INTERACTION OF SULFATE ION WITH A CRITICAL TYROSINE RESIDUE IN YEAST PHOSPHOGLYCERATE KINASE DETECTED THROUGH THE TETRANITROMETHANE REACTION

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1. Introduction

There is evidence from the kinetics of enzyme catalysis [1] and from NMR studies [2] that sulfate ion interacts strongly with yeast phosphoglycerate kinase (PGK) (3-phospho-D-glycerate 1-phosphotransferase EC 2.7.2.3). The binding is to specific sites, apparently in the catalytic region of the enzyme, and affects substrate interaction and the conformation of the enzyme. NMR spectra [2] and ultraviolet difference spectra (unpublished results) suggest that a tyrosine residue is very close to the sulfate binding site. Others [3] have shown that there is a critical tyrosine residue at the 3-phosphoglycerate site whose modification also affects ATP binding. Knowledge of a strong interaction of sulfate ion with a residue at the substrate binding site may be a critical factor in interpreting X-ray models of the enzymes which have been obtained from crystals in $(NH_4)_2SO_4$ solution [4].

In the present report we show that 4 mM $(NH_4)_2SO_4$ will completely protect one tyrosine residue of PGK from nitration by tetranitromethane (TNM). We also partially characterize a residual activity refractory to TNM in the absence of sulfate. During the course of this work a substantial investigation of the reaction of TNM with PGK was published [5]. The present report is limited to data which supplement that work.

2. Materials and methods

PGK was obtained from Boehringer-Mannheim Ltd., initially as specially selected batches of high specific activity. The enzyme was found to give a single band on SDS–gel electrophoresis with molecular weight of 47 000 and to give a single peak in the analytical ultracentrifuge. Later work was with ordinary stock enzyme from Boehringer but results were not significantly different. The specific activity was between 560 and 590 at 25°C. Centrifugation of the crystals followed by dialysis was used to remove $(NH_4)_2SO_4$.

The enzyme was assayed at 25°C as previously described [2], including removal of $(NH_4)_2SO_4$ from the coupling enzyme glyceraldehyde-3-phosphate dehydrogenase.

Tetranitromethane was purchased from Sigma Chemical Corporation and diluted 1:10 into ethanol before further dilution into the reaction mixture. Nitration of the enzyme was usually performed at 25°C, in 0.05 M Tris–HCl buffer, pH 8.0 with enzyme concentration between 4 and 40 μM and TNM concentrations from 5–300 times higher. The enzyme concentration was determined by its absorption at 278 nm using an extinction coefficient of 0.5/mg/ml [6]. Production of nitroformate was measured at 350 nm and production of nitrotyrosine was measured at 428 nm using extinction coefficients of 14 000 and 4000 respectively [7,8]. Absorbance of nitrotyrosine at 350 nm was found negligible, but absorbance of nitroformate at 428 nm was significant, equal to 3% of its absorbance at 350 nm. Readings at 428 nm were corrected for nitroformate absorbance and the correction was very significant, amounting to 25–30% of the total 428 nm absorbance.

3. Results

A typical time-course for the inactivation of PGK with TNM is shown in fig.1. The half-time of the reaction shown is about 2.5 min; doubling or halving the TNM concentration changed the rate of reaction proportionately. When the reaction was measured at four temperatures from 0–30°C, the Q_{10} was found to be 2.5 ± 0.2 and the inactivation proceeded to the same extent at all temperatures. It should be noted that there is appreciable activity which is not lost although the molar ratio of TNM to PGK is over 300:1. A control experiment showed that the rate of nitroformate production in the absence of protein was only about 1.5%/10 min under the same conditions. The presence of excess TNM at the end of activity loss was confirmed in other experiments when addition of excess β-mercaptoethanol produced a massive increase in nitroformate concentration.

The effect of as little as 4 mM $(NH_4)_2SO_4$ on the reaction is striking. Figures 2A, B and C show enzyme inactivation, nitrotyrosine production and nitroformate production in the presence and absence of $(NH_4)_2SO_4$. In order to monitor all these changes concurrently, the TNM concentration had to be reduced. In this experiment the TNM is in only a 5-fold molar excess over the enzyme. It can be seen that 4 mM $(NH_4)_2SO_4$ completely protected the enzyme against inactivation and reduced the production of nitrotyrosine and nitroformate. At 35 min, when inactivation has reached its fullest extent, the dotted curve of fig.2B shows that the concentration of nitrotyrosine produced is about 20 μM more in the unprotected sample than in the protected sample. In fig.2C, the dotted curve shows a corresponding difference in nitroformate production of about 43 μM. Two observations may be made about the reaction which occurs only in the absence of $(NH_4)_2SO_4$. First, the production of nitroformate is twice that of nitrotyrosine, indicating that one mole of TNM is reacting with another residue than tyrosine and that this residue is equally protected by sulfate. Second, the ratio of nitrotyrosine produced to PGK present 0.57 (20–35 μM) corresponds closely to the degree of

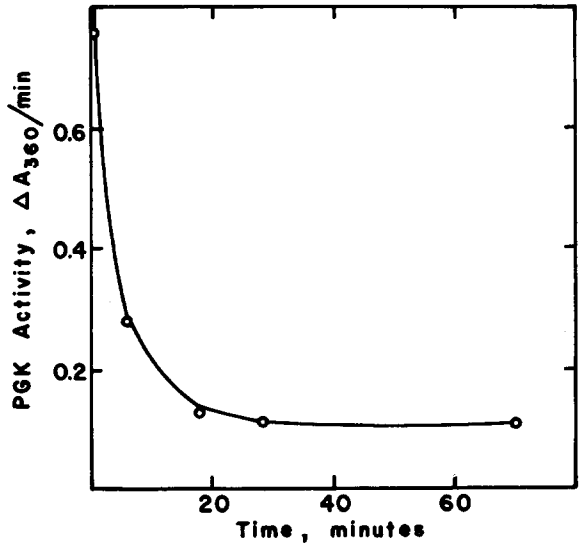
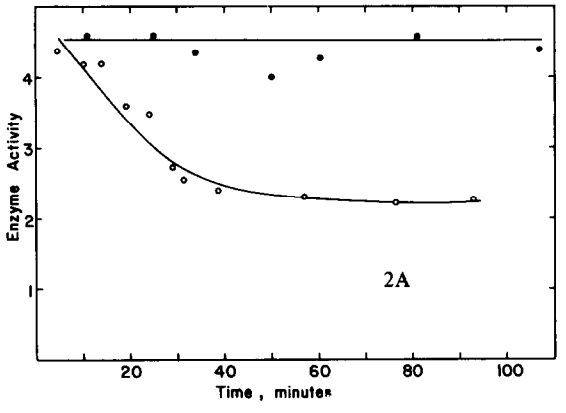
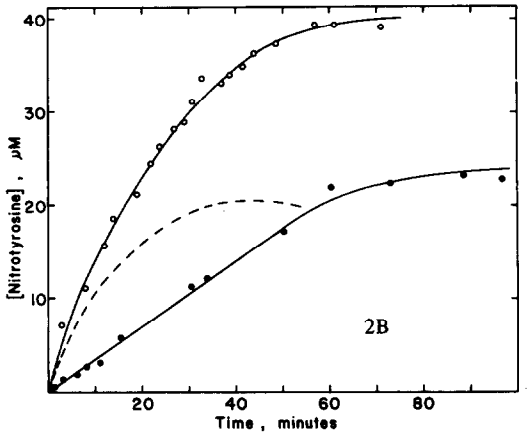


Fig.1. Time course of PGK inactivation by TNM. The reaction was run in 50 mM Tris–HCl buffer pH 8.0, 27°C; 1.35 mM TNM, 4 μM PGK.





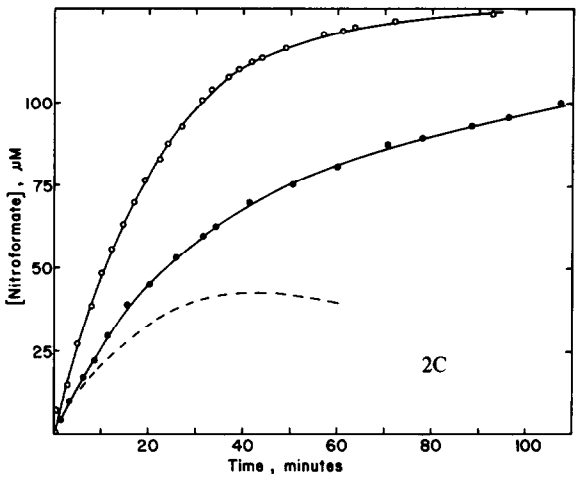


Fig.2. Reaction of TNM with PGK with and without 4.0 mM $(NH_4)_2SO_4$ present. The reaction was in 0.05 M Tris–HCl buffer pH 8.0, 25°C, 0.16 mM TNM, 0.035 mM PGK. To measure catalytic activity a 15 μl aliquot of reaction solution was diluted into 300 μl of ice-cold assay buffer and 15 μl of the diluted solution was added to the standard 2.5 ml assay solution. Nitrotyrosine and nitroformate concentrations were calculated as in Materials and methods from absorbances of the reaction solution at 350 and 428 nm. Open circles, no $(NH_4)_2SO_4$; closed circles, with 4 mM $(NH_4)_2SO_4$, dashed lines in B and C are measured differences between the curves obtained with and without $(NH_4)_2SO_4$.

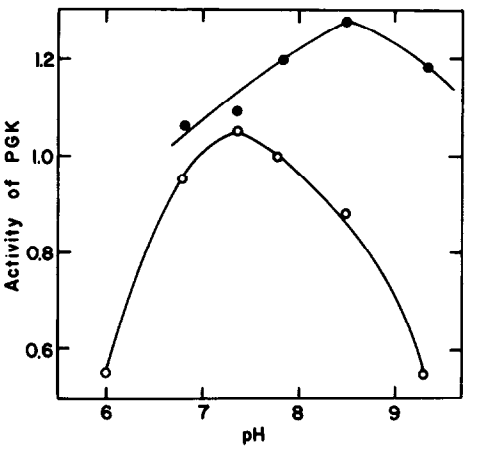


Fig.3. pH profiles of modified and native enzyme. ATP 4 mM, PGA 10 mM, EDTA 0.08 mM, Mg(acetate)$_2$ 4 mM, NADH 0.025 mM, GAPDH 100–500 μg per 2.5 ml assay solution: Buffers were 50 mM Tris–HCl buffer down to pH 7.4 and 50 mM MES buffer below pH 7.4, 30°C. The ordinate is absorbance change per minute. Rates for the modified enzyme are adjusted approximately 5 × higher than obtained directly so that the shapes of the pH profiles can be more easily compared.

inactivation achieved in this particular reaction, 0.55. While the exactness of these ratios may be fortuitous the correspondences suggest possibilities that will be discussed.

In a separate experiment to examine the nature of the residual activity, pH profiles of native and partially inactivated enzymes were obtained and at fixed pH values saturation curves for 3-phosphoglycerate (PGA) or ATP were measured. Figure 3 shows the pH profiles of the native and modified enzymes at substrate concentrations that are saturating at pH 8.0. It can be seen that the pH maximum of the residual activity occurs at a distinctly lower pH and that it appears to be somewhat narrower than that for the native enzyme.

Since substrate saturation curves have been studied in detail [9] our data are not shown but can be summarized as follows. Complete curves for the saturation of modified and unmodified enzyme with PGA were obtained at 4 mM ATP at pH 6.3, 7.8 and 9.2. Saturation curves for both enzymes were obtained with ATP at 10 mM PGA, pH 7.8. In no case was there any apparent change in the K_m parameter. It appeared that only V_{max} or enzyme concentration had been altered.

4. Discussion

The 3.5 Å resolution X-ray structural determination for yeast PGK [4] does not show the substrate binding site in great detail but at the resolution achieved the molecule seems to have the same structural features as horse muscle PGK for which higher resolution results have been obtained [10]. Peculiar features of that structure are that the γ-phosphate of ATP appears to stick out into solution, and that no site for 3-PGA was detected. Iodination of a specific tyrosine in yeast PGK inhibits the enzyme and appears to eliminate 3-PGA binding and weaken ATP binding [3]; since the tyrosine is protected from iodination by 3-PGA it appears likely that it is close to the binding site for the 3-PGA and perhaps to the

binding site for the γ-phosphate of ATP. A previous study with TNM [5] showed also that a critical tyrosine residue could be protected from nitration by substrates. It seems likely that the tyrosine detected by those studies is the same one that we find protected by $(NH_4)_2SO_4$, and therefore the positioning of substrate in crystals of the enzyme in concentrated $(NH_4)_2SO_4$ solution may be significantly different from the catalytically productive state. The results of Larsson-Raznikiewicz [1], in fact, show that although the enzyme is considerably activated by SO_4^{2-} concentration below about 20 mM, it is fully inhibited by concentrations approaching 0.2 M. It has been previously found by Tanswell et al. that 4 mM $(NH_4)_2SO_4$ is enough to cause a major change in the NMR resonances of 2 imidazole groups of PGK [2] and that inhibition of the catalytic activity and changes in the ultraviolet spectrum of the enzyme are readily detected at such low sulfate concentrations (unpublished data). The discrepancy [2] between the X-ray data and the NMR data regarding the configuration of bound ATP might be caused by the differences in sulfate concentration.

The correspondence between the degree of inactivation and the degree of nitration of the protectable tyrosine residue is in complete agreement with other modification studies showing such stoichiometry [3,5]. Our observations that a second protectable residue also reacts with TNM is most likely explained by the fact that there is a single sulfhydryl group on the enzyme which can be modified by a variety of reagents without causing inactivation [11]. However the sulfhydryl group appears to be far from the nucleotide binding site in the 'open' configuration of PGK determined by X-ray crystallography. It is not apparent why this group should be protected by substrate, and it does not appear to be protected from reaction with mercurybenzoate [11]. It may be that under our conditions of low sulfate concentration the cysteine is located very close to the tyrosine and perhaps interacting with it. Certainly the tyrosine in question is in a special state as shown by its high reactivity [3,5] and low pK (unpublished data).

The problem of the residual activity could be explained by one of two very recent observations. The occurrence of variable amounts of very tightly bound 3-PGA has been demonstrated in preparations of yeast PGK [12]. Tightly bound 3-PGA would protect a corresponding fraction of the enzyme from TNM, but a question then remains as to why some molecules of enzyme should demonstrate such very high affinity for one of the substrate molecules. Our pH profiles appear to show quite definitely that the molecules resistant to TNM are a sub-population of the original enzyme molecules present. Another explanation is suggested by the recent discovery of 3 isozymes of the enzyme differing in isoelectric point [13]. It appeared likely that one of these is a form that is loosely membrane-associated. Further work will be needed to solve this problem, but we will not be continuing work on this enzyme beyond the present report.

Acknowledgements

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