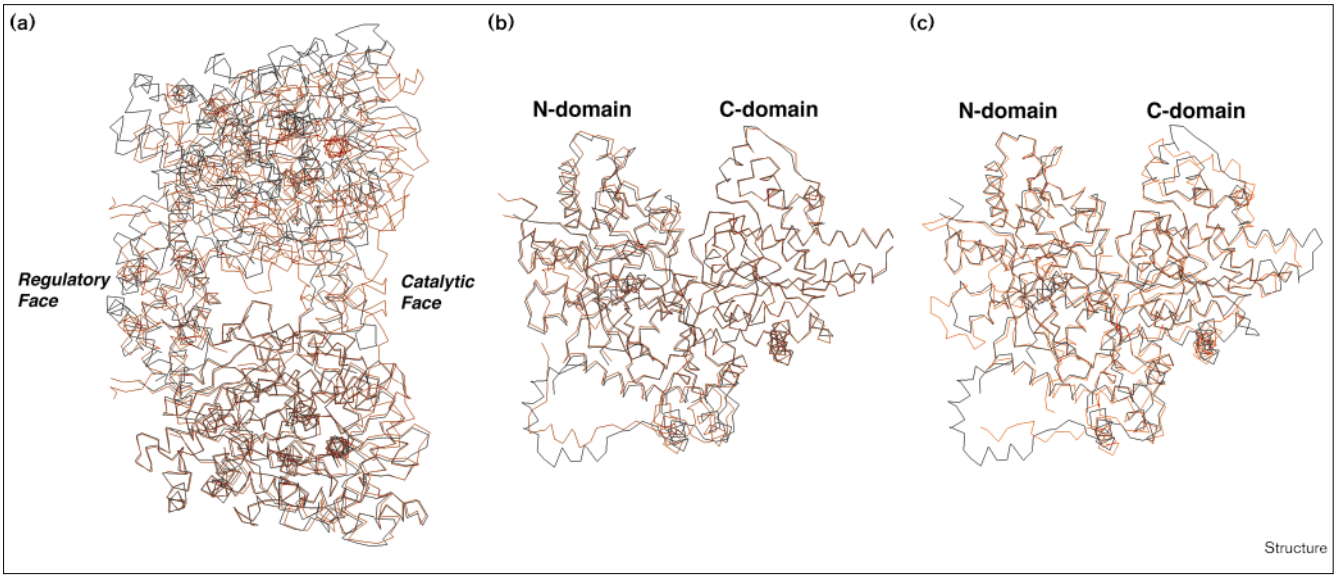
Figure 1



Enzyme allosteric site as an ON/OFF phosphorylation switch. **(a)** Structure of unphosphorylated (inactive) yeast GP dimer. The allosteric site is occupied by Glc-6-P. One subunit of the homodimer is colored green and the other blue. Catalytic face view and side view are related by a 90° rotation about a vertical axis. The GP dimer has been functionally described as having two 'faces': the regulatory face, at which the allosteric effectors and the phosphorylated N termini bind, and the catalytic face, on the opposite side, where the substrates enter. The 39-residue N-terminal extension that stretches from the regulatory face of one subunit to the catalytic face of the other subunit is shown in red. **(b)** Structure of phosphorylated (active) yeast GP dimer with the N-terminal 22 residues deleted. The mutant has similar kinetic properties as the full-length form. The allosteric site is occupied by the refolded N-terminal segment. The discontinuous break within the GATE loop of the phosphorylated GP is due to disordering of residues 282 and 283. **(c)** Close-up view of the allosteric site, comparing unphosphorylated and phosphorylated states. The CAP loop is colored orange and the neighbouring subunit is colored gray. The 'effector' molecules, Glc-6-P in the unphosphorylated structure and the phosphorylated N-terminal segment in the phosphorylated structure, are colored red. Sidechains are colored blue.

and the phosphorylated structure (with the phosphorylated N terminus bound) is shown in Figure 1c. In the unphosphorylated structure, Glc-6-P helps stabilize the inactive conformation of the enzyme by reinforcing subunit

Figure 2



Subunit and domain movements. **(a)** Phosphorylation induces subunit separation in yeast GP. The lower subunits of the unphosphorylated (red trace) and phosphorylated (black trace) yeast GP dimer are superimposed, shown in side view. **(b)** Phosphorylation does not induce large inter-domain movement in yeast GP. The C-terminal domains of the unphosphorylated (brown trace) and phosphorylated (black trace) yeast GP subunit are superimposed. **(c)** Domain-to-domain separations are similar between sulfate-activated mammalian GP and phosphorylated yeast GP. The C-terminal domains of the ammonium sulfate activated mammalian GP (red trace) and phosphorylated yeast GP (black trace) subunit are superimposed.

contacts through extensive hydrogen-bond interactions [20]. Substitution of the bulkier phosphorylated N terminus at the allosteric site expands the site by pushing the CAP region of one subunit away from the other subunit. The phosphoryl group functions as the register. In both structures, interactions at the phosphate-binding subsite (with Arg309, Arg310 and Lys313) are essentially the same and presumably are not responsible for the different regulatory responses between the binding of Glc-6-P and the phosphorylated N terminus. The specific hydrogen-bond interactions formed by the glucose portion of Glc-6-P and the hydrophobic sidechains of the phosphorylated N-terminal segment at the allosteric site are responsible for the distinct regulatory responses observed.

The hydrophobic sidechains involved in the activation of the yeast GP are not conserved in the GP family. This observation, plus the distinct phosphorylation switch of the mammalian GP, suggest that different family members have evolved distinct structural motifs for initiation of activation. The enzyme of *Dictyostelium* is also activated by phosphorylation and possesses an even longer N-terminal extension, in which the phosphorylation site may reside. Although the site(s) of phosphorylation has not yet been determined, sequence information indicates a phosphorylation mechanism distinct from those of the mammalian and yeast GPs. How do different phosphorylation signals arrive at the conserved catalytic site?

Phosphorylation induces quaternary structural changes

The phosphorylated N-terminal segment refolds into a wedge at the dimer interface and forces the subunits of the yeast GP to move apart. The comparison of the subunit-to-subunit separation between the Glc-6-P inhibited structure and phosphorylation activated structure is shown in Figure 2a. There is a progressive increase in subunit-to-subunit separation from the regulatory face to the catalytic face. The distance between the centers of mass of the subunits increases by 3.4 Å upon activation. The dimer interface contact area decreases from 5200 $Å^2$ in the unphosphorylated structure to 3300 $Å^2$ in the phosphorylated structure.

Activation of mammalian GP also correlates with a similar increase in subunit separation. Unlike yeast GP, in which wedging of the phosphorylated N terminus on the regulatory face causes a greater subunit separation on the catalytic face, the mammalian subunits are drawn together on the regulatory face upon binding of the phosphorylated N terminus to trigger a subunit separation on the catalytic face [12,15].

There is a negligible change in the relative position of the N- and C-terminal domains upon activation of yeast GP (Figure 2b). In contrast, mammalian GP exhibits N- and C-terminal domain separation upon activation which widens the catalytic cleft for the substrates to enter [21–23].

Superposition of the sulfate-activated mammalian GP and phosphorylated yeast GP reveals that their domain-to-domain separations are similar (Figure 2c), indicating that the relatively static domains of yeast GP are already in the 'open' conformation before phosphorylation.

A relay links the upstream phosphorylation switch to the downstream catalytic site

The large subunit displacement upon activation of yeast GP is brought about mainly as a result of rigid-body movement of one subunit relative to another. Several surface structures undergo flexible rearrangement to stabilize the rigid subunit movement (refer to Figure 1). Firstly, the 380s loop, located near the active site entrance, moves away from the active site to provide room for the substrate to enter. Secondly, the N-terminal extension refolds and wedges into the subunit interface upon phosphorylation. Thirdly, the CAP loop, which forms part of the allosteric site, moves in order to accommodate the bulky phosphorylated N terminus. Fourthly, the TOWER reorients and points towards the catalytic center of the symmetry-related subunit upon phosphorylation. Finally, in the active site GATE loop, the sidechain of Tyr280 flips towards the catalytic center. The proposed sequence of events which lead to these structural rearrangements is described below. Excluding these flexible structures from calculation, the root mean square deviation of Cα position between the superimposed unphosphorylated and phosphorylated subunit is 0.7 Å, indicating an essentially rigid body.

The locations of these structural elements which undergo rearrangement upon activation provide a clue as to how the phosphorylation signal progresses. An activation relay which accounts for all the structural movements observed is shown in Figure 3. Firstly, phosphorylation-induced refolding of the N terminus disrupts the contact between the 380s loop and the N terminus, and thus allows the 380s loop to move away from the active site. Secondly, the 'activation switch' is turned on by the phosphorylated N terminus as a result of its refolding and wedging into the allosteric site, thereby pushing the subunits apart through the CAP interactions. Thirdly, the signal is then transmitted through the distorted dimer interface towards the catalytic face, where the symmetry-related TOWERs respond by rearranging their positions in order to stabilize the new dimer interface. Finally, the TOWER rearrangement then induces the active site GATE loop to assume the active conformation. The structural elements along this activation pathway are analyzed in the following sections.

Protein dimer interface transduces signal of phosphorylation

The dimer interface of yeast GP may function as a dual molecular imprint, evolved to recognize two distinct ways of complimentary packing between the neighboring subunits — one arrangement stabilizes the inactive state and the other stabilizes the active state. The allosteric site, located within the dimer interface, then functions as a 'switch' between these two packing modes. It is likely that a similar strategy was employed by mammalian and other regulated GPs during evolution to transmit the regulatory signals from the regulatory face to the catalytic face. Comparison of the dimer interfaces from GP homologs reveals important mechanistic information.

Figure 3

Schematic presentation of phosphorylation initiated activation relay in yeast GP. Side views of the dimer, corresponding to those in Figures 1a and b, are shown. The sequence of events after phosphorylation can be described in five steps. Firstly, disruption of the contacts of the 380s loop, TOWER and CAP with the extended N-terminal extension. Secondly, refolding and wedging of the phosphorylated N-terminal segment in the allosteric site. Thirdly, transmission of the activation signal across the dimer interface. Fourthly, rearrangement of the TOWER. Finally, activation of catalytic site. (The five events are numbered sequentially on the figure.)

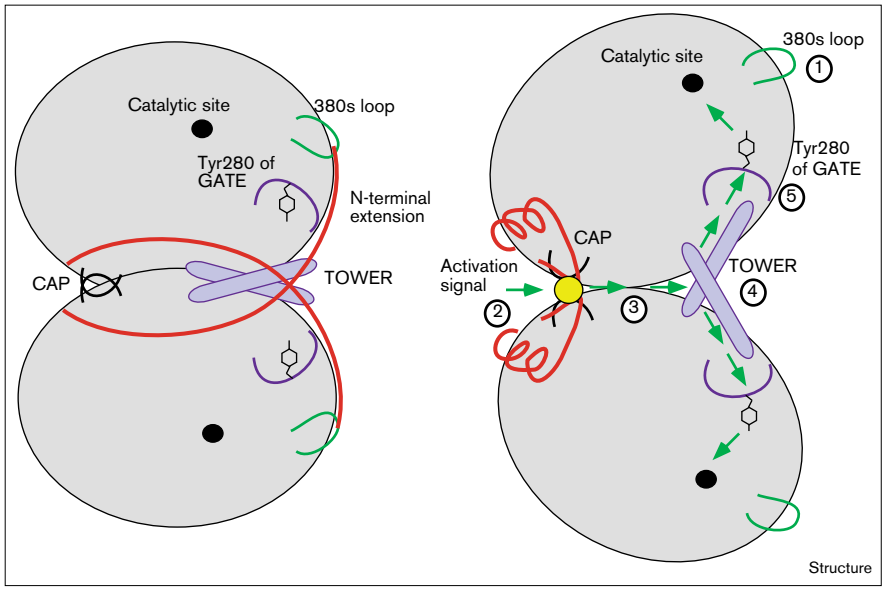
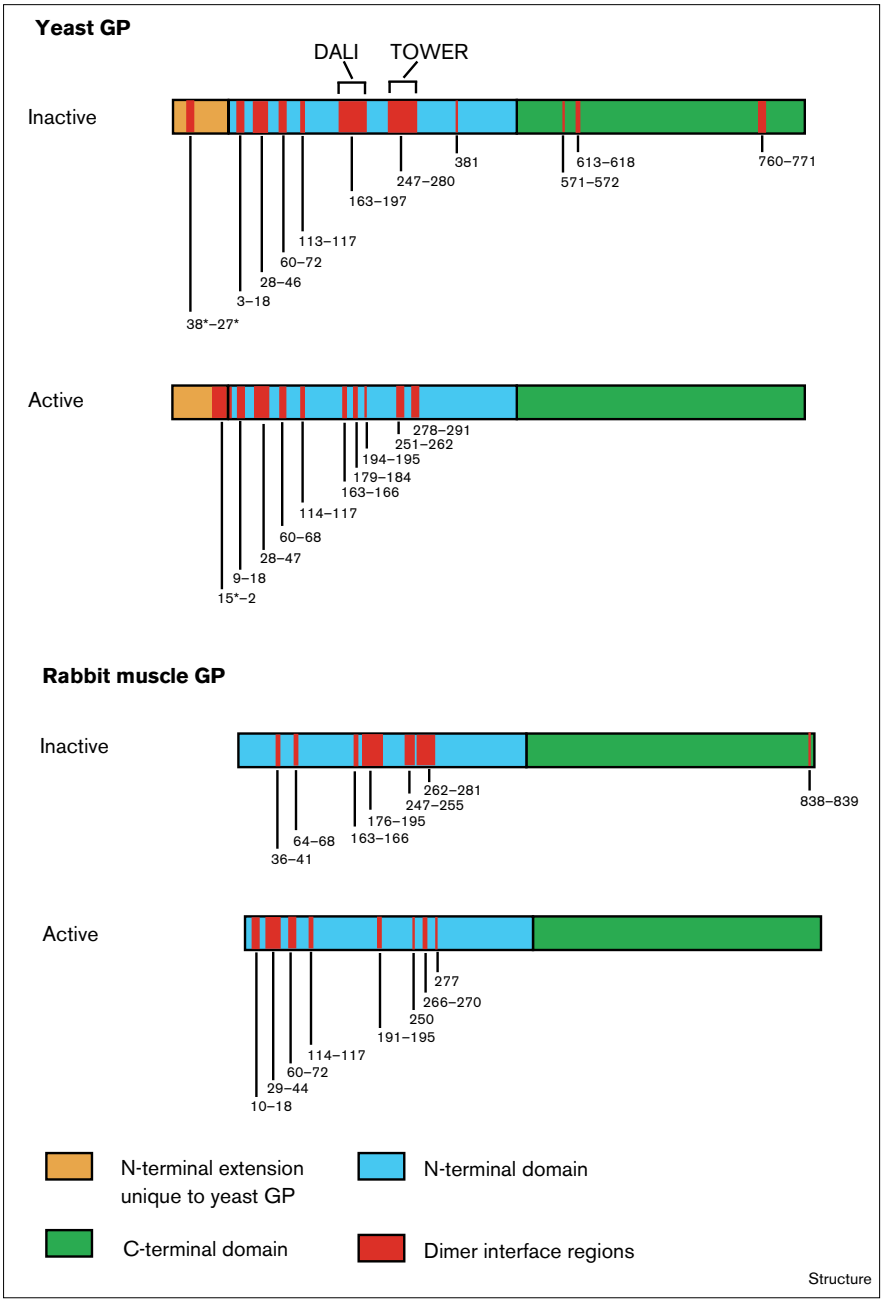


Figure 4



Comparison of the subunit-to-subunit contacts in the active and inactive states of the yeast and mammalian GPs. The dimer interface region includes all residues within 4 Å of the neighbouring subunit.

The subunit-to-subunit contact in yeast GP, before and after activation, is mapped onto its linear sequence in Figure 4. The corresponding change in the rabbit muscle enzyme is also shown. In both enzymes, the dimer interface is located mainly between the N-terminal domains. Although there are similar regional patterns of involvement in the dimer interface between the two enzymes, the identity of the residues involved are poorly conserved. In both the yeast and mammalian enzyme, activation results in a decrease in the dimer-interface area and therefore in the number of contacting residues within the DALI (residues 165–195) and TOWER regions. The DALI loop can be considered as 'rest platform' for the TOWER of the symmetry-related subunit in the inactive states of both enzymes, stabilizing the symmetry-related TOWER in the inactive position. Activation results in major disruption of TOWER–DALI interactions, allowing the TOWERS to assume the activated position. The DALI loop does not undergo structural rearrangement but moves as a rigid body with the rest of the subunit

structure. Moreover, activation of rabbit muscle enzyme results in a significant increase in the subunit contact area within the N-terminal 120 residues. The two effects together results in greater similarity in the subunit-contact pattern between the active states than between the inactive states of the two enzymes, indicating a convergence of their activation pathways. Only one hydrogen-bond interaction at the subunit interface, which is between the sidechain of Arg60 and the backbone of residue 37, is conserved in both the active states of yeast and mammalian GP. As Arg60 is conserved in all GPs sequenced so far, this hydrogen-bond interaction might be a key contribution to the registration of the subunit position in the active state.

The analysis indicates that distinct activation signals of yeast and mammalian GPs begin to converge at the level of subunit positioning. It is possible that refined adjustment of the active-state constellation occurs downstream of the subunit positioning to generate a precise catalytic center. The freedom from constraint on the specific subunit-to-subunit interactions during evolution may be an important criterion for the development of versatile control features in GPs, as it provides the flexibility for a variety of ligands and/or phosphopeptides to be recruited as useful effectors within the subunit interface.

Distinct activation signals of GP family converge at hydrophobic TOWER–GATE interactions

Refolding and wedging of the N-terminal segment on the regulatory face is recapitulated on the catalytic face where another dramatic and extensive structural movement occurs upon yeast GP phosphorylation. The symmetry-related TOWERs disengage from their inactive position and rotate towards the catalytic site of the neighbouring subunit (Figure 1). In this process, TOWER interactions specific to the inactive state are broken, and new interactions specific to the active state are established. Because the TOWERs are located on the catalytic face of the enzyme, their rearrangement in response to the wedging of the phosphorylated N terminus on the regulatory face should directly influence the catalytic activity of the enzyme. To determine which components of the TOWER rearrangement play a dominant role in activation, TOWER interactions specific to the inactive and active states were analyzed. Residues involved in this subset of interactions have been checked for conservation among thirteen other known GP sequences. The assumption is that residues involved in interactions which are generally important for GP activation should be more conserved during evolution.

Firstly, packing of the TOWER was analyzed. Table 1 summarizes the residues that are within van der Waals contact distance with the TOWER and are unique to the active or inactive state, together with their occurrences in other GPs. The result indicates that the active-state environment of the TOWER is more conserved among GPs than the inactive state environment. Strictly conserved residues are Tyr280, involved in the inactive state contact with the TOWER, and Leu291, Phe166 and Gly288 which form contacts with the TOWER in the active state. The dramatic structural rearrangement of the TOWER coupled with the simultaneous increase in the conservation of the TOWER environment upon activation strongly suggest that distinct activation signals converge at the TOWER structure in all members of the GP family. The lack of constrain of the TOWER environment in the inactive state may provide the flexibility for distinct upstream activation triggers to evolve.

Table 1

The TOWER environment in the active state is more conserved than that in the inactive state.

Unphosphorylated state		Phosphorylated state	
Residue*	Occurrences†	Residue*	Occurrences†
Ala(–11)	0/13		
Thr(–10)	0/13	Ile165′	7/13
Gly(–9)	0/13	Phe166′	13/13
Phe(–8)	0/13	Phe285′	7/13
Glu162	7/13	Gly288′	13/13
Tyr280	13/13	Leu291′	13/13
Ile171′	0/13	Pro611′	10/13
Val176′	6/13		
Asn184′	1/13		
Gln270′	1/13		
Ala277′	1/13		

*Residue number followed by a prime indicates that the residue is from the neighbouring subunit. †Occurrences in thirteen GP sequences other than yeast GP [24].

Table 2 summarizes an analysis of specific hydrogen-bond interactions of the TOWER, listing only those unique to the active or inactive state, and the occurrence frequency of the involved residues in other GPs. The result shows that none of the residues involved in hydrogen-bond interactions is highly conserved. Although most of the hydrogen-bond interactions unique to the inactive state are between the TOWER and the surrounding residues, most of the hydrogen-bond interactions unique to the active state link residues within the TOWER, which probably serve to strengthen the TOWER structure. The result predicts that hydrogen-bond interactions of the TOWER will be different in other members of the GP family, and that they have evolved independently to stabilize the inactive and active conformation of the enzyme.

Table 2

Non-conservation of the hydrogen bonds associated with the TOWER region which are unique in either the phosphorylated or the unphosphorylated state.

Unphosphorylated State				Phosphorylated State			
H-bond donor*	Occurrences†	H-bond acceptor*	Occurrences†	H-bond donor*	Occurrences†	H-bond acceptor*	Occurrences†
220:NH2	0/13	251:OE1	3/13	250:N		268:OE1	1/13
254:N		252:O		269:NE2	1/13	251:O	
255:N		–10:O		163:OH′	7/13	251:OE1	3/13
262:OH	11/13	177:O′	253:N		265:O		
268:NE2	1/13	265:OG	0/13	262:N		260:O	
271:NH1	2/13	268:OE1	1/13	264:N		261:O	
181:OH′	2/13	269:OE1	1/13	265:N		261:O	
247:NH1	3/13	184:O′		265:OG	0/13	262:O	
273:OE2	12/13	163:OH′	7/13	270:N		267:O	
				271:N		268:O	

*Residue number followed by a prime indicates that the residue is from the neighbouring subunit. †Occurrences in thirteen GP sequences other than yeast GP [24].

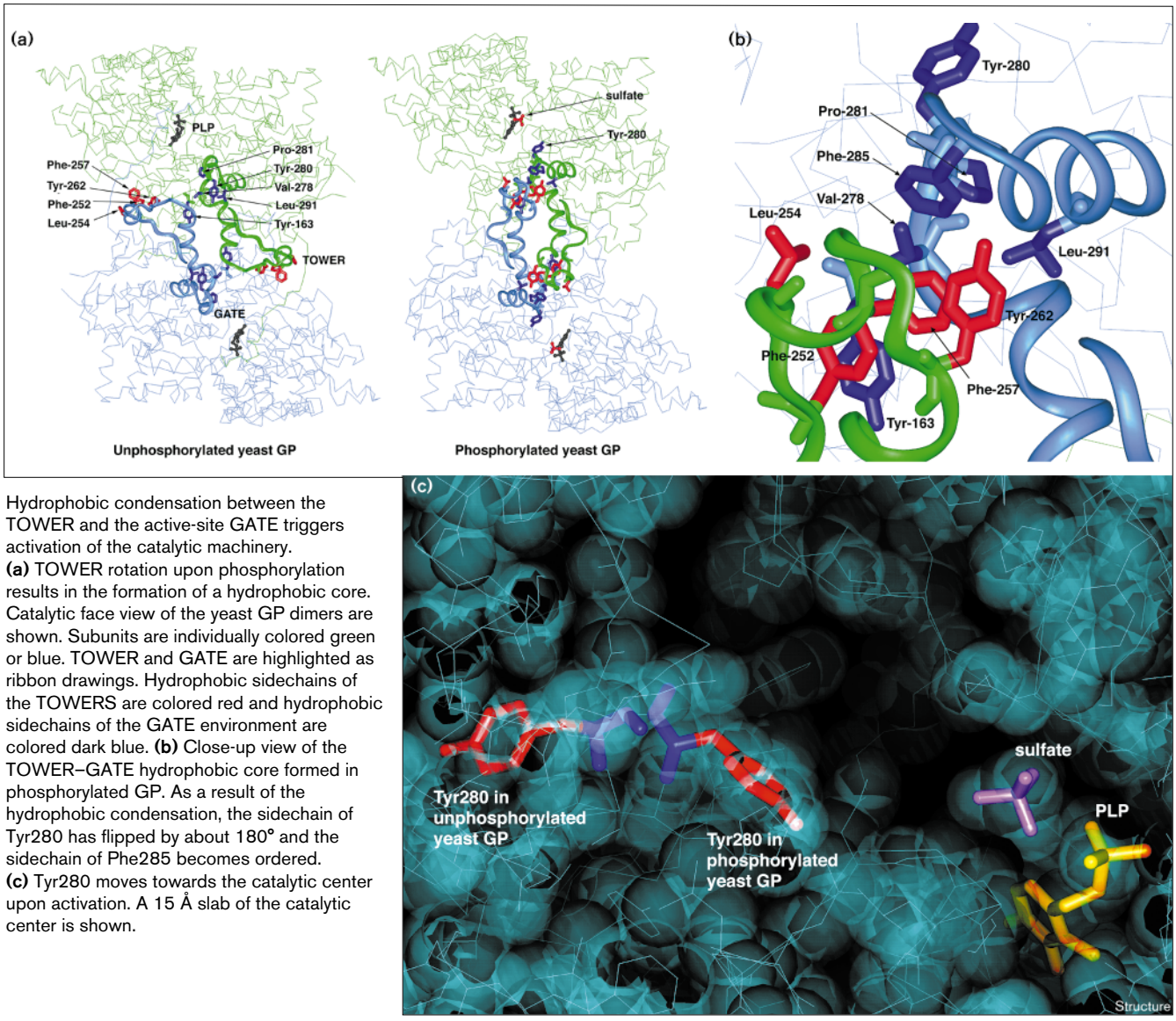
Finally, hydrophobic residues within the TOWER and their environment in the active and inactive structures were examined. The analysis revealed a hydrophobic core, unique to the active state, formed between the residues of the TOWER (Phe252, Leu254, Phe257 and Tyr262) of one subunit and residues near the catalytic cleft of the symmetry-related subunit (Tyr163, Val278, Try280, Pro281, Phe285 and Leu291) (Figures 5a and b). Conservation of these hydrophobic residues among other GPs is summarized in Table 3. High conservation of these residues and their interactions upon activation suggest an important function of hydrophobic packing. Intriguingly, the sidechain of Tyr280, a strictly conserved residue on the active site GATE loop, flips by approximately 180° and points towards the catalytic center upon the hydrophobic condensation (Figure 5c). Also, Asn282 and Asp283 of the GATE become disordered accompanying the rotation of Tyr280. The hydrophobic condensation also results in the disorder-to-order transition of Tyr285, which becomes part of the hydrophobic core near the active site in the phosphorylated structure (Figure 5b). Not all GP homologs listed in Table 3 are allosterically regulated, for example, *E. coli* maltodextrin phosphorylase is constitutively active. The conservation of the hydrophobic TOWER residues in this *E. coli* enzyme suggests that other allosterically regulated forms evolve from a constitutively active ancestral form which has the TOWER stabilized only in the active state. Probably, allosteric regulation evolves through a mechanism that allows the TOWER to move away from its active position, responding to binding of an allosteric inhibitor, and back to the active position upon activator binding.

One caveat is that it is often difficult to deconvolute residue conservation resulting from participation in regulation from conservation that would be due to the strict structural requirements associated with an active site. In fact, the conserved hydrophobic response element described above in GPs represents a mechanism that links regulation to catalysis, as activation involves bringing two halves of the response element (the TOWER and the GATE) together through hydrophobic interactions to complete the active site. High conservation of the TOWER hydrophobic residues cannot be explained merely by their proximity to the active site, as residues involved in hydrogen-bond interactions with the TOWER, also close to the active site, are poorly conserved.

Activation of the catalytic machinery

In vitro, GPs catalyze the reaction: $glucose_n$ + Pi ↔ $glucose_{n-1}$ + Glc-1-P, in either direction depending on the whether inorganic phosphate (Pi) or Glc-1-P is provided as substrate. Although 20 mM Glc-1-P is present in the crystallization conditions, electron density consistent with a sulfate ion (a mimic of a phosphate ion) was found in the substrate-binding site of phosphorylated yeast GP near the PLP cofactor, presumably due to the high concentration of ammonium sulfate in the crystallization conditions. Not surprisingly, the relative positions of the sulfate and PLP phosphate is the same as that found in the sulfate activated mammalian GP, as both enzymes utilize the same catalytic mechanism [14]. Interestingly, at a similar ammonium sulfate concentration, sulfate ion was not found in the catalytic site of the unphosphorylated yeast GP [19]. Examination of the structure indicates that the entrance to

Figure 5



Hydrophobic condensation between the TOWER and the active-site GATE triggers activation of the catalytic machinery. **(a)** TOWER rotation upon phosphorylation results in the formation of a hydrophobic core. Catalytic face view of the yeast GP dimers are shown. Subunits are individually colored green or blue. TOWER and GATE are highlighted as ribbon drawings. Hydrophobic sidechains of the TOWERS are colored red and hydrophobic sidechains of the GATE environment are colored dark blue. **(b)** Close-up view of the TOWER–GATE hydrophobic core formed in phosphorylated GP. As a result of the hydrophobic condensation, the sidechain of Tyr280 has flipped by about 180° and the sidechain of Phe285 becomes ordered. **(c)** Tyr280 moves towards the catalytic center upon activation. A 15 Å slab of the catalytic center is shown.

the catalytic site is blocked in the unphosphorylated structure as a result of the interaction between the 380s loop and the N terminus which constrains the 380s loop, so that it is directed towards the active center (Figure 6a). Also, in the unphosphorylated structure, the sidechain of Arg569 extends over the substrate-binding site to create a further block (Figure 6a). In the activated yeast GP, Arg569 rearranges and becomes part of the high affinity sulfate-binding pocket. These rearrangements upon activation show why the active site of the unphosphorylated enzyme does not bind substrate.

The comparison of the active-site constellations of the unphosphorylated (no substrate bound) and phosphorylated (with sulfate bound) yeast GP is shown in Figure 6b. Three major changes occur in the active site as a result of activation. Firstly, the sidechain of Arg569 rearranges to bind the sulfate ion. This basic residue is strictly conserved among all GPs. The importance of this residue in substrate binding is further reinforced by the crystal structure of the activated rabbit muscle GP, which reveals similar function of Arg569 [14]. Secondly, rotation of the backbone of the GATE loop puts the sidechain of Tyr280 into the active site. Despite strict conservation of Tyr280 and its articulate movement into the active site upon yeast GP activation (see also Figure 5c), the corresponding change in the position of Tyr280 has not yet been observed upon activation of the rabbit muscle GP. Examination of the muscle GP structure suggests that tetrameric association of the activated mammalian GP accounts for this difference. Unlike yeast GP

Table 3

Conservation of hydrophobic residues in the TOWER region environment.

GP homologs*	Hydrophobic residues†									
Yeast	Tyr163	Phe252	Leu254	Phe257	Tyr262	Val278	Tyr280	Pro281	Phe285	Leu291
Rabbit muscle	Phe	•	•	•	•	•	•	•	•	•
Rat muscle	Phe	•	•	•	•	•	•	•	•	•
Human muscle	Phe	•	•	•	•	•	•	•	•	•
Rat brain	Phe	•	•	•	•	•	•	•	•	•
Human brain	Phe	•	•	•	•	•	•	•	•	•
Rat liver	•	•	•	•	•	•	•	•	•	•
Human liver	•	•	•	•	•	•	•	•	•	•
Dictyostelium I‡	•	•	•	•	•	•	•	•	Thr	•
Dictyostelium II‡	Phe	•	•	•	•	•	•	•	Thr	•
Potato H§	•	•	•	•	•	•	•	•	Thr	•
Potato L§	•	•	•	•	His	•	•	•	Ser	•
E. coli	•	•	•	•	•	•	•	•	Thr	•
E. coli MP#	•	•	•	•	Phe	•	•	•	His	•

*Sequence information obtained from a previous publication [24]. †Filled circle indicates identity. ‡*Dictyostelium* has two GP isozymes, type I and II. §Potato has two GP isozymes, type H and L. #*E. coli* maltodextrin phosphorylase.

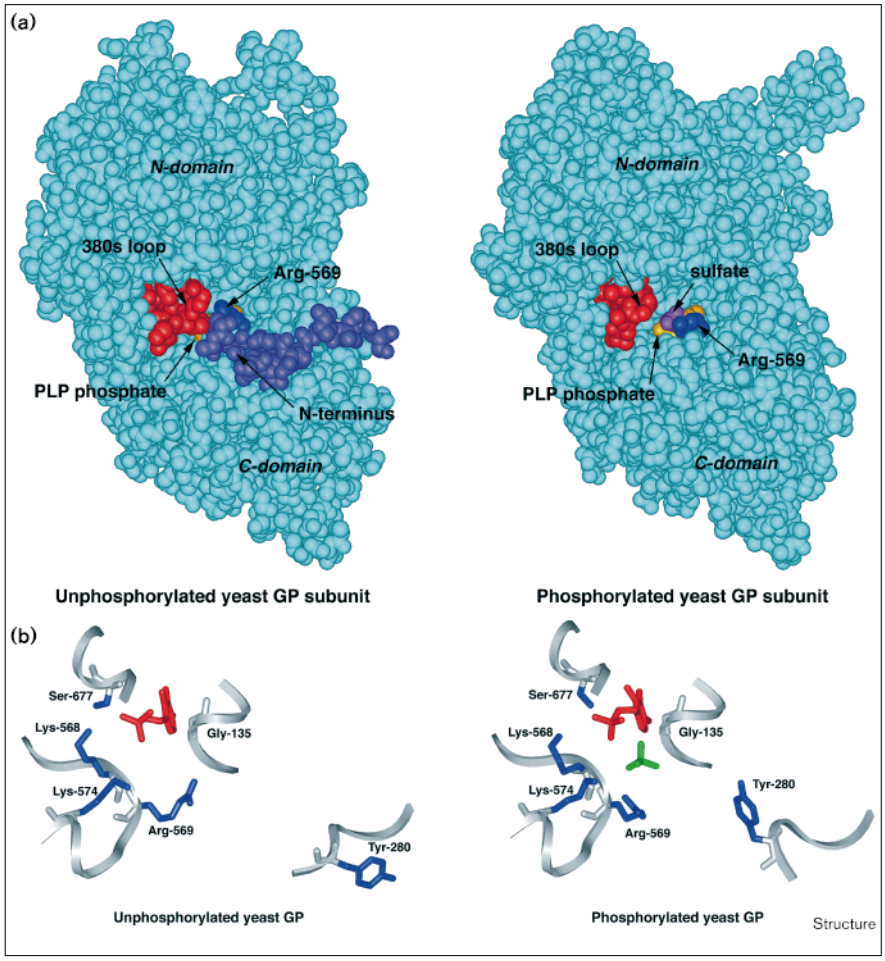
which exists as a functional dimer when activated, mammalian GP forms a tetrameric structure from a pair of functional dimers. The TOWERs on the catalytic face are involved in the dimer-to-dimer contacts within the tetramer interface [18], which may prevent them from reaching the fully activated position. *In vivo*, anchoring of the enzyme to glycogen through the catalytic face maintains the activated rabbit muscle GP as a dimer and prevents tetramerization. The tetrameric structures of mammalian GP therefore do not represent truly active state. The exact role of Tyr280 in GP catalysis remains to be elucidated. Although there is an empty pocket next to Tyr280 in the activated yeast GP (Figure 5c), the structures of the rabbit muscle enzyme suggest that this pocket does not bind Glc-1-P or Pi. Whether this pocket accommodates part of the polymeric glucose chain of glycogen remains to be determined. Finally, the sidechain of Ser677 rotates about the Cα–Cβ bond and forms a hydrogen bond with the PLP phosphate. Ser677 is not conserved among GPs; in all mammalian GPs and *E. coli* maltodextrin phosphorylase, a glycine is located at this position. Crystal structure of the rabbit muscle GP revealed no significant function for Gly677; therefore, interaction of Ser677 with PLP phosphate upon activation of yeast GP may play an ancillary role, such as modification of catalytic rate.

Discussion

A new theory explaining how distinct activation mechanisms of the GP family converge to turn on the conserved catalytic site is presented. The study reveals that distinct activation signals are transmitted across the protein subunit interface to a conserved hydrophobic response element leading to the catalytic site. In the inactive state, this conserved element consists of ten hydrophobic residues dispersed over two subunits of the homodimer on the catalytic face. Phosphorylation induces hydrophobic condensation of these residues via structural rearrangement, leading to enzyme activation. Structural components along the relay, which link phosphorylation to enzyme activation, in yeast GP becomes increasingly conserved downstream towards the catalytic center. This observation suggests that diverse regulation in the GP family came about as a result of the evolutionary constraint on the downstream hydrophobic response element, coupled with flexibility and variability in elements of the upstream relays. The mechanism by which diverse regulation in the GP family evolved can serve as a model for understanding how other diverse biological controls have evolved.

Cellular signaling pathways are typically played out with phosphorylation controlling a set of modular protein components, which link upstream receptor molecules to downstream working molecules through protein–protein relays. The phosphorylation control mechanism of GP is reminiscent of this scenario, with phosphorylation initiating an intramolecular relay of conformational effects culminating in catalytic activation. Thus, the molecule represents a complete signaling system, with its own submolecular

Figure 6



Activation of catalytic site. **(a)** In unphosphorylated yeast GP, the entrance to the catalytic site is blocked through interaction between 380s loop and N-terminal extension. In the activated yeast GP, Arg569 rearranges and becomes part of the sulfate-binding pocket. Space-filling presentation of one subunit is shown. **(b)** Comparison of the catalytic-site constellation between the unphosphorylated and phosphorylated yeast GP. The cofactor PLP is colored red. Active site substrate mimic, sulfate, is colored green. Sidechains are colored blue.

components in the roles of ligand (phosphorylated N terminus), receptor (allosteric site), transducer (dimer interface, TOWER and GATE) and working site (catalytic site).

The analysis and comparison of yeast and mammalian GP structures presented in this study now enable us to define a cascade of coordinated events that activate yeast GP. The initial phosphorylation of Thr(–10), located in a non-polar environment, expels the N terminus from the catalytic cleft of the symmetry-related subunit. As a result, the previous contacts of the 380s loop, TOWER and CAP with the N terminus are disrupted, enabling these regions to assume their active-state conformation in the succeeding events. The phosphorylated N terminus refolds compactly and wedges into the allosteric site within the dimer interface, forcing the subunits to separate. New interactions between the subunits are generated and stabilized through an alternative packing of the dimer interface and reorientation of the symmetry-related TOWERs, which in addition trigger the conformational change of the active site GATE loop of the neighbouring subunit. The final event of the cascade involves the docking of conserved hydrophobic residues between the TOWER and the active-site GATE, which induces rotation of the Tyr280 sidechain into the substrate-binding site to complete the activation process.

The distinct phosphorylation control mechanisms of yeast and mammalian GPs embed records of their evolutionary history. The allosteric site of both enzymes, evolved originally to bind the inhibitor Glc-6-P [24], has taken distinct paths in the evolution of activation switches. In mammalian GP, the allosteric site has evolved to elicit activation by AMP, whereas activation by phosphorylation has evolved at another site within the dimer interface, 14 Å away. In contrast, yeast GP did not evolve the AMP switch, and phosphorylation has evolved to become the only activator operating in the allosteric site. Despite the differences, their subunit interfaces are preferentially chosen to become locations of phospho-regulation. The mechanism of yeast GP suggests that a pre-evolved allosteric site within the dimer interface provides an evolutionary advantage for

phospho-regulation, probably because a pocket for binding phosphate was already present and phospho-peptide binding resulted in direct competition with inhibitor binding. As a result, phosphorylation exerted an immediate functional modification by rejecting the inhibitor. In mammalian enzyme, a different evolutionary course was followed. It is likely that activation by AMP has evolved before phosphorylation control, and has selected the allosteric site for the same advantages as the yeast enzyme. Subsequent development of phospho-regulation for sensing extracellular stimuli acquired a new site working in concert with AMP activation. The new site has also evolved within the dimer interface in the neighborhood of the allosteric site, and phosphorylation works by triggering similar subunit rotation as AMP binding. Presumably, adaptability of the subunit interface towards phospho-peptide binding during evolution contributes to the success of subunit interface becoming a site of phospho-regulation in mammalian and yeast GPs. This evolutionary strategy also makes the phosphorylation reaction of a multisubunit protein cooperative, a principle shared with the action of allosteric ligands in oligomeric proteins.

Biological implications

How does the activation mechanism of yeast glycogen phosphorylase (GP) illuminate an understanding of regulation in the family of GPs and in other protein phosphorylation control systems? The observation that yeast GP structural components involved in the activation process become increasingly more conserved among the GP family downstream in the activation relay is highly significant. The activation signal starts with phosphorylation of the completely unique N terminus on the regulatory face, causing it to refold into a wedge and force the subunits to move apart. In this way, the signal is passed on to the subunit interface, which is conserved among GPs only in the overall contact pattern but not the specific interactions. The signal is then received by the TOWER on the catalytic face; here, structural rearrangement of a group of highly conserved hydrophobic residues induces condensation of these residues to form a core which triggers the formation of a productive catalytic site. This analysis suggests a mechanism for the development of versatile control features in the GP family during evolution, with strict evolutionary constraint only on the hydrophobic TOWER–GATE interactions between the subunits. Variations along the dimer interface, which permit binding of new ligands and phosphorylated sidechains, are allowed during evolution as long as the hydrophobic TOWER–GATE interactions are preserved in the active state. The large dimer interface then provides the capacity to integrate a greater variety of signals and allows divergent control features to emerge [25]. Evolution of divergent control switches on a conserved protein machinery is a general biological phenomenon. One example is the evolution of divergent activation loops in controlling the conserved catalytic site in the family of protein kinases [5]. Another example is the family of G-protein coupled seven transmembrane helix receptors, which have evolved divergent extracellular ligand–receptor interactions to activate the conserved intracellular G-proteins. The principle by which diverse control switches evolved in the family of GPs can serve as a paradigm for other biological systems.

How does a simple mechanism of reversible protein phosphorylation fulfill a versatile role in biological regulation? The phospho-regulatory mechanism of yeast and mammalian GPs illustrates the general principle by which phosphorylation exerts its diverse regulatory functions. By attaching a phosphoryl group on different peptidyl sequences, each phospho-peptide serves as a unique 'ligand' to initiate specific protein functional modification by binding to the target protein. The mechanism thus has the potential to provide a gigantic pool of phospho-peptidyl ligands for regulation, which utilize their common phosphoryl groups as the register and the diverse peptide sequences for binding specificity. The analogy of protein phosphorylation switches to classical ligands implies that their actions can be antagonized through binding competition. In yeast GP, Glc-6-P antagonizes the effect of phosphorylation by binding competitively with the phosphorylated N-terminal segment at the allosteric site and, most importantly, exposing the phosphorylation site to protein phosphatase which leads to faster rate of dephosphorylation (KL, PKH and RJF, unpublished data). Whether other proteins undergoing reversible phosphorylation control are regulated *in vivo* by their corresponding antagonizing partners remains to be determined. Dephosphorylation facilitated by specific antagonist binding, however, could potentially lead to more specific dephosphorylation control *in vivo* and make up for the apparent lack of sequence specificity of protein phosphatases [26]. Furthermore, this study points to the possibility of structural-based phosphorylation antagonist design which can specifically block the effect of phosphorylation of a single-target protein. This could be a potential drug-design strategy to specifically block abnormal pathways in cellular signal transduction.

Materials and methods

Crystallization of the phosphorylated active state yeast GP

The phosphorylated form of yeast GP was prepared from a *E. coli* expression system as described previously [9]. The phosphorylated form of yeast GP was crystallized in the presence of the substrate, Glc-1-P, by hanging-drop vapour-diffusion technique using the Linbro plates. The protein concentration was 32 mg/ml and was in 130 mM sodium succinate pH 6.0, 2 mM DTT, 0.2 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotonin, 1 μg/ml pepstatin A, 0.5 mM PMSF and 0.02% sodium azide. The well solution contains 100 mM Bis Tris (pH 5.0), 50 mM NaCl, 50 mM Glc-1-P, 400 mM ammonium sulfate and 4.5% PEG4000. Hanging drops were prepared by mixing 5 ml of

protein solution with 5 ml of the well solution. The plates were then incubated at 18°C for 1 week without disturbance. Small crystals appeared after a week. Large crystals were grown by seeding using the small crystals as seeds. For seeding, the drops were equilibrated for a day and then seeds were introduced using a cat whisker. The crystal dimensions are typically 0.2 × 0.2 × 0.6 mm.

X-ray data measurement and reduction

Data was collected at room temperature on Raxis II image plate detector mounted on an 18 kW rotating anode generator operating at 50 kV and 180 mA at a detector distance of 140 mm and $2\theta = 0$. Oscillation images of 1° per frame and 30 min exposure were collected and the data were processed using the Raxis II data reduction software. The completeness of the data to 2.8 Å is 72% and the R_{merge} is 10.5%.

Structure determination and refinement

The procedures have been published previously [10].

Structure analysis

Comparative structure analysis was performed using INSIGHT II (Biosym Technologies), RasMol [27] and GEM [28]. Superposition of enzyme subunit was performed using residue range 20–38, 48–104, 114–209, 214–240, 290–375, 385–745 and 749–831. Superposition of C-terminal domain was performed using residue range 484–745 and 749–831. The cut-off distance for hydrogen bonds was 3.0 Å. The cut-off distance for van der Waals contacts was 4 Å.

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