Site-Directed Mutagenesis of Histidine-388 in the Hinge Region of Yeast 3-Phosphoglycerate Kinase: Effects on Catalytic Activity and Activation by Sulfate†

Maria T. Mas,* Jerome M. Bailey, and Zenaida E. Resplandor

Physical Biochemistry Section, Division of Biology, Beckman Research Institute of the City of Hope, Duarte, California 91010

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ABSTRACT: It has been proposed that the catalytic mechanism of 3-phosphoglycerate kinase (PGK) and the regulation of its enzymatic activity by sulfate ions involve relatively large conformational changes. We have applied site-directed mutagenesis to assess the role of the interactions between glutamate-190 and histidine-388, located in the interdomain hinge region, in the substrate- and sulfate-induced conformational transitions. We have shown previously that substitutions of Glu-190 with either glutamine or aspartate resulted in a complete loss of sulfate activation and in decreased activities; corresponding to 26% and 36% of the activity of native PGK, respectively [Mas, M. T., Resplandor, Z. E., & Riggs, A. D. (1987) *Biochemistry 26*, 5369–5377]. In contrast, the Lys-388 and Ala-388 mutants retain the ability to undergo sulfate-induced activation and exhibit a larger decrease in activity (relative activities of 6% and 13%, respectively). The decrease of the enzymatic activities of these mutants and the relatively small changes of the K_m values for the substrates imply that both residues participate in the catalytic mechanism by contributing to the conformational flexibility of the enzyme. Our results lead to the following conclusions: (1) in contrast to glutamate-190, histidine-388 is not critical for the sulfate-induced activation; (2) the critical role of Glu-190 is not due to the specific interactions with histidine-388 but to new contacts with other amino acid residue(s) that occur as the result of sulfate-induced rearrangements of the structural elements of the hinge; (3) the substrate- and sulfate-induced conformational transitions seem to occur via different mechanisms; (4) a cumulative effect of multiple interactions of the side chains in the hinge region, rather than individual residues(s), is likely to be responsible for the efficient mechanism of ligand-induced domain movement.

Analysis of the crystallographic structures of horse and yeast PGK[1] (Banks et al., 1979; Watson et al., 1982) suggested that the ATP and phosphoglycerate binding sites were located on two separate domains, corresponding to the carboxy- and amino-terminal halves of the molecule, respectively. On the basis of the large distance between the substrate binding sites a hinge-bending domain movement has been postulated to explain the catalytic mechanism of this enzyme (Banks et al., 1979; Watson et al., 1982). It was shown that the activity of the enzyme is affected by salts (Larsson-Raźnikiewicz & Jansson, 1973; Scopes, 1978). The salt activation effect, investigated extensively for sulfate ions, was shown to be principally due to the specific interactions of anions with the enzyme (Scopes, 1978). Complex salt activation effects have also been ascribed to changes in the conformation of the enzyme (Larsson-Raźnikiewicz & Jansson, 1973; Roustan et al., 1980). Experimental evidence for the substrate- and sulfate-induced conformational changes has been obtained from sedimentation (Roustan et al., 1980), low-angle X-ray scattering (Pickover et al., 1979), and NMR (Tanswell et al., 1976) studies. Examination of the crystallographic model of the open conformation of the enzyme led to a hypothesis (Watson et al., 1982) that interactions of the Glu-190 and His-388 residues in the hinge region of PGK (Figure 1) might be involved in the mechanism of the hinge-bending conformational transition. We have applied site-directed mutagenesis to evaluate relative contributions of these residues to the substrate- and sulfate-induced conformational transitions and to characterize the nature of Glu-190–His-388 interactions.

We have recently reported that the replacement of glutamate-190 with a glutamine or an aspartate resulted in a decreased activity of the enzyme and in a complete elimination of the sulfate-induced activation (Mas et al., 1987). This paper describes the effects of the mutations of histidine-388 (His → Lys and His → Ala) on the catalytic properties of PGK and on the sulfate activation effect.

Experimental Procedures

Reagents and Enzymes. Glyceraldehyde-3-phosphate dehydrogenase, TPCK-trypsin, ATP, 3-phosphoglycerate, NADH, leupeptin, pepstatin, Tris base, and triethanolamine were from Sigma Chemical Co. HPLC solvents were from Pierce Chemical Co. Molecular weight standards and electrophoresis reagents were from Bio-Rad. The reagents used in the mutagenesis experiments and in protein purification were described previously (Mas et al., 1987). The YEp9T-PGK plasmid and yeast strains were kindly provided by Dr. R. A. Hitzeman (Department of Cell Genetics, Genentech, Inc.).

Site-Directed Mutagenesis. Ala-388 and Lys-388 mutations were introduced by oligonucleotide-directed mutagenesis of the *Kpn*I–*Bam*HI fragment excised from the YEp9T-PGK

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[1] Abbreviations: PGK, phosphoglycerate kinase; NADH, nicotinamide adenine dinucleotide, reduced form; ATP, adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TPCK, *N*-tosylphenylalanine chloromethyl ketone; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid.

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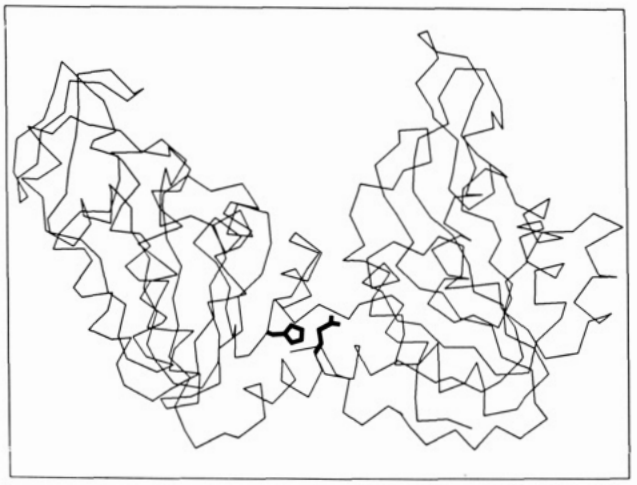

FIGURE 1: Interdomain localization of His-388 and Glu-190. The left and right lobes correspond to the carboxy- and amino-terminal domain, respectively. The side chains of His-388 and Glu-190 are shown in boldface.

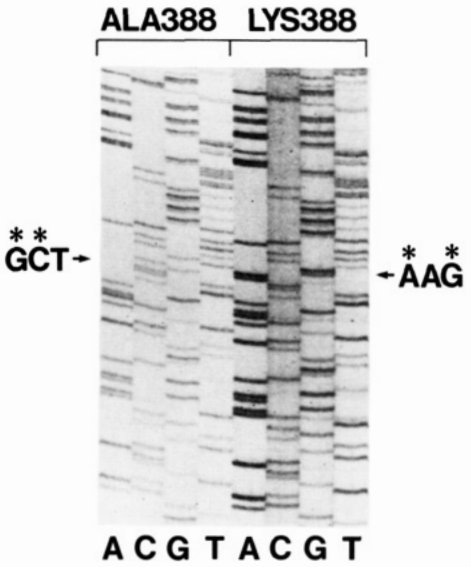

FIGURE 2: DNA sequence data for the Ala-388 and Lys-388 mutants. The original codon in yeast PGK gene, CAT (His), has been changed to GCT (Ala) and AAG (Lys). The asterisks indicate mutated bases. DNA sequencing was carried out by the dideoxynucleotide chain termination method (Sanger et al., 1977), as described previously (Mas et al., 1987).

plasmid (Chen et al., 1984) and subcloned in the M13mp19 vector, as described previously for the Asp-190 mutant of PGK (Mas et al., 1987). Oligonucleotide primers were synthesized and purified at the DNA Synthesis Facility of the City of Hope. The following 20-mers were used to produce the His-388 → Ala-388 and His-388 → Lys-388 mutations, respectively:

5′-AAGATCTCCG*C*TGTCTCTAC-3′
5′-AAGATCTCCA*AG*GTCTCTAC-3′

where the asterisk indicates a base change. The presence of the mutations was confirmed by DNA sequencing (Figure 2).

Protein Purification. The genes of both mutants were expressed in *Saccharomyces cerevisiae* strain XSB44-35D (Chen et al., 1984; Mas et al., 1987). Enzymes were purified according to the previously published procedure (Mas et al., 1986), with the following modifications. The ammonium sulfate precipitation step was omitted, and the yeast extract, following protamine sulfate treatment, was desalted by gel filtration on an Ultrogel AcA 202 column (LKB). Leupeptin

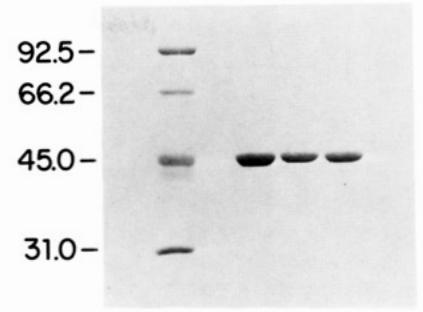

FIGURE 3: SDS–PAGE of the native and mutant phosphoglycerate kinases. Lanes: (1) low molecular weight standards (Bio-Rad), whose sizes are shown in kilodaltons; (2) native PGK; (3) Lys-388 mutant; (4) Ala-388 mutant.

and pepstatin (1×10^{-7} M) and 2 mM EDTA were included in all buffer solutions. The purity of the enzyme preparations was checked by SDS–PAGE (Figure 3), according to the method of Laemmli (1970).

HPLC Analysis and Mass Spectrometry. To confirm the presence of the desired mutations in the purified proteins, the HPLC profiles of the tryptic peptides were compared for the native and the mutant enzymes. A total of 1 mg of each protein was dialyzed overnight against two changes of 50 mM NH_4HCO_3. Dialyzed enzymes were incubated at 37 °C in the presence of TPCK-trypsin [trypsin to PGK ratio of 1:100 (w/w)] in 50 mM NH_4HCO_3 (1 mL) for 4 h, followed by a second addition of trypsin and incubation for an additional 20 h. A total of 300 μL of each sample was analyzed on a reverse-phase C18 HPLC column (Vydac), in a Waters HPLC apparatus, equipped with a Model 6000A pump and a Model 660 solvent programmer. The column was equilibrated with 0.1% trifluoroacetic acid (solvent A) and eluted with a linear gradient of 0–60% solvent B (90% acetonitrile containing 0.1% trifluoroacetic acid) over 120 min. The column temperature was maintained at 40 °C. The absorbance was monitored at 214 nm with a Model 441 absorbance detector (Waters Associates). Comparison of the elution profiles obtained for the tryptic digests of the native, Ala-388 mutant, and Lys-388 mutant samples revealed the presence of one distinct peak in each HPLC profile. The elution positions of these peaks corresponded to 27.5%, 28.75%, and 27% concentration of solvent B, respectively (data not shown). Fractions containing these peptides were isolated, lyophilized, and analyzed on a positive ion fast atom bombardment mass spectrometer (JEOL HX100HF). the monoisotopic mass values (MH^+) of 1754.5 and 1688.4, obtained for the individual peptides isolated from the native (His-388) and the Ala-388 mutant enzyme, respectively, were in agreement with the calculated values for the tryptic fragments corresponding to residues 386–403 of PGK. The amino acid sequence of this peptide in native yeast PGK (Hitzeman et al., 1982) is Ile-Ser-His^{388}-Val-Ser-Thr-Gly-Gly-Gly-Ala-Ser-Leu-Glu-Leu-Leu-Glu-Gly-Lys. The distinct peptide isolated from the tryptic digest of the Lys-388 mutant PGK yielded the MH^+ value of 1417.3, in agreement with the size of the expected tryptic peptide corresponding to residues 389–403.

Kinetic Studies. The Michaelis constants K_m and V_{max} were determined for the reaction of PGK in the direction of the formation of 1,3-diphosphoglycerate, by a coupled assay with glyceraldehyde-3-phosphate dehydrogenase (Bücher, 1947). The initial rate measurements, based on the decrease in absorbance of NADH at 340 nm, were carried out at 25 °C in a Uvikon 860 spectrophotometer (Kontron). Assays were conducted in 20 mM triethanolamine–acetate buffer, pH 7.5, containing 50 mM sodium sulfate, 0.2 mM NADH, and 150

Table I: Comparison of the Effects of Mutations of His-388 and Glu-190 on the Catalytic Properties of Phosphoglycerate Kinase[a]

PGK	K_m(ATP) (mM)	K_m (3-phospho-glycerate) (mM)	V_{max} (units/mg)
native (His-388, Glu-190)	0.30	0.50	826
mutant (Lys-388, Glu-190)	0.13	0.31	40
mutant (Ala-388, Glu-190)	0.12	0.23	120
mutant (Gln-388, Glu-190)[b]	0.11	0.50[c]	165[c]
mutant (His-388, Gln-190)[d]	0.51	1.10	98[e]
mutant (His-388, Asp-190)[d]	0.25	1.10	120[e]

[a] Kinetic measurements were conducted at 25 °C in 20 mM triethanolamine buffer, pH 7.5, containing 50 mM Na_2SO_4 and 1 mM magnesium acetate, as described under Experimental Procedures. The values of the kinetic parameters represent averages of at least two determinations. Amino acid substitutions are underlined. [b] From Wilson et al. (1987). [c] Estimated from the data of Wilson et al. (1987). [d] From Mas et al. (1987). [e] At 50 mM concentration of Na_2SO_4 the V_{max} values for the Gln-190 and Asp-190 mutants, which lack sulfate activation, are approximately 2.6-fold lower than the corresponding values determined in the absence of sulfate (Mas et al., 1987).

μg/mL glyceraldehyde-3-phosphate dehydrogenase. The concentration of one substrate was kept constant (5 mM ATP or 10 mM 3-phosphoglycerate), whereas the concentration of the other substrate covered the range of approximately 0.1–10 times the K_m value. The concentration of free magnesium ion was maintained constant at 1 mM (Scopes, 1978; Mas et al., 1987).

Salt Activation Experiments. Enzymatic activities were measured as a function of Na_2SO_4 concentration (0–70 mM) at 25 °C, in 50 mM Tris-HCl buffer, pH 7.8, containing 1 mM $MgCl_2$, 0.2 mM NADH, and 150 μg/mL glyceraldehyde-3-phosphate dehydrogenase, under conditions similar to those described by Larsson-Raźnikiewicz and Jansson (1973). The concentrations of ATP and 3-phosphoglycerate were 1 mM and 2 mM, respectively, in one set of experiments and 0.25 mM each in another set of experiments.

Computer Modeling. The computer graphics picture (Figure 1) was generated with an Evans & Sutherland graphics system (Model PS330), a Talaris laser printer (Model 800), and a graphics program, BIOGRAF (Biodesign, Inc., Pasadena, CA). Yeast PGK coordinates were from the Protein Data Bank, Brookhaven National Laboratory.

RESULTS

Determination of the Catalytic Properties of the Ala-388 and Lys-388 Mutants of PGK. The Michaelis constants for the Ala-388 and Lys-388 mutants and for the native enzyme were determined as described under Experimental Procedures, in 20 mM triethanolamine–acetate buffer, pH 7.5, in the presence of 50 mM sodium sulfate. The experimental conditions were identical with those used previously for the determination of the kinetic parameters for the Gln-190 and Asp-190 mutants of PGK (Mas et al., 1987). Under these conditions, simple Michaelis–Menten kinetics are observed. The results of the determination of the K_m values for ATP and phosphogylcerate and the maximum velocities (V_{max}) are shown in Table I. The corresponding values for the Gln-388 mutant, reported recently by Wilson et al. (1987), and for the Gln-190 and Asp-190 mutants (Mas et al., 1987) are shown for comparison. The maximum velocities for the Ala-388 and Lys-388 mutants (V_{max} = 120 units/mg and 40 units/mg, respectively) were decreased approximately 7- and 20-fold, respectively, as compared to that of the native enzyme. The Michaelis constants for the substrates were not changed significantly, indicating that binding of ATP and phosphoglycerate was not perturbed as the result of amino acid substitutions at position 388. In fact, a slight decrease (about 2-fold) of the K_m values was observed for both Ala-388 and Lys-388 mutants, suggesting an increase in the substrate binding affinities. A similar result has been reported for the Gln-388 mutant (Wilson et al., 1987).

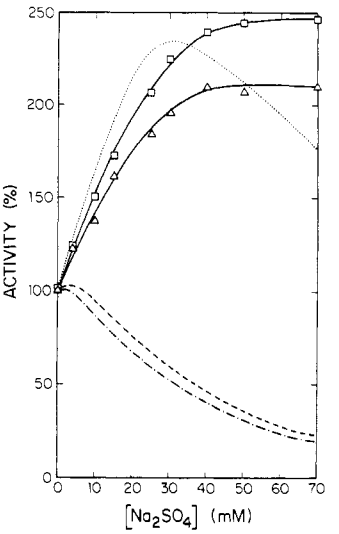

FIGURE 4: Effect of Na_2SO_4 on the enzymatic activities of the native and the mutant phosphoglycerate kinases determined in the presence of 1 mM ATP and 2 mM 3-phosphoglycerate, as described under Experimental Procedures. Enzymatic activities are expressed relative to the activity of each enzyme in the absence of sulfate. (△) Lys-388 mutant; (□) Ala-388 mutant. The data for native PGK (···), the Asp-190 mutant (– –), and the Gln-190 mutant (–·–) (Mas et al., 1987) are shown for comparison.

Effect of Sulfate Ions on the Catalytic Activity of the Ala-388 and Lys-388 Mutants of PGK. Enzymatic activities (v_{obsd}) of the Ala-388 and Lys-388 mutants were measured as a function of Na_2SO_4 concentration. Figure 4 shows the results of the determinations under the conditions used previously (Mas et al., 1987) to characterize the Gln-190 and Asp-190 mutants (2 mM phosphoglycerate, 1 mM ATP, and 0.2 mM NADH). The results for the native enzyme and for the latter two mutants are shown for comparison. The pronounced sulfate activation effect exhibited by native PGK is also observed for the Ala-388 and Lys-388 mutants, although the shapes of the activation curves are different. The activation curves of both mutants reach a plateau at about 50 mM sulfate concentration, whereas native PGK exhibits a maximum at about 30 mM Na_2SO_4, followed by a decrease of enzymatic activity at higher sulfate concentrations (Figure 4). In contrast, a complete loss of salt activation was observed (Mas et al., 1987) when the proximal glutamate-190 has been replaced by an aspartate or a glutamine. Figure 5 shows the results of a similar experiment conducted at 0.25 mM ATP, 0.25 mM phosphoglycerate, and 0.2 mM NADH for the Ala-388 and Lys-388 mutants and for the native enzyme. Under these conditions the shapes of the activation profiles are similar for all three enzymes with a maximum at low salt concentration (at 15, 20, and 10 mM sulfate, respectively) and a gradual decrease at higher sulfate concentrations. The Ala-388 mutant exhibited the highest degree of activation and Lys-388 the lowest (Figure 5). A similar qualitative trend was observed at higher substrate concentrations (Figure 4). In the absence of sulfate, enzymatic activities of the Lys-388 and Ala-388 mutants, measured at saturating substrate concentrations in the presence of 20 mM triethanolamine–acetate buffer at pH 7.5, represented 6% and 13%, respectively, of the specific activity of the native enzyme under identical conditions.

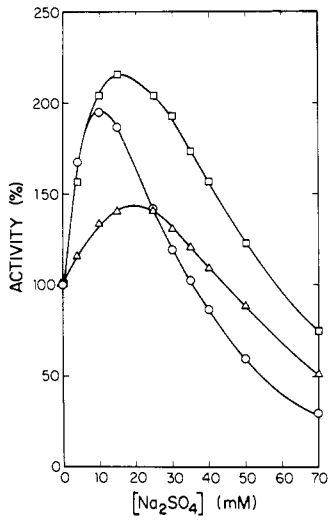

FIGURE 5: Effect of Na_2SO_4 on the enzymatic activities of native PGK and of the Lys-388 and Ala-388 mutants determined in the presence of 0.25 mM ATP and 0.25 mM 3-phosphoglycerate. (○) Native PGK; (□) Ala-388 mutant; (△) Lys-388 mutant. Experimental conditions are given under Experimental Procedures.

DISCUSSION

This work is a continuation of our studies of the structural basis of the substrate and sulfate-induced domain movement in phosphoglycerate kinase. It has been proposed by Watson et al. (1982) that the interactions between histidine-388 and glutamate-190 (see Figure 1) might be essential for controlling the conformational state of PGK. The observation that these residues are located in a hydrophobic environment led to a suggestion that a salt link formation might be important for the mechanism of conformational transition in PGK (Watson et al., 1982). We have tested this hypothesis by site-directed mutagenesis of glutamate-190 (Mas et al., 1987) and of histidine-388, as described in this paper.

Effect of Mutations in the Hinge on Salt Activation Effect. Recent experiments of Wilson et al. (1987), in which histidine-388 was replaced with a glutamine, and the results presented in this paper (histidine → alanine substitution) have demonstrated that these mutant enzymes exhibit sulfate activation and thus provided evidence against the importance of charge–charge interactions. Moreover, the ability of the Ala-388 mutant to undergo a sulfate-induced activation (Figures 4 and 5) argues against a possible role of the hydrogen-bonding interactions between these residues. We have shown recently that glutamate-190 is critical for the sulfate-induced activation of phosphoglycerate kinase (Mas et al., 1987). Substitution of glutamate-190 with either aspartate or a glutamine resulted in a complete loss of sulfate activation. Since the mutant enzymes containing the intact glutamate-190 and either glutamine (Wilson et al., 1987) or lysine or alanine (this paper) at position 388 all exhibit sulfate-induced activation, the critical role of glutamate-190 cannot be ascribed to its specific interactions with the side chain of histidine-388. It can be proposed from these results that the critical interactions of glutamate-190 are likely to involve contacts with other amino acid residues occurring during the path of the sulfate-induced conformational transition. This implies a reorientation of structural elements of the hinge, in the vicinity of Glu-190. A relative shift of two helices in the hinge region has been proposed by Blake et al. (1986) on the basis of a computer simulation of the domain movement in PGK. A "helix-scissors" movement of the helix V (residues 185–199) and helix XIII (residues 393–401) has been postulated from these studies (Blake et al., 1986). Recent experiments conducted in this laboratory (M. T. Mas and Z. E. Resplandor, unpublished data) indicate the importance of the carboxy-terminal elements of the hinge for the mechanism of domain movement.

Effect of the Mutations in the Hinge on the Catalytic Properties of Phosphoglycerate Kinase. Similarity of the K_m values observed for the PGK mutants with the amino acid substitutions at positions 388 and 190 (Table I) indicates that the mutations of either of these two residues do not affect significantly the ATP and phosphoglycerate binding sites. It can therefore be inferred that the reduced catalytic activities of these mutants have resulted from perturbations of their conformational flexibility, which is an essential part of the catalytic mechanism of PGK. In the absence of sulfate ions the relative catalytic activities of the Gln-190 and Asp-190 mutants were higher than those of the histidine-388 mutants. In the presence of saturating substrate concentrations, the former two enzymes exhibited 26% and 36% of the activity of the native enzyme, respectively (Mas et al., 1987), whereas the corresponding activities of the Lys-388 and Ala-388 mutants were 6% and 13% and that of the glutamine-388 mutant was about 20% (Wilson et al., 1987). Thus, in contrast to a dramatic effect of the mutations of glutamate-190 on the salt activation phenomenon and a moderate effect on catalytic activity, the mutations at position 388 had a more pronounced effect on catalytic activity and a relatively small effect on sulfate activation. These results support our earlier hypothesis (Mas et al., 1987) that the substrate- and sulfate-induced conformational transitions in PGK are likely to occur via different mechanisms, although the elements of an interdomain hinge region participate in both mechanisms.

It has been shown previously that chemical modifications of a tyrosine (Markland et al., 1975; Bacharach et al., 1977) and glutamate (Brevet et al., 1973; Mas et al., 1987), located in the interdomain region, caused inactivation of the enzyme, while the binding of both substrates was not affected. These results suggested that the presence of a bulky reagent might hinder conformational flexibility of the enzyme. A relatively low activity of Lys-388 mutant could in part be due to the large size of a lysine side chain. A precise packing of the structural elements of the hinge region seems to be essential for an efficient propagation of the ligand-induced conformational changes. It is conceivable that any amino acid substitutions in this region might interfere with the efficiency of the conformational transitions and thereby reduce enzymatic activity of the enzyme. The importance of the precise nature of the amino acid residues in this region can also be expected from a high degree of conservation of PGK sequences in different species (Hitzeman et al., 1982; Mori et al., 1986; Mas et al., 1986). Thus a critical role can be attributed to multiple interactions of several amino acyl side chains in this region, rather than to an individual residue or a pair of residues. Of the existing models, describing the mechanisms of domain closure in proteins (Lesk & Chothia, 1984), the "helix-interface shear mechanism"—which involves cumulative effects of small shifts and rotations of packed segments, accomodated by small conformational changes in side chains—seems most suitable to account for the observed effects of the mutations and chemical modifications in the hinge region of PGK. A simple hinge-bending mechanism, in which rigid body motion of the domains is assumed (Pickover et al., 1979; Blake et al., 1986), seems less likely because of a relatively large interdomain interface in this enzyme. Moreover, the effect of substrates (Stinson, 1974) and sulfate (Roustan et al., 1980) on the rates of chemical modification of a single cysteine, distant from the

hinge and from the active site, implies a delocalization of ligand-induced conformational transitions. Studies are in progress to evaluate the extent of delocalization of conformational changes in PGK.

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p10, a Low Molecular Weight Single-Stranded Nucleic Acid Binding Protein of Murine Leukemia Retroviruses, Shows Stacking Interactions of Its Single Tryptophan Residue with Nucleotide Bases†

Jose R. Casas-Finet, Nam-In Jhon, and August H. Maki*

Department of Chemistry, University of California, Davis, Davis, California 95616

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ABSTRACT: Room temperature fluorescence and low-temperature phosphorescence studies of the association of p10, a basic low molecular weight single-stranded DNA binding protein isolated from murine leukemia viruses, point to the involvement of its single tryptophan residue in a close-range interaction with single-stranded polynucleotides. Optically detected triplet-state magnetic resonance (ODMR) techniques applied to the complex of p10 protein with the heavy atom derivatized polynucleotide poly(5-HgU) demonstrate the occurrence of stacking interactions of Trp^{35} with nucleic acid bases, thus agreeing with earlier reports that this residue is involved in the binding process [Karpel, R. L., Henderson, L. E., & Oroszlan, S. (1987) *J. Biol. Chem. 262*, 4961-4967].

Most retroviruses can be distinguished by their pathogenicity [for a review, see Bishop (1978)]. Many are oncogenic (Oncovirinae) and are commonly identified according to the type of neoplasm they induce and the species from which the virus has been isolated (Bishop, 1978). Given the pathogenic role of these viruses, considerable effort has been directed to obtain a better understanding of their biology, which may suggest therapeutic and preventive measures against infection. Genomes of endogenous retroviruses reside in the germ lines of many, if not all, species and segregate as normal genetic elements within these species (Bishop, 1978). Both endogenous (baboon) and exogenous (bovine, feline) retroviruses are known to induce enzootic leukosis (lymphosarcoma, leukemia) in their respective hosts (Burny et al., 1980; Essex, 1980). Bovine leukemia virus (BLV) infects 20% of all domesticated cattle in the United States (Ferrer, 1979); infectious virus has been demonstrated in milk of dairy cows (Ferrer et al., 1981) and has proven infectious in other species including chimpanzee (Van Der Maaten & Miller, 1976). Feline leukemia virus (FLV) infects most free-roaming cats and is responsible for

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* Author to whom correspondence should be addressed.

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