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Halibut Muscle 3-Phosphoglycerate Kinase. Chemical and Physical Properties of the Enzyme and Its Substrate Complexes†

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ABSTRACT: An efficient procedure for the purification of 3-phosphoglycerate kinase (PGK) from Pacific halibut muscle is described. The molecular weight (43 500) and specific activity are similar to those of other species of PGK. The isoelectric point (>9.5) is more than 1.4 pH units higher than that reported for mammalian muscle PGK. The reaction of the seven thiol groups with 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) is kinetically biphasic; reaction at a single fast-reacting thiol inactivates the enzyme. The binding of all substrates and products to PGK was observed by ^{31}P NMR. 1,3-Diphosphoglycerate (1,3-P_2G) is more tightly bound than is any of the other reaction components. Unlike 1,3-P_2G in aqueous solution, the complex with PGK is protected from hydrolysis over a period of weeks. The ^{31}P chemical shifts of this complex are insensitive to pH which suggests that solvent water is excluded from the substrate-bound cleft. As with yeast PGK, the equilibrium constant for the phosphoryl transfer reaction is near unity in the enzyme site environment in contrast to a value of approximately 10^3 (in favor of ATP) in aqueous solution. Since the ternary complex equilibrium ^{31}P NMR spectrum can be accounted for entirely on the basis of the various binary complex spectra, there is no compelling evidence for the involvement of a stoichiometrically substantial phosphoenzyme intermediate.

3-Phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) catalyzes the reversible, magnesium ion dependent phosphoryl transfer from 1,3-diphosphoglycerate (1,3-P_2G)[1] to ADP (eq 1) in an important

$$1,3\text{-}P_2G + ADP \overset{Mg^{2+}}{\rightleftharpoons} 3\text{-}PG + ATP \qquad (1)$$

ATP generation step of the glycolytic pathway. PGK has been purified from several sources (Krietsch & Bücher, 1970; Yoshida & Watanabe, 1972; Okonkwo et al., 1973; Scopes, 1975a,b; Johnson et al., 1976b; De & Kirtley, 1977). Insight into the structure and function relationships operative in PGK catalysis has been advanced by X-ray crystallographic studies of the enzymes from yeast (Bryant et al., 1974) and from horse muscle (Blake & Evans, 1974), by recent mechanistic studies (Johnson et al., 1976a; Wrobel & Stinson, 1978; Scopes, 1978a,b; Webb & Trentham, 1980) of both yeast and muscle PGK, and by the total sequencing of horse muscle PGK (Banks et al., 1979).

This laboratory has previously studied the detailed catalytic properties of several glyceraldehyde-3-phosphate dehydrogenase (GPDH) enzymes with most recent emphasis on the enzymes from sturgeon and halibut muscle (Seydoux et al., 1973; Seydoux & Bernhard, 1974; Schwendimann et al., 1976; Bernhard et al., 1977; Malhotra & Bernhard, 1981). The sarcoplasmic fluid of white fish muscle is an abundant source of the glycolytic enzymes and is nearly devoid of other protein components. For reasons discussed elsewhere (Seydoux et al., 1973), the fish muscle glycolytic enzymes are particularly useful for investigations of the regulatory processes which modulate the catalytic function.

The molecular events which occur during the sequence of reactions by which glyceraldehyde 3-phosphate, inorganic

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[1] Abbreviations: 1,3-P_2G, 1,3-diphospho-D-glycerate; 3-PG, 3-phospho-D-glycerate; NAD^+ and NADH, nicotinamide adenine dinucleotide, oxidized and reduced forms, respectively; PGK, 3-phosphoglycerate kinase; GPDH, glyceraldehyde-3-phosphate dehydrogenase; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid, disodium salt; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CM, carboxymethyl; $NaDodSO_4$, sodium dodecyl sulfate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Nbs, 2-nitro-5-thiobenzoate.

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phosphate, and ADP are converted to ATP and 3-PG are currently of interest to us (Weber & Bernhard, 1982). In order to better assess results obtained from similar investigations of the coupled two-step system, we felt it desirable to prepare both GPDH and PGK from the same fish muscle. In this paper, we describe a convenient, high-yield procedure for the purification to homogeneity of PGK from Pacific halibut muscle and some characteristic physical and chemical properties.

The equilibrium binding of substrate and product to halibut PGK has been investigated by ^{31}P NMR. All reaction components contain at least one phosphate moiety. ^{31}P NMR is particularly suited for detailed mechanistic studies with this kinase (Nageswara Rao et al., 1978; Lerman & Cohn, 1980). The nearly complete resolution of the various phosphorus resonance lines affords both binding and kinetic information about the unique reactive centers.

Experimental Procedures

Materials. Pacific halibut (*Hippoglossus stenolepis*) was purchased locally as frozen steaks and was stored at −20 °C prior to use. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was from Boehringer Mannheim Biochemicals, and pig heart lactate dehydrogenase was from Whatman Biochemicals, Ltd.

Ammonium sulfate (special enzyme grade) and urea (ultrapure) were obtained from Schwarz/Mann Biochemicals. $NaDodSO_4$, Nbs_2, pyruvic acid, NAD^+, NADH, and CM-Sephadex (C-50-120) were purchased from Sigma Chemical Co., as were the sodium salts of AMP, ADP, ATP, 3-PG, 2,3-diphosphoglycerate, and phosphoenolpyruvate. 1,3-P_2G was prepared and purified according to the procedure of Furfine & Velick (1965). 1,3-P_2G stocks (5–10 mM, 0.5 mL) were stored at −80 °C. D_2O (99.8 mol %) was obtained from Bio-Rad Laboratories. Reagents and buffers used were analytical grade or the highest quality commercially available. All solutions were prepared by using glass double-distilled water and were passed through a Millipore filter (0.45 μM).

Isolation and Purification of Halibut Muscle PGK. All steps in the isolation and purification procedure were carried out at 4 °C. (See Table I for a summary.)

(*I*) *Extraction* (*Fraction I*). Frozen halibut steaks were trimmed free of skin, bone, and red tissue and then weighed. Frozen muscle tissue was homogenized with two parts of 25 mM EDTA, pH 8.5, in a Waring blender (pausing frequently in order to minimize heating the homogenate). The homogenate was then stirred for 30 min. After centrifugation at 16000*g* for 45 min, the supernatant was filtered through glass wool.

(*II*) *Ammonium Sulfate Fractionation* (*Fraction II*). Solid ammonium sulfate was added slowly to fraction I to obtain 60% saturation (2.35 M), and the solution was stirred for an additional 1 h. The precipitate was removed by centrifugation at 16000*g* for 1 h and discarded. After the supernatant was again filtered through glass wool, the kinase activity was precipitated by the slow addition of solid ammonium sulfate to obtain 83% saturation (3.25 M) and stirred for an additional hour or more. After centrifugation at 16000*g* for 1 h, the pellet was resuspended in a minimal volume of 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and 100 mM KCl and dialyzed exhaustively against this same buffer.

(*III*) *CM-Sephadex Chromatography* (*Fraction III*). After removal of any precipitated protein by centrifugation, fraction II was applied at a flow rate of 50 mL/h to a column (2.1 × 35 cm) of CM-Sephadex (C-50-120) previously equilibrated with the Tris-HCl buffer. The column was washed at the same flow rate with the Tris-HCl buffer until the absorbance at 280 nm of the effluent was ≤0.1 OD unit. The enzyme was then eluted with a linear KCl gradient (100–220 mM, 550 mL/reservoir) of the Tris-HCl buffer at a flow rate of 40 mL/h. Fractions (12.5 mL each) of high, nearly constant, specific activity were pooled.

(*IV*) *Ammonium Sulfate Suspension Storage* (*Fraction IV*). The enzyme (fraction III) was precipitated either by the addition of solid ammonium sulfate to 100% saturation (3.9 M) or by exhaustive dialysis against the Tris-HCl buffer containing ammonium sulfate maintained at 100% saturation. Chloroform (2–5 drops) was added to retard bacterial growth, and the enzyme suspension was stored at 4 °C.

Assays of Enzymatic Activity. 3-Phosphoglycerate kinase turnover was assayed by coupling the Mg^{2+}-dependent formation of 1,3-P_2G from ATP and 3-PG to the glyceraldehyde-3-phosphate dehydrogenase catalyzed reduction by NADH by using a procedure similar to that of Bücher (1955) in 50 mM 2-methylimidazole hydrochloride buffer, pH 7.4, 25 °C. Halibut muscle GPDH, isolated by using a procedure similar to that developed for sturgeon muscle GPDH (Seydoux et al., 1973), was not used in these assays because it copurifies with a small amount of background PGK activity which reduces the assay sensitivity. The assay mixture also contained ammonium sulfate (ca. 30 mM) introduced as a consequence of the storage medium of the GPDH used for these assays. Kinase activity was observed as an absorbance decrease at 340 nm due to the oxidation of NADH. A molar extinction coefficient of 6200 M^{-1} cm^{-1} (Horecker & Kornberg, 1948) was used.

GPDH activity was determined by the Ferdinand (1964) assay. Adenylate kinase and phosphoglyerate mutase activities were determined according to the procedures of Price et al. (1973) and Sutherland et al. (1949), respectively.

Protein Determinations. Protein was determined either (1) by the method of Bradford (1976), using commercially prepared protein assay dye solution (Bio-Rad Laboratories) and bovine γ-globulin as the standard, or (2) by measurement of the absorbance at 280 nm. The average of ten dry weight determinations gave an extinction coefficient ($E^{0.1\%}_{280nm}$) of 0.64 mg^{-1} for purified halibut muscle PGK.

Gel Electrophoresis and Isoelectric Focusing. Polyacrylamide gel electrophoresis to assess purity and to estimate molecular weight was carried out by using 7.5% Bio-Phore tube gels (Bio-Rad Laboratories) according to the manufacturer's procedures. Gels were fixed and stained for protein at 60 °C with 0.2% Coomassie Brilliant Blue G-250 (Eastman) in 5:5:1 H_2O:methanol:acetic acid. Densitometric traces of the stained gels were obtained at 540 nm. Proteins of known subunit size were used as molecular weight markers. Isoelectric focusing was performed at 10 °C with an LKB Multiphor 2117 flatbed apparatus and commercially prepared pH range 3.5–9.5 PAG slab plates (LKB). The pH gradient data accompanying each PAG plate lot were assumed correct.

Analytical Ultracentrifugation. Sedimentation velocity measurements were conducted in a Beckman Model E ultracentrifuge equipped with a UV scanner set at 280 nm. Enzyme samples (0.5–2.5 mg/mL) in 10 mM Hepes, pH 7.0, were run at 20 °C in an AN-D rotor at 44 000 rpm by using a 12-mm double-sector boundary centerpiece and quartz cell windows. Sedimentation coefficients (uncorrected for density and viscosity) were estimated from linear regression analyses of semilog plots of the boundary distance vs. time.

Amino Acid Analysis. Analyses were performed with a Technicon TSM amino acid analyzer according to the two-

column method of Moore et al. (1958). Enzyme samples were hydrolyzed under reduced pressure in 6 N HCl for 24 and 72 h at 110 °C. Where necessary, the data have been corrected for decomposition by extrapolation to zero time. Certain amino acids (glycine, alanine, valine, isoleucine, and leucine) increased in concentration with time; for these, only data from the 72-h hydrolysates were used. Cysteine content was determined by titration of freshly prepared enzyme with Nbs_2 (Ellman, 1959) in 8 M urea, pH 7.0. Tryptophan, NH_3, and the amide (Asn + Gln) content were not determined.

Steady-State Kinetics. Steady-state kinetic constants were determined for the reaction of ATP with 3-PG by using the coupled assay described above. In the determination of K_m for ATP, concentrations of 3-PG and Mg^{2+} (either Cl^- or SO_4^{2-}) were both maintained at 10 mM, while ATP concentration was varied from 0.05 to 10 mM. In the determination of K_m for 3-PG, concentrations of ATP and Mg^{2+} were maintained at 4 and 10 mM, respectively, while 3-PG concentration was varied from 0.05 to 35 mM. The data were analyzed by linear regression fits to Lineweaver–Burk plots.

Reaction of the Enzyme with Nbs_2. The number of free thiol groups was determined by titration of the enzyme (3–5 μM) with excess Nbs_2 (200 μM) in 8 M urea, pH 7.0. It was found that the absorbance of 2-nitro-5-thiobenzoate [ϵ_{412} = 13600 M^{-1} cm^{-1} (Ellman, 1959)] is unchanged in this solvent. Reaction under these conditions was complete in ca. 0.5 min.

Studies of the kinetics of the Nbs_2 reaction with native PGK at 25 °C were carried out in 100 mM Hepes, pH 7.0, containing 1 mM EDTA and 20–200 μM Nbs_2. Numerical analyses of the kinetic data were carried out with a computer program based on the iterative fitting procedure described by Cleland (1968). Data points representative of at least 70% of the total reaction amplitude were fit to either a single or a sum of two exponential decay function(s).

For investigation of the effect of thiol group modification on PGK activity, aliquots taken at various times from the reaction mixtures were assayed for kinase activity without prior dilution. Data analyses were from semilog plots of activity vs. time.

^{31}P NMR Procedures. Fourier-transform (FT) ^{31}P NMR spectra were obtained at 40.5 MHz with a Varian XL-100 NMR spectrometer equipped with a 12-mm bore probe insert. NMR samples contained 10–15% D_2O (v/v) as the heteronuclear field frequency lock signal and were not spun during data acquisition. All spectra were proton broad-band decoupled by using a heteronuclear noise bandwidth of 1000 Hz and 5-W decoupler power.

All chemical shifts are given in parts per million (ppm) relative to external 85% H_3PO_4 at the same temperature. As per convention, negative shifts were assigned to resonance lines downfield from 85% H_3PO_4. Hexamethylphosphoramide (HMPA) was employed as a nonionizing and noninteraction internal line-width (field homogeneity) standard; the HMPA chemical shift (singlet) in aqueous solution is −29.9 ppm. Most spectra were acquired by using a 20-μs pulse width (ca. 50° flip angle) at a repetition rate much faster than $1/T_1$ of the slowest relaxing nuclear spins. As a consequence, absolute line intensity comparisons among different spectra usually were not significant. Determination of longitudinal (T_1) relaxation times anticipated to be less than 1 s was accomplished by using a modified inversion–recovery method. Alternatively, when T_1 times were expected to exceed 1 s, the progressive saturation method (Freeman & Hill, 1971) was employed.

^{31}P NMR Enzyme Samples. PGK stored as an $(NH_4)_2SO_4$ suspension was pelleted by centrifugation and was taken up in buffer (usually 100 mM Hepes, pH 7.0, containing 1 mM EDTA). Equilibration was achieved by exhaustive dialysis at 5 °C. Enzyme solutions were made 10–15% in D_2O (v/v) and were concentrated to 0.6–3.0 mM (ca. 25–130 mg/mL) in 1.4–2.0 mL by vacuum dialysis at 1 °C using a collodion bag apparatus. Only enzyme having ≥95% maximal specific activity was used. The pH of the enzyme samples (read prior to and at the conclusion of an experiment) was assumed to be that indicated by the pH meter with no correction for the presence of D_2O.

Table I: Purification of 3-Phosphoglycerate Kinase from Halibut Muscle[a]

fraction	vol (mL)	total protein (g)	sp act. (units/mg)	yield (%)
(I) extract[b]	2185	127[c]	4.1	100
(II) 60–83% ammonium sulfate pellet	348	40[c]	9.7	75
(III) CM-Sephadex column pool	324	0.310[d]	825	49
(IV) ammonium sulfate suspension	185	0.298[d]	810	46

[a] All steps were performed at 4 °C. [b] Starting from 1055 g of frozen, trimmed muscle. [c] By the method of Bradford (1976). [d] From the absorbance at 280 nm by using an extinction coefficient ($E^{0.1\%}_{280nm}$) of 0.64 cm^{-1} mg^{-1} mL.

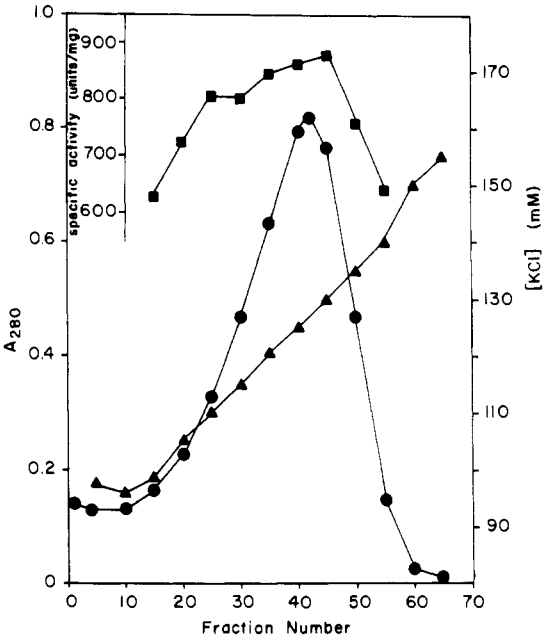

FIGURE 1: KCl gradient elution profile of halibut muscle PGK from a CM-Sephadex column at pH 8.0. Fractions 25–50 were pooled for ammonium sulfate suspension storage: 280-nm absorbance (●); specific activity (■); KCl concentration gradient (▲).

Results

Enzyme Isolation Procedure. The summary of a typical preparation of purified halibut muscle 3-phosphoglycerate kinase is given in Table I. Approximately 300 mg of enzyme[2] (nearly 200-fold purified from its state in the extraction medium) can be isolated from 1 kg of frozen halibut muscle at an overall yield of 46%. This one-column procedure has been

[2] An additional 70 mg of PGK/kg of muscle has more recently been obtained by the inclusion of 1.0×10^{-4} M phenylmethanesulfonyl fluoride in the extract buffer (Weber & Bernhard, 1982).

used for a wide range of starting tissue amounts (0.3–5.0 kg), resulting in the same high fractional yield of enzyme. The CM-Sephadex column KCl gradient elution profile is shown in Figure 1. At pH 8.0 and 100 mM KCl (Tris-HCl buffer), only a small fraction (ca. 2%) of the applied protein adsorbs to the column resin. The peak enzyme fractions elute with buffer containing 125 mM KCl. The high pH and ionic strength required for the CM-Sephadex column chromatography represent a significant departure from the conditions found suitable for ion-exchange purification of PGK from yeast and other muscle sources (Scopes, 1975a).

The PGK specific activity is nearly constant across the protein elution peak; deviations on either side were attributable to both protein contamination (detected by gel electrophoresis in the presence of $NaDodSO_4$) and nucleotide (inferred from the 280:260-nm absorbance ratio). Pooled activity fractions, stored as an ammonium sulfate precipitate at 5 °C, are quite stable, retaining greater than 90% of the initial activity after 3 months.

Assessment of Purity. Under reducing and denaturing conditions, a single band was detected on 7.5% polyacrylamide–$NaDodSO_4$ gels. By comparison with protein standards of known size, halibut muscle PGK migrated in the $NaDodSO_4$ gels with a molecular weight of 43 500 ± 1500.

Further evidence of the homogeneity of the enzyme was the symmetrical boundary observed in analytical sedimentation velocity studies (sedimentation coefficient = 3.3 S, independent of native enzyme concentration). The 280:260-nm absorbance ratio of purified halibut muscle PGK is 1.67.

Our preparation of PGK contained no detectable GPDH activity, although halibut GPDH prepared by ammonium sulfate fractionation at above 83% saturation followed by CM-cellulose column chromatography at pH 6.5 still contains approximately 0.2% PGK. This PGK activity is equivalent to ca. 2% of the yield of purified PGK, taking into consideration that the yield of GPDH from muscle extract is about 10-fold greater than PGK. Only trace amounts of both adenylate kinase and phosphoglycerate mutase activities (<0.01% and 0.002%, respectively) were found. The presence of 10 μM P^1,P^5-bis(5′-adenosyl)pentaphosphate, a potent reversible inhibitor of adenylate kinase activity, was without effect on PGK turnover rates.

Amino Acid Composition. The finding that halibut PGK strongly absorbs to CM-Sephadex at pH 8.0 indicates that the enzyme is a basic protein. This is consistent with our finding that on isoelectric focusing gels over the pH range 3.5–9.5, the enzyme invariably exited the gel at the alkaline pH end. No attempt was made to carry out focusing experiments above pH 9.5. The amino acid composition of halibut muscle PGK is shown in Table II and compared with the enzymes from rabbit muscle, horse muscle, and yeast. Note the lower glutamic acid content of *halibut* muscle PGK. This may contribute to the observed higher isoelectric point of halibut muscle PGK relative to the value of 7.2 found for the yeast and rabbit muscle enzymes (Krietsch & Bücher, 1970; Stinson, 1974). However, we have not determined the amide content (Asn + Gln) of halibut PGK.

Steady-State Kinetics. At pH 7.4 and 25 °C, halibut PGK has a specific activity of 810 ± 20 units/mg. K_m values of 1.33 and 0.51 mM were obtained for 3-PG and ATP, respectively, at 10 mM Mg^{2+} and either 4 mM ATP or 10 mM 3-PG as the invariant substrate. Lineweaver–Burk plots of initial rate vs. 3-PG or ATP concentration showed no significant deviation from linearity. The turnover number (k_{cat}) in the direction of ATP utilization is 570 ± 25 s^{-1}. Near pH neutrality, this value is nearly insensitive to variations in pH. At 8 mM divalent metal ion, the substitution of Mn^{2+} results in ca. 20% slower initial rates independent of pH over the range 6.0–8.0.

Table II: Amino Acid Composition of 3-Phosphoglycerate Kinase from Halibut Muscle As Compared with Rabbit Muscle, Horse Muscle, and Yeast

amino acid	residues/mol of enzyme			
	halibut muscle[a]	rabbit muscle[d]	horse muscle[e]	yeast[d]
Lys	42.9	46	42	46
His	8.2	8	6	9
Arg	8.2	12	11	14
Asp	44.0[f]	44[f]	23	41[f]
Thr	18.7	17	18	18
Ser	17.3	24	24	25
Glu	26.4[f]	33[f]	26	38[f]
Pro	15.0	18	16	18
Gly	45.2	40	40	37
Ala	42.3	39	41	43
Cys	7.1[b]	8	7	1
Val	40.0	38	40	38
Met	14.3	13	13	3
Ile	20.8	18	18	23
Leu	35.5	38	38	41
Tyr	4.7	4	4	8
Phe	14.6	16	16	19
Trp	ND[c]	4	4	2
Asn	ND	ND	22	ND
Gln	ND	ND	7	ND

[a] Data were obtained for 24- and 72-h hydrolysates and are the average of two analyses. The molecular weight of halibut PGK is 43 000. [b] Determined by reaction of the halibut enzyme with Nbs_2 in 8 M urea, pH 7.0. [c] Not determined. [d] From Krietsch & Bücher (1970). [e] From Banks et al. (1979). [f] Amide content was not distinguished.

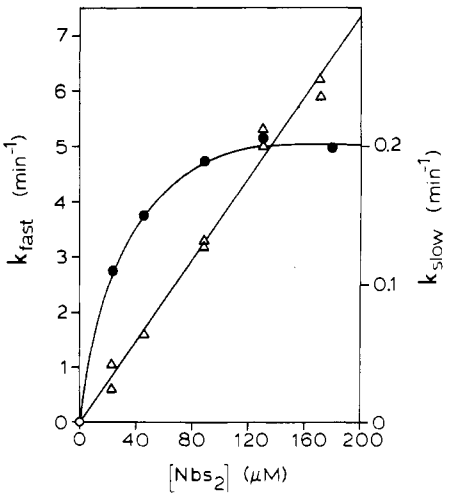

FIGURE 2: Reaction of halibut PGK with Nbs_2. The apparent first-order rate constants are shown as functions of Nbs_2 concentration at pH 7.0 and 25 °C: (△) fast process; (●) slow process. Enzyme concentration was 3 μM. Under these conditions, light-scattering absorbance due to enzyme precipitation was observed when the chemical modification was 85–90% completed.

Reaction of Enzyme Thiol Groups with Nbs_2. In 8 M urea, 7.1 ± 0.2 equiv of Nbs_2 reactive thiol per mol of PGK was found by using five freshly prepared enzyme stocks. With the native enzyme, the Nbs_2 reaction is kinetically biphasic. The effect of excess Nbs_2 concentration on both the fast (k_f) and slow (k_s) first-order rate constants is shown in Figure 2. The fast rate is first order in Nbs_2; the bimolecular rate constant is 625 ± 40 M^{-1} s^{-1}. At all Nbs_2 concentrations tested, the total calculated absorbance change ($A_f + A_s$) is 99 ± 3% of

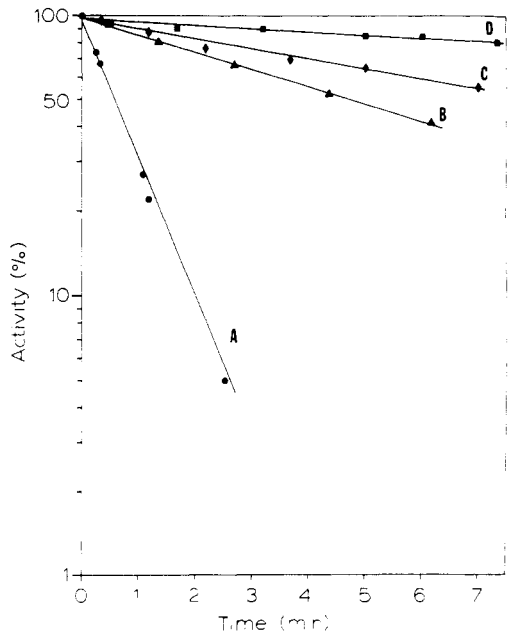

FIGURE 3: Kinetics of inactivation of halibut muscle PGK by reaction with Nbs_2 with and without substrates present. Enzyme (1.5 μM) was incubated with 55 μM Nbs_2 at pH 7.0 and 25 °C: (A) no substrate present; (B) 9.7 mM 3-PG present; (C) 7.7 mM ATP present; (D) both 9.7 mM 3-PG and 7.7 mM ATP present.

that found for the reaction of an identical amount of enzyme in 8 M urea. The fast and slow reaction processes are well resolved at Nbs_2 concentrations above 100 mM. Amplitude values of 29% and 71% are found for the fast and slow phases, respectively. At the higher Nbs_2 concentrations, the Nbs_2-independent slow rate constant is 0.20 min^{-1}.

In addition, Nbs_2 modification experiments were carried out in the presence of high concentrations of ATP and/or 3-PG (7.7 and 9.7 mM, respectively). Either substrate blocks the fast phase of the Nbs_2 reaction. In the presence of substrates, the kinetics of Nbs_2 reaction data are not described by a sum of two exponential decays; in the presence of ATP, there is a pronounced lag in Nbs production.

Inactivation of the Enzyme by Reaction with Nbs_2. Incubation of PGK with excess Nbs_2 results in the loss of enzyme activity. The kinetics of enzyme inactivation are first order (Figure 3). The pseudo-first-order rate constant for inactivation at 55 μM Nbs_2 is 1.2 min^{-1}, a value very nearly the same as the fast rate constant for the Nbs_2 reaction (k_f = 1.8 min^{-1}). Although the inactivation rate is slower in the presence of either ATP or 3-PG, in the presence of both substrates the rate of inactivation is markedly still slower.

^{31}P NMR Studies. (A) Binary ES Complexes. At high enzyme concentration (ca. 1 mM) and slightly substoichiometric 3-PG concentration, a single ^{31}P resonance line is observed. This line is shifted downfield 2 ppm from the position for free 3-PG. In addition, the line width was increased by 10–15 Hz relative to aqueous 3-PG. When the 3-PG concentration was adjusted to severalfold above that of enzyme sites, the lone NMR signal had a chemical shift very near that for the free ligand, indicating rapid exchange between bound and free 3-PG. An equilibrium study of the binding of 3-PG to PGK, at pH 7.0 and 7.5 °C, was carried out (data not shown). The reversible binding of a ligand to a single site on an enzyme obeys eq 2, provided that the chemical shifts are

$$L_0 = \frac{\Delta E_0}{\delta_{obsd} - \delta_f} - K_d \qquad (2)$$

in "fast exchange" and $L_0 \gg$ [EL] (Dahlquist & Raftery, 1968). Here, L_0 and [EL] are the chemical concentrations of total ligand and enzyme–ligand complex, respectively. δ_f and δ_{obsd} are the chemical shifts (in hertz) of the free ligand and the observed resonance, respectively (relative to a given standard). Δ is the difference (in hertz) between the free and bound chemical shifts, and K_d is the dissociation constant. Approximate linear fits to the data from titrations at two different enzyme concentrations (1.3 and 0.5 mM) gave an apparent K_d = 150 ± 50 μM and Δ = 80 ± 5 Hz (1.9–2.1 ppm). There was no evidence of chemical shift artifacts which can arise from bulk magnetic susceptibility effects in the presence of a high concentration of enzyme (Blumenstein, 1975). ^{31}P chemical shifts for enzyme-bound 3-PG and for the other bound substrates are presented in Table III.

Table III: ^{31}P NMR Chemical Shifts and Coupling Constants for PGK Substrates Free and in the Presence of PGK[a]

	shift[b] (ppm)				
substrate	1-P	3-P	α-P	β-P	γ-P
1,3-P_2G[c]	2.0	−3.2			
3-PG[c]		−3.3			
ADP			11.0	7.3	
MgADP			10.4	6.4	
ATP			11.3	22.2	7.0
MgATP			10.8	19.5	5.8
E·1,3-P_2G	−0.1	−5.5			
E·3-PG		−5.4			
E·ADP			11.0	7.8	
E·ATP			11.2	21.9	6.7
E·MgATP			11.1	20.1	5.8

[a] Buffer conditions were 100 mM Hepes-1 mM EDTA, pH 7.0, 7.5 °C. [b] Relative to 85% H_3PO_4. [c] No chemical shift difference was observed in the presence of 10 mM Mg^{2+}.

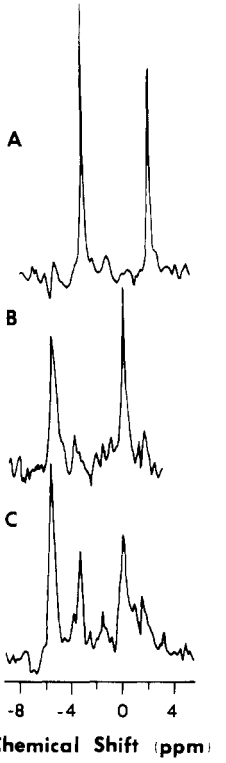

FIGURE 4: E·1,3-P_2G binary complex. Conditions were 50 mM Hepes, 50 mM imidazole, and 1 mM EDTA, pH 7.0, 7.5 °C. (A) No enzyme, 1.4 mM; KCl, 28 mM. (B) Enzyme, 1.1 mM; 1,3-P_2G, 0.7 mM; KCl, 13 mM. (C) Enzyme, 1.0 mM; 1,3-P_2G, 1.3 mM; KCl, 25 mM. NMR parameters: pulse width, 20 μs; sweep width, 2500 Hz; line broadening, 6.4 Hz. Acquisition time: (A) 1.0 s; (B and C) 0.75 s. Number of scans: (A) 8000; (B) 8500; (C) 9500.

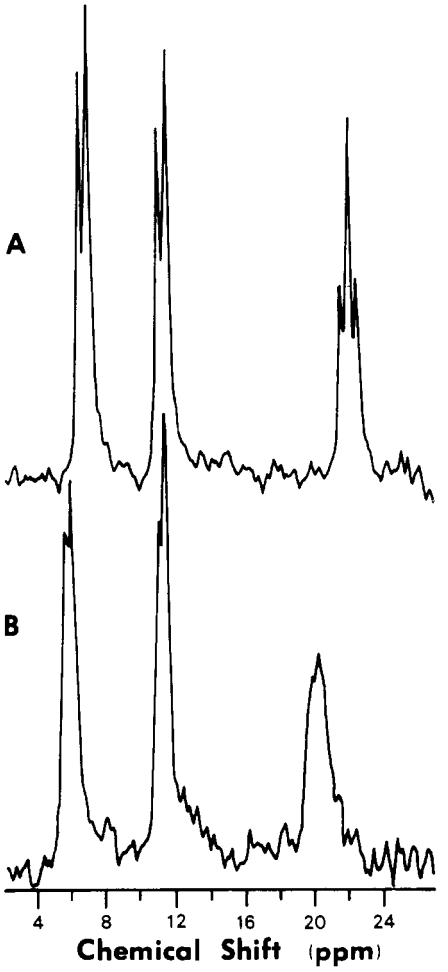

FIGURE 5: E·ATP and E·MgATP binary complexes. Conditions were 100 mM Hepes and 1 mM EDTA, pH 7.0, 1.5 °C. (A) Enzyme, 2.2 mM; ATP, 1.9 mM. (B) $MgCl_2$, 4.0 mM, added to sample A. NMR parameters: acquisition time, 1.0 s; pulse width, 20 μs; sweep width, 2500 Hz; line broadening, 6.4 Hz. Number of scans: (A) 6000; (B) 5500.

The ^{31}P NMR spectrum of free 1,3-P_2G is shown in Figure 4A. The 1-P resonance is at 2.0 ppm and is upfield from the 3-P signal (−3.2 ppm). Line assignments were made by analogy with 3-PG. When enzyme sites are in excess of 1,3-P_2G (Figure 4B), both resonance signals are shifted downfield by 2 ppm. That this new magnetic environment reflected the fully bound ligand was confirmed by the addition of 1,3-P_2G to above the enzyme site concentration (Figure 4C). No further shift occurs in either resonance, and signals corresponding to free 1,3-P_2G are seen. Therefore, unlike E·3-PG, enzyme-bound and free 1,3-P_2G are in slow exchange at pH 7.0 and 7.5 °C. Under these conditions, 1,3-P_2G binds stoichiometrically to PGK, with a K_d too small to measure by ^{31}P NMR. It is important to note that when the spectrum of sample 4C was retaken after having been stored for 5 days at 5 °C, a considerable amount of E·1,3-P_2G was still observed, whereas all of the free ligand had hydrolyzed to 3-PG and P_i.

The ionic strength dependence of E·1,3-P_2G formation was examined. Under identical conditions of pH and temperature, but with 115–125 mM KCl present, the lines are broadened considerably. Upon addition of more ligand, the NMR signals approach coalescence near the respective chemical shifts for free 1,3-P_2G. Therefore, ligand exchange is faster at the higher ionic strength, and, most likely, the binding is weaker. Note that the 1-P and 3-P lines react in concert to this solution environmental change. An interpretation that is consistent with this behavior is that 1,3-P_2G is bound to the enzyme but is not chemically transformed from the point of view of the phosphate moieties. In this regard, the ^{31}P NMR spectrum obtained on addition of P_i to E·3-PG does not resemble that of E·1,3-P_2G. Additionally, the chemical shifts corresponding to 1,3-P_2G bound to PGK were found to be pH independent over the range 5.9–8.0.

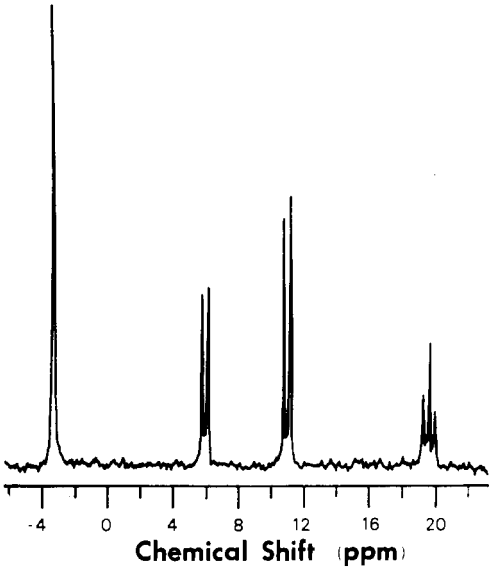

FIGURE 6: ^{31}P NMR spectrum of the PGK-catalyzed substrate–product equilibrium. 3-PG, ATP, and Mg^{2+} concentrations were 10, 4, and 8 mM, respectively, identical with steady-state conditions. Enzyme concentration was 12.4 μM. The reaction mixture was buffered with 100 mM Hepes and 0.2 mM EDTA, pH 7.1, and was equilibrated at 7.5 °C for 1 h prior to the start of NMR data acquisition. NMR parameters: acquisition time, 1.0 s; pulse width, 20 μs; sweep width, 2500 Hz; line broadening, 1.1 Hz; number of scans, 4000.

(*B*) *E·ATP and the Effect of* Mg^{2+}. ^{31}P NMR spectra of ATP and MgATP bound to the enzyme at 1.5 °C are shown in Figure 5. The chemical shift data are summarized in Table III. Only small chemical shift differences occur upon binding; at most, the shifts differ from the free resonances by 0.5 ppm. Such small differences precluded the use of chemical shift titration for K_d determinations. When ATP is added to severalfold above the enzyme site concentration, the observed chemical shifts approximate those of the free ligand, thereby indicating the nucleotide is bound under fast exchange conditions whether or not Mg^{2+} is present. There was no evidence that either ATP or MgATP was hydrolyzed by the enzyme.

Line broadening also is apparent when ATP is bound to PGK. If it is assumed that nearly all of the ATP was bound (here, [E] > [ATP]), the increase in line width due to the presence of enzyme is 10–15 Hz. The ^{31}P spin–spin coupling, with constants 15 and 20 Hz in the presence and in the absence of Mg^{2+}, respectively, is still resolved and is unchanged from that of free ATP.

(*C*) *Ternary* ES_2 *Complexes*. Under conditions of catalytic amounts of PGK, the chemical equilibrium constant, K_{eq}, is

$$K_{eq} = \frac{[\text{MgADP}][1,3\text{-}P_2G]}{[\text{MgATP}][3\text{-PG}]} \quad (3)$$

Enzyme at 12.5 μM was incubated with high concentrations of ATP, 3-PG, and Mg^{2+} at 7.5 °C for 1 h, after which time the ^{31}P NMR spectrum was obtained. The result is shown in Figure 6. No signals other than those corresponding to free MgATP and 3-PG are seen even after repetitively acquiring spectra for several more hours. The same result was obtained

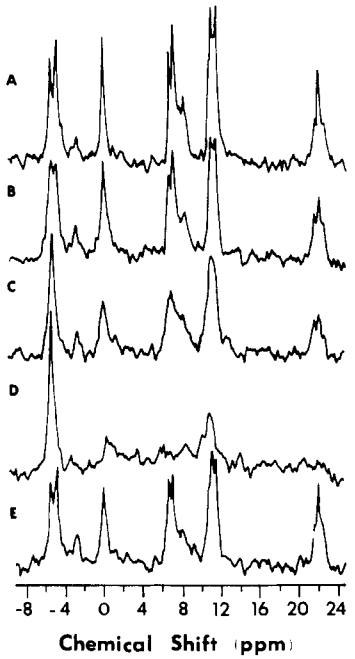

FIGURE 7: ^{31}P NMR spectra of catalytically competent PGK ternary complexes at equilibrium. (A) 2.5 mM enzyme was incubated with 2.1 mM 3-PG and 2.2 mM ATP in 100 mM Hepes and 1 mM EDTA, pH 7.0 at 7.5 °C, for 1 h prior to the start of ^{31}P NMR data acquisition. $MgCl_2$ was added to sample A at a total concentration of (B) 1.4, (C) 2.8, and (D) 4.2 mM. (E) EDTA, 11.1 mM, was added to sample D. NMR parameters: acquisition time, 1.0 s; pulse width, 20 μs; sweep width, 2500 Hz; line broadening, 6.4 Hz; number of scans, 8000.

when catalysis was initiated in a sample which contained MgADP and 1,3-P_2G (ignoring the small amount of AMP which arose from trace adenylate kinase contamination). With the assumption of similar relaxation rates, an upper limit of 2% (from the observed signal to noise ratio in Figure 6) is placed on the amount of 1,3-P_2G at equilibrium. Therefore, $K_{eq} \leq 8 \times 10^{-4}$ from these data.

With the assumption that phosphoryl transfer occurs in ternary complexes of PGK and cosubstrates, under conditions where $[E]_0 > [MgATP] \simeq [3\text{-PG}] \gg K_d$ for either substrate, the following situation is maximized (eq 4a,b), where K_{eq}' is

$$E\cdot MgATP\cdot 3\text{-PG} \overset{K_{eq}'}{\rightleftharpoons} E\cdot MgADP\cdot 1,3\text{-}P_2G \quad (4a)$$

$$K_{eq}' = \frac{[E\cdot MgADP\cdot 1,3\text{-}P_2G]}{[E\cdot MgATP\cdot 3\text{-PG}]} \quad (4b)$$

the equilibrium constant relating productive enzyme ternary complexes (Nageswara Rao & Cohn, 1977). ^{31}P NMR spectra of a mixture of 2.5 mM PGK, 2.1 mM 3-PG, and 2.2 mM ATP are shown in Figure 7. At equilibrium, with no added Mg^{2+}, ^{31}P signals corresponding to all four substrates are observed (Figure 7A). The same result was obtained with an enzyme sample to which slightly substoichiometric amounts of 1,3-P_2G and ADP had been added. The chemical shift data indicate that the exchange characteristics of the different substrates to and from their bound environments are qualitatively unchanged from the individual behavior in binary complexes with the enzyme. To a good approximation, the concentrations of free ligands, binary E·L complexes, and nonproductive ("wrong way") ternary complexes in this sample accounted for less than 25% of the observed signals. Furthermore, the ^{31}P longitudinal (T_1) relaxation times measured under these conditions for bound 3-PG and 1,3-P_2G are similar, thereby permitting K_{eq}' to be estimated from the integrated line intensities. At pH 7.0 and 7.5 °C, K_{eq}' is 1.0 ± 0.5, more than 1000-fold greater than K_{eq}. Thus, a model consistent with the available data is that interconversion of substrates and products occurs within productive ternary complexes of nearly identical free energy. Others (Nageswara Rao et al., 1978) have concluded that yeast PGK behaves likewise.

Upon addition of Mg^{2+} to the enzyme sample (Figure 7B,C), the ^{31}P signals broaden to such an extent that nearly all of the spectral features are lost. When an excess of EDTA is added (Figure 7E), the ^{31}P NMR spectrum closely approximates that observed prior to the addition of Mg^{2+}. These results are consistent with a Mg^{2+}-induced enhancement of the rate of chemical exchange between specific pairs of ^{31}P nuclei. The pattern of broadening observed in these spectra strongly argues that all of the substrates are undergoing exchange, a situation consistent with the presumption that a majority fraction of the enzyme exists in productive ternary complexes at equilibrium. The lack of a significant buildup of AMP (−2.8 ppm) from the trace adenylate kinase contaminant in these enzyme samples further indicates that most of the ADP was bound to PGK. From the chemical shift difference between the 3-P resonances of 1,3-P_2G and 3-PG (−5.0 and −5.5 ppm, respectively) in Figure 7A, where the bound reactants are in slow chemical exchange, an extreme upper limit of 125 s^{-1} is established for the interconversion rate in the absence of added Mg^{2+}. Since (a) the 1-P (1,3-P_2G) and γ-P (ATP) signals do not overlap and are unchanged from the chemical shift values in binary complexes and since (b) no significant line broadening is observed when Mg^{2+} is added to those binary complexes, the additional line width caused by the onset of rapid chemical exchange when Mg^{2+} is added to the ternary mixture is related to the phosphoryl transfer rate enhancement according to eq 5 (Pople et al., 1959) where

$$\tau_M^{-1} = \Pi(\Delta\nu_+ - \Delta\nu_-) \quad (5)$$

$\Delta\nu_+$ and $\Delta\nu_-$ are the line widths (in hertz) in the presence and in the absence, respectively, of Mg^{2+}. For example, relative to Figure 7A, the spectrum shown in Figure 7C reflects an increase of ca. 55 s^{-1} in the phosphoryl transfer rate from 1,3-P_2G. The severity of the line broadening with excess Mg^{2+} present (Figure 7D) precludes the possibility of determining the final rate enhancement.

^{31}P NMR spectra of ternary mixtures with high, stoichiometric concentrations of enzyme were also obtained at pHs of 8.0 and 5.9 (data not shown). The absence of chemical shift differences in both the 1-P and 3-P of 1,3-P_2G was the most significant feature of these spectra. Hence, by this criterion, the nature of the 1,3-P_2G binding site environment does not differ from that revealed by the spectrum of the binary enzyme complex (E·1,3-P_2G) over this range of pHs. In addition, when KCl (92 mM) is added to the ternary mixture at pH 5.9, all of the ^{31}P resonance lines narrow markedly, presumably as a result of lowering the fraction of enzyme-bound ligands.

Discussion

The isolation and purification to virtual homogeneity of halibut muscle 3-phosphoglycerate kinase have been described. By this procedure, it is possible to prepare gram quantities of the enzyme. The purified enzyme has a specific activity of 810 units/mg when assayed with ATP and 3-PG as substrates. At 25 °C, this activity is comparable to that of PGK from other sources. The broad pH optimum for halibut PGK activity is similar to that reported for the yeast and rabbit muscle enzymes which function at near-maximal velocity over the pH range 6–9 (Krietsch & Bücher, 1970).

The comparison of amino acid composition among the PGK enzymes from various sources (Table II) shows considerable conservation, especially among those residues most commonly found in the regular, secondary structural elements of globular proteins (Chou & Fasman, 1974). These comparative amino acid contents are significant in light of the known and common three-dimensional structural features of yeast and horse muscle PGK (Blake, 1975). On the basis of catalytic activity and its modulation by environmental changes, we presume a similar three-dimensional structure for halibut PGK. Variations in the amino acid composition without changing the three-dimensional folding pattern have been noted previously (Rossmann et al., 1975; Sigler et al., 1968).

The low Glx content is probably reflected in the high isoelectric point of halibut muscle PGK. It is unlikely that the greater positive charge associated with halibut muscle PGK is related to the primary events of catalysis. Indeed, the values of K_m and k_{cat} we report are nearly identical with those reported for other PGK enzyme species. However, the nature of the control of this enzyme's in vivo function could be influenced by the details of the surface topology and charge, most notably, as regards its potential for interaction with other enzymes of the glycolytic pathway. In this regard, we note that a small amount of PGK remains associated with GPDH purified from halibut muscle. This result is of considerable interest to us since it may occur as a consequence of specific protein–protein interactions.

All enzymes of the glycolytic pathway, but particularly GPDH, are present in high concentration in sarcoplasmic fluid. Specific interactions among these enzymes may direct the flow of metabolites in this rather viscous medium. Preliminary hydrodynamic evidence which indicates the occurrence of glycolytic multienzyme complexes in *Escherichia coli* has appeared (Mowbray & Moses, 1976) although the physiological significance has not been established. Specific interactions between glycolytic enzymes which alter the kinetics of catalysis have been reported (Ovádi & Keleti, 1978; Ovádi et al., 1978).

Our investigation of the reactivity of Nbs_2 with halibut muscle PGK establishes the presence of seven cysteine residues. From the kinetic data presented in Figure 2, it appears likely that two cysteines are initially accessible to Nbs_2. The fast-reacting thiols appear to react at a rate (k = 625 M^{-1} s^{-1}) characteristic of an unhindered thiol in aqueous solution (glutathione, k = 375 M^{-1} s^{-1}) (Warren & Cheatum, 1966). This value is almost 10^3-fold greater than that reported for the reaction of Nbs_2 with the single thiol of yeast PGK (k = 0.7 M^{-1} s^{-1}) under similar conditions (Wrobel & Stinson, 1978). In light of both the observed precipitation of the enzyme before reaction with Nbs_2 is complete (see legend to Figure 2) and the Nbs_2 concentration independence, it is reasonable that the slower reaction rate is concomitant with an unfolding of the modified enzyme which exposes the buried thiol groups.

From the Nbs_2 inactivation study (Figure 3), it can be concluded that a single modified cysteine residue is responsible for the inactivation of PGK and that this reaction is inhibited by bound substrates. The kinetic method proposed by Ray & Koshland (1961) was employed for determining the number of essential enzyme thiol residues. The greater protection when both substrates are present could result either from "substrate synergism" or from the presence of still more tightly bound products. No precise role for the essential thiol group has been elucidated. With the assumption that the phosphoryl transfer mechanism is the same for yeast and halibut muscle PGK, the nonessential role demonstrated for the single (slow Nbs_2 reacting) thiol group of the yeast enzyme (Krietsch & Bücher, 1970; Markland et al., 1975) argues against the direct participation of a thiol group in the phosphoryl transfer pathway.

As yet, we do not have a complete understanding of the mechanism by which 1,3-P_2G is stabilized by the enzyme. On the basis of the ^{31}P NMR spectra obtained from the binary complexes, the integrity of the acyl phosphate bond is maintained in the absence of ADP. In particular, no evidence was obtained to suggest the presence of a stoichiometric phosphoenzyme species at equilibrium. The bound 1-P and 3-P signals exhibit similar and concerted NMR behavior in response to an increase in the solvent ionic strength. Similarly, no significant differences in the 1-P and 3-P line widths or T_1 relaxation rates were found. Such results might also arise from a mechanism in which the reactive phosphoryl group can exchange very rapidly between covalent and noncovalent ES complexes. However, the results of Webb & Trentham (1980), which demonstrate inversion of γ-phosphorus chirality in ATP, provide further arguments against intermediary phosphoryl transfer to PGK.

The tight binding of 1,3-P_2G coupled with an equilibrium constant near unity (see below) for the enzyme-bound reactants and products might be important for glycolytic regulation. The in vivo condition of high enzyme and low (three-carbon) metabolite concentrations makes it likely that a large fraction of the metabolites in the sarcoplasmic fluid of resting muscle are enzyme bound. In particular, 1,3-P_2G is bound to PGK, and covalent 3-phosphoglyceroyl is bound to GPDH (Bloch et al., 1971).

The solvent conditions employed for the spectra shown in Figure 5 are very similar to those used in a recent parallel study of yeast PGK (Nageswara Rao et al., 1978). There the authors found ^{31}P NMR chemical shift evidence to suggest that ATP exists in two slowly exchanging bound environments which differ in affinity for Mg^{2+} by no less than a factor of 30-fold. Our results with halibut muscle PGK do not reveal any magnetically distinguishable ATP binding sites.

We find that the equilibrium constant for halibut PGK bound reaction components in productive complexes is comparable to the results of Nageswara Rao et al. (1978). The equilibrium constant near unity for the reaction on the enzyme surface is as predicted for catalytic efficiency by Albery & Knowles (1976).

The turnover number, k_{cat}, at 25 °C for the phosphoryl transfer from ATP is ca. 575 s^{-1} and reflects the slowest rate process after the ternary complex has been formed. A similar k_{cat} value was determined for yeast PGK (Krietsch & Bücher, 1970). In comparison, these authors determined that the rate of turnover reaction in the direction of glycolysis was ca. 1400 s^{-1}. Thus, the forward and reverse steady-state catalytic rates differ by less than a factor of 3-fold. From $K_{eq}' \simeq 1$, it is certain that the forward and reverse rate components associated with the chemical transformation event are themselves not very different and may be rate determining. Unfortunately, it was not possible to measure these rates from our ^{31}P NMR spectra under conditions of excess (saturating) Mg^{2+}. k_{cat} is somewhat slower than the inferred rate for the phosphoryl transfer from ATP. This indicates that the rate of desorption of 1,3-P_2G, which binds more tightly than any other substrate, is likely a significant factor in this reaction direction (Weber & Bernhard, 1982). We calculate that the specific rate of PGK·1,3-P_2G dissociation has an upper limit on the order of 1 s^{-1}, assuming K_d < 10 nM (Scopes, 1978b; this work), and $k_{association}$ is ca. 10^8 M^{-1} s^{-1}. Hence, other ligands

or other proteins in the GPDH–PGK coupled assay system for PGK activity must strongly enhance the 1,3-P_2G transfer velocities. The role of such effectors is discussed in the following paper (Weber & Bernhard, 1982).

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