Cooperative Binding Is Not Required for Activation of Muscle Phosphorylase†

Michelle F. Browner,* Peter K. Hwang, and Robert J. Fletterick*

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94141-0448

Received February 4, 1992; Revised Manuscript Received August 19, 1992

ABSTRACT: Muscle and liver glycogen phosphorylase isozymes differ in their responsiveness to the activating ligand AMP. The muscle enzyme, which supplies glucose in response to strenuous activity, binds AMP cooperatively, and its enzymatic activity becomes greatly enhanced. The liver isozyme regulates the level of blood glucose, and AMP is not the primary activator. In muscle glycogen phosphorylase, the residue proline 48 links two secondary structural elements that bind AMP. This amino acid residue is replaced with a threonine in the liver isozyme; unlike the muscle enzyme, liver binds AMP noncooperatively, and the enzymatic activity is not greatly increased. We have substituted proline 48 in the muscle enzyme with threonine, alanine, and glycine and characterized the recombinant enzymes kinetically and structurally to determine if proline at this position is critical for cooperative AMP binding and activation. Importantly, all of the engineered enzymes were fully activated by phosphorylation, indicating that enzymatic activity was not compromised. Only the mutant enzyme with alanine at position 48 responds like the wild-type enzyme to the presence of AMP, indicating that proline is not absolutely required for full cooperative activation. The substitution of either threonine or glycine at this position, however, creates enzymes that no longer bind AMP cooperatively. The enzyme with threonine at position 48 further mimics the liver enzyme, in that the maximal enzymatic activity is also reduced. Significantly, the glycine substitution caused the enzyme to be fully activated by AMP, although binding was not cooperative. The hyperactivation of the glycine mutant by AMP suggests that the total free energy of activation has decreased. Structural analysis of the inactive forms of the threonine and glycine mutant enzymes showed that the domain containing the AMP binding site has moved toward the position found in structures of activated enzymes. By a single amino acid substitution, glycogen phosphorylase was redesigned to be fully activated without AMP binding energy spent altering the second binding site, as required for a cooperative interaction. Thus, this work suggests that AMP binding energy is efficiently spent on changing the active site conformation.

Muscle glycogen phosphorylase has long been the focus of intense investigation by biochemists and biophysicists with the aim of understanding its properties and fundamental mechanisms of allosteric regulation. Muscle phosphorylase is allosterically activated by covalent modification, phosphorylation of serine 14, and by the noncovalent interaction of ligands, such as AMP. X-ray crystallographic studies have shown that the AMP binding and the phosphorylation sites are within 16 Å of each other, contained within the activation domain (N-terminus, amino acid 120) (Sprang et al., 1991). Binding of either activator involves the strengthening of subunit contacts across the phosphorylase dimer (Sprang et al., 1988), as proposed in the Monod, Wyman, and Changeux model of allostery (Monod et al., 1965).

The rate at which the muscle enzyme breaks down glycogen, producing glucose 1-phosphate (Glc-1-P), for the mobilization of chemical energy is controlled by the intracellular products of ATP hydrolysis, specifically the AMP concentration. The muscle enzyme binds AMP cooperatively, and AMP regulates its activity over a narrow concentration range. In contrast, the liver isozyme, which is responsible for maintenance of whole body glucose levels, binds AMP noncooperatively. Full activation of the liver enzyme in the presence of AMP is only 10% of that of the muscle enzyme with AMP bound (Coats et al., 1991; Kobayashi et al., 1982). Careful examination of the 171 amino acid differences between the muscle and liver isozymes led to the suggestion that proline 48, which is a threonine in liver, could be important for determining the conformational response of the phosphorylase isozymes to AMP activation (Rath et al., 1987). Proline 48 is not directly involved in AMP binding but joins two structural elements that form subunit contacts (Figure 1A). The two AMP binding sites are linked by the 2-fold symmetrical association of these structural elements (Figure 1B). We hypothesized that amino acid side chains which influence the main-chain flexibility at this site would result in altered AMP binding, subunit interactions, and enzyme activation. To test this hypothesis, we substituted proline 48 in rabbit muscle phosphorylase (Pro48) with alanine (Pro48Ala), glycine (Pro48Gly), and threonine (Pro48Thr). The native and engineered enzymes were assayed to determine if activation by phosphorylation or AMP binding had been affected by the single amino acid substitutions. The atomic structures of dephosphorylated Pro48Thr and Pro48Gly were analyzed to determine if a structural change was correlated with altered enzymatic function.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression. All mutagenesis, DNA sequencing, and expression of variant phosphorylase constructs were carried out using the pTACTAC/phosphorylase plasmid described previously (Browner et al., 1991). In summary, this plasmid contains the full-length rabbit muscle phosphorylase cDNA (Nakano et al., 1986), the hybrid *trp–lac* promoter, the repressor gene *lacI*^{q}, and the M13 origin for making single-stranded DNA (Muchmore et al., 1989). The proline residue at position 48 of rabbit muscle phosphorylase was replaced with alanine, glycine, and threonine using a DNA mutagenesis procedure similar to that of Kunkel (Browner et

† This work was supported by Grants AM32822 and DK26081 from the National Institutes of Health (to R.J.F.).

* Authors to whom correspondence should be addressed.

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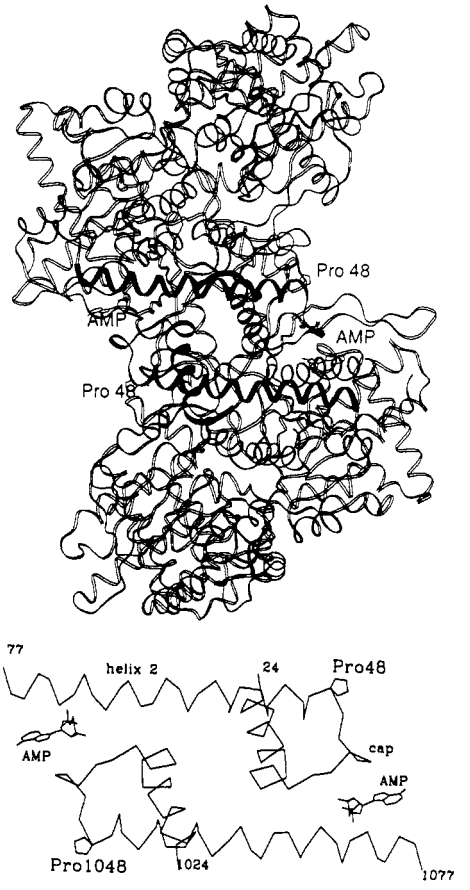

FIGURE 1: (A, top) Glycogen phosphorylase dimer represented by a ribbon tracing of the α-carbon atoms. The 2-fold axis of symmetry is perpendicular to the page. Traced in the thicker ribbon are residues 19–79 of each monomer. The position of proline 48 is noted, and the AMP binding site is shown (Sprang et al., 1987). (B, bottom) Closeup of amino acids 24–77 from both subunits of the glycogen phosphorylase dimer again showing the position of proline 48 and AMP. The two secondary structural elements important in the activation are labeled, helix 2 and the cap. The subunit interactions in this portion of the structure occur around the bound AMP molecules.

al., 1991; Kunkel, 1985). Oligonucleotides, 36 bases in length, which incorporated the necessary base substitutions, were synthesized on an Applied Biosystems PCRmate synthesizer. DNA constructs containing the appropriate base substitutions were first identified by restriction enzyme digestion, and the presence of the correct codon at position 48 and the surrounding sequence was confirmed by double-stranded DNA sequence analysis using Sequenase (U.S. Biochemicals) (Tabor & Richardson, 1987).

Native phosphorylase and the variant proteins were expressed in *Escherichia coli* strain 25A6 [W3110; *ton*A, *lon*Δ, *qal*E, *htp*P^{ts}] as described previously (Browner et al., 1991). In brief, bacterial cells containing the expression plasmid were grown in standard LB/ampicillin in the presence of 250 μM IPTG (isopropyl β-D-thiogalactopyranoside), 3 mM $MnCl_2$, and 0.5 mM pyridoxine, at 22 °C for 48 h. Under these growth conditions greater than 25 mg of phosphorylase protein was expressed per liter of bacterial culture.

Protein Purification and Assays. All phosphorylase proteins were purified to greater than 95% purity using standard chromatographic procedures (Browner et al., 1991). The purified protein was stored in 25 mM β-glycerophosphate, 1 mM EDTA, and 1 mM β-mercaptoethanol at 4 °C. The identity and purity of the proteins were always confirmed by denaturing SDS–PAGE (Laemmli, 1970), Coomassie blue staining, and Western blot analysis. Protein concentrations were determined using a modified Bradford assay (Protein Assay Reagent, Pierce).

Both the native phosphorylase protein and the variants were assayed in solution using a colorimetric assay that determines the amount of inorganic phosphate released when glucose, from glucose 1-phosphate (Glc-1-P), is incorporated into glycogen (Cori et al., 1943). All protein samples were passed over a Sephadex G-50 column to replace the glycerophosphate storage buffer with 10 mM BES, pH 7.0. The final enzyme reaction contained 100 mM BES, pH 7.0, 75 mM Glc-1-P, 1% glycogen (60 mM glucose), 0.5 mM EDTA, and 0.5 mM DTT. Enzyme which had not been phosphorylated was assayed in the presence of 10 μM to 1 mM AMP. Typically, determination of the kinetic constants for AMP activation included multiple measurements at ten AMP concentrations (0.01, 0.012, 0.015, 0.02, 0.025, 0.03, 0.05, 0.1, 0.5, and 1.0 mM). Samples were assayed at 0, 5, 10, 15, and 30 min, and the release of inorganic phosphate was quantitated by a color change of molybdovanadate measured spectrophotometrically at 310 nm. The specific activity is calculated as micromoles of phosphate released per minute per milligram of protein.

Multiparameter least-squares analysis for the determination of kinetic constants was performed using the statistical analysis and modeling (SAAM) package (Berman et al., 1962).

Phosphorylation. The purified recombinant phosphorylase proteins were phosphorylated using rabbit muscle phosphorylase kinase (Sigma Biochemicals). The reaction buffer was 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, and 60 mM NaF, and the final reaction contained 0.7 mg of phosphorylase (14 pmol/μL), 3.0 μg of phosphorylase kinase (1 unit), and 0.2 mM ATP (0.1 nCi/pmol). The presence of γ-^{32}P-radiolabeled ATP (Amersham) allowed the phosphotransfer reaction to be quantified. The reaction was carried out at 30 °C for 3.5 h, and the incorporation of phosphate was monitored at 30-min intervals by spotting 10 μL of the reaction mixture onto phosphocellulose filters which were washed three times in 75 mM phosphoric acid (Roskoski, 1983). The filters were dissolved in 10 mL of BioFluor (New England Nuclear) and counted using a Beckman LS300 scintillation counter. On average, 26% of the native and variant proteins were phosphorylated. The specific activities of the phosphorylated proteins were calculated as a function of the percent of phosphorylated protein.

X-ray Crystallographic Structure Determination. Protein crystals of both Pro48Thr and Pro48Gly (nonphosphorylated protein) were grown by using microseeds of native phosphorylase crystals. X-ray diffraction quality crystals were obtained by additional growth using macroseeds obtained from the microseeding (Browner et al., 1991). All crystals were grown in 10 mM BES, pH 7.0, 0.1 mM EDTA, 1.0 mM DTT, 10 mM $MgCl_2$, and 1.0 mM AMP (Eagles et al., 1972). Crystals from which X-ray diffraction data were collected averaged 0.2 mm × 0.2 mm × 1.0 mm in size; two crystals were used for each data set. Diffraction data (Table I) were recorded to 2.7 and 2.8 Å, respectively, using a Xentronics multiwire area detector and a Rigaku RU-200 rotating anode; the Kα radiation (50 kV, 60 mA) was reflected from a graphite monochromator. Pro48Thr data were reduced using the XENGEN software package (Nicolet Instrument Co.), and Pro48Gly data were reduced with BUDDHA (Blum et al., 1987).

Phase information for the structure determination of Pro48Thr was obtained using a model of dephosphorylated phosphorylase with no solvent molecules (Acharya et al., 1991). Although AMP is present in the crystallization buffer, electron

Table I: Structure Analysis

	Pro48Gly	Pro48Thr
	Crystal Data	
space group	$P4_32_12$	$P4_32_12$
cell dimensions (Å)	$a = b = 128.5$, $c = 116.3$	$a = b = 128.5$, $c = 116.3$
	Diffraction Data	
resolution (Å)	2.8	2.7
total observations	123 951	94 843
observations $>2\sigma$	94 864	74 047
unique reflections	22 228	24 972
R_{sym}[a]	0.10	0.09
	Refinement Data	
refinement (cycles)[b]	100	93
R_{cryst}[c]	0.190	0.205
rms difference		
bond lengths (Å)	0.016	0.013
bond angles (deg)	2.8	1.7
no. of atoms	6578	6582
no. of solvent molecules	307	382

[a] $R_{sym} = \sum_h\sum_i|\langle F_h\rangle - F_{hi}|/\sum_h F_h$, where $\langle F_h\rangle$ is the mean structure factor magnitude of i observations for symmetry-related reflections with Bragg index h. [b] PROLSQ refinement included all data 6.0 Å and higher. [c] $R_{cryst} = \sum_h\sum_i||F_o| - |F_c||/\sum|F_o|$, where F_o and F_c are the observed and calculated structure factor magnitudes.

density corresponding to AMP has not been observed. Initially, the Pro48Thr data scaled to the starting model at R_{cryst} = 32.5%. The amino acid side chain at position 48 was removed from the starting model. The resulting F_c values were refined (Hendrickson & Konnert, 1980) for 20 cycles, and then the missing atoms were fitted to an $F_o - F_c$ electron density map with FRODO (Jones, 1978, modified by W. Pflugrath, M. Saper, R. Hubbard, and P. R. Evans). Other atoms were moved and removed as needed to be correctly positioned with the electron density, or when possible, side chains were moved to the position of an acceptable rotamer (Ponder & Richards, 1987). Solvent molecules were added by listing electron density peaks from an $F_o - F_c$ map, checking each symmetry-equivalent peak and positioning a water molecule such that there was no steric conflict and that the solvent molecule was within hydrogen-bonding distance of another atom (S. Sprang, unpublished results). The position of solvent molecules was checked graphically using FRODO.

The X-ray diffraction data from Pro48Gly were initially scaled to the final Pro48Thr model, with no solvent molecules and threonine side-chain atoms at position 48 removed (R_{cryst} = 30.8%). Refinement was done essentially as described for Pro48Thr.

Structural Analysis. Comparative structure analyses were performed using InsightII (BioSym) and Gem (E. Fauman, unpublished results). The principal axes description was done by calculating the moment of inertia for three domains in the phosphorylase monomer, such that the eigenvalues are the moments of inertia about each principal axis and the eigenvectors are the direction of the axes (Goldstein, 1950). The principal axes were shape weighted according to the mass distribution to more accurately represent the shape of the protein domain (Browner et al., 1992).

RESULTS

The four phosphorylase proteins, Pro48 (wild-type rabbit muscle phosphorylase), Pro48Ala, Pro48Gly, and Pro48Thr, used in this study were purified from a bacterial expression system to approximately 95% homogeneity as determined by polyacrylamide gel analysis.

Kinetic Analysis. Rabbit muscle phosphorylase can be activated by covalent phosphorylation of serine 14 or by the noncovalent binding of AMP. The role of the specific amino acid at position 48, in response to both modes of activation, was examined. The enzymatic activities of either the phosphorylated or AMP-bound proteins were determined by measuring the rate of glycogen synthesis; inorganic phosphate is released from Glc-1-P as glucose is incorporated into glycogen. In all experiments, the substrate concentrations (Glc-1-P and glycogen) were saturating.

Table II: Kinetic Analysis

	AMP activation				phosphorylation
protein	V_{max} (units/mg)[a]	K_a (μM)	α[b]	EOF[c]	V_{max} (units/mg)[a]
Pro48	32 ± 3	>200	<0.01	1.7	120 ± 14
Pro48Ala	29 ± 1	81 ± 72	0.05	1.7	87 ± 16
Pro48Gly	30 ± 1	16 ± 2	0.6	0.3	77 ± 20
Pro48Thr	17 ± 3	45 ± 9	0.7	0.7	128 ± 25

[a] units = nmol of phosphate released/min. [b] α: interaction coefficient measuring homotropic cooperativity between AMP sites. [c] EOF: error of fit = $\sum(1/w_i)(v_c - v_o)^2/(n - p)$, where n is the number of unique observations, p the number of parameters, v_c the calculated velocities, v_o the observed velocities, and w_i the standard deviation of the unique observations.

(A) Phosphorylation. The enzymatic activity of all four enzymes was determined after covalent modification by phosphorylation of Ser14 in vitro, using phosphorylase kinase. The efficiency of phosphorylation was determined by the incorporation of radioactively labeled phosphate from ATP into the protein substrates. There was no evidence that any of the proteins were better or worse substrates for phosphorylase kinase. The mutant enzymes Pro48Ala, Pro48Gly, and Pro48Thr were fully activated by covalent phosphorylation (Table II). The V_{max} values of the phosphorylated, engineered enzymes were the same as that for the wild-type enzyme, within experimental error.

(B) AMP Activation. The ability of muscle phosphorylase and the three variants to be activated by AMP was determined at 10 concentrations. Activation by AMP was dramatically affected by the substitution of either a glycine or a threonine at position 48 of muscle phosphorylase. The replacement of proline with threonine reduced enzymatic activity in the presence of AMP to half that of the wild-type enzyme and also significantly reduced the cooperativity of AMP binding (Figure 2). Substitution of glycine at this position caused the loss of cooperative AMP binding, but this enzyme was still fully activated. In fact, the mutant enzyme, Pro48Gly, had higher activity at lower AMP concentrations than the native enzyme (Figure 2).

The kinetic constants for the enzymes (Table II) were determined by multiparameter, nonlinear least-squares refinement (Berman et al., 1962; Kasvinsky et al., 1978). The model used to fit the experimental data assumed that the enzyme dimer was fully bound to substrate, because the assays were done with Glc-1-P at saturating concentrations, and that the substrate-saturated dimer without AMP bound was inactive. The following equation was derived for the reaction velocity, according to Segel (1975).

$$v = \frac{V_{max}(\beta[A]/K_a + [A]^2/\alpha K_a)}{1 + 2[A]/K_a + [A]^2/\alpha K_a^2}$$

K_a is the intrinsic dissociation constant for AMP from the first site on the dimer, and α, the interaction factor, is the factor by which the dissociation constant has been decreased for the second AMP molecule; thus αK_a is the dissociation constant for the second AMP site, and β is the ratio of velocities

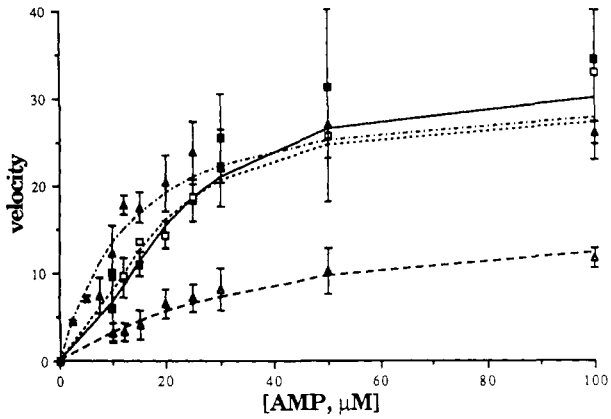

FIGURE 2: Plot of activator (AMP) concentration versus velocity. For each enzyme the observed velocities are shown by squares (opened, Pro48, and filled, Pro48Ala) or triangles (filled, Pro48Gly, and open, Pro48Thr) with the associated standard deviations shown by error bars. The curves represent calculated velocities from the nonlinear least-squares refinement (Table I). The solid line is Pro48, the small dashed line Pro48Ala, the dash-point line Pro48Gly, and the large dashed line Pro48Thr.

of product formation for the half-saturated dimer to that for the dimer with two AMP molecules bound. The parameters K_a, α, and V_{max} were refined at various values of β ranging from 0 to 1.0. For all of the enzymes tested, the value of β did not affect the interpretation of the other refined parameters. The error of fit was low, indicating good agreement between the refined parameters and the observations (Table II, β = 1). For a fully cooperative enzyme, K_a and α are inversely correlated; this correlation results in an increased parameter redundancy and an expected large error in the refined kinetic parameters. This was true for both Pro48 and Pro48Ala. For example, the calculated K_a for muscle phosphorylase was >200 μM because the interaction factor was very small (<0.01). The velocity plot (Figure 2) indicates, however, that the binding constant for AMP was 20 μM, as expected for muscle phosphorylase. The K_a and α for Pro48Gly and Pro48Thr did not, however, show this interdependence. The K_a and α for these two enzymes refined with low errors and confirmed that these mutant phosphorylases have greatly reduced cooperative AMP binding (Table II).

The interaction factor (α) can be related to the Hill coefficient ($n_{calc} = 2/(1 + \sqrt{\alpha})$) (Segel, 1975) such that the cooperative enzymes (wild type and Pro48Ala) have a Hill coefficient of 1.8. The weakly cooperative enzymes, Pro48Gly and Pro48Thr, have Hill coefficients of 1.2 and 1.1, respectively. The calculated Hill values were the same as those determined from conventional Hill plots. The Hill coefficient has been shown to change linearly with respect to the energy of intersubunit interaction over a wide range of values (Wyman, 1964). The relationship between the free energy of subunit interaction and the interaction factor is described by the equation (Levitzki, 1978):

$$\Delta G_I = \frac{-2.3RT \log (1/\alpha_2)}{2}$$

The free energy of subunit interaction for the enzymes which bind AMP cooperatively is −1.2 kcal/mol, but for the enzymes which are weakly cooperative, the free energy is calculated to be −0.1 kcal/mol.

Structural Analysis. The loss of cooperative AMP binding to Pro48Gly and Pro48Thr warranted a structural analysis of these variants to determine if there were changes relative to the native, dephosphorylated structure. The ground-state, inactive structures of Pro48Gly and Pro48Thr were determined using the structure of dephosphorylated rabbit muscle phosphorylase as the starting model (Acharya et al., 1991). The final models at 2.8 Å (Pro48Gly) and 2.7 Å (Pro48Thr) were refined to crystallographic *R*-factors of 19.0% and 20.5%, respectively (Table I). These variant structures were compared with the wild-type, dephosphorylated structure at 1.9 Å (Acharya et al., 1991); the overall rms deviation was 0.57 Å for Pro48Gly and 0.53 Å for Pro48Thr. The rms deviation for main-chain atoms was 0.3 Å for both structures, and the rms deviation of side chains was slightly larger, 0.74 Å for Pro48Gly and 0.69 Å for Pro48Thr. Furthermore, the substitution of proline 48 with either glycine or threonine did not significantly alter the relative crystallographic temperature factors for any region of the protein.

The most dramatic difference, within the activation domains of the mutant phosphorylase models and the native structure, was at position 117, which is a leucine approximately 8 Å from proline 48. The carbonyl oxygen of leucine 117 was flipped 180°, which changes the torsional angle of the backbone angle, ψ, to 151.3°, rather than the less favorable angle of −17.7°. A water that is conserved in the phosphorylated phosphorylase structures (S. Sprang, E. Goldsmith, and R. Fletterick, unpublished results) fills the hole left by repositioning the carbonyl oxygen. The carbonyl oxygen is now within hydrogen-bonding distance of another water. All of these atoms refine well and the *B*-factor for the leucine 117 carbonyl oxygen is only slightly above average (37.9 Å^2, in Pro48Thr) for the variant structures, whereas in the native structure the *B*-factor was 147.8 Å^2 (Acharya et al., 1991). The new position of the leucine 117 atoms does not result from the amino acid replacement at position 48, as we have observed that the new position is consistent with electron density maps of several other phosphorylase structures analyzed in our laboratory.

Figure 3 shows a portion of the electron density map for the Pro48Gly and Pro48Thr structures in the vicinity of amino acid 48. The atomic coordinates for the wild-type, dephosphorylated phosphorylase are also shown. The atom positions in the variant structures are virtually identical with those of the native structure. Proline 48 is in the trans conformation, and both glycine and threonine easily substitute at this position with nearly identical ϕ and ψ values. The ψ angle for glycine is the most different (18°), but all angles fall well within the α-helical range (Ramachandran & Sasiekharan, 1968).

Evidence of global structural changes in a phosphorylase dimer can be found by analyzing the relative orientations of the separate domains. Three domains are important to consider in this case: the activation domain (defined as N-terminus, amino acid 120), the N-terminal domain (amino acid residues 190–481), and the C-terminal domain (residues 483–714) (Sprang et al., 1991). To determine if the amino acid replacements at position 48 affected the domain configurations, the three principal axes of each domain were determined and used to define the orientation of the domain (Browner et al., 1992). Values for the three angles, which describe the relative orientation of two domains in a variant phosphorylase dimer, were subtracted from the angle values of the principal axes for the same domains in wild-type phosphorylase. A comparison of the activation domains showed that this domain was rotated in both Pro48Gly and Pro48Thr relative to its configuration in native phosphorylase. The rotation for the activation subdomain, averaging the three difference values, was 0.7° for Pro48Gly and 0.5° for Pro48Thr. When the same comparison was done for the N-terminal domain, the rotation was 0.3° and 0.2°, respec-

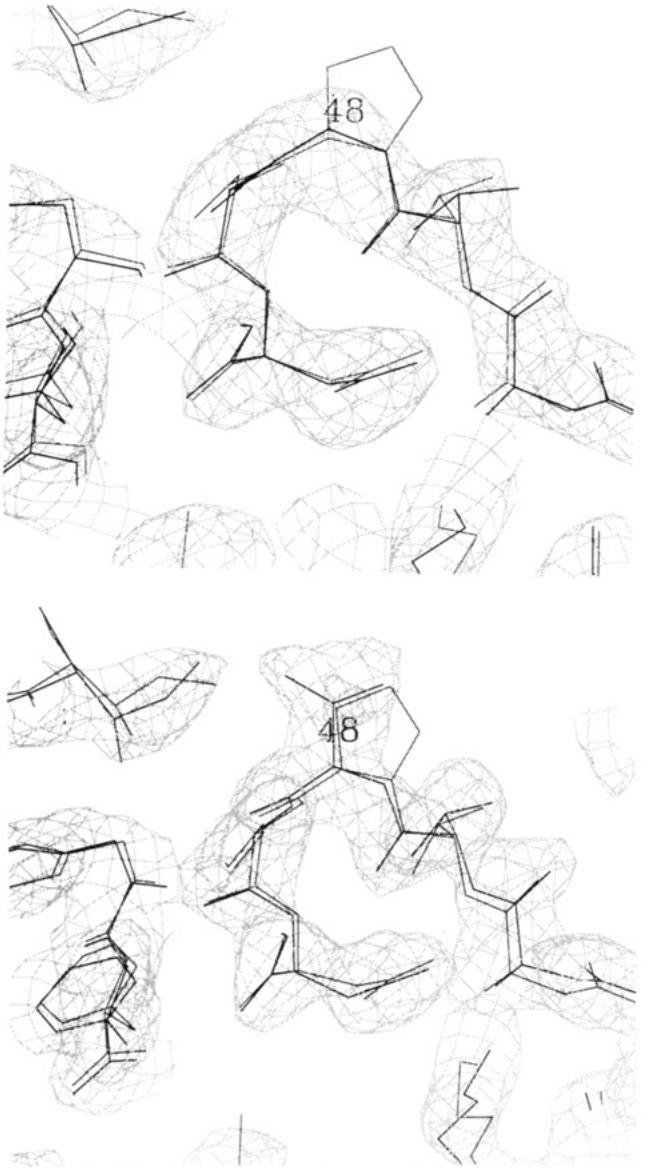

FIGURE 3: $2F_o - F_c$ electron density maps of Pro48Gly (A, top) and Pro48Thr (B, bottom) with the atomic coordinates represented by solid lines. The atomic positions of wild-type phosphorylase are shown by the broken lines.

tively. An estimate of the error in these angular differences was provided by examining the rotation of the C-terminal domain, which was not expected to move as it is a large distance from the site of mutation. The rotation of the C-terminal domain for both variant structures was only 0.05° in both mutant structures.

DISCUSSION

Detailed structural studies of phosphorylated or dephosphorylated rabbit muscle phosphorylase in different complexes with its allosteric effectors have provided a clear picture of the structural changes which occur upon activation (Browner & Fletterick, 1992). Whether activation is mediated by covalent modification, phosphorylation of serine 14, or by AMP binding, the conformational change is similar, although the magnitude of the quaternary movements differs. In particular, the symmetrical movement of the CAP structure toward helix 2 on the opposite subunit (Figure 1) is a common theme in all the activated structures (Barford et al., 1991; Sprang et al., 1988, 1991). The importance of proline 48 in the mechanism of AMP activation was first suggested from a comparison of amino acid differences between the muscle and liver isozymes (Rath et al., 1987). The correct positioning of the CAP relative to helix 2 in the same monomer was hypothesized to be critical in the proper configuration of the cooperative AMP binding sites. Proline 48 is at the junction of the CAP and helix 2, approximately 12 Å from the AMP binding site. Liver phosphorylase, which has a threonine at position 48, binds AMP noncooperatively. The replacement of proline 48 in the muscle enzyme with alanine, glycine, and threonine clearly demonstrated the critical importance of this residue in AMP activation and also revealed the precise role of cooperative AMP binding in the activation of muscle phosphorylase.

Substitution of proline 48 had no effect on activation by covalent modification; all mutant enzymes were fully activated by serine 14 phosphorylation. In contrast, the amino acid residue at position 48 had dramatic effects on AMP activation, in some cases. The notion that proline might be necessary as a hinge between the two symmetrical structural elements that cooperatively bind AMP (Figure 1) was rejected. Substitution of alanine at position 48 had no effect on cooperative binding or activation of the muscle enzyme. The original hypothesis, that the threonine substitution at position 48 could modify cooperative AMP binding in the liver enzyme (Rath et al., 1987), however, was confirmed.

The engineered proteins with either glycine or threonine substituting for proline 48 bound AMP noncooperatively; only Pro48Thr showed reduced enzymatic activity. Compared to the muscle isozyme, liver phosphorylase is a less active enzyme, whether activated by phosphorylation or AMP binding (Coats et al., 1991; Kobayashi et al., 1982). Thus, the single amino acid substitution of threonine at position 48 in the muscle enzyme results in an enzyme that mimics some of the behavior of the liver isozyme, loss of cooperative AMP binding and reduced maximal enzymatic activity. The complementary mutagenesis experiment in the liver enzyme, replacing threonine 48 with proline, however, was not sufficient for restoring enhanced enzymatic activity, as judged qualitatively in crude extracts (Coats et al., 1991). The loss of cooperative AMP binding and the decrease of enzymatic activity in Pro48Thr suggest that this amino acid substitution both perturbs the configuration of the cooperative AMP binding sites and interferes with the transmission of the allosteric signal from AMP to the active site. The structural analysis of inactive Pro48Thr showed no major atomic rearrangements, although the activation domain has moved relative to the position in the wild-type structure. The fact that activation by covalent modification was not affected by substitution of proline 48 is evidence that the mechanisms of activation by AMP and phosphorylation are, in part, independent.

The most intriguing finding, that cooperative AMP binding is not required for full activation of phosphorylase, was found from analysis of the glycine-substituted protein. Pro48Gly was fully activated by AMP, although the binding was not cooperative. As in the case of Pro48Thr, the rotation of the activation domain in Pro48Gly is in the same direction as in the phosphorylated enzyme, relative to the wild-type, inactive structure (Browner et al., 1992). The rotation of the activation domain is larger for Pro48Gly than Pro48Thr. The kinetic analysis indicates that the effect of a glycine at position 48 is a decrease in total energy required for activation, due to the loss of the free energy associated with a cooperative binding interaction. Altering the ground state (inactive) of the enzyme in the direction of the activated enzyme, as evidenced by the activation domain rotation, allows the hyperactivation of

Pro48Gly at lower AMP concentrations. Thus, the Pro48Gly enzyme is to wild-type phosphorylase as myoglobin is to hemoglobin. A similar loss of AMP cooperativity, as observed in the single amino acid proline 48 variants, was observed when dephosphorylated, rabbit muscle phosphorylase was modified by glutaraldehyde (Wang & Tu, 1970).

We have shown that the allosteric effects of AMP binding, activation and cooperativity, can be uncoupled. In the native enzyme the free energy from binding a single AMP molecule is available both for alteration of the second binding site at the subunit interface and also for inducing global structural changes. In Pro48Gly, energy from binding AMP noncooperatively is effectively transmitted only within a subunit of the dimer to the active site. Thus, we can assume that in phosphorylase cooperative binding energy is also efficiently transmitted within the subunits to the active sites and not lost on altering the second binding site. Cooperative AMP binding does not modulate the energy required for activation but rather is a biological mechanism for tuning the activity of the enzyme to physiological AMP concentrations. As shown in both the native liver and muscle isozymes and our engineered variants, the enzymatic activity and sensitivity to AMP are adjustable by substituting only a few amino acids in glycogen phosphorylase.

ACKNOWLEDGMENT

We thank Stuart Tugendreich for constructing the phosphorylase variants, Christine Luong and Hayyah Muller for protein purification and kinetic analyses, and Paul Thomas for crystallization of Pro48Gly, Eric Fauman for structure analysis programs (GEM), and Dr. Louise Johnson for providing the coordinates and F_o for dephosphorylated, muscle phosphorylase.

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Registry No. Thr, 72-19-5; Ala, 56-41-7; Gly, 56-40-6; Pro, 147-85-3; AMP, 61-19-8; glycogen phosphorylase, 9035-74-9.