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# SPECTROPHOTOMETRIC pH TITRATIONS AND NITRATION WITH TETRANITROMETHANE OF THE TYROSYL RESIDUES IN YEAST PHOSPHOGLYCERATE KINASE

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## Summary

Spectrophotometric pH titrations of phosphoglycerate kinase (EC 2.7.2.3) reveal seven tyrosyl residues. In the native state one tyrosyl residue has $pK_{app}$ equal to 9.3, another has $pK_{app}$ of about 12.9, and five have $pK_{app}$ values close to 11.0. Titration above pH 10 causes concomitant reduction of the catalytic activity. Reactivation of the enzyme occurs during storage at pH 7.8. In 6 M guanidine · HCl seven tyrosyl residues with $pK_{app}$ values equal to 10.0 appear.

Nitration of three tyrosyl residues occurs easily when tetranitromethane is used in excess. Four tyrosyl residues appear to be masked or buried.

The tyrosyl residue having $pK_{app}$ equal to 9.3 can be selectively nitrated. Simultaneously the enzyme loses 40% of its catalytic activity. No change in the $K_m$ value for one or the other of the two substrates, MgATP or 3-phospho-D-glycerate, was observed in the mononitrated enzyme. On the other hand MgATP protects the tyrosyl residue from nitration whereas 3-phospho-D-glycerate at corresponding condition appears harmless. These results suggest the low ionizing tyrosyl residue to be situated close to the binding site of MgATP, possibly in a pocket just behind. Circular dichroism measurements indicated that minor successive changes occur in the secondary structure, mainly the $\beta$-structure, when the enzyme is being nitrated. It is reasonable to think that these structural changes, possible in combination with steric hindrance, are responsible for the decrease in catalytic activity.

Dimerization of the enzyme occurs if the single thiol group is not masked before the tetranitromethane treatment.

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Abbreviations: GdmCl, guanidine · HCl; 3-*P*-glycerate, 3-phospho-D-glycerate.

## Introduction

In our studies on the structure and function relationships of yeast phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) the tyrosyl residues were selected the second of interest after the single cysteinyl residue (cf. refs. 1—3). Recent reports by others show that iodination [4] or modification with tetranitromethane [5] of the tyrosyl residues affects the catalytic activity of phosphoglycerate kinase. Our results have been presented earlier [6] in a preliminary form.

## Materials and Methods

*Reagents.* Phosphoglycerate kinase was prepared from baker's yeast [7] and component 2 was used. A molecular weight of 45 000 and a molar absorptivity of 22 500 $M^{-1} \cdot cm^{-1}$ were used [8,1]. The catalytic activity was assayed at condition described earlier [8,9,1]. All chemicals were of analytical grade. Only glass-distilled water was used.

*Spectrophotometric titrations.* A Zeiss PMQ II and a Gilford 240 spectrophotometer were used. Phosphoglycerate kinase was added to the different buffers at 25°C to get a final enzyme concentration of 1.2—1.5 mg/ml. pH was measured on a Radiometer 25 pH meter before and after the addition. Corrections were made for high $Na^+$ and $K^+$ concentrations. The molar absorptivity change at 295 nm (cf. ref. 10) when one tyrosyl residue ionizes was taken as 2400 $M^{-1} \cdot cm^{-1}$ [11] for the native enzyme, and as 2450 $M^{-1} \cdot cm^{-1}$ [12] in 6 M guanidine · HCl (GdmCl). The following buffer systems were used: Tris · HCl (pH 7.4—8.6); glycine-OH (pH 8.6—10.4); lysine-OH (pH 10.5—11.2); and NaOH (pH 11.2—13.8). The ionic strength was adjusted with KCl to 0.1, except above pH 12.8 when the ionic strength was increased to 1.25.

*Chemical modifications.* Modification of the single thiol group was carried out in 0.05 M phosphate buffer (pH 8.0) at 25°C with a molar ratio of *p*-hydroxymercuribenzoate to enzyme equal to two (cf. ref. 3). Excess of *p*-hydroxymercuribenzoate was removed by dialysis.

Nitrations were performed in the same phosphate buffer using enzyme concentrations of 8—10 mg/ml. Tetranitromethane in 96% ethanol was added to the protein solution. Aliquots were repeatedly withdrawn and were passed through a Sephadex G-25 column (1 × 20 cm) before the final determinations of the 3-nitrotyrosine content. Simultaneously aliquots were withdrawn for tests of the catalytic activity. The following absorbance coefficients at pH 8.0 have been used to estimate the extents of nitration: $\epsilon_{275}$(3-nitrotyrosine) 4000 $M^{-1} \cdot cm^{-1}$ [13]; $\epsilon_{428}$(3-nitrotyrosine) 4100 $M^{-1} \cdot cm^{-1}$ [13]; $\epsilon_{275}$(tyrosine) 1360 $M^{-1} \cdot cm^{-1}$ [13]; and $\epsilon_{275}$(tryptophan) 5431 $M^{-1} \cdot cm^{-1}$ [14]. The following empiric equations were found valid for estimation of the extent of nitration:

$$Abs_{275} = Y[2\epsilon_{trp} + X\epsilon_{3\text{-nitrotyr}} + (7 - X)\epsilon_{tyr} + 2068]$$

$$Abs_{428} = Y[X\epsilon_{3\text{-nitrotyr}} + 1006]$$

$X$ is the number of nitrotyrosines per enzyme molecule, $Y$ the protein concen-

tration and $\epsilon$ the molar absorptivity. The above equations give

$$X = \frac{(\text{Abs}_{428} \times 22\,450) - (\text{Abs}_{275} \times 1006)}{(\text{Abs}_{275} \times 4100) - (\text{Abs}_{428} \times 2640)}$$

This equation showed excellent agreements with the corresponding values obtained by amino acid analyses. Both methods have in parallel been used in this work. The amino acid analyses were performed according to Sokolovsky et al. [13].

*Fingerprinting.* Tryptic digestion and high voltage paper electrophoresis were performed as described earlier [7]. Chromatography was carried out for 16 h in *n*-butanol/acetic acid/water (4 : 1 : 5, v/v). Peptides containing 3-nitrotyrosine were identified according to Cuatrecasas et al. [15]. The other peptides were stained using the cadmium-ninhydrin method [16].

*Molecular weight determinations.* Gel filtrations were carried out on a Sephadex G-100 column (dimensions: 2.5 × 100 cm). The protein markers were fungal laccase (EC 1.10.3.2), native phosphoglycerate kinase, and human carbonic anhydrase B (EC 4.2.1.1).

*Circular dichroism.* The same CD equipment and mean residue weight were used as earlier [7]. CD measurements between 300 and 250 nm were performed in a 1 cm quartz cell with enzyme concentration of about 3 mg/ml. Below 250 nm a 1 mm quartz cell and enzyme concentrations of about 0.5 mg/ml were utilized. A 0.05 M phosphate buffer (pH 8.0) was used.

## Results

*Spectrophotometric titrations*

Difference spectra of phosphoglycerate kinase in buffers of pH 8.0 and 10.0 show absorption maxima at 245 and 295 nm. When the absorbance of the enzyme with increasing pH values is followed at 295 nm (Fig. 1) or 245 nm three distinct stages are observed, and the entire change in absorbance corresponds to ionization of seven tyrosyl residues. The first stage shows titration of a tyrosyl residue with an $pK_{app}$ of 9.3. The increase in absorbance occurs instantaneously with the change in pH. In the next stage ionization of five tyrosyl residues appears. The $pK_{app}$ of those was estimated to 11.0. The increase in absorbance in this second stage is time dependent and the experimental absorbance values given in Fig. 1 for pH values above 10 are obtained by extrapolation to zero time. The last tyrosyl residue appears to have an $pK_{app}$ of about 12.9.

To test whether storation of phosphoglycerate kinase at alkaline pH values does affect the enzymatic activity samples were taken exactly 1 min after the enzyme was applied to a selected pH and were then applied to 50 mM Tris · HCl buffers (pH 7.8). Aliquots were withdrawn at different times for activity measurements. Extrapolation to zero time gave the activity of phosphoglycerate kinase after incubation at the selected pH for 1 min. These results are presented in Fig. 1, which shows that the catalytic activity is unaffected during titration of the most easily ionized tyrosyl residue but does successively approach zero when the remaining six tyrosyl residues are being deprotonated. Renaturation of phosphoglycerate kinase was shown to occur at pH 7.8. After

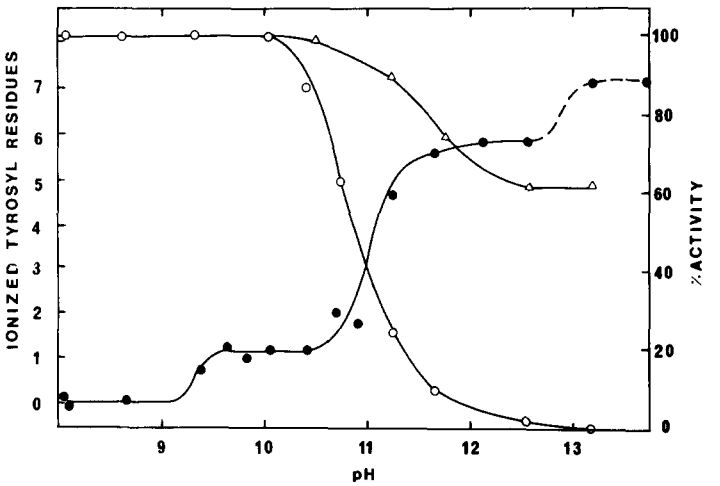


Fig. 1. Spectrophotometric pH titration of the tyrosyl residues in phosphoglycerate kinase identified at 295 nm. Number of ionized residues per enzyme molecule (●); and by enzymatic activity 1 min after addition of the enzyme to a selected pH (○), and after renaturation of the titrated enzyme in 50 mM Tris · HCl buffer (pH 7.8) for 24 h (△). The dashed curve represents a part of the experiment performed at elevated ionic strength (see Materials and Methods).

24 h at this pH 60% of the original activity was regained in samples that earlier appeared completely inactive (Fig. 1).

Phosphoglycerate kinase denaturated in 6 M GdmCl gives a titration curve corresponding to seven tyrosyl residues all having $pK_{app}$ values close to 10.0.

*Nitration with tetranitromethane*

*Selective nitration of three tyrosyl residues.* To avoid dimer formation (cf. below) the thiol group in phosphoglycerate kinase was modified by *p*-hydroxymercuribenzoate treatment prior to the nitration. At a 16-fold molar excess of tetranitromethane three tyrosyl residues easily react. Simultaneously a 50% reduction of the enzymatic activity occurs. No 3,5-dinitrotyrosine was detected by amino analysis. Fingerprint technique utilized on the modified enzyme (cf. Materials and Methods) showed three yellow spots after exposure to ammonia vapour. The cadmium-ninhydrin staining method gave indications for a good tryptic digestion (cf. ref. 7).

As a maximum value by an average 3.6 tyrosyl residues were found to react when tetranitromethane was used in a very large excess (Fig. 2). In the initial phase three tyrosyl residues react in parallel with a 50% reduction of the catalytic activity. A slow nitration follows and the activity concomitantly drops to 15%.

*Selective nitration of one tyrosyl residue.* A 4-fold molar excess of tetranitromethane causes nitration of one specific tyrosyl residue in phosphoglycerate kinase (Fig. 3). The modified enzyme loses 40% of its catalytic activity. Fingerprinting technique showed one 3-nitrotyrosine containing spot which consisted of only one peptide.

Spectrophotometric pH titration of the mononitrated enzyme at 295 nm followed above pH 10.5 the pattern seen in Fig. 1 for the native enzyme, corre-

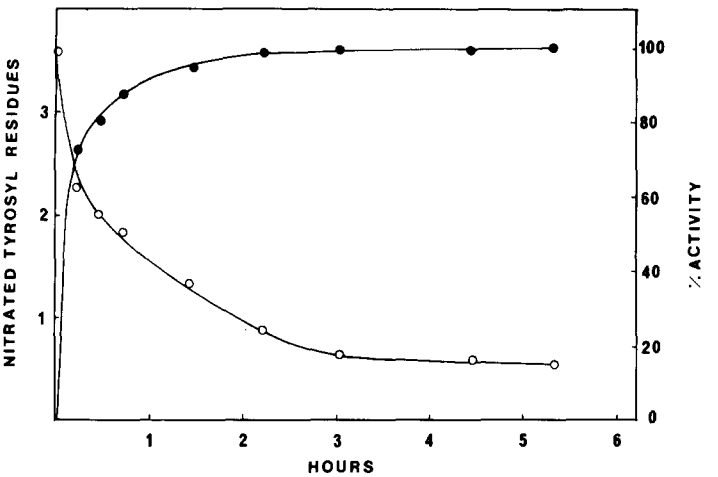


Fig. 2. Nitration of the tyrosyl residues in *p*-hydroxymercuribenzoate-treated phosphoglycerate kinase using 100-fold molar excess of tetranitromethane. Number of nitrated tyrosyl residues per enzyme molecule (●); the catalytic activity (○).

sponding to the ionization of six tyrosyl residues. On the other hand while the tyrosyl residue with an $pK_{app}$ of 9.3 is in Fig. 1 represented by a change in absorbance corresponding to 2400 $M^{-1} \cdot cm^{-1}$ the nitrated enzyme shows a change in the molar absorptivity of 340 $M^{-1} \cdot cm^{-1}$ when pH increases from 8.0 to 10.0. This value is in excellent agreement with the change in the molar absorptivity for pure 3-nitrotyrosine, which separately was determined to 340 $M^{-1} \cdot cm^{-1}$ at corresponding conditions. Evidently the tyrosyl residue having the $pK_{app}$ of 9.3 is the most reactive one towards tetranitromethane.

Fig. 4 presents nitration of phosphoglycerate kinase using a 4-fold molar excess of tetranitromethane in the presence of 11 mM 3-*P*-glycerate or 7 mM

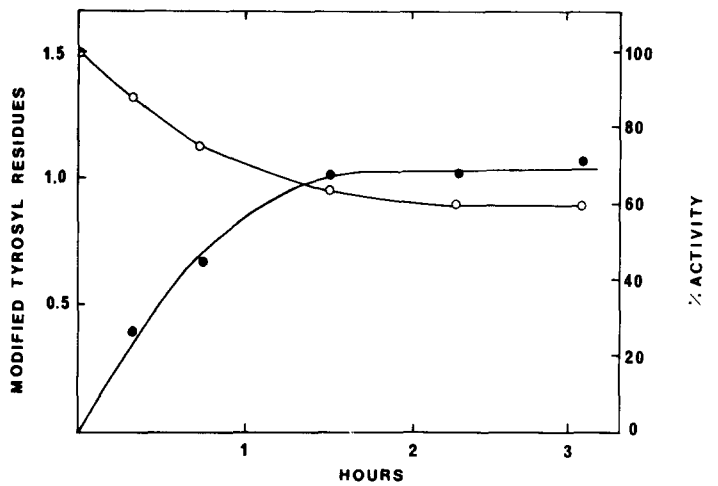


Fig. 3. Nitration of the tyrosyl residues in *p*-hydroxymercuribenzoate-treated phosphoglycerate kinase using 4-fold molar excess of tetranitromethane. Number of nitrated tyrosyl residues per enzyme molecule (●); the catalytic activity (○).

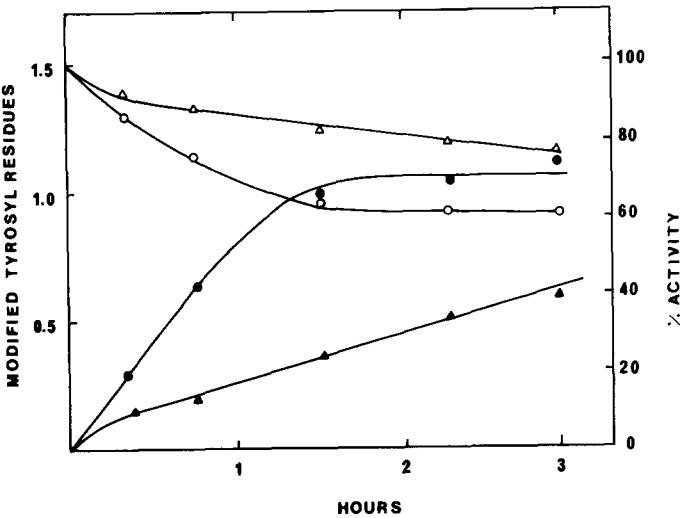


Fig. 4. Effects of 3-*P*-glycerate or MgATP on the nitration of phosphoglycerate kinase at the experimental conditions described in Fig. 3. Number of nitrated tyrosyl residues per enzyme molecule in the presence of 11 mM 3-*P*-glycerate (●); and 7 mM MgATP (▲). The catalytic activity of the enzyme at corresponding conditions of nitration, 11 mM 3-*P*-glycerate (○), and 7 mM MgATP (△).

MgATP. 3-*P*-glycerate appears neither to affect the nitration nor the concomitant decrease in the catalytic activity. On the other hand MgATP delays the nitration and the parallelly occurring decrement of the enzymatic activity. The fingerprint technique, showed that the same tyrosine is nitrated in the presence as in the absence of MgATP or 3-*P*-glycerate.

*Kinetics and conformation of the nitrated enzyme*

Substrate kinetics on both native and mononitrated enzyme at 10 mM $Mg^{2+}$

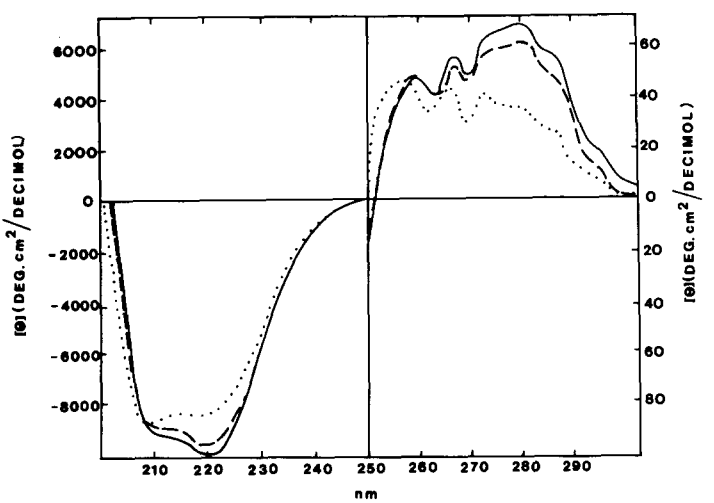


Fig. 5. Circular dichroism spectra of *p*-hydroxymercuribenzoate-treated phosphoglycerate kinase having none (———), one (- - - - -), or three (· · · · · ·) tyrosyl residues nitrated.

resulted in biphased Lineweaver-Burk plots, which are typical for phosphoglycerate kinase (cf. ref. 17). The intersections with the abscissa axis and thus also the $K_m$ values for both MgATP and 3-*P*-glycerate were unaffected by the nitration.

CD measurements were performed on *p*-hydroxymercuribenzoate-labelled phosphoglycerate kinase, which had none, one or three tyrosyl residues nitrated. The results are presented in Fig. 5. The spectra in the 200—250 nm region suggest the nitration procedures to cause minor changes in the secondary structure. The values at 208 nm are the same in all three cases, giving evidence for the spectral changes mainly to reflect changes in the β-structure (cf. ref. 18).

If the native enzyme was treated with a 16-fold molar excess of tetranitromethane three tyrosyl residues were nitrated as above. In addition the SH group became unavailable for reaction with *p*-hydroxymercuribenzoate, and gel filtration on a Sephadex G-100 column showed a protein component with double the molecular weight of the native enzyme, thus about 100 000. Cleland's reagent (2,3-dihydroxy-1,4-dithiobutane) had no apparent effect on this large molecule.

## Discussion

Kinetic studies performed on phosphoglycerate kinase in native or mononitrated form indicate that both forms of the enzyme have the same affinity for MgATP and 3-*P*-glycerate, respectively, giving evidence for nitration to affect the maximal initial velocity only and not the binding of the substrates. MgATP delays the nitration of the most reactive tyrosyl residue suggesting this residue as being located in a pocket behind the nucleotide binding site (cf. ref. 19). Others [5] have reported that MgATP protects the tyrosyl residues of phosphoglycerate kinase from being nitrated. It is, however, evident from, for example, the fingerprint analysis of those authors that more than one tyrosyl residue react at the conditions used. As a complement to our results one may note that a very high 3-*P*-glycerate concentration (0.32 M) also was reported [5] to protect tyrosyl residues from being nitrated by tetranitromethane. As we mainly are interested in the structure and function relationships of the enzyme, and thus want to avoid non-specific substrate binding in our studies on substrate protection of a specific amino acid residue, we have not used substrate concentrations much higher than about 10 times the Michaelis constant. At those conditions the rate of the reaction was not affected by 3-*P*-glycerate (Fig. 4).

Evidence for changes in the β-structure of the enzyme is presented in Fig. 5, to occur in parallel with the modifications of the tyrosyl residues. The importance of tyrosyl residues as β-sheet nucleation formers in proteins has earlier been suggested by Chow and Fasman [20]. X-ray crystallographic studies on yeast [21] and on horse muscle [22] phosphoglycerate kinase indicate that the nucleotide binding site of these enzymes is associated with regions of β-structure (cf. ref. 23). Thus it appears that steric shielding of the catalytic groups of the enzyme in combination with conformational changes is responsible for the effects of tetranitromethane modification on the catalytic activity of yeast phosphoglycerate kinase. The most important role of the tyrosyl residues is

probably to maintain the structural integrity of the enzyme and not to be actively involved in the catalytic mechanism or in substrate binding.

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