AN ESSENTIAL ARGINYL RESIDUE IN PHOSPHOGLYCERATE KINASE FROM YEAST

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1. Introduction

The basic amino acids are known to have important roles in enzyme catalysis. Some of these residues have already been chemically modified in yeast phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3.). The results show that there are at most three essential lysyl residues per enzyme molecule [1] whereas a direct implication of a histidyl residue in the catalytic reaction of phosphoglycerate kinase seems improbable [2]. In this report the arginyl residues have been modified with 2,3-butanedione in borate buffer. This reagent has been introduced to protein studies mainly by Riordan [3] and it has been successfully used to identify essential arginyl residues in several enzymes (cf. [3–13]). Our results suggest an essential arginyl residue in yeast phosphoglycerate kinase.

2. Materials and methods

Phosphoglycerate kinase from yeast was prepared as described previously [14]. A molecular weight of 45 000 and a molar absorptivity of 22 500 $M^{-1}cm^{-1}$ were used [15,16]. The catalytic activity was assayed under conditions described earlier [15,16,17]. All chemicals were of analytical grade. Only glass-distilled water was used.

An aliquot from a freshly prepared and pH adjusted butanedioneborate solution was added to phosphoglycerate kinase in borate buffer to initiate the modification reaction. Borate buffer has to be used due to the participation of borate in the modification reaction [3]. To stop the reaction, samples (25 μl) were withdrawn from the reaction mixture at different times and added to (500 μl) 50 mM Tris-HCl buffer (pH 7.8). One minute later 10 μl was taken for activity measurement. This dilution procedure did not result in reactivation and no change in activity could be seen even if the incubation time in the Tris-HCl buffer was increased to 30 min. Samples for amino acid analysis were passed through a Sephadex G-25 (Fine) column (1×48 cm), equilibrated with 50 mM borate buffer (pH 7.5), at 22°C to remove excess reagent. After the protein had been hydrolyzed in evacuated, sealed ampoules with 6 M HCl at 110°C for 20–22 h the amino acid contents were analysed on a Beckman Unichrom analyser. The degree of modification was followed through losses in the arginine content [3]. Yeast phosphoglycerate kinase has been reported to contain 42 lysines [16] and 8 histidines [16] and using these values as a reference an average of 13 arginines were found.

The proton NMR experiments were performed at 270 MHz using a Bruker spectrometer operating in the Fourier transform mode. All solutions were prepared in ^2H_2O (> 99.8%) and pH was estimated by the addition of 0.4 to the pH meter reading.

3. Results

3.1. *Chemical modification with butanedione in borate buffer*

Phosphoglycerate kinase is rapidly inactivated at different concentrations of butanedione as shown in fig.1. In the concentration range studied the inactivations follow pseudo-first order kinetics with respect

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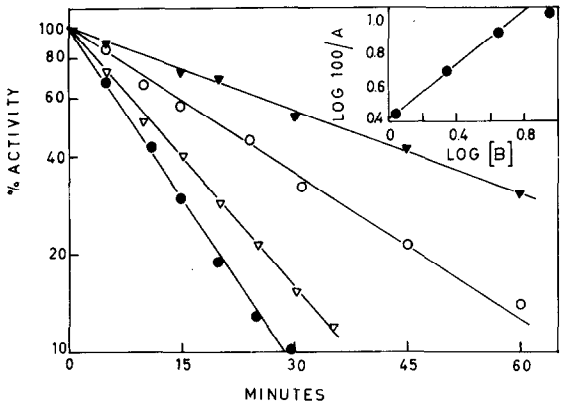

Fig.1. Inactivation of phosphoglycerate kinase with different concentrations of butanedione. Phosphoglycerate kinase (1 mg/ml) was incubated at 25°C in 50 mM borate buffer, (pH 7.5), with the following concentrations of butanedione: (▼) 1.11 mM; (○) 2.22 mM; (▽) 4.44 mM; (●) 8.89 mM. The results are presented as semi-log plots of % residual activity *vs.* time. The activity of a control sample, containing no butanedione, remained constant during the reaction time. Insert: The data of fig.1. are plotted as log 100/A *vs.* log B, where A is the half-time (min) of inactivation and B the butanedione concentration (mM).

to enzyme active sites until 90 to 95% inactivation has occurred, and prolonged incubation results in complete inactivation. Several authors [18–22,12] have used a plot of log $(1/t_{½})$ against log inhibitor concentration, where the reaction order with respect to inhibitor can be determined from the slope of the plot. Such a plot (fig.1 insert) shows a slope of 0.82. However, a deviation from a linear relationship in the plot is observed and this is obvious at 8.9 mM butanedione. The slope at lower concentrations of butanedione, i.e. the initial slope, is closer to 1.0 than to 0.82. These results indicate that not more than one molecule of butanedione per active site of the enzyme is necessary for inactivation. A similar saturation effect as above has been reported with alcohol dehydrogenase [6] at higher concentrations (10 mM) of butanedione.

To test the reversibility of the modification, phosphoglycerate kinase was modified with 4.44 mM butanedione, under conditions described in fig.1, until a residual activity of 10% was obtained. At this time the reaction mixture was passed through a Sephadex G-25 column (1×48 cm), which was equilibrated with 50 mM Tris-HCl buffer (pH 7.5). Incubation of the eluted enzyme at 25°C for 6 to 7 h reactivates phosphoglycerate kinase to 90% of the original activity. However, no reactivation is observed if 50 mM borate buffer (pH 7.5) is used in the gel filtration instead of the Tris-HCl buffer.

3.2. *Amino acid analyses of the modified enzyme*

A correlation of the decrease in activity with the modification of arginyl residues is demonstrated in fig.2. More than two arginyl residues seem to be modified before complete inactivation is obtained. However, extrapolation to zero activity of the initial slope of the plot, which is responsible for the major decrease in activity, shows that the loss of activity is the result of 1.0 modified arginyl residue per enzyme molecule. The ratio of histidine to lysine remained constant throughout these experiments. No detectable change could be observed for any other amino acid.

3.3. *The effect of substrates on the reaction with butanedione*

MgATP, MgADP and 3-phospho-D-glycerate are each effective in slowing down the inactivation reactions as illustrated in fig.3. The degree of protection is in reasonable agreement with the degree of substrate binding if one assumes that the previously reported K_m values [23,24] approximately represent

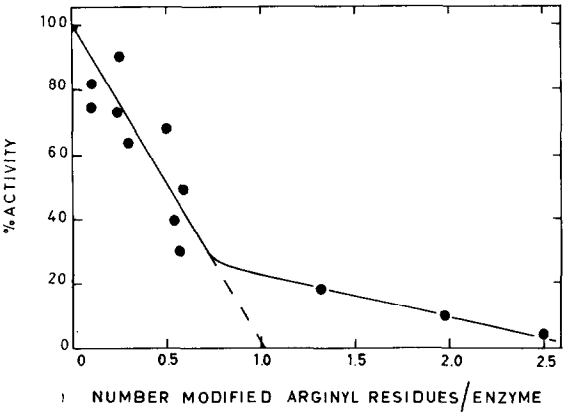

Fig.2. Correlation of the inactivation of phosphoglycerate kinase with the modification of arginyl residues. Samples were withdrawn for amino acid analyses (see Materials and methods) from the reaction of phosphoglycerate kinase with 4.44 mM butanedione (1.11 mM were used occasionally) under conditions described in fig.1. The points represent the average values from two independent runs.

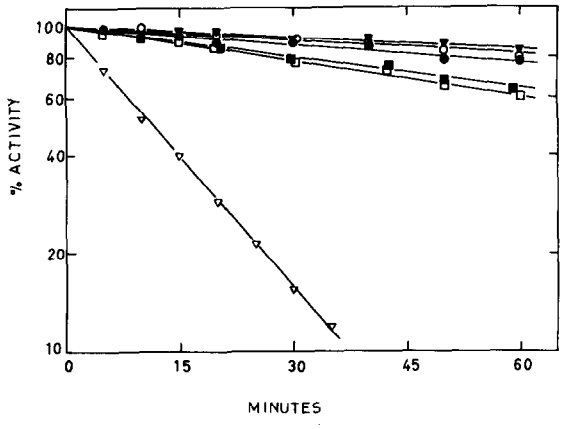

Fig.3. Inactivation of phosphoglycerate kinase with butanedione in the presence of substrates. The enzyme was modified with 4.44 mM butanedione, under conditions described in fig.1, with the following concentrations of substrates: (▽) none; (□) 7 mM MgATP; (○) 21 mM MgATP; (■) 11 mM 3-phospho-D-glycerate; (▼) 33 mM 3-phospho-D-glycerate; (●) 8 mM MgADP. The results are presented as semi-log plots of % residual activity *vs.* time. Some experimental values have been left out of the figure for clarity.

Table 1
Modification of arginyl residues in phosphoglycerate kinase in the presence and absence of substrates

Substrate	Number of arginyl residues per enzyme molecule	
	Modified	Protected
None	1.6 ± 0.30	
21 mM MgATP	0.5 ± 0.14	1.1 ± 0.44
33 mM 3-phospho-D-glycerate	0.3 ± 0.09	1.3 ± 0.39

Phosphoglycerate kinase (1 mg/ml) was modified with 4.44 mM butanedione in 50 mM borate buffer (pH 7.5) at 25°C for 35 min in the presence of different substrates. The sample standard deviations (S.D.) are calculated from three independent runs.

the dissociation constants. A similar protection is found with a dead-end mixture of a substrate and a product. Thus, a solution containing 9 mM ADP, 9 mM Mg and 33 mM 3-phospho-D-glycerate delays the reaction with butanedione to the same extent as 33 mM 3-phospho-D-glycerate does under corresponding conditions. Apparently all of these substrates are capable of protecting an arginyl residue from being modified. This is further demonstrated by a direct comparison of the degree of modification in the presence and absence of substrates. The results are summarized in table 1. Although the standard deviations are rather high it seems clear that at least one arginyl residue less is modified in the presence of either MgATP or 3-phospho-D-glycerate.

To test for a possible interaction of borate with the substrates, NMR spectra at 270 MHz of 21 mM MgATP, 33 mM 3-phospho-D-glycerate and 50 mM NAD^+, respectively, were recorded in both 50 mM borate buffer (pH 7.5) and 50 mM Tris-DCl (pH 7.5). No changes in the spectra of MgATP and 3-phospho-D-glycerate could be detected, implying that the decrease in reaction rate in the presence of substrates does not result from a decrease in the borate concentration. The NAD^+ spectra, on the other hand, showed noticeable differences. Borate is known to interact with NAD^+, preferentially with the ribose adjacent to the nicotinamide moiety [25,26].

4. Discussion

The observation of pseudo-first order kinetics during 90 to 95% of the inactivation reaction, the known selectivity of butanedione and the high degree of reversibility of the modification reaction strongly suggest that no major conformational changes occur parallel with the modification. The reaction order with respect to butanedione suggests that inactivation is the result of one modified arginyl residue per active site of the enzyme. The amino acid analyses show that the loss of activity is correlated with the modification of one arginyl residue per enzyme molecule. Furthermore, MgATP and 3-phospho-D-glycerate can each protect approximately one arginyl residue from modification and simultaneously nearly no activity is lost. The evidence presented in this work strongly support the idea of an essential arginyl residue in phosphoglycerate kinase. However, the exact function of this residue is not known at present. Borders and Riordan [11] have postulated that arginyl residues are generally involved in the binding of ATP by providing a positive charge to interact with the negative oligophosphate moiety of the nucleotide (cf. [16]).

Our results are in accordance with this postulate. However, the fact that 3-phospho-D-glycerate protects to an almost similar degree as MgATP suggests an even further involvement of this essential residue in the catalytic mechanism of phosphoglycerate kinase.

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