Chemical Modification of Yeast 3-Phosphoglycerate Kinase*

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SUMMARY

Sulphydryl reagents, as well as mild hydrogen peroxide oxidation, do not inhibit the activity of yeast phosphoglycerate kinase, indicating that these sulphydryl group and 3 methionine residues present in the enzyme are not essential for activity. Nitration of phosphoglycerate kinase by tetranytrimethane inhibits the enzyme by reaction with a single tyrosine residue. Substrates provide partial protection against inactivation by nitration. Circular dichroism spectra indicate that no conformational changes occur upon nitration. However, perturbation of the microenvironment surrounding the aromatic amino acid residues, particularly tyrosine, was observed. The same perturbation was observed on addition of the substrate 3-phosphoglycerate kinase to native phosphoglycerate kinase.

The role of the enzyme in the action of yeast phosphoglycerate kinase has been studied by modification with O-methylisourea, 2-methoxy-5-nitrotropon, and pyridoxal phosphate. Guanidination shows there are lysines essential for phosphoglycerate kinase; extrapolation to zero activity indicates that there are three essential lysines as judged by nitrotyrosination and three essential lysines when the enzyme is reacted with pyridoxal phosphate. Substrates afford partial protection and extrapolation to total protection indicates that up to three lysines are protected by MgI TP and one lysine by 3-phosphoglycerate. Spectrofluorescence and optical rotatory dispersion measurements show that there is no detectable conformational change for the guanidinated phosphoglycerate kinase and that there are slight changes in the spectra suggesting that there may be slight conformational changes for the nitrotyropropylated and the pyridoxal phosphate-modified enzymes.

3-Phosphoglycerate kinase (EC 2.7.2.3) is a glycolytic enzyme that catalyzes the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate with the concomitant phosphorylation of ADP to ATP. The equilibrium of this reaction is favorable to ATP formation (1) thereby helping give direction to the glycolytic pathway while being one of the two sites of ATP formation.

At the time our work was initiated there was very little physicochemical data on phosphoglycerate kinase. A molecular weight of 34,000 had been reported (2) but there was little additional detailed structural information available for this enzyme. With the commercial availability of yeast phosphoglycerate kinase this enzyme appeared to be a good candidate for the investigation of both primary structure and catalytic mechanism. Subsequent to the development of our interest in this enzyme, several reports appeared revising the molecular weight to between 45,000 (3) and 50,000 (4) and giving the amino acid composition of yeast phosphoglycerate kinase (5).

There has been, however, little work on the structural requirements of the enzyme necessary for function. In this paper we report the results of modification studies on cysteine, methionine, tyrosine, and lysine residues aimed at establishing whether these residues are critical to enzyme activity.

EXPERIMENTAL PROCEDURE

Materials

The following materials were purchased commercially: yeast phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, and ATP, ADP, ITP, IDP, NADH, and 3-phosphoglycerate were all purchased from Boehringer-Mannheim Corp.; enzyme grade ammonium sulfate, urea (ultrapure grade), thioglycolic acid, and guanidine HCl (high purity, low ultraviolet absorbance) from Mann Research; aminepeptidase M from Henley and Co., Inc., N.Y.; dansyl chloride from Sigma; carboxypeptidase A, from Boehringer-Mannheim Corp.; trypsin from屐sh enzyme grade; and ammonium persulfate from Reagent Products, Inc., N.Y.

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1 The abbreviations used are: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; SDS, sodium dodecyl sulfate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TPCK, trypsin phenylalanine chloromethyl ketone.
Methods

Purification—Several batches of commercial phosphoglycerate kinase were found to be impure by standard polyacrylamide disc gel electrophoresis (6). These were dialyzed against 0.055 M sodium acetate, pH 6.0, containing 0.00065 M EDTA and purified by chromatography on carboxymethylcellulose (1.8 X 55 cm) using a linear gradient in sodium acetate from 0.10 to 0.70 M, pH 6.0. Protein concentrations were determined either by absorbance at 280 nm or by the method of Lowry et al. (8). Except where otherwise stated all of the operations were performed at 4°C.

Gel Electrophoresis—The method of Davis (5) was used for standard polyacrylamide disc gel electrophoresis. The method of Laemmli (11) was used for SDS-polyacrylamide gel electrophoresis at ambient temperature.

Phosphoglycerate Kinase Assay—The assay couples phosphoglycerate kinase to glyceraldehyde-3-phosphate dehydrogenase and utilizes the decrease in absorbance at 340 nm resulting from the oxidation of NADH as an index of activity. The reaction mixture contained 0.1 M Tris buffer, pH 7.5, 0.0165 M ATP, 0.33 M MgSO4, 0.32 M 3-phosphoglycerate, 8.6 mM NADH, and 2 mg per ml of a crystalline suspension of glyceraldehyde-3-phosphate dehydrogenase. Aliquots (10 μl) of enzyme solution (0.1 to 0.5 μg of phosphoglycerate kinase) were added to 0.99 ml of the above stock assay solution. The assays were performed at ambient temperature and activities were corrected to saturation conditions by the method of Larsson-Raznikiewicz (8).

Determinations of Extinction Coefficient and Molecular Weight—Molecular weights were determined by high speed sedimentation equilibrium as described by Yphantis (10) using native or denatured (dialysis against 6 M guanidine-HCl containing 0.0005 M EDTA at pH 8.6) enzyme. The final volume was adjusted to 1 ml and the protein concentration was determined either by absorbance at 280 nm using an extinction coefficient of ε280 (A412) = 7000 (3) or by the method of Lowry et al. (8). Except where otherwise stated all of the operations were performed at 4°C.

Quantitative Determination—Quantitative determination was achieved by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH as an index of activity. The reaction mixture contained 0.1 M Tris buffer, pH 8.0, containing 5 X 10^-4 M EDTA and either 30 or 150 mg of the enzyme 0.16 mM Tris-chloride, pH 8.5, at ambient temperature. Aliquots were removed at varying times and diluted with 0.1 M sodium acetate, pH 6.0, containing 5 X 10^-4 M EDTA prior to activity determination. After elution for 30 min, the reaction mixture was applied to a Sephadex G-100 column (2.5 X 140 cm) and eluted with 0.1 M Tris-buffer, pH 7.6, containing 5 X 10^-4 M EDTA. Gel filtration served to remove both the excess reagent and any phosphoglycerate kinase polymers which may have formed during the nitration reaction. The nitratd enzyme thus obtained was pooled and concentrated by ultrafiltration and appropriate aliquots were removed for activity assay, amino acid analysis, standard and SDS-polyacrylamide gel electrophoresis, molecular weight determination by sedimentation equilibrium analysis, and tryptic digestion followed by peptide mapping. In large scale experiments, 120 mg of purified phosphoglycerate kinase were utilized for tryptic digestion and isolation of the single nitrated peptide.

Phosphoglycerate kinase was also nitrated with a 10-fold molar excess of tetranitromethane in the presence of either 0.32 M 3-phosphoglycerate or 0.0165 M ATP and 0.022 M MgSO4, or in the absence of substrates. Each experiment was performed using 2 mg of enzyme and nitration was continued for 30 min. Aliquots were withdrawn during the reaction and enzyme activity was determined.

Lysine Modification with O-Methylisourea—To 2.5 ml of phosphoglycerate kinase (2.25 mg per ml) in 0.2 M glycine-NaOH, pH 10.5, were added 2.5 ml of freshly neutralized 0.6 M O-methylisourea, pH 10.5. A control with the same buffer and at the same pH was run under identical conditions. At appropriate intervals aliquots were removed from the mixture at ambient temperature. Aliquots were removed from both sample and control at various time intervals and were immediately dialyzed against 10 mM Na2HPO4, pH 8.5. These samples were desalted on Sephadex G-25, and aliquots were removed for assay and amino acid analysis. Quantitative determination was achieved by determining the absorbance at 420 nm using an extinction coefficient of ε420 (A280) = 17000 (10). Lysine modification with 3-mercaptopentoehanol (down to 10 mM used for substrate protection studies) was accomplished by determination of the absorbance at 420 nm using an extinction coefficient of ε420 (A280) = 20,700 (10). Lysine Modification with Pyridoxal Phosphate—To 0.5 ml of a phosphoglycerate kinase solution (containing 2 to 7 mg per ml of phosphoglycerate kinase) in 50 mM phosphate buffer, pH 8.5 (down to 10 mM used for substrate protection studies) was added an equal volume of the buffer (also 30 mM IPTG-60 mM MgSO4, or 30 mM 3-phosphoglycerate for the substrate protection studies) and this solution was allowed to incubate for 10 min at 25°C. To
this solution was added 1.0 ml of the phosphate buffer containing the desired molar excess of pyridoxal phosphate. The reaction was carried out for 10 min at 25°. For time course studies, 100-μl aliquots were withdrawn at 1-min intervals. The reaction was stopped and the Schiff base reduced by addition of a 100-fold molar excess of solid NaBH₄ with respect to pyridoxal phosphate. After a 1-hour incubation, the reduced samples were dialyzed against 50 mM phosphate buffer, pH 8.5, by conventional means or by ultrafiltration. A control was run in the absence of pyridoxal phosphate. If substrate protection studies were performed there was an excess of solid NaBH₄ with respect to pyridoxal phosphate. After a 1-hour incubation, aliquots were removed from the final solutions for assay and protein determination. Quantitative determination of the difference absorptions at 325 nm using the molar extinction coefficient of 8.5 of ε₃25 (325 nm) = 7500 (20). Purification of the components of the reaction mixture of pyridoxal phosphotase-modified phosphoglycerate kinase was accomplished by the method of electrophoresis using a LKB 1100 apparatus (110 m column), 1% ampholine carrier ampholytes, pH range 3 to 10. A total of 7.2 mg of modified phosphoglycerate kinase (nine-tenths of the native and modified protein, at a concentration of 10 mg per ml, was performed essentially as described by Butler et al. (21) using a 100-fold molar excess of maleic anhydride. The protein was denatured by dialysis against 6 M urea in 0.2 M borate buffer at pH 9.0. Solid maleic anhydride was added with a slow stirring solution of enzyme, but lacking substrate. Aliquots were removed from the final solutions for assay and protein determination. Quantitative determination of the moles of lysine modified was accomplished by determination of the difference absorption at 325 nm using the molar extinction coefficient for pH 8.5 of ε₃25 (325 nm) = 7500 (20).

**RESULTS**

Cysteine and Methionine Modification—Cysteine titration by the method of Eilman (16) gave the following results: in the presence of denaturing agents 1.0 residue for cysteine per mol of protein is readily available to DTNB. However, in the absence of denaturating agents, DTNB reacts very slowly with phosphoglycerate kinase and even after 4 hours only 0.5 residue of the sulfhydryl group had reacted. These results are in accord with the results of amino acid analysis (Table I) which show 1 mol of cysteic acid after performic acid oxidation (24). That the single, slowly reacting sulfhydryl group is not necessary for phosphoglycerate kinase activity was shown by the finding that after dialysis of the DTNB-treated enzyme 50 to 70% of the activity was regenerated. This occurred even with enzyme denatured in urea or guanidine HCl in which the sulfhydryl group had fully reacted. Controls incubated under similar conditions but without DTNB showed similar loss in activity. Only in the case of SDS as denaturant was there complete loss of activity after dialysis and this was attributed to irreversible denaturation by SDS.

Mild hydrogen peroxide oxidized yeast phosphoglycerate kinase resulted in no loss of enzyme activity over the 140-min time period of incubation. Duplicate amino acid analyses on the other hand indicate quantitative conversion of the 3 methionine residues present in the enzyme to methionine sulfone and the oxidation of cysteine to cysteic acid.

Tyrosine Modification—Nitration of phosphoglycerate kinase with 21-fold molar excess of tetranitromethane for 90 min resulted in a 93% loss of enzyme activity. Gel filtration of the mixture produced three yellow bands, the first of which contained all of the protein and separated into two distinct protein peaks. Equilibrium sedimentation of the first peak indicated aggregated forms of nitratated enzyme in the molecular weight range 120,000 to 150,000 and gel electrophoresis confirmed the presence of at least two and probably three species. The second peak contained the bulk of nitratated phosphoglycerate kinase and was shown to contain a single species on gel electrophoresis. Activity of this material was less than 3% of the native enzyme and sedimentation equilibrium gave a molecular weight of 35,300. The amino acid composition of this material was identical with that of native enzyme except that 3 to 4 tyrosine residues had been nitrated.

Nitration of phosphoglycerate kinase (both 30- and 180-μg samples) with a 10-fold molar excess of tetranitromethane for 30 min resulted in the loss of approximately 84% of the enzyme activity (Fig. 1). Gel filtration of the mixture gave a single yellow protein peak. Standard and SDS gel electrophoresis indicated the presence of a single protein species. Sedimentation equilibrium analysis confirmed this and indicated a molecular weight of 43,500. For comparison, sedimentation equilibrium analysis of
Amino acid composition of native and nitrated phosphoglycerate kinase

<table>
<thead>
<tr>
<th>Residue</th>
<th>Native enzyme</th>
<th>Nitrated enzyme*</th>
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<tr>
<td>Residues</td>
<td>Integral</td>
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<tr>
<td>Lysine</td>
<td>40.30</td>
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<tr>
<td>Histidine</td>
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<tr>
<td>Arginine</td>
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<tr>
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<td>17</td>
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<tr>
<td>Serine</td>
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<td>25</td>
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<tr>
<td>Glutamic acid</td>
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<td>37</td>
</tr>
<tr>
<td>Proline</td>
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</tr>
<tr>
<td>Glycine</td>
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<td>Alanine</td>
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<td>Valine</td>
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</tr>
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<td>Methionine</td>
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<td>Isoleucine</td>
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<td>Leucine</td>
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<tr>
<td>Tyrosine</td>
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<td>6</td>
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<tr>
<td>Phenylalanine</td>
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<td>18</td>
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<tr>
<td>Cysteic acid</td>
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<td>1</td>
</tr>
<tr>
<td>Sum</td>
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<td>403</td>
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</table>

* Composition calculated on the basis of 40 lysines and 13 arginines.
* Amino acid analysis of the nitrated protein gave the identical composition as the native protein except the nitrated enzyme contained 5 tyrosine residues and 1 residue of nitrotyrosine compared to 6 tyrosine residues in the native enzyme. Results of amino acid analyses (using 24- and 72-hour hydrolys) are given in Table 1. Values for duplicate analyses agreed in all cases to within ±3%.

The specific activity of control phosphoglycerate kinase was 875 units per mg. Nitration in the absence of substrate resulted in an 85% loss of enzyme activity. In contrast, nitration in the presence of either 3-phosphoglycerate or MgATP resulted in a loss of only 50% of activity (Fig. 1).

Peptide mapping of the tryptic digest of maleylated, nitrated phosphoglycerate kinase consistently gave approximately 16 peptides visible after staining with ninhydrin. This is in good agreement with the expected number of 13 or 14 peptide fragments based on the arginine content of yeast phosphoglycerate kinase. (Tryptic digestion of native, nonmaleylated protein gave 45 or 46 peptides visible on staining with ninhydrin.) Spraying with 50% ammonia spray prior to ninhydrin staining indicated a single nitrated peptide, however, elution from the paper with 30% acetic acid failed to produce a homogenous product. Gel filtration on Sephadex G-75 and ion exchange chromatography on SP-Sephadex was successful in that it permitted a separation of the peptide mixture into approximately three to four fractions, each containing several components.

The circular dichroism spectra of native and nitrated phosphoglycerate kinase are shown in Fig. 2. Values of α helix, β structure, and random coil configurations over the range of 200 to 260 nm calculated according to the method of Greenfield and Fasman (22), are for the native enzyme 27%, 63%, and 10%, respectively, and for the nitrated enzyme 28%, 59%, and 13%, respectively. On the basis of this data it can be seen that there is no gross conformational difference between the native and the nitrated enzymes suggesting, perhaps, a small conformational change localized in the region of the modified tyrosine residue. This is substantiated by qualitative examination of the circular dichroism spectra over the range 250 to 400 nm which suggests a small
but definitive change in the tertiary structure around the aromatic amino acid residues, particularly tyrosine, when native phosphoglycerate kinase is compared to nitrated phosphoglycerate kinase.

Addition of the substrate 3-phosphoglycerate to the native enzyme causes a perturbation of the CD spectra over the range 250 to 400 nm suggesting a small change in tertiary structure particularly around the aromatic residues, and inferring that an aromatic residue(s) may somehow be involved in the binding site of the enzyme.

Lysine Modification—The time course of inhibition of phosphoglycerate kinase by each of the three reagents is shown in supplemental Figs. 1 to 3. Although the time of reaction varies with each reagent there is a rapid inhibition of yeast phosphoglycerate kinase by each of these lysine-specific reagents. Except in the case of guanidination, where total inactivation is obtained, it has not been possible to achieve better than 75% over-all inhibition. Amino acid analysis of the various guanidinated samples showed a loss of only lysine with a concomitant appearance of homoguanine. Up to 36 lysines were modified; extrapolation to zero activity suggests that there are at most 12 “essential” lysine residues.

The stoichiometry of modified lysines for nitrotrptonylated phosphoglycerate kinase was determined by the absorbance at 420 nm (the spectrum of the reaction product was that expected for nitrotrptonylated lysine (19)) and amino acid analysis showed that no other residues were affected by the reagent. Extrapolation to zero activity (Fig. 3) suggests that there are at most three essential lysines.

The stoichiometry of lysines modified by pyridoxal phosphate was determined by measuring the difference in absorbance at 325 nm of modified versus native phosphoglycerate kinase. The spectrum of the reaction product was consistent with protein-bound N-phosphopyridoxal lysine (20). Amino acid analysis of the pyridoxal phosphate-modified enzyme after borohydride reduction showed no losses in any of the other amino acids. Extrapolation to zero activity (Fig. 4) also suggests that there are at most three essential lysines for yeast phosphoglycerate kinase.

For the reactions with 2-methoxy-5-nitrotropon and with pyridoxal phosphate it is possible to calculate a pseudo-first order rate constant ($k_{app}$) from the log plots, and by plotting $1/k_{app}$ versus $1/t$ to show that there is a preliminary formation of an enzyme-inhibitor complex for both 2-methoxy-5-nitrotropon and for pyridoxal phosphate according to the analysis of Petra (28) and Fiszkiiewicz and Smith (29). For 2-methoxy-5-nitrotropon the apparent $K_i = 0.1$ mm and for pyridoxal phosphate the apparent $K_i = 0.54$ mm. These values are close to the $K_m$ values for substrates of yeast phosphoglycerate kinase (5) suggesting that there is a fairly selective binding of the reagents at least in the active site region.

Although the pH of the guanidination reaction precludes any substrate protection studies, the reaction with 2-methoxy-5-nitrotropon and pyridoxal phosphate occurs at a pH for which substrates do bind. MgITP was used instead of MgATP for

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**Fig. 2.** CD spectra of yeast phosphoglycerate kinase. For experimental details see “Methods.” Curve A, spectra of native enzyme; Curve B, spectra of nitrated enzyme (same curve obtained with native enzyme in the presence of 3-phosphoglycerate).

**Fig. 3.** Residual activity of yeast phosphoglycerate kinase versus moles of lysine modified per mol of enzyme by 2-methoxy-5-nitrotropon. Average of six runs. The initial rate of inhibition is extrapolated to zero activity. PGK represents phosphoglycerate kinase.
FIG. 4. Residual activity of yeast phosphoglycerate kinase versus moles of lysine modified per mol of enzyme by pyridoxal phosphate. Average of three runs. The initial rate of inhibition is extrapolated to zero activity.

FIG. 5. Protection of yeast phosphoglycerate kinase activity by substrates against inhibition by 2-methoxy-5-nitrotroponone. Shown are the time courses of inhibition for control (○—○), 100:1 pyridoxal phosphate in 50 mM phosphate buffer, pH 8.5, but with no substrate added (□—□), with 10 mM 3-phosphoglycerate present (■—■), with 10 mM MgITP present (△—△). See text for details.

The determination of the number of lysine residues protected by substrate against modification is complicated by the fact that there are "side reactions" with nonessential lysines and because often under the conditions of the experiment it is not always possible to achieve total protection. Therefore, the results from the determination of the number of lysines modified in the presence of substrate have been plotted as follows: the protection of activity is plotted against the difference in moles of lysine modified or the protection of lysine residues by substrate. Protection of activity, P, is defined as $P = 1 - \frac{I_p}{I_u}$ where $I_p$ = the inhibition in the presence of substrate and $I_u$ = the inhibition in the absence of substrate, where the activity of a control sample is defined as 0% inhibition. Such a plot is given in Fig. 7, A and B for 2-methoxy-5-nitrotroponone modification using MgITP and phosphoglyceric acid as protecting substrates and shows that three lysines are protected by MgITP and one lysine by phosphoglyceric acid. Fig. 8 shows a similar set of experiments for pyridoxal phosphate modification, which indicates that MgITP protects only two of the lysines that react with pyridoxal phosphate. Phosphoglyceric acid does not protect phosphoglycerate kinase activity from pyridoxal phosphate inhibition.

If there are indeed several essential lysines which have nearly equal reactivity with each other and with several nonessential lysines it would be expected that there are multiple lysines modified even when only 1.3 mol of lysine per mol of enzyme are nitrotroponylated. To check this, peptide mapping experiments were undertaken. The tryptic digest of maleylated nitrotroponyl-enzyme gave one major yellow peak on Sephadex G-75 gel filtration chromatography. This peak contained a number of peptides as is seen in Fig. 9. Prior to ninhydrin staining there are three clearly defined yellow spots represented by hatching. The modified peptides were eluted in subsequent experiments, hydrolyzed, and subjected to amino acid analysis. The yellow spot with the greatest electrophoretic and the least chromatographic mobility seems to be a peptide of 17 amino acids of the following composition: Lys3, Asn2, Ser4, Gly6, Ala3, Val0, Phe, Arg, containing one modified lysine.

Acrylamide gel electrophoresis of 2-methoxy-5-nitrotroponone-modified phosphoglycerate kinase shows that the product is not homogeneous but is a mixture of two proteins, unmodified and modified; the modified enzyme appears to be mononitrotroponylated. A more complex pattern is obtained for the pyridoxal phosphate-modified phosphoglycerate kinase for which the in-
Fig. 7. Plot of protection of activity versus the protection of moles of lysine modified per mol of yeast phosphoglycerate kinase by 2-methoxy-5-nitrotroponone when protection is afforded by 3-phosphoglycerate (A) and by MgITP (B). Data are averages from at least two runs. Plots are extrapolated to full protection to determine the number of lysines protected by substrate. See text for definition of $P$. These experiments were conducted in 0.1 M Tris-NO$_3$ buffer, pH 8.5, with 2-methoxy-5-nitrotroponone to protein ratios varying from 50:1 to 100:1.

dividual bands seen on electrophoresis have been isolated by electrofocusing and characterized. It appears that the difference in mobility for each fraction is due to the difference in the amount of pyridoxal phosphate incorporated (see Fig. 10 and Table II). It also can be seen that the per cent of specific activity in each fraction decreases systematically as the number of lysines modified increase. Each fraction was subjected to acrylamide gel electrophoresis and each ran as a homogeneous band corresponding to the bands observed in the original electrophoresis pattern. The modified proteins were also subjected to SDS gel electrophoreses. For all of the modified species a single band was observed that was indistinguishable from that of the native protein.

The molecular weights of the 2-methoxy-5-nitrotroponone- and the pyridoxal phosphate-modified proteins were determined by sedimentation equilibrium and found to be, within experimental error, the same as the unmodified protein, consistent with the SDS gel electrophoresis results.

The $[m']_{233}$ values obtained for native and each of the modified proteins were determined and it is observed that within the limits of experimental error guanidination does not cause a change in the $[m']_{233}$ whereas the larger reagents 2-methoxy-5-nitrotroponone and pyridoxal phosphate, do cause a change slightly greater than experimental error, suggesting that there may be a slight concomitant conformational change.

The suggestion of a slight conformational change based on the optical rotatory dispersion results for the nitrotroponylated phosphoglycerate kinase is confirmed by spectrofluorescence measurements, as is the absence of any detectable change upon guanidination. There is no change in the emission spectrum of the guanidinated phosphoglycerate kinase even when up to 36 lysines have been modified. For the nitroponylated protein, on the

Fig. 8. Plot of $(1 - P)$ versus the protection of moles of lysine modified per mol of yeast phosphoglycerate kinase by pyridoxal phosphate when protection is afforded by MgITP. Data represent average of two runs. See text for definition of $P$. Fig. 9. Peptide map of 2-ml aliquots of the pooled fractions from the main 420-nm absorbing peak from the Sephadex G-75 column. Electrophoresis at 1700 volts for 83 min at pH 1.9 and 37° was in the horizontal dimension and chromatography (butanol-pyridine acetic acid-water) for 9 hours at 25° was in the vertical dimension. Hatching indicates peptides that were yellow prior to ninhydrin staining.
Fig. 10. Elution profile from the electrofocusing fractionation of a pyridoxal phosphate reaction mixture which had, over-all, 0.9 mol of lysine modified per mol of yeast phosphoglycerate kinase. The separation required 72 hours with a maximum voltage of 500 volts. Each tube contained 4 ml; pH in each fraction (O-O); absorbance at 280 nm, $A_{280} (A-A-A)$, and total activity, OD/min, (●●●) are given for each tube. The heavy bars indicate the fraction pooled for further analysis.

Table II
Characterization of fractions obtained by electrofocusing on reaction mixture of yeast phosphoglycerate kinase modified by pyridoxal phosphate

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Mol lysine modified/mol enzyme</th>
<th>Residual activity</th>
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<tbody>
<tr>
<td>I</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>II</td>
<td>1.0 ± 0.1</td>
<td>60.5</td>
</tr>
<tr>
<td>III</td>
<td>2.2 ± 0.2</td>
<td>33.7</td>
</tr>
<tr>
<td>IV</td>
<td>3.4 ± 0.3</td>
<td>5.3</td>
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</table>

other hand, there is a shift of 3 to 4 nm of the tryptophan emission spectrum indicating a change in the environment of one or both of the tryptophans that could be due either to a conformational change or to a nitropropanol moiety protecting tryptophan from solvent. Circular dichroism experiments also indicate a slight change in the conformation near aromatic residues.

Digestion of denatured phosphoglycerate kinase with combinations of carboxypeptidases A or B or both indicated that the COOH-terminal residue is lysine and that alanine is probably the penultimate residue. This COOH-terminal lysine is also removed by carboxypeptidase B for native enzyme.

**DISCUSSION**

Reagents specific for sulphydryl groups, even at high concentrations have little effect on the activity of yeast phosphoglycerate kinase. This is in marked contrast to the muscle enzyme, which has been shown to be sensitive to sulphydryl reagents (31). When the yeast enzyme is denatured, DTNB reacts rapidly and stoichiometrically with one sulphydryl group, however, after dialysis, the modified enzyme regains approximately 70% activity comparable with the regain of activity observed for the urea or guanidine-HCl exposed control enzyme with intact sulphydryl group. Thus, it appears that cysteine in yeast phosphoglycerate kinase does not play an essential role in catalysis, binding of substrate, or conformation of the enzyme. (A recent report (32) substantiates these findings.) On the basis of mild hydrogen peroxide oxidation of yeast phosphoglycerate kinase, which converted the 3 methionine residues to methionine sulfone without loss in activity, the same argument may be made for the methionine residues as has already been proposed for the single thiol group, namely that they do not play an essential role in catalysis by the enzyme.

Tetranitromethane was found to be a potent inhibitor of yeast phosphoglycerate kinase. It is known that tetranitromethane is not specific for tyrosine residues (33), however, amino acid analysis excluded the possibility of residues other than tyrosine being modified by tetranitromethane in yeast phosphoglycerate kinase. Although there is evidence of oxidation of the single sulphydryl residue by tetranitromethane, our inhibitor studies rule out a role for cysteine in the active site. Nitration at low molar ratios of tetranitromethane to protein (10 fold m excess) results in the formation of only 1 residue of nitrotyrosine with the concomitant loss of approximately 85% of the enzyme activity. Furthermore, recovery of tyrosine plus nitrotyrosine is almost quantitative under these conditions. Since substrate provided partial protection, it may be assumed that the single tyrosine residue nitrated under these conditions is involved in the binding or catalytic site of phosphoglycerate kinase. Nitration at higher levels of tetranitromethane to protein results in the loss of approximately 30% of tyrosine plus nitrotyrosine as indicated by amino acid analysis, presumably due to tyrosine condensation resulting in cross-linking and aggregation as has been found with other proteins (34, 35). This supposition was confirmed by sedimentation equilibrium analysis (high molecular weight aggregates) as well as by fractionation on Sephadex G-100 (high molecular weight peak) of phosphoglycerate kinase nitrated under these conditions.

It is of interest to note that arginine kinase (36) was inactivated by nitration with essentially complete loss of activity on reaction of a single tyrosine residue although maximally 4 residues of tyrosine were available. It is interesting to speculate that a single tyrosine in yeast phosphoglycerate kinase may be reacting similarly to that in arginine kinase. However, in the case of arginine kinase there was a distinct conformational change upon nitration of the single tyrosine residue whereas in yeast phosphoglycerate kinase no conformational change could be demonstrated by means of circular dichroism. There were, however, subtle but definite changes in the aromatic amino acid region of the CD spectra of the yeast enzyme after nitration or upon the addition of substrate suggesting the involvement of an aromatic residue(s) (presumably tyrosine) in the binding site of the enzyme. This interpretation is in agreement with a similar proposal made by Roustan et al. (37) in a study of the binding sites of yeast phosphoglycerate kinase on the basis of differential ultraviolet absorption spectra.

Lysine modification experiments demonstrate that there are at most three essential lysines which function in binding the phosphorylated substrates of yeast phosphoglycerate kinase. The modifying reagents used vary in their rate of reaction and in their selectivity. An analysis of the kinetics of inhibition for the 2-methoxy-5-nitro trope and the pyridoxal phosphate reactions shows in both cases that an enzyme-inhibitor complex forms before the reaction, which is consistent with the greater selectivity of these reagents over O-methylisourea. Similar results have been obtained for pyridoxal phosphate inhibition of glutamate
dehydrogenase (29), phosphoglucone isomerase (38), and phosphorylase (39) and for the Woodward’s reagent K reaction with carboxypeptidase A (28).

The pattern of substrate protection is somewhat complex. For the 2-methoxy-5-nitrotropane reaction it appears that three lysines are protected by MgITP and only one by 3-phosphoglycerate. At this point it cannot be ascertained whether the lysine protected by 3-phosphoglycerate is also one of the three protected by MgITP. The pyridoxal phosphate results indicate that 3-phosphoglycerate affords little if any protection against inhibition thus pyridoxal phosphate appears not to react with the lysine that helps bind 3-phosphoglycerate. In contrast, MgITP protects only 2 residues. This may indicate that pyridoxal phosphate does not react covalently with all of the critical lysines; possibly a third lysine can be tied up by electrostatic interaction with the phosphate on one of the pyridoxal phosphates covalently bound. These interactions plus steric crowding may account for the low overall levels of pyridoxal phosphate incorporation actually observed, in contrast to 2-methoxy-5-nitrotropane which has steric bulk only and O-methylisourea which introduces a minimal steric crowding. This may also explain why only O-methylisourea of the three reagents was capable of producing 100% inhibition. It is possible that the basic lysine residues are positioned such as to act as a “charge cluster” for binding the phosphorylated substrates.

Because there was an incomplete physicochemical characterization of yeast phosphoglycerate kinase when our work was initiated, we felt that it would be of interest to determine some of the physicochemical properties of both native and chemically modified enzymes. These data would be useful in establishing conformations of the enzyme as determined by sedimentation equilibrium analysis is 46,800. Furthermore, after denaturation, sedimentation equilibrium analysis gave a molecular weight of 44,500 indicating that the enzyme is composed of a single polypeptide chain. Sedimentation velocity and diffusion studies indicate a molecular weight of 49,100 for the native enzyme. This was substantiated by SDS gel electrophoresis which gave a molecular weight of 47,800. The amino acid composition of the yeast enzyme presented in Table I is in good agreement with earlier reports (3, 5) and gave a molecular weight of 44,500.

Our chemical modification and physical characterization studies indicate that for yeast phosphoglycerate kinase cysteine and methionine are not essential for the action of the enzyme. In contrast, it appears that one tyrosine and up to three lysines are essential. These residues are not involved in maintaining the structural integrity of phosphoglycerate kinase but seem to function in substrate binding.

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**SPECIAL MATERIAL**

**Chemical Modification of Yeast 3-Phosphoglycerate Kinase**


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**Figure 1:**
Time course of inhibition of yeast 3-phosphoglycerate kinase by D-methylthioimid. Average of three runs. See text for details.

**Figure 2:**
Time course of inhibition of yeast phosphoglycerate kinase by 2-mercapto-1,3-propanediol. Average of three runs. See text for details.

**Figure 3:**
Time course of inhibition of yeast phosphoglycerate kinase by peridol phosphate at a molar ratio of 20:1 (3.2 x 10^{-6} peridol phosphate) and 100:1 (8.8 x 10^{-6} peridol phosphate). Average of three runs. See text for details.
Chemical modification of yeast 3-phosphoglycerate kinase.
F S Markland, A D Bacharach, B H Weber, T C O'Grady, G C Saunders and N Umemura


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