Site-Directed Mutagenesis of Glutamate-190 in the Hinge Region of Yeast 3-Phosphoglycerate Kinase: Implications for the Mechanism of Domain Movement†

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ABSTRACT: In order to evaluate a possible contribution of glutamate-190, situated in the hinge region of yeast 3-phosphoglycerate kinase (PGK), to the mechanism of the substrate- and sulfate-induced domain movement, we have constructed two point mutants, Gln-190 and Asp-190, using oligonucleotide-directed in vitro mutagenesis. The Michaelis constants of the mutants for ATP and 3-phosphoglycerate were not significantly altered, whereas the catalytic activities were decreased, both in the absence and in the presence of sulfate ions. In the absence of sulfate, the Gln-190 and Asp-190 mutants exhibited 26% and 36% of the activity of the native enzyme. In the presence of 30 mM Na_2SO_4, a concentration at which native PGK exhibits maximum activation, the relative activities of the Gln-190 and Asp-190 mutants were 6% and 9%, respectively. In contrast to the native enzyme, which undergoes activation at low sulfate concentrations and inhibition at high concentrations, both mutants showed a complete loss of the salt activation effect. These results suggest that Glu-190 is not directly involved in the binding of substrates but might be important for conformational flexibility. We have also demonstrated that, similarly to native PGK, both mutants are completely inactivated by the incorporation of 1 mol of glycine ethyl ester/mol of enzyme. Appreciable protection against inactivation is observed in the presence of both substrates, MgATP and 3-phosphoglycerate. Only limited protection is observed in the presence of the individual substrates, suggesting that the modification does not occur at the substrate binding sites. The rates of inactivation of the mutant enzymes are increased 3-fold, indicating that the environment of a reactive glutamate has been altered as the result of the mutations introduced in the hinge. Thus our results demonstrate that two distinct glutamates, remote from the active site, are important for the catalytic function of PGK.

Yeast 3-phosphoglycerate kinase catalyzes a reversible transfer of a phosphate group between ATP[1] and 3-phosphoglycerate (Scopes, 1973):

MgATP + 3-phosphoglycerate ⇔ MgADP + 1,3-diphosphoglycerate

The enzyme is a monomer composed of 415 amino acids, arranged in two globular domains separated by a "hinge" (Figure 1A). The interdomain hinge region (Figure 1B) contains an α-helix (amino acids 185–199) and the carboxy-terminal end of the polypeptide chain (residues 386–415). It has been proposed that a substrate-induced conformational change, resulting in the closure of the active-site cleft between the two domains, is essential for the catalytic mechanism of the enzyme (Banks, 1979; Anderson et al., 1979). The available crystallographic structures determined for yeast (Watson et al., 1982) and horse muscle (Blake and Rice, 1981) phosphoglycerate kinases represent the open conformation. Strong evidence for a large-scale conformational change has been obtained from studies in solution. Low-angle X-ray scattering (Pickover, 1979) and equilibrium sedimentation (Roustan, 1980) studies have indicated a large change in the shape of the molecule upon binding of both substrates. It has also been demonstrated that sulfate ions, in the absence or in the presence of the substrates, cause similar changes in the hydrodynamic properties of the enzyme and induce an increase in its catalytic efficiency (Roustan et al., 1980).

It has been proposed by Watson et al. (1982) that the interaction between glutamate-190 and histidine-388 situated in the hinge region of PGK (Figure 1B,C) might be important for controlling the conformational transition between the open and closed forms of the enzyme. Also, a single glutamyl residue, distant from the substrate binding sites, has been implicated as critical for the catalytic process on the basis of chemical modification studies (Brevet et al., 1973; Desvages et al., 1980).

In this study we have used site-directed mutagenesis to examine a possible role of glutamate-190 in the mechanism of domain movement. We have also investigated the relationship between glutamate-190 and the critical glutamyl residue postulated from the chemical modification studies.

Experimental Procedures

Materials. Yeast phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, ATP, 3-phosphoglycerate, NADH, MES, Tris, triethanolamine, and nonradioactive glycine ethyl ester hydrochloride were from Sigma Chemical Co. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodi-

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[1] Abbreviations: PGK, 3-phosphoglycerate kinase; NADH, nicotinamide adenine dinucleotide (reduced form); ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; NTPs, nucleoside triphosphates; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; GEE, glycine ethyl ester; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance; AMV, avian myeloblastosis virus; bp, base pair; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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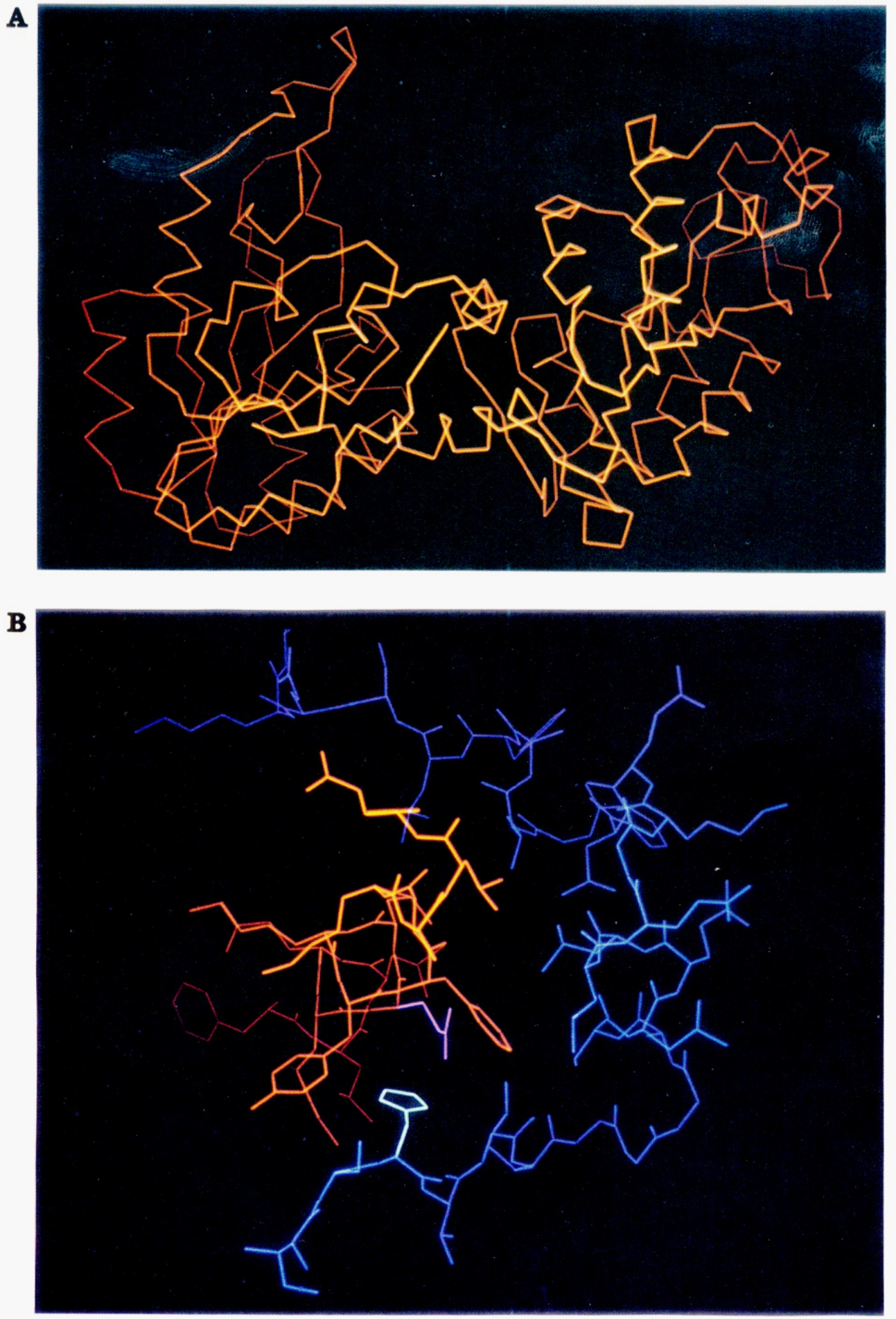
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B

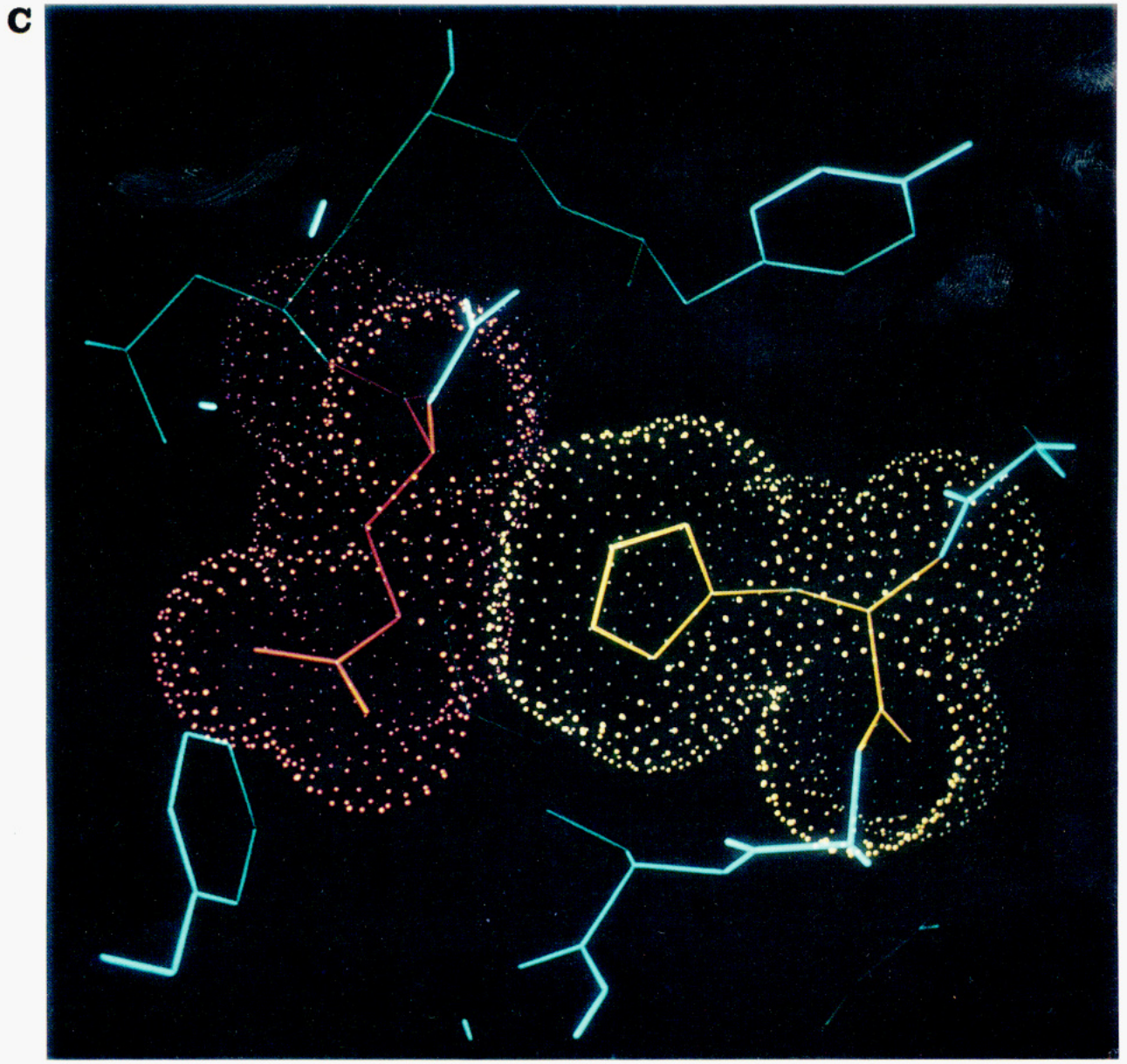

FIGURE 1: Computer graphics representation of the structure of yeast PGK. (A) α-Carbon backbone. Two domains, amino terminal (left) and carboxy terminal (right), are separated by a hinge region containing two α-helices. The bottom helix consists of residues 185–199. The top helix (residues 393–401), shown end on, is a part of the carboxy-terminal fragment of the polypeptide chain. The remainder of the chain (residues 402–415) extends toward the amino-terminal domain. A view of the interdomain region, in a different orientation, is shown in part B. (B) The hinge region: the bottom interdomain α-helix is shown in red; the carboxy-terminal fragment of the polypeptide chain (residues 386–415) is shown in dark blue (Lys-415 at the top); the side chains of Glu-190 and His-388 are colored purple and light blue, respectively. (C) van der Waals surfaces calculated for Glu-190 and His-388. Computer graphics pictures were generated by using an Evans & Sutherland Color Graphics System and the Biograf graphics program (Biodesign Inc., Pasadena, CA).

imide metho-*p*-toluenesulfonate was purchased from Fluka. [1-^{14}C]Glycine ethyl ester hydrochloride and AMV reverse transcriptase were from New England Nuclear. Other reagents used for ^{35}S sequencing were from Pharmacia. Enzymes used in mutagenesis experiments were from Bethesda Research Laboratories. [γ-^{32}P]ATP was from Amersham.

Site-Directed Mutagenesis. Oligonucleotide-directed in vitro mutagenesis [Razin et al., 1978; Hutchinson et al., 1978; for review see Smith and Gillam (1981)] was performed according to the protocols described by Messing (1983) and Zoller and Smith (1983). Mutagenic oligonucleotides (20-mers) were synthesized chemically (Sinha et al., 1984) at the DNA Synthesis Facility of the City of Hope. Crude oligonucleotides were purified by using Sep-Pak C18 cartridges (Waters Associates) and polyacrylamide gel electrophoresis, as described by Lo et al. (1984). The YEp9T plasmid (Chen et al., 1984) containing yeast PGK gene (YEp9T-PGK) was a gift from Dr. R. A. Hitzeman (Department of Cell Genetics, Genentech, Inc.). A scheme of the construction of the expression vector containing the Gln-190 mutant PGK gene is shown in Figure 2. The YEp9T-PGK vector was cut into two fragments at the unique *Kpn*I and *Bam*HI restriction sites. The restriction fragments were separated by polyacrylamide gel electrophoresis and isolated by electroelution. The smaller (1389-bp) *Kpn*I–*Bam*HI fragment was subsequently cut with *Xba*I, yielding two fragments (Figure 2). The 455-bp *Kpn*I–*Xba*I fragment, containing the prospective mutation site, was subcloned into M13mp19 phage DNA. The single-stranded phage DNA containing the PGK *Kpn*I–*Xba*I insert was purified from the supernatant of the transfected *Escherichia coli* JM107 cells and used as a template for mutagenesis. The in vitro synthesis of the complementary strand was carried out according to the

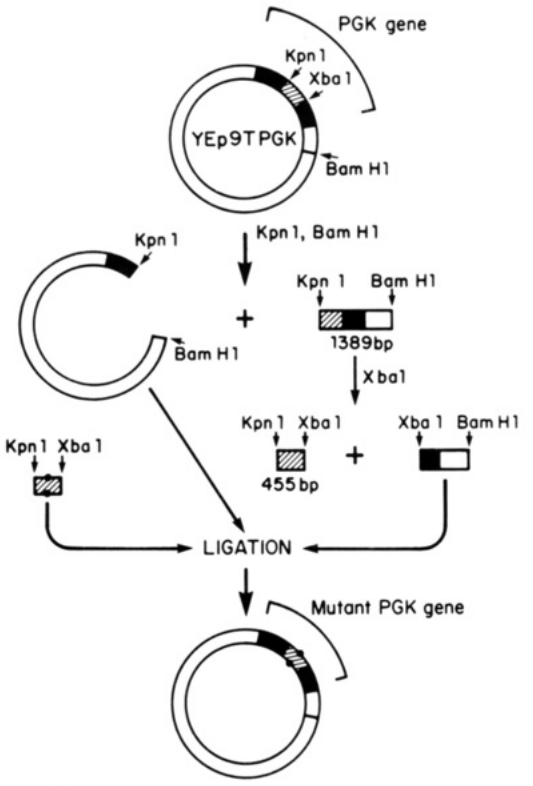

FIGURE 2: Schematic representation of the construction of the glutamine-190 mutant PGK. The 455-bp region of the PGK gene used for mutagenesis is shown as a hatched fragment. The rest of the PGK coding region is shown as solid black segments. The final step of the construction is a three-fragment ligation.

published procedures (Messing, 1983; Zoller & Smith, 1983), in the presence of the large fragment of DNA polymerase I (Klenow), dNTPs, the mutagenic oligonucleotide (containing the GAA → CAA codon change), and the universal sequencing primer. The gaps were closed with T4 DNA ligase. The resulting double-stranded DNA was used for transformation of competent *E. coli* JM107 cells. Single-stranded phage DNA, isolated from 100 individual plaques, were screened by dot blot hybridization on nitrocellulose filters (Zoller & Smith, 1983), using the ^{32}P-labeled mutagenic oligonucleotide as a hybridization probe. Positives were plaque-purified and rescreened. The presence of the mutation was confirmed by sequencing selected clones (Sanger et al., 1977; Biggin et al., 1983; Zagursky et al., 1985) (Figure 3). The RF DNA, containing the mutation, was digested with *Kpn*I and *Xba*I and used for the construction of the Gln-190 expression vector (Figure 2). For the construction of the Asp-190 mutant (GAA → GAC codon change), the entire *Kpn*I–*Bam*HI fragment was used for M13 mutagenesis and construction of the expression vector. The mutant PGK genes were expressed in *Saccharomyces cerevisiae* strain XSB44-35D (*a pgk1 trp1 ade1 leu1 gal1*) (Chen et al., 1984), kindly provided by Dr. R. A. Hitzeman. Yeast transformations were performed according to the method of Ito et al. (1983).

Protein Purification. The mutant PGK proteins were purified according to the procedures developed for the purification of human yeast chimeric phosphoglycerate kinases (Mas et al., 1986). The final enzyme preparations were at least 98% pure, as estimated from the SDS–PAGE (Laemmli, 1970) stained with Coomassie Blue.

Kinetic Studies. The kinetic parameters K_m and k_{cat} were determined for the reaction of PGK in the direction of the formation of 1,3-diphosphoglycerate, using a coupled assay with glyceraldehyde-3-phosphate dehydrogenase (Bücher, 1947). The initial rate measurements were conducted at 25 °C with a Cary 219 spectrophotometer (Varian). The decrease of NADH absorption at 340 nm was monitored as a function of time. Assays were carried out in a 1-mL solution containing 20 mM triethanolamine–acetate buffer, pH 7.5, 50 mM magnesium acetate, 0.2 mM NADH, and 150 μg/mL glyceraldehyde-3-phosphate dehydrogenase. The kinetic parameters were determined from linear Lineweaver–Burk plots, at constant ATP concentration (5 mM) or constant 3-phosphoglycerate concentration (10 mM). The concentration of the other substrate varied in the range of approximately 0.1–10 times the K_m value. The concentration of free magnesium ions was kept constant at 1 mM (Scopes, 1978a). ATP concentrations were determined spectrophotometrically at 259 nm, with an extinction coefficient of 15 400 M^{-1} cm^{-1}. The concentration of 3-phosphoglycerate was determined enzymatically at 366 nm (Larsson-Raźnikiewicz, 1972).

Salt Effect Experiments. Enzymatic activity was measured as a function of Na_2SO_4 concentration (0–150 mM) at 25 °C in 50 mM Tris-HCl buffer, pH 7.8, containing 1 mM $MgCl_2$,

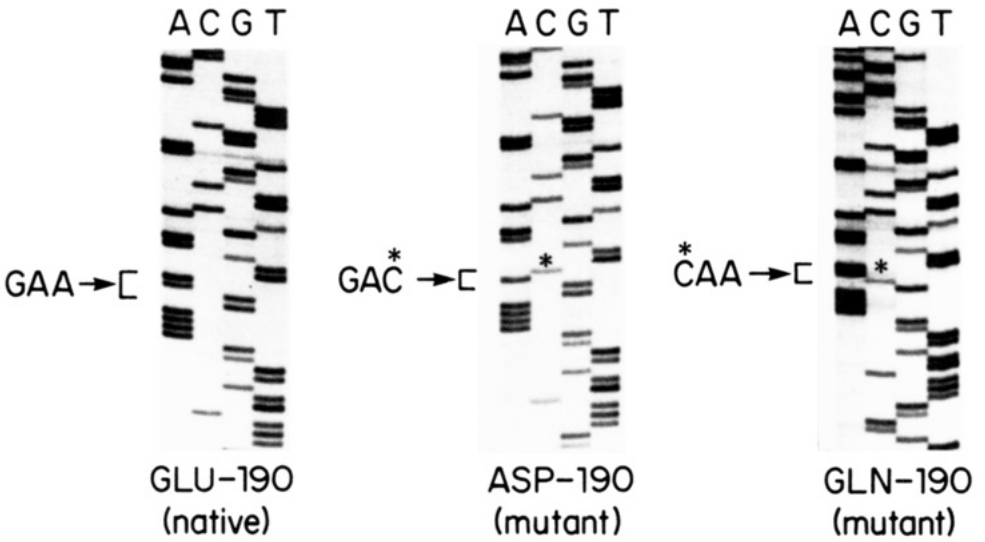

FIGURE 3: DNA sequence data for native and mutated genes of yeast PGK. The *Kpn*I–*Xba*I fragment of the Gln-190 mutant DNA and the *Kpn*I–*Bam*HI DNA fragments of the Asp-190 mutant and the native PGK were sequenced by the dideoxy method as described under Experimental Procedures. The universal sequencing primer was annealed close to the *Kpn*I site. An asterisk above a band indicates a position of a base change (GAA → GAC for the Asp-190 and GAA → CAA for the Gln-190 mutation).

Table I: Determination of Catalytic Constants for Native and Mutant Phosphoglycerate Kinases[a]

amino acid at position 190	ATP			3-phosphoglycerate		
	$K_m \times 10^{-3}$ (M)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	$K_m \times 10^{-3}$ (M)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Glu (native)	0.30	598.0	2.0×10^6	0.5	606.7	1.2×10^6
Gln (mutant)	0.51	73.8	1.45×10^5	1.1	53.7	4.9×10^4
Asp (mutant)	0.25	88.0	3.52×10^5	1.1	65.0	5.9×10^4

[a] The kinetic measurements were performed in 20 mM triethanolamine–acetate buffer, pH 7.5, containing 50 mM Na_2SO_4 and 1 mM magnesium acetate, as described under Experimental Procedures. Errors of the K_m and k_{cat} determinations were less than 10%.

1 mM ATP, 2 mM 3-phosphoglycerate, 0.2 mM NADH, and 150 μg/mL glyceraldehyde-3-phosphate dehydrogenase (Larsson-Raźnikiewicz & Jansson, 1973). Assays were run in duplicate. In some experiments the enzymes were preincubated for 3 h at a given salt concentration prior to the activity assay under identical conditions.

Reaction with CMC and GEE. The enzymes (0.4 mg/mL) were incubated with 0.04 M CMC in the presence of 0.25 M glycine ethyl ester in 20 mM MES buffer, pH 6.1. In the protection experiments, substrates were added separately or together (2.5 mM MgATP and 5 mM 3-phosphoglycerate). The enzyme sample without the reagents was always included as a control. At specified time intervals, aliquots were withdrawn and diluted 10-fold (mutant enzymes) or 100-fold (native PGK) into ice-cold, 20 mM MES buffer, pH 6.5. Aliquots were assayed for phosphoglycerate kinase activity by using a conventional assay (Scopes, 1973).

Incorporation of [1-^{14}C]Glycine Ethyl Ester in the Presence of CMC. The enzymes were incubated with 0.04 M CMC and 0.25 M [1-^{14}C]glycine ethyl ester under conditions described above. At specified time intervals, 0.5-mL aliquots (in duplicates) of the reaction mixture were withdrawn, diluted 2-fold with 10 M urea in 20 mM MES, pH 6.5, and to separate excess of reagents, immediately filtered through two consecutive columns with Sephadex G-50–80 (Sigma Chemical Co.), packed in 3-cm^3 plastic syringes and equilibrated with 5 M urea in the same buffer. A column centrifugation procedure (Penefsky, 1977) was used for rapid gel filtration. The filtered solutions, containing modified enzyme, were assayed for radioactivity and protein concentration. Radioactivity was measured in a Beckman LS 6800 liquid scintillation counter. Protein concentrations were determined by using the Bio-Rad protein assay (Bradford, 1976). Native PGK was used to prepare standard curves in the presence of 5 M urea in 20 mM MES, pH 6.5.

Computer Graphics Modeling. Computer graphics pictures (Figure 1) were obtained with a PS330 Color Graphics System (Evans & Sutherland) and an interactive graphics program, Biograf (Biodesign, Inc., Pasadena, CA). Yeast PGK coordinates were from the Protein Data Bank, Brookhaven National Laboratory.

Results

Comparison of Kinetic Properties of the Native and Mutant Phosphoglycerate Kinases. It has been shown that yeast PGK exhibits anomalous kinetics, dependent on the composition of the assay solution (Larsson-Raźnikiewicz, 1966; Scopes, 1978a). The presence of an anion-binding, activatory site has been postulated to account for the nonlinear double-reciprocal plots (Scopes, 1978a). It has been shown that linear double-reciprocal plots can be obtained at concentrations of Na_2SO_4 above 20 mM and at a constant concentration (1 mM) of free magnesium ions (Scopes, 1978a). A random sequential type of mechanism has been postulated with sulfate acting as a competitive inhibitor (Scopes, 1978a). In order to compare the catalytic constants (K_m and k_{cat}) for the native and the Gln-190 and Asp-190 mutants of PGK, assays were performed in 0.02 M triethanolamine–acetate buffer, pH 7.5, in the presence of 50 mM sodium sulfate and at a constant (1 mM) concentration of free magnesium ions, as described under Experimental Procedures. Under these conditions simple Michaelis–Menten kinetics were observed (not shown). The kinetic parameters, determined from double-reciprocal plots, are presented in Table I. The Michaelis constants for ATP are similar for the native and the Asp-190 mutant PGK (0.30 mM and 0.25 mM, respectively). The K_m for ATP of 0.51 mM, obtained for the Gln-190 mutant, is about 2-fold higher. The k_{cat} values for the Gln-190 and Asp-190 PGK mutants, measured at saturating concentrations of 3-phosphoglycerate, are about 8-fold and 7-fold lower, respectively, in comparison to the k_{cat} of about 600 s^{-1} for the native PGK. The K_m values for 3-phosphoglycerate for both mutants (K_m = 1.1 mM) are about 2-fold higher than the corresponding Michaelis constant for the native enzyme (K_m = 0.5 mM). The corresponding k_{cat} values for the mutants are decreased by about 1 order of magnitude under these conditions (Table I).

Effect of Sulfate Ions on the Catalytic Activity of the Native and Mutant Phosphoglycerate Kinases. Sulfate behaves as an activator of yeast PGK at low concentrations and as an inhibitor at high concentrations. It has been postulated that a change in conformation of the enzyme is responsible for the activation (Larsson-Raźnikiewicz & Jansson, 1973; Roustan et al., 1980). At high concentrations, sulfate ion acts as a competitive inhibitor (Scopes, 1978a). In order to examine whether the mutations in the hinge region have affected the ability of the enzyme to undergo a sulfate-induced activation, we measured enzymatic activities of the native PGK and of both mutants as a function of Na_2SO_4 concentration. Extensive studies of the effect of salt on the activity of yeast PGK (Scopes, 1978a) have demonstrated that the shape of the activation curve, the extent of activation, and the sulfate concentration required for maximum activation depend on the relative concentrations of the substrates. Generally, at lower concentrations of the substrates the maximum activation occurs at lower sulfate concentrations (Scopes, 1978a; Larsson-Raźnikiewicz & Jansson, 1973). Activity measurements were carried out at 1 mM MgATP and 2 mM 3-phosphoglycerate concentrations, as described under Experimental Procedures. At these concentrations appreciable activation of yeast PGK has been observed (Larsson-Raźnikiewicz & Jansson, 1973). The results obtained for the Gln-190 and Asp-190 mutants and for the native PGK, in the concentration range of 0–150 mM Na_2SO_4, are shown in Figure 4. In agreement with the studies of Scopes (1978a), the native PGK exhibits activation at lower concentrations of sulfate, followed by inhibition at higher concentrations. The maximum activation of approximately 135% is observed for the native enzyme at about 30 mM concentration of Na_2SO_4 (Figure 4). In contrast, the Gln-190 and Asp-190 mutants exhibit inhibition throughout the entire range of sulfate concentration. The same results were obtained when the mutant enzymes were preincubated for 3 h at a given salt concentration prior to the activity

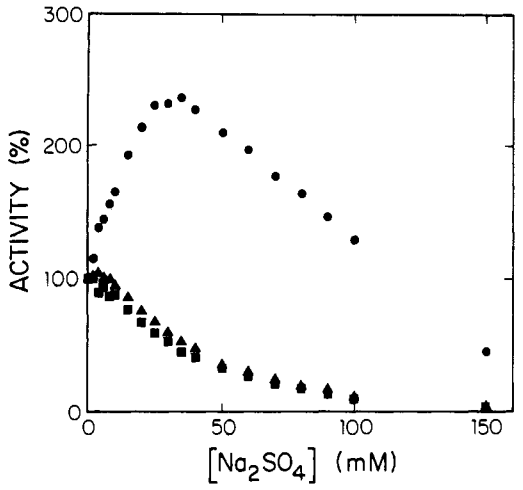

FIGURE 4: Effect on Na_2SO_4 on enzymatic activity of the native and the mutant phosphoglycerate kinases: (●) native PGK; (■) Gln-190 mutant; (▲) Asp-190 mutant. Activities are expressed relative to the activity of each enzyme in the absence of sulfate. Each point represents an average of two to three determinations. Conditions of the activity assays are described under Experimental Procedures.

Table II: Comparison of Relative Enzymatic Activities of Native and Mutant Phosphoglycerate Kinases in the Absence and Presence of 30 mM Na_2SO_4[a]

	relative activity	
amino acid at position 190	0 mM Na_2SO_4	30 mM Na_2SO_4
Glu (native)	1.00	1.00
Gln (mutant)	0.26	0.06
Asp (mutant)	0.36	0.09

[a] Enzymatic activities (v_{obsd}) were determined as described in the legend to Figure 4. Relative activities of the mutants are expressed as a fraction of the activity of the native enzyme measured under identical conditions.

measurements. A comparison of the relative enzymatic activities of the native enzyme and both mutants, in the absence and in the presence of 30 mM Na_2SO_4, is shown in Table II. In the absence of sulfate, the Gln-190 and Asp-190 mutants exhibit 26% and 36% of the activity of the native enzyme under identical conditions. In the presence of 30 mM Na_2SO_4, when the maximum activation of the native enzyme is observed, the relative activities of the Gln-190 and Asp-190 mutants are 6% and 9%, respectively. Similar relationships were also obtained under the conditions of the kinetic measurements described in the preceding section, at saturating concentration of both substrates (5 mM MgATP and 10 mM 3-phosphoglycerate). In the absence of Na_2SO_4, the Gln-190 and Asp-190 mutants exhibited 30% and 36% activity, respectively, relative to the native enzyme, whereas in the presence of 50 mM Na_2SO_4, the corresponding relative activities were 10% and 14%.

Chemical Modification of the Native and Mutant Phosphoglycerate Kinases with Glycine Ethyl Ester in the Presence of Carbodiimide. It has been demonstrated previously (Brevet et al., 1973; Desvages et al., 1980) that the chemical modification of yeast phosphoglycerate kinase by glycine ethyl ester in the presence of a soluble carbodiimide results in a stoichiometric incorporation of 1 mol of the nucleophile/mol of protein. The modification was accompanied by a complete loss of activity. The substrates, MgATP and 3-phosphoglycerate, added together at saturating concentrations, provided considerable protection against inactivation, whereas only slight protection was observed when they were added separately (Brevet et al., 1973; Desvages et al., 1980). It has also been demonstrated that the modified enzyme retains the ability to bind substrates. From these results it has been postulated that a single glutamyl residue, located outside of the substrate binding sites, is involved in the catalytic process (Brevet et al., 1973; Roustan et al., 1980). Although the sequence of the modified peptide has been reported (Desvages et al., 1980), and one of the glutamates located in the carboxy-terminal fragment of the molecule was implicated as the modified residue, the reported sequence does not match exactly the known primary sequence elucidated later from the cDNA sequence of yeast PGK (Hitzeman et al., 1982). More recently, an analysis of the crystallographic structure of the yeast enzyme led to the proposal that glutamate-190 might be important for the mechanism of conformational transition between the open and closed forms of the enzyme (Watson et al., 1982). In order to elucidate the relationship between the critical glutamic acid residue implicated from the chemical modification studies and glutamate-190, we conducted chemical modification studies for the Gln-190 and Asp-190 mutants and for the native phosphoglycerate kinase, using CMC and glycine ethyl ester as a nucleophile (Hoare & Koshland, 1967).

Table III: Rates of Inactivation of the Native and Mutant Phosphoglycerate Kinases by CMC and GEE in the Absence and Presence of Substrates[a]

	$k \times 10^2$ (min^{-1})		
additions	Glu-190	Asp-190	Gln-190
none	4.1	12.4	11.6
3-phosphoglycerate (5 mM)	2.9	7.4	8.6
MgATP (2.5 mM)	2.1	5.0	6.6
3-phosphoglycerate + MgATP	1.1	2.2	3.3

[a] The inactivation reaction was carried out at 25 °C in 20 mM MES buffer, pH 6.1, in the presence of 0.04 M CMC, 0.25 M GEE, and substrates, as indicated. At regular time intervals, aliquots were withdrawn and assayed as described under Experimental Procedures. The pseudo-first-order rate constants were calculated from the equation $\ln E/E_0 = -kt$, where E/E_0 is the fraction of residual activity of the enzyme.

(1) Inactivation Rates in the Absence and in the Presence of Substrates. The enzymes (0.45 mg/mL) were incubated at 25 °C in 20 mM MES buffer, pH 6.1, in the presence of a large molar excess of CMC and GEE (0.04 and 0.25 M, respectively). Under these conditions a rapid inactivation was observed in the absence of the substrates for the native phosphoglycerate kinase and for the Asp-190 and Gln-190 mutants. The activities of the controls incubated under similar conditions in the absence of these reagents were not significantly altered during the time of the experiment. The inactivation in the absence and in the presence of saturating concentrations of the substrates obeyed pseudo-first-order kinetics. The rate constants for the inactivation are shown in Table III. In the absence of the substrates both mutant enzymes were inactivated approximately 3 times faster than the native PGK. In the presence of both substrates, 3-phosphoglycerate and MgATP, an appreciable protection against inactivation was observed for the native enzyme and for the Asp-190 and Gln-190 mutants (73%, 82%, and 72%, respectively). Smaller protection effects were observed in the presence of the individual substrates (Table III).

(2) Incorporation of [1-^{14}C]Glycine Ethyl Ester. In order to determine the number of modified amino acid residues responsible for the inactivation, the correlation between the loss of activity and a covalent incorporation of [1-^{14}C]glycine ethyl ester into the native and the mutant enzymes was measured as described under Experimental Procedures. The incorporation is directly proportional to the extent of inactivation, as shown in Figure 5. For both native and mutant enzymes, a stoichiometry of approximately 1 mol of GEE

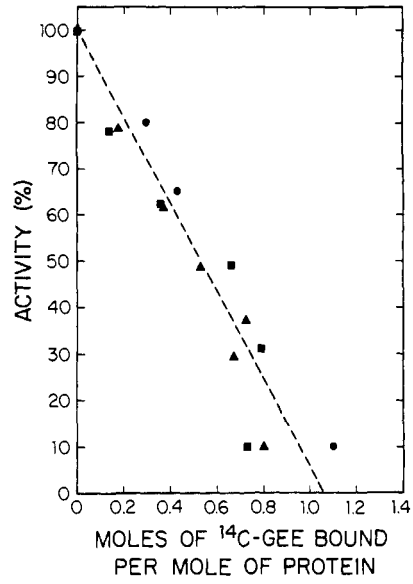

FIGURE 5: Incorporation of [1-^{14}C]glycine ethyl ester as a function of inactivation: (●) native PGK; (■) Gln-190 mutant; (▲) Asp-190 mutant. The enzymes were incubated at 25 °C in 20 mM MES buffer, pH 6.1, in the presence of 0.04 M CMC and 0.25 M [1-^{14}C]GEE. During the course of inactivation, aliquots of the reaction mixture were withdrawn, rapidly filtered through Sephadex G-50 columns, and assayed for radioactivity and protein content, as described under Experimental Procedures.

incorporated/mol of enzyme was obtained from the linear extrapolation of the incorporation data to a complete activity loss.

Discussion

A critical role has been postulated for a single glutamyl residue in yeast PGK, in the mechanism of a large-scale conformational change occurring during catalysis. This hypothesis was based on the results of crystallographic (Watson et al., 1982) and chemical modification studies (Brevet et al., 1973; Desvages et al., 1980). In order to evaluate a possible contribution of glutamate-190, situated in the hinge region, to the mechanism of the domain movement, we replaced this residue with a glutamine and an aspartate, using site-directed mutagenesis. In the Glu → Gln mutation a charge has been eliminated, without altering the geometry of the side chain, whereas in the Glu → Asp substitution the distance between the carboxylate at position 190 and histidine-388 has been increased. In the open conformation of yeast PGK, glutamate-190 and histidine-388 are in a close van der Waals contact, as shown in Figure 1C. The formation of a hydrogen bond and/or a salt bridge seems possible between a nitrogen of His-388 and an oxygen of Glu-190. The results presented in this study clearly demonstrate that substitution of Glu-190 with a glutamine or an aspartate significantly decreases catalytic efficiency and eliminates the ability of the enzyme to undergo a sulfate-induced activation. A similar effect of both mutations on the properties of the enzyme indicates that a combination of several factors—charge, hydrogen bonding, and side-chain packing—might be important for the optimal interaction required for the efficient conformational change.

Despite numerous kinetic, ligand binding, and spectroscopic studies of the interactions of sulfate with PGK, a controversy still exists with respect to the number and the location of the sulfate binding site(s). From the kinetic experiments, carried out under a variety of conditions (Scopes, 1978a,b), it has been concluded that the nonlinear kinetics and the anion activation effect can both be explained by the presence of an activatory site close to the catalytic phosphate transfer site. Support for this hypothesis has been obtained from the substrate and anion binding studies (Scopes, 1978b). Sulfate ion has been shown to compete with the binding of phosphate and of the substrates. These studies led to a conclusion that both the substrate and the sulfate activation effect can be ascribed to the acceleration of the dissociation rate of the product, 1,3-diphosphoglycerate (Scopes, 1978b). One sulfate binding site has been determined from spectral studies of the perturbation of a chromophoric reagent bound to a single sulfhydryl group, located far from the active site (Wrobel & Stinson, 1978). It has been concluded from these studies that the anion binding site is located in a region distant from the active site. Roustan et al. (1980) investigated the effect of sulfate on the modification of the environment of the substrates, using UV difference spectroscopy. A specific perturbation of the spectra produced by the binding of MgATP and 3-phosphoglycerate was demonstrated. The K_s values for MgATP and for 3-phosphoglycerate, determined from the spectral perturbations, have been shown to be sulfate independent (Roustan et al., 1980). Evidence for the binding of sulfate in the proximity of the active site has been obtained from ^{31}P NMR studies (Nageswara Rao et al., 1978). A significant perturbation of the environment of 3-phosphoglycerate, but not 1,3-diphosphoglycerate, has been observed, in addition to the shift in the equilibrium of the enzyme-bound substrates in favor of the enzyme–MgATP–3-phosphoglycerate complex.

In view of the fact that the precise location of the sulfate binding site has not been unambiguously determined, it is not possible to distinguish whether the lack of sulfate activation observed for the mutants of PGK is the result of a direct perturbation of the sulfate binding site or a perturbation of a distinct site responsible for the transmission of the sulfate-induced conformational change. Despite the fact that the shape of the molecule determined from the sedimentation studies (Roustan et al., 1980) appears to be similar, it seems rather unlikely that the mechanisms of the sulfate- and substrate-induced conformational changes are identical. In the former case a conformational change is triggered by binding of a small ion, whereas in the latter case the presence of both substrates (Watson et al., 1982; Scheffler & Cohn, 1986) is necessary to produce the closed conformation of the enzyme. Even if the binding of sulfate at or near the active site is assumed, in agreement with the results of most of the studies discussed above, the path of the conformational transition between the open and closed forms of the enzyme is likely to be different in these two cases. A dramatic effect of the Gln-190 and Asp-190 mutations on the sulfate activation pattern, and a relatively small effect on the catalytic efficiency, might reflect the differences between the substrate- and sulfate-induced mechanisms.

Although it has been reported that the glutamyl residue, covalently modified by glycine ethyl ester, resides in the carboxy-terminal peptide of PGK (Desvages et al., 1980), its precise location within the amino acid sequence of yeast PGK has not been unequivocally established. It was therefore important to determine whether glutamate-190 and the GEE-modifiable group represent two distinct amino acid residues. The kinetics of the inactivation in the presence of CMC and GEE (Table III) and the covalent incorporation data (Figure 5) demonstrate that the chemical modification of a single glutamate in the native PGK, as well as in both mutant enzymes, is responsible for a complete loss of activity.

The increased rates of inactivation observed for the mutants suggest that substitution of Glu-190 in the hinge of PGK with

a glutamine or with an aspartate affects the reactivity and/or accessibility of the other glutamyl residue to chemical modification. This observation is in agreement with a carboxy-terminal location of the chemically modified residue, as postulated by Desvages et al. (1980). It is conceivable that the amino acid substitutions at position 190 might have resulted in a weakening of the interactions of the α-helical segment of the hinge with the carboxy-terminal fragment (Figure 1B) and in a subsequent increase in the susceptibility of the carboxy-terminal glutamate to chemical modification. Incorporation of a bulky chemical group into this residue probably interferes with the substrate-induced conformational change occurring in this region. Since the modification of either of these glutamates, chemically or mutagenetically, has resulted in a perturbation of the original catalytic function of the enzyme, both residues may be viewed as "critical". Since these residues are remote from the substrate binding sites, it can be postulated that their modification has affected conformational flexibility of the molecule. A 2-fold increase of the K_m value for 3-phosphoglycerate might be due to an indirect effect on the binding of this substrate. During protein purification we have observed an increased susceptibility of the Asp-190 and the Gln-190 mutants to proteolytic degradation, reflected by the appearance of the minor satellite bands on the SDS–polyacrylamide gels of the enzyme fractions, migrating slightly faster than the main PGK band (unpublished results). The addition of inhibitors of carboxypeptidase activity completely eliminated degradation. This observation suggests an altered conformation of the carboxy terminus, as the result of the weakening of the interactions with the hinge and/or with the N-terminal domain (see Figure 1A,B), and is also consistent with the increased susceptibility of one of the carboxy-terminal glutamic acid residues to chemical modification.

Analysis of an X-ray model of the PGK structure revealed a considerable distance between the inferred binding sites for the substrates, each located on a different lobe, and led to a hinge-bending model, involving relative movement of the two domains via a rotation around a single bond in the hinge region, to describe the catalytic mechanism of PGK (Anderson et al., 1979; Watson et al., 1982). Examination of the hinge reveals a close packing of the structural elements in this region (Figure 1A,B). A "helix–scissors" mechanism has been proposed recently to explain a hinge-bending conformational change (Blake et al., 1986). According to this model, based on computer modeling, the gap between the rigid domains can be effectively closed by the relative rotation of two closely packed helices at the domain interface. It has been pointed out by Lesk and Chothia (1984) that the molecular mechanism of the domain movement is likely to depend on the structure and the extent of contacts at the domain interfaces. The results presented in this paper provide evidence for the contribution of the side chain of glutamate-190 to the catalytic mechanism of PGK and to the ability of the enzyme to undergo sulfate-induced activation. It can be proposed from this study that the multiple interactions of the side chains, contributed by several amino acid residues in the hinge region, might be important for the transmission of the substrate-induced conformational change in PGK. The helix interface shear mechanism described by Lesk and Chothia (1984), in which small displacements of the side chains at the interfaces between the secondary structure elements are responsible for the propagation of conformational changes, might more adequately describe the mechanism of the domain movement in PGK. Studies are in progress to evaluate the contribution of other structural elements to the mechanism of the domain movement in PGK.

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Yeast DNA Primase Is Encoded by a 59-Kilodalton Polypeptide: Purification and Immunochemical Characterization†

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ABSTRACT: The DNA primase from the yeast *Saccharomyces cerevisiae* has been purified 9200-fold to homogeneity. The yeast DNA primase is a monomeric protein of molecular weight 59 000, and under conditions described in this report, it is stable at 4 or −80 °C. The primase does not bind to DEAE-cellulose, is not inhibited by a high concentration of α-amanitin (4 mg/mL), and is capable of synthesizing small (up to 15 nucleotides in length) ribo or ribo–deoxy mixed initiator RNA primers. The primer synthesis is stimulated by ATP; however, other ribonucleotides could be replaced by deoxynucleotides without any measurable effect on the overall DNA synthesis. Thus, the purified primase is distinct from the RNA polymerases of *S. cerevisiae*. Immunoblot analysis of the polypeptides in a crude cell extract using a mouse polyclonal antibody prepared against the highly purified primase indicates that the 59-kilodalton polypeptide is the native form and not a degraded form of a larger polypeptide; however, primase is degraded rapidly to smaller polypeptides by yeast proteases especially in the absence of protease inhibitors.

The DNA primase activity has been shown to be essential for the synthesis of primers that enable DNA polymerases to initiate synthesis of Okazaki fragments in the lagging strand of the replication fork (Kornberg, 1980; Oertel & Goulian, 1977). The primase activity was demonstrated in *Escherichia coli* (*E. coli*)[1] to be distinct from the RNA polymerase, and the purified primase has been shown to be a product of the DnaG gene of *E. coli* (Bouche et al., 1975; Zechel et al., 1975; Rowen & Kornberg, 1978). Recent studies in several laboratories (Conaway & Lehman, 1982a,b; Tseng & Ahlem, 1982; Yagura et al., 1983) demonstrated that in eukaryotes, the DNA primase activity is associated at least in part with the DNA polymerase α. Vishwanatha and Baril (1986) have demonstrated recently that HeLa cell DNA primase is a 70-kDa polypeptide and could be separated from primase–polymerase complex by hydrophobic chromatography.

In the yeast *Saccharomyces cerevisiae*, a large fraction of the cellular primase activity remains free (Singh & Dumas, 1984; Wilson & Sugino, 1985; Jazwinski & Edelman, 1985). However, attempts toward purification of this free primase activity have led to ambiguities with respect to its mass, specific activity, subunit structure, and physical properties (Jazwinski & Edelman, 1985; Wilson & Sugino, 1985; Plevani et al., 1985). As primase is a major component of the chromosomal apparatus, purification and characterization of primase are important in deciphering the mechanism of eukaryotic chromosomal DNA replication. The present study has been directed toward developing an appropriate purification protocol for primase, establishing its subunit structure and size, and preparing a polyclonal antibody against primase for immunological characterization and molecular cloning of its gene.

Materials and Methods

Yeast. S. cerevisiae (wild-type bakers' yeast) was obtained as a gift from the American Yeast Corp., Baltimore, MD, and was removed from the fermenter during mid log phase.

Nucleotides, Enzymes, and DNA. All ribo- and deoxynucleotides were obtained from ICN. All radioactive nucleotides were obtained from New England Nuclear. Single-stranded M13 phage DNA was prepared according to the published procedure (Ray, 1969). Calf thymus DNA, yeast tRNA, and α-amanitin were obtained from Sigma Chemical Co. Calf thymus DNA was activated with DNase I according to the procedure of Fansler and Loeb (1974). Poly(dT), de-

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[1] Abbreviations: *E. coli*, *Escherichia coli*; *S. cerevisiae*, *Saccharomyces cerevisiae*; PMSF, phenylmethanesulfonyl fluoride; pepA, pepstatin A; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ssDNA, single-stranded DNA; Me_2SO, dimethyl sulfoxide; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; Bis, *N,N'*-methylenebis(acrylamide); kDa, kilodalton(s); pol I, polymerase I; NEM, *N*-ethylmaleimide.

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