# The Fast-Reacting Sulfhydryl Group of Rat Muscle Phosphoglycerate Kinase Is Necessary for Activity and Maintenance of Tertiary Structure†

Harmesh K. Sharma

ABSTRACT: Phosphoglycerate kinase from rat muscle has no disulfide linkage but possesses four –SH groups—one fast reacting and three slow reacting to 5,5′-dithiobis(2-nitrobenzoate). The enzyme is inactivated by reaction of the fast-reacting –SH with iodoacetate. There is a stoichiometric relationship between modification of this group and enzyme inactivation. The reaction follows pseudo-first-order kinetics and is dependent upon the iodoacetate concentration; the value of the second-order rate constant is 2.6 $M^{-1}$ $min^{-1}$. Amino acid analysis of the enzyme after treatment with iodo[2-$^{14}$C]acetate reveals that cysteine is the only amino acid to undergo modification. Moreover, the p$K$ for the fast-reacting –SH has been found to be 9.1, a normal value for a cysteinyl residue. MgADP and ADP protect the enzyme against iodoacetate inactivation, whereas 3-phosphoglycerate, MgATP, ATP, or $Mg^{2+}$ has no effect on the reaction. The dissociation constant ($K_d$) for MgADP as determined by iodoacetate inactivation kinetics is the same as the $K_m$ value, thus indicating that the fast-reacting –SH is in the MgADP binding domain of the enzyme. On storage, there is a loss of enzyme activity and a concomitant loss of fast-reacting –SH. Three protein peaks with $M_r$ values of 76 000, 43 000 and 36 000 are obtained after Sephadex G-200 chromatography of the stored enzyme. The evidence indicates that the protein with $M_r$ 43 000 is produced by unfolding of the native enzyme ($M_r$ 36 000). The large protein ($M_r$ 76 000) is a dimer resulting from formation of a disulfide linkage between the fast-reacting –SH of two molecules of $M_r$ 43 000. The dimeric protein is catalytically inactive but can be fully reactivated by reducing agents. Physical and immunological properties of the three forms of the enzyme are also described.

On the basis of altered reactivity of –SH groups, stability, antibody titer, and spectral studies, it has recently been shown that phosphoglycerate kinase of rat muscle exists in a different form in "young" and "old" animals (Sharma et al., 1980). Conformational differences have been shown to be the cause of age-related changes in enolase from the free-living nematode *Turbatrix aceti* (Sharma & Rothstein, 1980) and a suggestion of a conformational difference particularly in the nicotinamide binding site of muscle glyceraldehyde-3-phosphate dehydrogenase from young and old rats has been proposed by Gafni (1981a,b).

As an approach toward unraveling the causes of age-associated differences in rat muscle phosphoglycerate kinase, a study of the contribution of –SH groups in catalysis and maintenance of tertiary structure of the enzyme has been undertaken. Evidence is presented which shows that the fast-reacting –SH group of the enzyme is essential for the catalytic activity and that it is located at or near the ADP binding site. This group is also important in the maintenance of the tertiary structure of the enzyme. Oxidation of the group leads to the formation of a dimeric form of the enzyme which is catalytically inactive.

## Experimental Procedures

*Materials.* Male Sprague-Dawley rats, weighing 300 g, were obtained from Charles River Breeding Laboratories. The animals were sacrificed by guillotine and leg muscles were removed. The ammonium sulfate suspension of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Sigma) was centrifuged and dissolved in either Tris–acetate buffer or phosphate buffer for the assay of phosphoglycerate kinase in the backward or forward direction, respectively. DL-Glyceraldehyde 3-phosphate (free acid) was obtained from Sigma. Complement and hemolysin were purchased from Pel-Freez Biologicals. Radioactive iodo[2-$^{14}$C]acetate (specific activity 54 mCi/mmol) was purchased from Amersham. Trypsin (TPCK[1] treated) was purchased from Worthington. Cellulose-coated glass plates (MN300, 20 × 20 cm) were purchased from Fisher and prewashed twice by ascending chromatography with 1-butanol–pyridine–acetic acid–water (1:1:1:1.5). Pyridine was refluxed for 4 h over ninhydrin and phthalic anhydride (each 1 g/L) and distilled twice. Analytical-grade 1-butanol, formic acid, and acetic acid were distilled twice. All other reagents were of analytical grade.

*Isolation of Phosphoglycerate Kinase and Its Dimeric Form.* The enzyme was purified according to the procedure described earlier (Sharma et al., 1980) except that EDTA was excluded during CM-cellulose chromatography since it prevented the binding of the enzyme to the absorbent. The pure enzyme preparation was stored at 4 °C in a screw-capped sterile tube in Tris-HCl buffer (10 mM, pH 7.0) containing 100 mM KCl. When the enzyme had lost 60–70% of its activity, it was loaded onto a precalibrated column of Sephadex G-200 (2.3 × 110 cm), the column was developed with the above buffer, and the fractions were monitored for absorbance at 280 nm and for enzyme activity. Fractions containing the dimeric form of the enzyme ($M_r$ 76 000) were pooled and concentrated on an Amicon PM-10 membrane. Generally a single or sometimes two chromatographic cycles yielded an electrophoretically pure preparation of the dimeric form of the enzyme.

*Enzyme Assay.* The enzyme was assayed in the forward direction by the method of Bucher (1955). To 1.0 mL of reaction mixture containing 50 mM phosphate buffer (pH 6.9),

† From the State University of New York at Buffalo, Division of Cell and Molecular Biology, Buffalo, New York 14260. *Received June 4, 1982.* This work was supported in part by Grant AG00618 from the National Institute on Aging, awarded to Morton Rothstein, State University of New York at Buffalo.

[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; TNB, 5-thio-2-nitrobenzoate; CD, circular dichroism; CNBr, cyanogen bromide; CM, carboxymethyl; EDTA, ethylenediaminetetraacetate; Isotris–BSA buffer, 10 mM Tris-HCl, pH 7.4, 0.14 NaCl, 0.5 mM $MgSO_4$, 0.15 mM $CaCl_2$, and 0.1% bovine serum albumin.

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0.415 mM NAD, 4 mM ADP, and 5 mM $MgSO_4$ was added 8–10 units of glyceraldehyde-3-phosphate dehydrogenase and a sufficient amount of DL-glyceraldehyde 3-phosphate so as to obtain an absorbance of 0.2 at 340 nm. The reaction was initiated by adding phosphoglycerate kinase, and the rate of reaction was monitored by the increase in absorbance at 340 nm.

In the backward direction, the enzyme was assayed in 1.0 mL of reaction mixture containing 50 mM Tris–acetate buffer (pH 7.3), 25 mM $MgCl_2$, 10 mM D-glycerate 3-phosphate, 5 mM ATP, 0.2 mM NADH, and 8–10 units of glyceraldehyde-3-phosphate dehydrogenase. The reaction was initiated by adding phosphoglycerate kinase, and the rate of reaction was monitored by the decrease in absorbance at 340 nm. Assay temperature was 30 °C in both the forward and backward directions. One unit of enzyme is the amount of enzyme required to catalyze the phosphorylation or dephosphorylation of 1 μmol of 3-phosphoglycerate or 1,3-diphosphoglycerate.

*Determination of "Free" and "Total" -SH Groups.* The determination of "free" SH groups was performed in 2.0 mL of reaction mixture containing 20.5 nmol of protein in buffer A (0.2 M Tris-HCl, pH 8.2, containing 0.02 M EDTA) and 680 nmol of DTNB. The reaction was measured by the increase in absorbance at 412 nm against a blank without the protein. The amount of -SH was calculated by using an $\epsilon_{412}$ of 13 600 $M^{-1}$ $cm^{-1}$ for TNB anions. Total cysteine was determined both as cysteic acid after oxidizing the protein with performic acid (Hirs, 1967) and by DTNB titration after reducing the protein with sodium borohydride (Cavillini et al., 1966).

*Reaction of Iodoacetate with Phosphoglycerate Kinase.* The kinetics of enzyme inactivation were studied by incubating the enzyme (0.12 nmol) at 30 °C in a 0.1-mL reaction mixture of buffer A with various concentrations of iodoacetate. At appropriate time intervals, aliquots of 5 μL of the reaction mixture were diluted to 0.1 mL of buffer A containing 16.4 mM DTT which was added to quench the reaction. The residual activity was assayed after 10 min. The reaction mixture without iodoacetate served as a control and the data was plotted as ln (% $E/E_0$) vs. time where $E$ and $E_0$ represent enzyme activities in the experiment and the control, respectively. The apparent rate constant was determined from the slope of the curve.

The stoichiometry of the carboxymethylation reaction and enzyme inactivation was determined in 2.0 mL of buffer A containing 13 nmol of protein and 14 μmol of iodoacetate at 30 °C. Aliquots of 0.4 mL were withdrawn at various time intervals, the reaction was arrested by adding DTT (10 mM), and the enzyme was assayed. The solution was dialyzed in the dark against distilled water for 48 h at 4 °C, lyophilized, and hydrolyzed with 6 N HCl at 105 °C overnight in a sealed, evacuated tube. The acid was removed under $N_2$ at 40 °C and the *S*-(carboxymethyl)cysteine was determined on a Beckman 121 MB amino acid analyzer.

In order to establish that iodoacetate reacts specifically with the cysteinyl residues of phosphoglycerate kinase, modification of the enzyme was performed as follows. To 15 nmol of the enzyme in 1.5 mL of buffer A was added 0.056 μmol of iodo[2-$^{14}$C]acetate and 8 μmol of nonradioactive iodoacetate. The reaction was followed by monitoring the loss of activity. When 80–90% of the activity was lost, DTT (10 mM) was added to stop the reaction. The modified enzyme was extensively dialyzed against distilled water, lyophilized, and prepared for amino acid analysis as described above. The acid hydrolysate was applied to a precalibrated column of a Beckman amino acid analyzer, 120-C, equipped with split-streaming device, and the fractions were collected and monitored for radioactivity and for determination of amino acids by the procedure of Moore & Stein (1954).

*Peptide Mapping.* Phosphoglycerate kinase (17–18 nmol) was labeled with 33.6 μCi of iodo[2-$^{14}$C]acetate in buffer A in the dark under $N_2$. The reaction was arrested with 2 mM DTT when the enzyme activity reduced 40% of its original value. The labeled enzyme and 50 nmol of unlabeled enzyme were mixed and completely carboxymethylated with 5 mM iodoacetate at 37 °C for 1 h in buffer A containing 2 mM DTT and 4 M guanidine hydrochloride. The enzyme was dialyzed in the dark at 0 °C against 0.2 M $NH_4HCO_3$ buffer, pH 8.5, and then digested overnight at 37 °C with 1% TPCK-treated trypsin (added in two aliquots at 12-h intervals). The digest was lyophilized 3 times using water. Peptide mapping was performed on cellulose-coated glass plates by using 2–3 nmol of the tryptic digest. The plates were developed by ascending chromatography in 1-butanol–acetic acid–water (4:1:5, organic layer), dried for 1 h, and electrophoresed in the second direction at 700 V for 1 h in acetic acid–formic acid–$H_2O$ (4:1:45, pH 1.9). Peptides were visualized by spraying with collidine–ninhydrin (100 mg of ninhydrin in 95 mL of absolute alcohol and 5 mL of collidine) and heating for 5 min at 105 °C. The untreated peptides were scraped off the plate and incubated with 0.2 mL of NCS tissue solubilizer overnight. Radioactivity was determined in toluene–base scintillation fluid.

*Microcomplement Fixation Assays.* Antiserum raised against the native enzyme was purified by immunoaffinity chromatography. The enzyme (15–20 mg) was attached to CNBr-activated Sepharose 4B at 4 °C in 0.1 M $NaHCO_3$ buffer, pH 8.0, for 18 h after which the exposed reactive groups of the Sepharose were blocked by overnight incubation with triethanolamine buffer (10 mM, pH 7.0). The unattached enzyme was removed by successive washing of the gel with acetate buffer (10 mM, pH 4.0) and Tris-HCl buffer (10 mM, pH 7.0), each containing 0.5 M KCl. The γ-globulin fraction of the antiserum was dialyzed against 10 mM Tris-HCl buffer (pH 7.0) containing 0.1 M KCl and centrifuged, and the supernatant was applied to the immunoabsorbent column (1 × 7 cm) which had been equilibrated with same buffer. The column was then washed with the buffer until the $A_{280}$ of the eluate reached zero. The pure antiphosphoglycerate kinase was eluted with glycine buffer (0.1 M, pH 2.5) containing 0.1 M KCl, brought immediately to pH 7.0 with dilute NaOH, and concentrated to 1 mg/mL protein. It was dialyzed against Isotris–BSA buffer (Stollar, 1978), centrifuged at 12000*g* for 10 min, and stored in small aliquots at −80 °C. Because of the low affinity of the antiserum for the rat muscle enzyme, microcomplement fixation assays were performed in a final volume of 1.2 mL as described by Stollar (1978). The analyses were carried out at various concentrations of antiphosphoglycerate kinase by using 1:200 complement and 1:500 of hemolysin stock solutions, respectively.

*Other Procedures.* Isoelectric focusing in sucrose columns, CD, and spectral studies were performed as described earlier (Sharma et al., 1980). Protein was assayed according to the procedure of Lowry et al. (1951). Estimation of molecular weight was carried out in 10 mM Tris-HCl buffer, pH 7.0, containing 100 mM KCl by using a Sephadex G-200 column (2.3 × 110 cm) at a flow rate of 15–20 mL/h. Determination of Stokes radius was carried out by the method of Nozaki et al. (1976). The sedimentation coefficient was estimated by

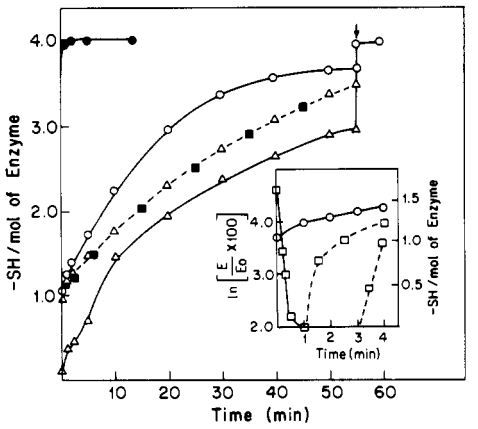


FIGURE 1: DTNB titration of phosphoglycerate kinase. The enzyme (0.72 mg) was incubated with DTNB (0.34 mM) in 2.0 mL of buffer A, and the increase in $A_{412nm}$ was monitored against a blank without the enzyme. (O—O) Enzyme alone; (●—●) enzyme denatured with 20 μL of 20% $NaDodSO_4$; (Δ—Δ) enzyme plus 13.4 mM of MgADP; (Δ---Δ) enzyme plus 13.4 mM MgATP; (■---■) enzyme plus 13.4 mM 3-phosphoglycerate. Arrow indicates the addition of 5 μL of 20% $NaDodSO_4$. The inset shows the loss of enzyme activity (□—□) in the initial stages of the DTNB reaction (O—O). The activity curve was obtained by running individual assays (100 μL containing the same proportions of the enzyme and DTNB mentioned above). At appropriate times, the activity was assayed by using 5 μL of the reaction mixture and 100 units of glyceraldehyde-3-phosphate dehydrogenase. (□---□) Recovery of the enzyme activity after adding 5 mM DTT at 1 and 3 min.

the procedure of Martin & Ames (1961) in a 5–20% sucrose gradient at 38 000 rpm at 4 °C for 23 h using a SW41 rotor. Pyruvate kinase, lactic dehydrogenase, malate dehydrogenase, and lysozyme served as markers.

## Results

*The Fast-Reacting -SH Is Essential for Enzyme Activity.* Figure 1 shows the titration of phosphoglycerate kinase with DTNB. The enzyme possesses one fast-reacting and three slow-reacting -SH groups. In agreement, total cysteines (determined as cysteic acid) amounted to 4.2 mol/mol of the enzyme, thus ruling out the presence of disulfide linkages. There is a complete loss of enzyme activity after reaction with DTNB (inset, Figure 1). Extrapolation of the linear part of the activity curve clearly shows that it is the blocking of the fast-reacting -SH group that leads to a total inactivation of the enzyme. As shown in the inset, the inactivation by DTNB can be reversed by DTT. The enzyme recovery depends upon the time of DTT addition; generally a minimum of 80% of the original activity is restored if DTT is added within 1 h during any part of the time course of the DTNB reaction. The effect of various ligands on the DTNB titration of the enzyme indicates that the fast-reacting -SH group is protected by MgADP, whereas the three slow-reacting -SH groups are protected by MgATP and 3-phosphoglycerate (Figure 1). Both classes of -SH groups are obviously protected by the combination of MgADP, MgATP, and 3-phosphoglycerate (data not shown).

The inactivation of the enzyme by sulfhydryl-specific reagents is shown in Table I. All the reagents inhibited the enzyme in terms of pseudo-first-order kinetics. *p*-(Chloromercuri)benzoate, DTNB, and mercuric chloride were the most potent inhibitors, but for each of these agents, the enzyme activity could be completely restored by DTT. Inaccurate measurement of phosphoglycerate kinase activity could result from the fact that DTNB also inactivates the auxiliary enzyme, glyceraldehyde-3-phosphate dehydrogenase. Therefore, the fast-reacting -SH groups of phosphoglycerate kinase was

Table I: Inactivation of Phosphoglycerate Kinase by Sulfhydryl-Specific Reagents[a]

| compound | concn (mM) | $k$[b] ($min^{-1}$) |
|---|---|---|
| iodoacetic acid | 13.9 | 0.034 |
| iodoacetamide | 13.9 | 0.33 |
| *N*-ethylmaleimide | 0.42 | 0.301 |
| *p*-(chloromercuri)benzoate | 0.0042 | 0.462 |
| 5,5'-dithiobis(2-nitrobenzoate) | 0.0042 | 0.462 |
| mercuric chloride | 0.042 | 0.266 |

[a] Enzyme inactivation (50 μg/mL) was performed in buffer A at room temperature. The reaction with iodoacetate, iodoacetamide, and *N*-ethylmaleimide was arrested by diluting aliquots 41-fold with buffer containing 15 mM DTT. The reaction with the other reagents was stopped by cooling in ice. Untreated enzyme served as a control. [b] $k$ is the pseudo-first-order rate constant.

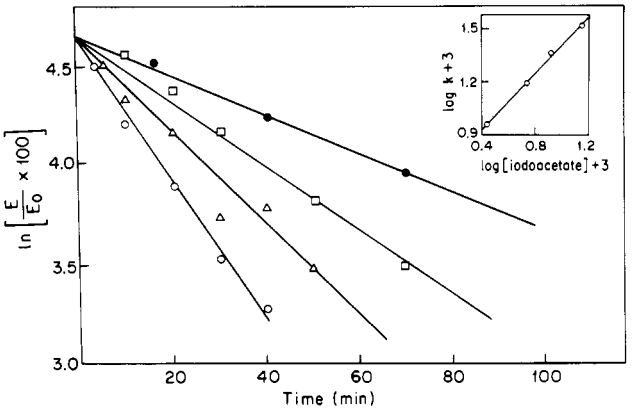


FIGURE 2: Inactivation of phosphoglycerate kinase by iodoacetate. The enzyme (50 μg/mL) was incubated in 100 μL of buffer A at an iodoacetate concentration of (●—●) 2.7, (□—□) 5.5, (Δ—Δ) 8.31, and (O—O) 13.85 mM. Inset shows the data plotted as log of pseudo-first-order rate constant of enzyme inactivation against log of iodoacetate concentration.

studied by using iodoacetate. In this case, excess reagent can be quenched by reducing agents before measuring residual enzyme activity.

When phosphoglycerate kinase from rat muscle was incubated at 30 °C with 14 mM iodoacetate in buffer A, the enzyme was inactivated by 80% in 35 min both in the backward and in the forward direction (Figure 2). Unless otherwise stated, the results described below were obtained by assaying the enzyme only in the backward direction. The reaction follows a pseudo-first-order kinetics with an apparent rate constant ($k$) of 0.032 ± 0.002 $min^{-1}$ in 14 mM iodoacetate. The rate of inactivation depends upon the concentration of iodoacetate, indicating that the overall reaction follows a second-order kinetics. A value of 2.6 $M^{-1}$ $min^{-1}$ for the second-order rate constant ($k'$) was obtained by plotting the value of $k$ against iodoacetate concentration. For determination of the number of iodoacetate molecules involved in the enzyme activation, the data of Figure 2 were analyzed by the equation of Hollenberg et al. (1971)

$$\log k = \log k' + n \log [I]$$

where $n$ is the number of inhibitor molecules reacting per active site of the enzyme. It is calculated from the slope of the line obtained by plotting log $k$ vs. log [I]. As shown in the inset of Figure 2, a value of 0.9 was obtained, demonstrating that the binding of one molecule of iodoacetate per active site results in total inactivation of the enzyme. Studies of the incorporation of iodoacetate vs. residual enzyme activity were carried out to determine the number of cysteinyl residues involved in the catalysis of the enzyme. The results show that there is

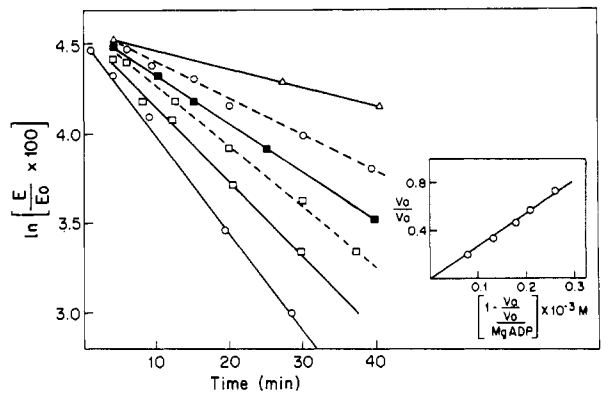


FIGURE 3: Effect of MgADP on iodoacetate inactivation of the enzyme. The enzyme (50 μg/mL) was incubated with 27.75 mM iodoacetate in buffer A (○—○) alone and in the presence of MgADP at concentration of 1 (□—□), 2 (□---□), 3 (■—■), 5 (○---○), and 10 mM (△—△). Inset shows the determination of the dissociation constant of the enzyme–MgADP complex where $V_A$ and $V_0$ are the respective pseudo-first rate constants of iodoacetate inactivation of the enzyme with and without MgADP.

a stoichiometric relationship in the incorporation of iodoacetate determined as (carboxymethyl)cysteine and the loss of enzyme activity. Extrapolation of the curve to zero activity shows that there was a modification of 1.08 mol of cysteine/mol of enzyme, thus confirming the results obtained by DTNB titration and kinetic analysis of inactivation by iodoacetate.

For identification of the amino acid residues modified, the enzyme was labeled with iodo[2-$^{14}$C]acetate as described under Experimental Procedures. The acid hydrolysate was subjected to amino acid analysis, and the modified amino acid was located by radioactivity measurements of the resulting fractions. More than 90% of the radioactivity was present in the peak corresponding to *S*-(carboxymethyl)cysteine, and no radioactivity was observed in any other peaks including those expected from (carboxymethyl)histidine and (carboxymethyl)lysine. These results clearly show that cysteine is the only amino acid of the enzyme which is modified by iodoacetate.

The effect of various ligands on the carboxymethylation reaction was studied to evaluate the involvement of the cysteinyl residue at the active site of the enzyme. Inactivation of the enzyme by iodoacetate is prevented by MgADP and ADP as indicated by the increase of $t_{1/2}$ from 12.5 min (control) to 25 min with MgADP and to 21 min with ADP. On the other hand, $Mg^{2+}$, MgATP, and 3-phosphoglycerate afforded no protection. The protective effect of MgADP depends upon its concentration (Figure 3), and the data were analyzed to determine the apparent dissociation constant ($K_d$) of the enzyme–nucleotide complex by the equation of Scrutton & Utter (1965)

$$\frac{V_A}{V_0} = \frac{k_2}{k_1} + K_d\left(\frac{1 - V_A/V_0}{[A]}\right)$$

where $V_A$ and $V_0$ are pseudo-first order rate constants in the presence and absence of ligand A; $k_1$ and $k_2$ are fractional order rate constants for the inactivation of E and EA in the equations

$$E + nI \xrightarrow{k_1} EI_n$$

$$E + A \rightleftharpoons EA \quad (K_d)$$

$$EA + nI \xrightarrow{k_2} EAI_n$$

when the values of $V_A/V_0$ are plotted against $(1 - V_A/A_0)/$[ADP], the slope is equal to $K_d$ and the ordinate intercept is equal to $k_2/k_1$. As shown in the inset of Figure 3, the $K_d$ for MgADP was calculated to be 2.67 mM, which agrees well with its $K_m$ value of 2.75 mM derived by saturation kinetics performed under identical conditions. The ratio of $k_2/k_1$ was obtained as zero, which suggests that the enzyme–ADP complex is not inactivated by iodoacetate. Furthermore, kinetic parameters like $K_m$ and $K_{cat}$ for MgADP, MgATP, 3-phosphoglycerate, and 1,3-diphosphoglycerate remain unaltered for the native as well as for the enzyme which is partially inactivated with iodoacetate (Table II). These results suggest that there are only two populations of enzyme molecules produced by the iodoacetate reaction, one native and the other catalytically inactive.

Table II: Kinetic Constants of Native and Iodoacetate-Modified Phosphoglycerate Kinase[a]

| | $V_{max}$ (mol min$^{-1}$ mL$^{-1}$) | | $K_{m,app}$ (mM) | | $K_{cat}$[b] | |
|---|---|---|---|---|---|---|
| | native | modified | native | modified | native | modified |
| forward direction | | | | | | |
| MgADP | 3.5 | 0.84 | 0.95 | 1.0 | 1988 | 1972 |
| 1,3-diphosphoglycerate | 2.62 | 0.66 | 0.042 | 0.036 | 1525 | 1541 |
| backward direction | | | | | | |
| MgATP | 0.91 | 0.22 | 0.83 | 0.91 | 519 | 548 |
| 3-phosphoglycerate | 0.42 | 0.1 | 2.22 | 2.72 | 239 | 236 |

[a] The enzyme (0.072 mg/mL) was incubated with 27.8 mM iodoacetate in buffer A at room temperature, and the reaction was arrested by diluting aliquots 41-fold with buffer containing 15 mM DTT. [b] $K_{cat}$ is moles of phosphorylation-dephosphorylation of 3-phosphoglycerate or 1,3-diphosphoglycerate per minute per mole of enzyme and is calculated by the equation $K_{cat} = V_{max}[E]^{-1}$ where [E] for the modified enzyme was determined from the residual activity of the iodoacetate-inactivated enzyme.

The pH-dependent kinetics of iodoacetate inactivation were analyzed to evaluate the p$K$ value of the cysteinyl residue of the enzyme. The following equation was used:

$$k = \frac{k'}{1 + H^+/K_A}$$

where $k'$ is the pH-independent absolute second-order rate constant, $k$ is the observed second-order rate constant, and $K_A$ is the proton dissociation constant of the cysteinyl residue and is obtained by plotting log $k$ vs. pH. The curve will be linear at low pH and flat at high pH. The intersection of the two lines will give the p$K$ value of the cysteinyl residue. Since the experiment was performed in a large excess of iodoacetate, for simplicity, the values of apparent first-order rate constants were substituted for those of second-order rate constants. By this analysis, a p$K$ of 9.1 was obtained which corresponds to the normal value of the unprotonated form of a cysteinyl residue.

*Evidence for Dimer Formation during Loss of Enzyme Activity.* On standing at 4 °C in 10 mM Tris-HCl buffer, pH 7.0, containing 100 mM KCl, phosphoglycerate kinase from rat muscle readily loses its activity. This loss is presumably due to the oxidation of the fast-reacting SH group as demonstrated above. Additional evidence is provided by the fact that the enzyme is stable for 2–3 weeks in the presence of 10 mM DTT (Figure 4); full activity was instantaneously restored when aliquots withdrawn along the time course of inactivation were incubated with the reducing agent; addition of cupric ions (0.1 μM) known to catalyze SH oxidation at substoichiometric levels (Olson & Massey, 1980 and references cited therein) rapidly inactivated the enzyme. As expected, the activity was

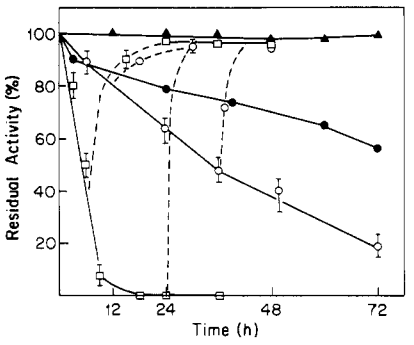


FIGURE 4: Loss of enzyme activity upon storage. The enzyme (1 mg/mL) was stored at 4 °C. (○—○) In 10 mM Tris-HCl buffer, pH 7.0, containing 100 mM KCl; (▲—▲) containing 5 mM DTT; (●—●) 0.2 mM MgADP; (□—□) 0.1 μM $CuSO_4$. Recovery of activity is shown by the broken lines after diluting 5 μL of the reaction mixture to 100 μL with the buffer containing 5 mM DTT.

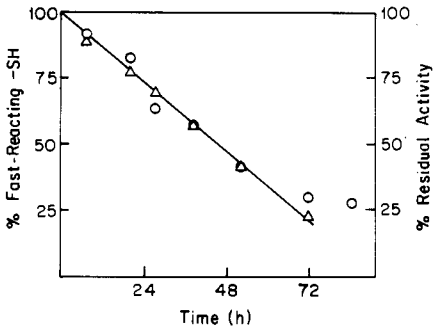


FIGURE 5: Effect of storage on the fast-reacting -SH of phosphoglycerate kinase. The enzyme (0.72 mg/mL) was stored at 4 °C in 10 mM Tris-HCl and 100 mM KCl, pH 7.0. Aliquots of 1 mL were monitored at various time intervals. (△—△) Enzyme activity; (○—○) fast-reacting -SH. Since an end point of the DTNB reaction could not be obtained at low pH, an increase in $A_{412\ nm}$ after 1 min was used in calculations of -SH content.

restored within 5–10 min after incubation with DTT (Figure 4); MgADP, which has been shown to protect the fast-reacting -SH group, stabilizes the enzyme. Loss of activity can also be prevented by incubating the enzyme under $N_2$ or with 1 mM EDTA (data not shown).

In order to determine whether the slow-reacting -SH groups are also involved in the loss of activity, the enzyme was incubated under the conditions described in Figure 4. Aliquots were withdrawn at appropriate time intervals and monitored for -SH content and enzyme activity. There was a decrease in the amount of fast-reacting -SH as the enzyme lost its activity, though the "total" -SH content determined after $NaBH_4$ treatment remained the same (4.2 mol/mol of enzyme). When the amount of fast-reacting -SH and the residual enzyme activity was plotted against time, a linear relationship was obtained (Figure 5), thus suggesting that both the reactions are concomitant; i.e., this group is responsible for the loss of enzyme activity. This conclusion is further confirmed by the analysis of tryptic digest of samples withdrawn at zero time and at later stages of the inactivation experiment described in Figure 4. The 0-h sample was labeled with iodo[2-$^{14}$C]acetate in buffer A until the enzyme activity had been reduced to 40% of the original activity, and the enzyme was totally carboxymethylated in 4 M guanidine hydrochloride with nonradioactive iodoacetate after reduction with 2 mM DTT and trypsinized. The sample containing partially active enzyme was carboxymethylated with nonradioactive iodoacetate in 4 M guanidine hydrochloride in buffer A at 37 °C for 1 h after which 2 mM DTT was added to reduce the disulfide linkages. The regenerated SH groups were

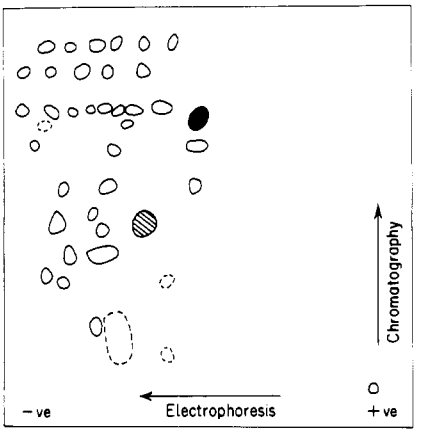


FIGURE 6: Peptide mapping of the tryptic digest of the native and partially active phosphoglycerate kinase. The enzyme (1 mg/mL) was stored at 4 °C in 10 mM Tris-HCl containing 100 mM KCl, pH 7.0, and aliquots of 1.0 mL of 0 and 60 h (20–30% of the original activity) were carboxymethylated with iodo[2-$^{14}$C]acetate and trypsinized, and the tryptic digests were analyzed by peptide mapping as described under the Experimental Procedures. The peptide shown by the closed circle contains the fast-reacting -SH. A tryptophan-containing peptide is shown by the hatched area and the peptides indicated by broken lines developed slowly with the ninhydrin spray.

then labeled with iodo[2-$^{14}$C]acetate and trypsinized as before. Peptide mapping of both the tryptic digests was carried out, and the peptides were analyzed for the distribution of radioactivity. As shown in Figure 6, only one peptide (closed circle), designated as the peptide possessing the fast-reacting -SH, contained all the radioactivity in both tryptic digests. These results show that it is the fast-reacting -SH which undergoes oxidation, leading to the loss of enzyme activity during storage.

Two protein peaks are obtained when pure, stored phosphoglycerate kinase, (containing 20–30% of the original activity) is subjected to chromatography on Sephadex G-200 (Figure 7A). Most of the enzyme activity appears at the end of peak b. When each fraction was incubated overnight with 5 mM DTT under $N_2$, the enzyme activity increased in peak a and at the beginning of peak b. However, no significant effect of the reagent was observed on the activity originally present at the end of peak b. This effect can be seen by the 20–25-fold increase in the ratio of enzyme activity with and without DTT treatment (Figure 7A). The $M_r$ values of the proteins in peak a at the beginning and at the end of peak b (indicated by arrows) are 76 000, 43 000 and 36 000, respectively. These proteins are designated as dimer, intermediate, and native enzyme. The peak a protein ($M_r$ 76 000) moved to the peak b position ($M_r$ 43 000) and not to the position of the native enzyme when gel filtration was performed in the presence of reducing agents. The apparent size of these proteins as indicated by their Stokes radii was determined by gel filtration on Sephadex G-200 column chromatography in 10 mM Tris-HCl buffer, pH 7.0, containing 100 mM KCl, and the results are shown in Figure 7B. The corresponding values of Stokes radii of 31, 27, and 24 Å were calculated for the proteins of $M_r$ values of 76 000, 43 000, and 36 000, respectively.

When partially inactive phosphoglycerate kinase is analyzed by isoelectric focusing on a sucrose column, three protein peaks with p*I*'s of 4.6, 6.0–6.5, and 7.6–7.8, respectively, are obtained (Figure 8). These proteins correspond to the dimer, the intermediate, and the native forms of the enzyme, respectively. Samples withdrawn at various times during the inactivation experiment of Figure 4 were analyzed by isoelectric focusing. Most of the protein from the early part of the curve focused at a p*I* of 7.6–7.8 and from the latter stages of the curve at

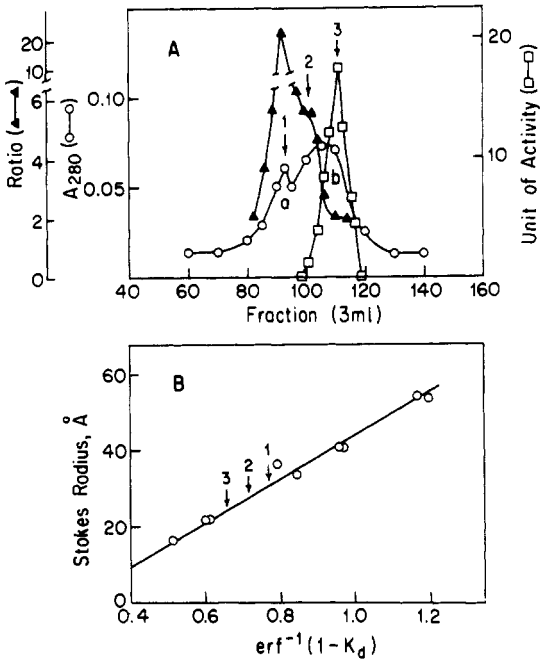


FIGURE 7: Sephadex G-200 chromatography of stored phosphoglycerate kinase. (A) The enzyme (20–30% of the original activity) was loaded onto a column (2.3 × 110 cm) of Sephadex G-200 which had been previously equilibrated with 10 mM Tris-HCl and 100 mM KCl, pH 7.0. Fractions were monitored by $A_{280}$ and enzyme activity. Each fraction was incubated overnight at 4 °C with 5 mM DTT under $N_2$ and reassayed. The ratio of enzyme activity with and without the reducing agents was determined. (B) shows the analysis of gel filtration data for the estimation of Stokes radius using as standards pyruvate kinase (54 Å), lactic dehydrogenase (41 Å), malate dehydrogenase (37 Å), chymotrysinogen (22.4 Å), and cytochrome *c* (16.4 Å). Arrows 1, 2, and 3 represent dimeric, intermediate, and native forms of the enzyme, respectively.

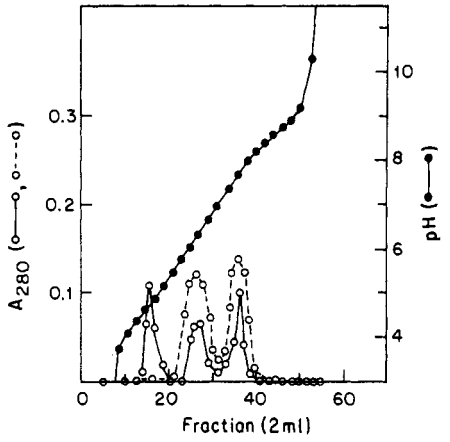


FIGURE 8: Isoelectric focusing of stored phosphoglycerate kinase. Enzyme containing 20–30% of the original activity was incubated overnight under $N_2$ with 5 mM DTT and focused on a sucrose gradient column: (O---O) in the presence of DTT; (O—O) without DTT treatment. The protein was monitored by $A_{280}$ and pH measurements were performed at room temperature.

a p*I* of 4.6. On the other hand, samples withdrawn in the middle of the curve had a p*I* in the range of 6.0–6.5. These results show that the protein with an approximate p*I* of 6.0–6.5 is an intermediate in the inactivation of the enzyme during storage.

The dimer does not bind either to CM-cellulose or to an ATP–Sepharose affinity column. Though it possesses no catalytic activity, full activity is regenerated after incubation with DTT. As shown in Table III, the degree of regeneration of enzyme activity by the reducing agent depends upon the

Table III: Regeneration of Enzyme Activity from the Dimer by DTT[a]

| | SH/mol of protein | | % of original sp act. | | |
|---|---|---|---|---|---|
| | free | total | untreated | after DTT | after denaturation-renaturation[b] |
| preparation 1 | 6.0 | 8.5–9.0 | 0 | 100 | 90 |
| preparation 2 | 3.5–4.0 | 8.5–9.0 | 0 | 50 | 90 |
| native enzyme | 4.2 | 4.6 | 100 | 100 | 90 |

[a] DTT was 10 mM. [b] The activity values are an average of four different experiments obtained by incubating the protein (0.5 mg/mL) under $N_2$ overnight at 0 °C in 5 mM Tris-HCl (pH 7.0) containing 100 mM KCl, 10 mM $MgSO_4$, 1 mM EDTA, 10 mM DTT, and 2 M guanidine hydrochloride. Renaturation of the enzyme was performed at 0 °C for 1 h by diluting a suitable aliquot 41-fold in the same buffer minus the denaturant.

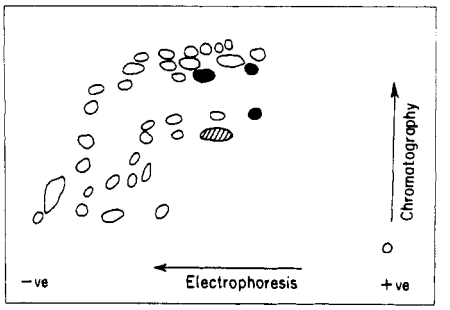


FIGURE 9: Peptide mapping of the dimer form of phosphoglycerate kinase. Partially oxidized dimer (four -SH per mole mol of protein) was carboxymethylated with iodoacetate for 1 h in the presence of 4 M guanidine hydrochloride after which it was reduced with 1 mM DTT and incubated for 30 min with 2.3 mM cold iodoacetate and 1.0 μCi of iodo[2-$^{14}$C]acetate. The peptides containing radioactivity are shown by closed circles. The tryptophan-containing peptide is shown by the hatched area. Details are given in the text.

free -SH content of the protein; i.e., full activity is restored in preparations containing 6 mol of free -SH whereas only 50% of the activity is restored in preparations containing 3–4 mol of free -SH. Full activity in the latter preparations could, however, be regenerated after reduction under denaturing condition and subsequent refolding without the denaturant. The dimer containing 3–4 mol of free -SH groups was carboxymethylated and trypsinized according to the protocol described for that of the partially active enzyme in Figure 6. The tryptic digest was analyzed by peptide mapping, and the distribution of radioactivity in various peptides was determined. As shown in Figure 9, there were two extra peptides other than the peptide containing the fast-reacting -SH group which possessed radioactivity, thus confirming that there were inter or intramolecular disulfide bridges in the greatly oxidized dimer.

Some of the properties of the native and the dimeric rat muscle phosphoglycerate kinase are listed in Table IV. Compared to gel filtration, the ultracentrifugation studies yielded a higher molecular weight for both proteins. Compared to the native enzyme, the dimer has a greater absorption at 280 nm and the ratio of $A_{280}/A_{260}$ is also higher (1.35 and 1.85, respectively). CD spectra in the near-UV region show a large difference in the absorption of the aromatic amino acid residues of the dimer and the native enzyme (Figure 10). The positive band at 256 nm which is present in the dimer but absent in the native enzyme is assigned to the disulfide linkage

Table IV: Properties of Native, Intermediate, and Dimer Forms of Rat Muscle Phosphoglycerate Kinase

| parameters | native | intermediate | dimer |
|---|---|---|---|
| $M_r$ | | | |
| ultracentrifugation | 45 900 | | 95 600 |
| gel filtration | 36 000 | 43 000 | 76 000 |
| $NaDodSO_4$ electrophoresis | 45 500 | 45 500 | 45 500 |
| average | 42 500 | 44 250 | 85 800 |
| sedimentation coeff ($s^0_{20,w}$) (S) | 4.0 | | 7.0 |
| diffusion coeff[a] ($D^0_{20,w}$) ($cm^2/s$) | $8.9 \times 10^{-7}$ | $8.3 \times 10^{-7}$ | $6.9 \times 10^{-7}$ |
| Stokes radius (Å) | 24 | 27 | 31 |
| isoelectric point | 7.6–7.8 | 6.0–6.5 | 4.6 |
| frictional ratio ($f/f_0$)[b] | 0.89 | 1.0 | 0.91 |
| partial specific volume | 0.742 | | |
| $A_{280}^{0.1\%}$ in 10 mM Tris-HCl (pH 7.0) and 0.1 M KCl | 0.6 | | 0.9 ± 0.1 |
| $A_{280}/A_{260}$ | 1.3 ± 0.1 | | 1.8 ± 0.1 |
| α-helical content[c] (%) | 13 | | 17 |
| immunodiffusion | complete identity | | complete identity |
| protein (μg) required for maximum $c'$ fixation | 0.35 | | 3.0 |

[a] Diffusion coefficient ($D$) was determined from the Stokes-Einstein equation: $D = kT/(6\pi\eta a)$ where $k$ is the Boltzmann constant, $T$ is absolute temperature, $\eta$ is the viscosity of the solution, and $a$ is Stokes radius. [b] Frictional ratio ($f/f_0$) was calculated from the equation $f/f_0 = a/[3\bar{v}M/(4\pi N)]^{1/3}$ where $M$ is the molecular weight, $N$ is Avogardro's number and $\bar{v}$ is the partial specific volume as calculated by the method of Schachman (1957). [c] A path length of 0.1 cm and a protein concentration of 0.45 mg/mL was used, and α-helical content was determined by the method of Greenfield & Fasman (1969).

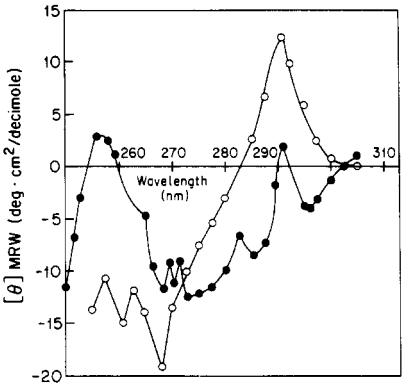


FIGURE 10: CD spectra of the native and the dimeric form of phosphoglycerate kinase. (O—O) Native enzyme; (●—●) dimeric form. The pathlength was 1.0 cm and protein concentration was 2–3 mg/mL. A mean residue weight of 107.8 was used for both the proteins.

(Strickland, 1974). The interaction of the disulfide linkage with a tryptophanyl residue is illustrated by the change of positive pseudo-band at 291 nm of the native enzyme into two negative bands at 285 and 295 nm. The phenylalanine absorption at 261 and 268 nm of the native enzyme was also affected by the disulfide linkage, appearing as a single band at 268 nm in the dimer form.

Immunological properties of both proteins were studied by using purified antiserum raised against the native enzyme. Double immunodiffusion analysis showed a pattern of complete identity. Microcomplement fixation studies showed that though the percent complement fixed remained unaltered, there is a lateral shift in the curves. That is, the amount of protein required for maximum complement fixation increased from 0.35 μg for the native to 3 μg for the dimeric forms of the enzyme. These studies indicate that there is a lower affinity of the antigenic determinants of the dimer toward the antiserum of the native enzyme (Levine & Vunakis, 1967).

## Discussion

From the results, it is clear that of four SH groups, the fast-reacting one is essential for the catalytic activity of phosphoglycerate kinase of rat muscle. The conclusion derives support from the carboxymethylation studies where it was shown that (a) cysteine is the only amino acid modified, (b) there is a mole:mole relationship between carboxymethylation and the loss of enzyme activity, (c) there is a selective labeling of a SH group in a single peptide when the enzyme is stoichiometrically S-alkylated by iodo[2-$^{14}$C]acetate, and (d) a p$K$ value of 9.1 for carboxymethylation reaction correlates well with the normal value for a cysteinyl group. The fact that the $K_d$ for MgADP (determined by steady-state kinetics of iodoacetate inactivation with and without the ligand) corresponds well with its $K_m$ (determined by saturation kinetics) suggests that this –SH group is present at or near the MgADP binding site. The identical value of $K_m$ and $K_{cat}$ for the native and the enzyme partially inactivated with iodoacetate lends further support to this conclusion. An essential catalytic role of the –SH group in phosphoglycerate kinase from other mammalian sources has also been reported. An –SH group in the vicinity of the MgADP binding site of horse muscle enzyme has been demonstrated by X-ray crystallographic studies (Blake et al., 1972). There are preliminary reports on the role of –SH groups in the activity of the enzyme from rabbit muscle (Kreitsch & Bucher, 1970) and bovine liver (Kulbe et al., 1975).

In contrast to the mammalian enzyme, the single –SH group of yeast phosphoglycerate kinase is not involved in catalysis, the binding of substrates, or the maintenance of tertiary structure (Markland et al., 1975; Arvidsson & Raznikiewicz, 1973). It is possible that either the catalytic role of the –SH group in the mammalian enzyme, conceivably as a general acid–base catalyst, is played by some other amino acid(s) in the yeast enzyme or the reaction mechanism is different in the two systems. This picture is reminiscent of yeast vs. rabbit muscle fructosebisphosphate aldolases (Lai et al., 1974; Lai & Horecker, 1972). More studies are therefore required to explain the differences in the mode of action of phosphoglycerate kinase from yeast and mammalian sources.

The evidence presented shows that the fast-reacting –SH is also involved in the maintenance of the tertiary structure of the rat muscle enzyme. There is a rapid and dramatic loss of activity in the absence of reducing agents. The loss of the fast-reacting –SH group parallels enzyme inactivation. All of the lost activity can be regenerated by the addition of DTT. During inactivation, a disulfide linkage is formed between the fast-reacting –SH of two enzyme molecules (Figure 5). The process is initiated by unfolding of the enzyme as characterized by an increase in apparent molecular weight and Stokes radius (Figure 7) and the lowering of isoelectric point from 7.6 to 6.0–6.5. The enzyme in this conformation contains the fast-reacting –SH in such an orientation that on one hand, it can readily return to its native form by treatment with reducing agents or it can get oxidized, resulting in the formation of an intermolecular disulfide linkage between two enzyme molecules. Oxidation eventually proceeds further with the formation of two to three extra disulfide linkages involving the slow-reacting –SH groups (Figure 9). The events up to and including the formation of the dimer produced by the –S–S– linkage of the fast-reacting –SH group are reversible. However, once the slow-reacting –SH groups are involved in the

formation of disulfide bridges, reactivation requires not only a reduction of these bridges but also an unfolding of the tertiary structure of the enzyme with guanidine hydrochloride and subsequent renaturation. That is, oxidation of the slow-reacting -SH groups must have brought about conformational changes which are not alleviated by a simple reduction process.

Compared to gel filtration, ultracentrifugation studies showed a higher molecular weight for the native and the dimeric forms of the enzyme which may be due to differences in the conformation of the protein molecules under the respective experimental conditions. Furthermore, weak binding of the enzyme to CM-cellulose in the presence of EDTA, together with the anomalous behavior of the yeast enzyme with various salts (Scopes, 1978; Wrobel & Stinson, 1980), suggests that the enzyme can exist in different conformational states under different ionic conditions. The same explanation would also account for the discrepancy in $M_r$ values of the rat muscle enzyme reported by Haas et al. (1974) and reported in this study.

Dimerization of rat muscle phosphoglycerate kinase involving -SH groups is not restricted to the pure enzyme but also occurs in the crude extract as shown by the fact that the protein unadsorbed to CM-cellulose (Sharma et al., 1980) contains all the three forms of the enzyme, namely, dimer, intermediate, and native (unpublished data). A similar phenomenon has been observed in the rat liver enzyme (Hiremath & Rothstein, 1982). Bojanovski et al. (1974) reported the presence of three species of phosphoglycerate kinase in crude extract of bovine liver with respective p*I*'s of 7.5, 5.8, and 3.8. The close similarity of the p*K*'s of bovine liver enzymes with those reported here suggest that the former may also be produced by dimerization of the native enzyme. The same phenomenon may explain the portion of the enzyme from *Turbatrix aceti* which is unadsorbed to DEAE-Sephadex (Gupta & Rothstein, 1976).

Partial proteolysis is another mechanism by which multiple species of the enzyme can be produced. Such is the case for phosphoglycerate kinase of human lymphoblasts (Tollefsbol & Gracy, 1980).

In previous studies, we reported a difference in the stability as well as in the conformation of the fast-reacting -SH groups of young and old phosphoglycerate kinase (Sharma et al., 1980). The experiments described here strongly suggest that the stability of the enzyme is determined by conformation of the fast-reacting -SH group. This factor may be involved in the greater stability of the old compared to the young form of the enzyme. However, it is not yet clear as to what might cause such a conformational change and its exact nature. The answer to this problem must await future structural studies.

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