Role of the Active Site Gate of Glycogen Phosphorylase in Allosteric Inhibition and Substrate Binding*

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The functional role in allosteric regulation of a flexible loop (residues 280–288) located near the active site of muscle glycogen phosphorylase was investigated. Mutations were made in residues 283–285 based on crystallographic studies that indicate that the loop functions as a gate controlling access of substrates to the active site and that these specific residues play distinct roles in mimicking the substrate and binding inhibitors when the enzyme is in an inactive conformation. Substitution of Ala or Asn for Asp-283, the putative substrate mimic, results in a 15-fold decrease in $V_{\text{max}}$; a 10-fold decrease in the $K_i$ for glucose 1-phosphate, a 10-fold increase in the $K_i$ for AMP, and a 10–20-fold increase in the $K_i$ for glucose. Substitution of Ala for Asn-284, which normally forms a hydrogen bond with the inhibitor glucose, reduces $V_{\text{max}}$ 3-fold, increases the $K_i$ for glucose 2-fold, but has little effect on AMP or glucose 1-phosphate binding or cooperativity. Substitution of Asp at 284, on the other hand, reduces $V_{\text{max}}$ 10-fold, elevates the $K_i$ for glucose 10-fold, decreases AMP cooperativity, but has little effect on the affinity of AMP or the cooperativity and binding of glucose 1-phosphate. Substitution of Leu for Phe-285, which forms aromatic stacking interactions with purine inhibitors, reduces $V_{\text{max}}$ 2-fold, decreases the affinity for caffeine at least 10-fold, raises the $K_i$ for AMP 3-fold, and decreases AMP cooperativity but has little effect on glucose 1-phosphate binding or cooperativity. The results of the mutagenesis studies show the importance of the 280’s loop for inhibitor binding and modulation of substrate affinity and suggest a role for the loop in allosteric activation. The propagation of allosteric effects across the domain interface may depend upon specific contacts between residues of the 280’s loop and the C-terminal domain.

Allosteric regulation of glycogen phosphorylase has traditionally been described in terms of the concerted model of Monod et al. (1) or the sequential model of Koshland et al. (2). Binding of effectors or substrates to the enzyme triggers long range quaternary and tertiary structural changes that alter substrate binding affinity and catalytic activity. Extensive biochemical (3–5) and crystallographic studies (6, 7) and, recently, site-directed mutagenesis (8–10) have begun to provide details of the molecular mechanism of allosteric control at the atomic level.

Glycogen phosphorylase catalyzes the phosphorolysis of glycogen to produce glucose 1-phosphate. The enzyme from muscle is activated by AMP and by phosphorylation at its N terminus and is inhibited by a variety of allosteric effectors that include glucose, glucose 6-phosphate, ATP, and purines (11–18). The active form of the enzyme is a dimer composed of two identical subunits, each of which contains an active site and five effector sites (7, 19–22). The monomer can be subdivided into N- and C-terminal domains. The AMP and phosphorylation sites are located at the dimer interface within 10 Å of each other in the N-terminal domain (23). The inhibitors, glucose 6-phosphate and ATP, compete with AMP for binding to the activation site (14, 15, 24, 25). A distinct glycogen activation site also lies within the N-terminal domain (21, 26). The active site resides within the subunit in a crevice between the N- and C-terminal domains (7, 21). Glucose binds at the active site where it competes for binding with glucose 1-phosphate (27–29). The purine inhibitory site lies just outside the active site (30).

Crystallographic studies have characterized a spectrum of conformational changes associated with phosphorylation of the enzyme and ligand binding, and have led to much speculation concerning the structural basis for the allosteric transition (23, 31–33). The 280’s loop (residues 280–288), which lies between $\beta$-strand 11 and $\alpha$-helix 8, is thought, in particular, to play a key role in the control of substrate and inhibitor binding (30, 33, 35–37). In structures of the enzyme in an inhibited state, the 280’s loop forms contacts with residues of the N- and C-terminal domains and blocks access to the catalytic cleft. Glucose and purine inhibitors form synergistic binding interactions with different residues of the 280’s loop and mediate intra- and interdomain contacts, which are presumed to stabilize the inactive conformation of the loop (23, 30, 37, 38). In structures of the enzyme in activated complexes with substrates or substrate analogues bound at the active site, the loop is disordered (33, 35, 36). Movement of the loop during activation is accompanied by rotation of the N- and C-domains apart by 5°. This reorientation of the 280’s loop and the opening of the catalytic cleft are thought to be required during the allosteric transition to allow phosphate and oligosaccharide to bind at the active site. For this reason, the 280’s loop is referred to as the active site gate.

Residues Asp-283, Asn-284, and Phe-285 form the apex of the gate and are thought to perform distinct functions in the allosteric response. Asp-283, a universally conserved residue in phosphorylases from prokaryotic and eukaryotic species (39, 40), behaves as a substrate mimic in the inactive state. Its carboxylate side chain occupies the active site in approximately the same position where phosphate binds when the enzyme is in an active conformation, and there it electrostatically and sterically interferes with substrate binding (33, 36). Residues Asn-284 and Phe-285, although not universally conserved, are found in all mammalian isozymes to participate in binding of inhibitors (30, 37–40). Crystallographic studies show that a hydrogen bond is formed between the C2 hydroxyl group of

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glucose and the Asn-284 side chain (38). The aromatic ring of Phe-285 forms aromatic stacking interactions with purine inhibitors (30). Asn-284 in addition contributes to the role of the loop as a gate by interfering sterically with substrate binding when the loop adopts its closed conformation. In the present study site-directed mutagenesis was used to test the role of the active site gate in allosteric regulation. Substitutions were made in residues Asp-283, Asn-284, and Phe-285 of the gate to perturb interactions thought to be important for allosteric inhibition and the stabilization of the inactive form of the enzyme. Kinetic analysis of the mutants shows the importance of gate residues for controlling the binding of substrates and inhibitors and suggests that proper positioning of the loop is necessary to achieve full allosteric activation.

EXPERIMENTAL PROCEDURES

Materials—Oyster glycogen type II was purchased from Sigma. Escherichia coli strain 25A6 was supplied by Genentech Inc. (South San Francisco, CA). E. coli strain NB42 was obtained from Dr. David Morgan at UCSF.

Mutagenesis—Enzyme variants of phosphorylase were made using the cDNA of rabbit muscle phosphorylase, cloned into the E. coli plasmid, pBluescript SK(+) (41). The procedure of Kunkel (53) with some modifications as described by Browner et al. (41) was used for oligonucleotide-directed mutagenesis. The following substitutions were made: alanine and asparagine at Asp-283, alanine and aspartate at Asn-284, and leucine at Phe-285. An Applied Biosystems PCRmate synthesizer was used for synthesis of the oligonucleotides needed for site-directed mutagenesis and DNA sequencing. Oligonucleotides were 27 to 38 bases in length and contained base substitutions for introduction of mutation sites as well as restriction sites neighboring the mutation sites to allow screening the DNA by restriction digests. Restriction sites were introduced using conservative base substitutions that did not alter the amino acid sequence of the protein. The mutant constructs were sequenced at the mutation sites and in the regions extending 300 base pairs on either side of the mutations to confirm the presence of correct substitutions.

Expression and Purification of Enzyme Variants—Most of the mutant constructs could be expressed in cultures of E. coli strain 25A6 (W3110; tonA, lacI, qaiE, htpF45). Cultures were grown at 22°C under conditions described previously for expression of the wild-type enzyme (41). From 1-liter cultures of E. coli, 1–10 mg of the enzyme variants were generally obtained. The N284A mutant was an exception and could not be expressed in the 25A6 strain. It was expressed more successfully in the lon-, E. coli strain, NB42, at a level of about 2 mg of protein per 1 liter of culture. Mutants were purified by chromatography on fast-flow metal chelating-Sepharose and fast-flow DEAE-Sepharose as described previously (42). The mutants were better than 95% pure as judged from SDS-polyacrylamide gel electrophoresis gels stained with Coomasie Blue. Enzyme variants were stored in 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM dithiothreitol, and 50% glycerol at -20°C. In preparation for kinetic assays, enzyme variants were desalted by chromatography on Sephadex G-25.

Kinetic Characterization of Phosphorylase Mutants—Protein concentrations were determined from absorbance measurements at 280 nm using the extinction coefficient for the wild-type enzyme of 1.32 cm²/mg (43). Glycogen was purified to remove contaminating phosphate for use in kinetic assays as described previously (10). Initial velocities were determined by measuring the phosphate produced from the reaction of glucose 1-phosphate and glycogen (44). The reaction mixtures contained 50 mM BES, pH 6.8, 1 mM EDTA, 1 mM, 1% glycogen in a final volume of 0.5 ml. Assays were performed at 30°C. The enzyme was first preincubated for 5 min at 30°C in assay mixtures containing everything but glucose 1-phosphate. Reactions were then initiated by addition of glucose 1-phosphate and quenched by addition of 10% trichloroacetic acid. The Km and Hill coefficients of AMP were determined for each of the mutants at a glucose 1-phosphate concentration of 10 mM. AMP was varied in the range of 0 to 1 mM for the D283A and D283N mutants and in the range of 0 to 6 mM for the wild-type enzyme and the N284A, N284D, and F285L mutants. The kinetic parameters for determination of allosteric activation were determined by nonlinear regression with the program JMP (SAS Institute Inc., 1989). The Km for glucose was determined for the mutants at an AMP concentration of 1 mM. The concentration of glucose 1-phosphate was varied in the range of 0 to 0.6 mM for the wild-type enzyme. Kinetic parameters for determination of the Ki values for glucose were fit to Equation 1 for a concerted model using the simplifying assumption that inhibitors bind exclusively to the inactive T-state (45).

RESULTS

Effects of Substitutions on Substrate Binding and Cooperativity—The mutants were characterized kinetically to determine the effects of mutations in the active site gate loop on allosteric regulation and catalytic activity. Amino acid replacements with alanine were designed to test the effects of substituting a residue with a neutral side chain incapable of forming normal hydrogen bonding interactions. Substitutions of either aspartate or asparagine were designed to test the effects of charge and hydrogen bonding potential. Substitution of leucine for Phe-285 was expected to weaken aromatic stacking interactions with inhibitors at the purine binding site. Kinetic parameters, determined from glucose 1-phosphate saturation curves for the enzyme variants, are shown in Table I. Removal of a negative charge at 283 apparently improves substrate binding. Substitution of either alanine or asparagine at 283 results in about a 16-fold decrease in the Vmax of glucose 1-phosphate but no loss in substrate cooperativity. The D283N mutant, in fact, shows a significant increase in cooperativity for glucose 1-phosphate. Substitution of alanine or asparagine at 284 has little effect on binding of glucose 1-phosphate. About a 1.6-fold decrease is observed in the S0.5 for glucose 1-phosphate
for both mutants, and substrate cooperativity is comparable with the wild-type enzyme. Substitution of Leu at Phe-285 has little effect on either the $S_{0.5}$ for glucose 1-phosphate or substrate cooperativity.

Activation of Phosphorylase Variants by AMP—Kinetic parameters determined from AMP saturation curves are shown in Table II. Surprisingly, substitutions in the gate loop have long range effects that extend to the AMP site 30 Å away. Mutations reduce maximal velocity and decrease the effectiveness of the obligatory activator, AMP. Substitution of alanine or asparagine at Asp-283 results in a 10-fold decrease in catalytic activity, an elevated $K_a$ for AMP, and a loss in cooperativity for AMP. Substitution of alanine at 284 reduces activity about 3-fold but has little effect on the apparent affinity or cooperativity of AMP binding. Substitution of aspartate at 284, however, reduces activity 10-fold, eliminates cooperativity for AMP, but has little effect on the $K_a$ for AMP. Substitution of leucine at 285 lowers $V_{max}$ 2-fold, raises the $K_a$ for AMP 3-fold, and decreases AMP cooperativity.

Inhibition by Glucose and Caffeine—The effects of mutations on inhibition of phosphorylase by glucose and caffeine were also investigated (see Table III). Substitutions of alanine or asparagine at Asp-283 raise the $K_i$ for glucose 20- and 10-fold, respectively. Substitution of alanine for asparagine at position 284 has little effect on glucose inhibition. Only a 2-fold increase in the $K_i$ for glucose is observed for this mutant; however, substitution of aspartate at 284 results in a 10-fold increase in the $K_i$ for glucose. Replacement of Phe-285 with Leu reduces the $K_i$ for caffeine at least 10-fold. The limited solubility of the inhibitor made it impossible to determine an accurate $K_i$; however, the F285L mutant shows no inhibition by caffeine at a concentration of 10 mM, which completely inhibits the wild-type enzyme. The combined results show that substitutions in the 280’s loop significantly alter regulatory behavior of glycogen phosphorylase.

**DISCUSSION**

Movement of the 280’s loop during the allosteric transition couples quaternary structural changes induced by the binding of AMP at the dimer interface to tertiary structural changes that reposition residues at the active site (see Fig. 1). In the inactive state, Asp-283 of the gate occupies the phosphate subsite of the active site where it hinders binding of the substrate. During allosteric activation, reorientation of the 280’s loop is accompanied by movement of Arg-569 into the active site where it contributes a salt-bridge interaction to phosphate in the substrate binding pocket (33, 35, 36). These structural changes during the allosteric transition may account for homotropic cooperativity for the substrate, glucose 1-phosphate. In order to test the role of Asp-283 of the gate, site-directed mutagenesis was used to replace Asp-283 with alanine and asparagine residues. Both mutants exhibit elevated $K_i$ values for AMP and diminished maximal velocities. Substrate cooperativity is comparable with the wild-type enzyme for the D283A mutant and is apparently increased for the D283N mutant. All of these observations suggest that these mutants have their conformational equilibria shifted toward an inactive state. The mutants, nevertheless, exhibit 10-fold decreases in the $S_{0.5}$ for glucose 1-phosphate. Removal of a negatively charged side chain from the substrate binding pocket thus appears to enable glucose 1-phosphate to bind with greater affinity to the inactive conformer presumably because of the loss in competition between the carboxylate side chain and the phosphate of the substrate. The asparagine side chain in the D283N mutant might have been expected to interfere sterically with substrate binding in the inactive state; however, this mutant shows a similar $S_{0.5}$ for glucose 1-phosphate to that of the D283A mutant. The asparagine side chain is likely displaced from the position normally found for Asp-283 in the wild-type enzyme. Substitutions of alanine or aspartate at neighboring Asn-284 have little effect on substrate binding or cooperativity. The 10–15-fold reduction in catalytic activities exhibited by the D283A and D283N mutants indicates that these substitutions either impair allosteric activation or more directly compromise catalytic function. The reduced cooperativity for AMP in the D283A and D283N mutants may reflect the inability of these mutants to achieve full activation. Cooperativity depends on the difference in ligand binding affinities and/or catalytic activities in inactive and activated conformational states. Diminished binding affinity for AMP in the activated state of a mutant, capable of only partial activation may, therefore, result in a corresponding loss in cooperativity. The gate may play a more important role in the active conformation than anticipated from crystallographic studies, which show the 280’s loop to be disordered in structures of activated complexes.
Although the Monod et al. (1) model is often invoked to describe allosteric behavior in glycogen phosphorylase (1), reality is probably more complicated, and it is possible that activation is not entirely concerted. Binding of AMP to the enzyme induces structural changes at the dimer interface and triggers the rotation of the subunits (46, 47). In the absence of substrates bound at the active site, these structural changes may only partially activate the enzyme. Binding of substrate at the active site may be necessary for triggering the local structural changes that are needed to achieve full activation. Electrostatic repulsion and steric interactions caused by binding of the substrate force the movement of the active site gate during the allosteric transition. In the mutants, the removal of the negatively charged carboxylate side chain eliminates the electrostatic repulsion between the gate and the substrate that normally drives movement of the loop during activation. Furthermore, the carboxylate group of Asp-283 normally forms an intradomain salt bridge interaction with His-571 as well as an intradomain interaction with the side chain of Asn-284 when the enzyme is in an inactive state (see Fig. 1). These contacts between the N- and C-terminal domains may be needed to transmit the structural changes across the domain interface that result in the rotation of the N- and C-terminal domains and the opening of the catalytic cleft.

Crystallographic evidence indicates that the enzyme can indeed adopt partially activated conformational states. In the structure of the phosphorylated enzyme with the inhibitor glucose bound at the active site, the N terminus of the enzyme shows structural changes characteristic of activation; however, the active site gate remains in its closed conformation and the N- and C-terminal domains have not rotated apart (23). The inhibitor glucose binds in about the same position as the substrate glucose 1-phosphate, but because it lacks the negatively charged phosphate, it does not trigger the movement of the active site gate, and so a hybrid conformational state is captured.

The universal conservation of Asp-283 and the proximity of its side chain to the C-terminal domain increases the probability that this residue plays a role in allosteric regulation (7, 21, 39). The substitution of Leu for Phe at 285 reduces binding of the enzyme.

Site gate assumes an as yet uncharacterized configuration in orthophosphate inhibition is contrast, elevates the $K_i$ for glucose 10-fold. The weaker binding of glucose in this mutant may be caused indirectly by displacement of the Asp-283 side chain due to unfavorable electrostatic interactions between the neighboring carboxyamide groups. Although crystallographic structures show that Asp-283 forms no direct interactions with the glucose inhibitor, hydrogen bonding interactions and van der Waals contacts with residues of the C-terminal domain and with the side chain of Asn-284, which does form a direct hydrogen bonding interaction with the glucose inhibitor, may be critical for maintaining the structure and positioning of the loop. Substitutions of asparagine and alanine at 283 increase the $K_i$ for glucose 10- and 20-fold, respectively. The sensitivity of glucose inhibition to substitutions at 283 indicates that interactions with the carboxylate side chain help to maintain the complementarity of the glucose binding pocket to the inhibitor.

The flexibility of the 280’s loop makes it ideally suited for its roles in modulating substrate and effector binding and for communicating regulatory signals between the subunit interface and the active site. Other allosteric proteins also possess loops that play key roles in conferring allosteric properties. In phosphofructokinase, the 6F loop plays a similar role in altering substrate affinity in R- and T-states (48). In the T-state, Glu-161 of the 6F loop protrudes into the active site and impedes binding of the substrate, fructose 6-phosphate. In the R-state, Glu-161 swings away and Arg-162 moves into a position where it can form a salt-bridge interaction with the phosphate of the substrate. Similarly, in aspartate transcarbamylase, activation is associated with the repositioning of residues of the 240’s loop and movement of Arg-229 into the active site where it forms a salt-bridge interaction with the β-carboxylate of aspartate (49–51). In cyclin-dependent kinase 2, the T-loop blocks the active site and hinders the binding of ATP and the peptide substrate when the enzyme is in an inactive state (52). Allosteric effects in all these enzymes are mediated by mobile loops that adopt alternate conformations in inactive and active states. The interchange in position of residues in flexible loops may prove to be a general structural mechanism for modulating substrate affinity during R- to T-state switching in allosteric proteins.

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