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YEAST 3-PHOSPHOGLYCERATE KINASE

ESSENTIAL ARGINYL RESIDUES AT THE 3-PHOSPHOGLYCERATE BINDING SITE

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Summary

Yeast 3-phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) is inactivated by phenylglyoxal. Loss of activity correlates with the modification of two arginyl residues, both of which are protected by all of the substrates. The modification is not accompanied by any significant conformational change as determined by optical rotatory dispersion. Ultraviolet difference spectrophotometry indicates that the inactivated enzyme retains its capacity for binding the nucleotide substrates whereas the spectral perturbation characteristic of 3-phosphoglycerate binding is abolished in the modified enzyme. The data suggest that at least one of the two essential arginyl residues is located at or near the 3-phosphoglycerate binding site. A likely role of this residue could be its interaction with the negatively charged phosphate or carboxylate groups of 3-phosphoglycerate.

Introduction

3-phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) plays an important role in the glycolytic system as it catalyses the first of the two ATP-forming reactions of this pathway. The tertiary structure of the single polypeptide chain of yeast 3-phosphoglycerate kinase has been established from X-ray data at 3.5 Å resolution [1]. The crystallographic data show that the enzyme is composed of two domains connected by a central waist. The nucleotide substrate binds to one of these domains on the inner surface of the deep depression forming the waist [1]. Chemical modification studies have pro-

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vided information on the catalytic properties of the enzyme. There is thus evidence for the involvement of a glutamyl residue in the catalysis [2,3]. It appears furthermore that one tyrosyl and as many as three lysyl residues are functional in substrate binding [4—7], whereas the participation of cysteinyl, methionyl or histidyl residues in the enzymic activity has been ruled improbable [5].

It is generally recognized that arginyl residues play an important role in the functional binding of anionic cofactors and substrates to enzyme active sites [8]. All of the substrates of 3-phosphoglycerate kinase bear negatively charged groups (carboxylate, phosphate), the existence of a positively charged recognition site(s) on this enzyme would therefore seem likely. Indeed it has recently been reported that 3-phosphoglycerate kinase is inactivated by arginine-selective reagents [9,10]. Hjelmgren et al. [9] suggest in a study using butanedione that the enzyme contains an essential arginyl residue. The exact function of this residue can however not be ascertained. Rogers and Weber [10] on the other hand, who also use the reagent butanedione and in addition cyclohexanedione, conclude from protection studies that at least one, possibly two, arginyl of 3-phosphoglycerate kinase are essential for its action on ATP. In the present paper we have examined the effect of the modification of arginyl residues by phenylglyoxal on the catalytic and physicochemical properties of 3-phosphoglycerate kinase. Our results indicate that at least one of the two essential arginyl residues modified by this reagent is situated at or near the 3-phosphoglycerate binding site.

Materials and Methods

Yeast 3-phosphoglycerate kinase was prepared according to the procedure described by Scopes [11]. Its specific activity was about 1300 units at 30°C, pH 7.5. Glyceraldehyde-3-phosphate dehydrogenase from yeast (80 units/mg at 25°C) was obtained from Boehringer Mannheim GmbH. Phenylglyoxal monohydrate was purchased from Aldrich Chemical Co. [7-^{14}C]Phenylglyoxal prepared from [7-^{14}C]acetophenone (ICN Corp.) by selenium oxidation [12] had a specific activity of 0.035 Ci/mol. ATP (sodium salt), ADP (lithium salt), AMP (sodium salt) and 3-phosphoglycerate were from Boehringer Mannheim GmbH, and NADH (sodium salt) was from Calbiochem. Insta-gel was obtained from Packard. All other chemicals were reagent grade.

Protein concentration and enzyme assay

3-Phosphoglycerate kinase concentration was determined by the microbiuret method or in some instances by absorbance measurements at 280 nm using $A^{1\%}_{1cm} = 5.0$ [13].

A molecular weight of 42 000 was used in the calculations [14]. The enzymic activity was determined spectrophotometrically according to the method of Bücher [15] where the phosphoglycerate kinase reaction is coupled to the reaction catalysed by glyceraldehyde-3-phosphate dehydrogenase.

Modification with phenylglyoxal

Modifications with phenylglyoxal were performed at 30°C. The reaction was initiated by adding an aliquot of a freshly prepared stock solution of phenylglyoxal in 35 mM veronal buffer, pH 7.5, to a solution of the enzyme (1—2 mg/

ml) in the same buffer. The final concentration of phenylglyoxal was 2 mM unless otherwise stated.

In samples destined for optical rotatory dispersion studies, differential spectrophotometric or amino acid analysis, the modification reaction was terminated by cooling the reaction mixture to 4°C followed immediately by exhaustive dialysis at 4°C.

In studies on the phenylglyoxal incorporation the enzyme was incubated with [^{14}C]phenylglyoxal as described above. At specified time intervals, two aliquots of the reaction mixture were simultaneously removed and assayed for respectively enzymic activity and incorporation of radioactivity. The modified protein was separated from excess reagent by precipitation with an equal volume of 30% trichloroacetic acid. The collected precipitate was washed 3 times with 15% trichloroacetic acid and dissolved in 10 ml Insta-gel for liquid scintillation counting.

Physical measurements

Ultraviolet difference spectra were obtained with a Cary model 15 spectrophotometer provided with thermostated compartments as previously described [16]. A matched pair of quartz cells of 0.437 cm light path were used. The dissociation constants and the molar absorption of the ligand protein complex were calculated from the variation of the maximal differential absorption at fixed wavelengths versus ligand concentrations according to the methods of refs. 16 and 17.

Optical rotatory dispersion measurements were performed on a FICA type Spectropol I spectropolarimeter using a 1-cm lightpath cell. All experiments were carried out in 0.05 M Tris/acetate buffer (pH 7.5) at room temperature. The protein concentrations were chosen as 0.1—0.2 mg/ml in the ultraviolet and about 2 mg/ml in the visible range. The rotatory parameters (a_0, b_0, λ_c) were calculated according to the procedure of Fasman [18].

Radioactivity was determined by liquid scintillation counting in a Intertechnique spectrometer Model SL 31.

Amino acid analysis

Amino acid analyses were conducted on a Beckman 120 C amino acid analyzer. The samples were hydrolyzed in evacuated, sealed ampoules with 6 M HCl at 110°C for 24 h.

Results

Inactivation of 3-phosphoglycerate kinase by phenylglyoxal

The inactivation of 3-phosphoglycerate kinase (2 mg/ml) with phenylglyoxal (2 mM) in 35 mM veronal buffer pH 7.5 and at 30°C follows pseudo-first-order kinetics with a k_{app} of 0.028 min^{-1}. The activity of control samples remained constant during the time allowed for inactivation. The order of inactivation with respect to reagent concentration was studied. In Fig. 1 is shown the variation of the inactivation rate with the concentration of phenylglyoxal. A plot of log $(1/t_{1/2})$ vs. log [inhibitor] as previously used by several authors (e.g. refs. 9, 19 and 20) results in a straight line with a slope of 1.05 indicating that the reac-

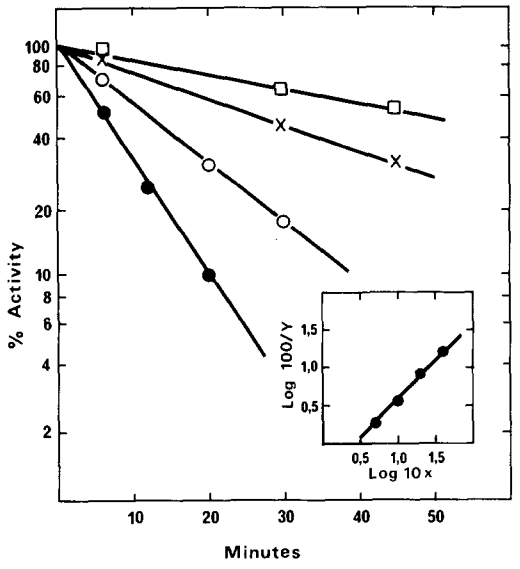

Fig. 1. Kinetics of 3-phosphoglycerate kinase inactivation at various phenylglyoxal concentrations. 3-Phosphoglycerate kinase (24 μM); 35 mM veronal buffer, pH 7.5; temperature, 30°C. The phenylglyoxal concentrations were 0.5 mM (□); 1.0 mM (X); 2.0 mM (○) and 4.0 mM (●). Inset: determination of the order of reaction with respect to phenylglyoxal. Log $10/x$ is plotted against log $100/y$ where x represents the phenylglyoxal concentration (mM) and y the half-time of inactivation (min).

tion is first-order with respect to reagent concentration and that 1 mol of phenylglyoxal per mol of enzyme is sufficient to produce inactivation.

Incorporation of [^{14}C]phenylglyoxal

The incorporation of phenylglyoxal into 3-phosphoglycerate kinase as a function of time was investigated by the aid of ^{14}C-labelled reagent. As show in

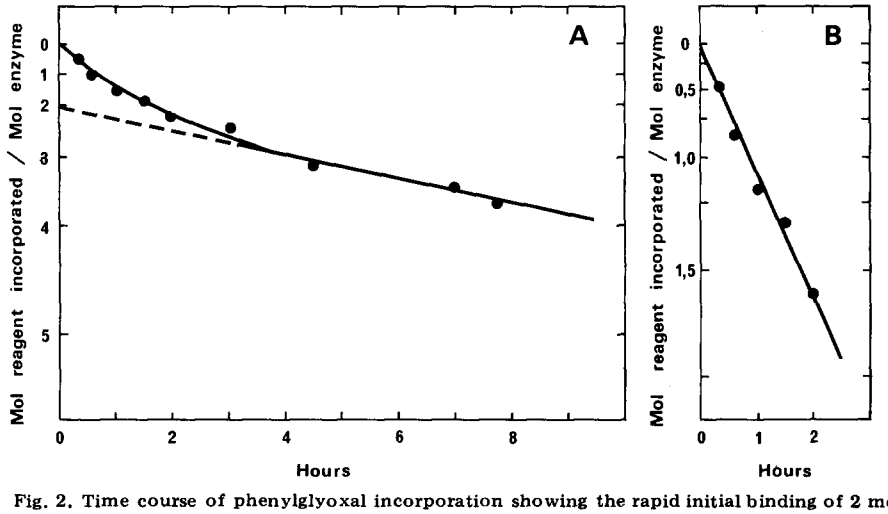

Fig. 2. Time course of phenylglyoxal incorporation showing the rapid initial binding of 2 mol of reagent. 3-Phosphoglycerate kinase (48 μM); phenylglyoxal (2 mM); 35 mM veronal buffer, pH 7.5; temperature, 30°C, (A). Determination of the constant rate of reaction for the first 2 mol of reagent incorporated according to the method of Ray and Koshland [21] (B).

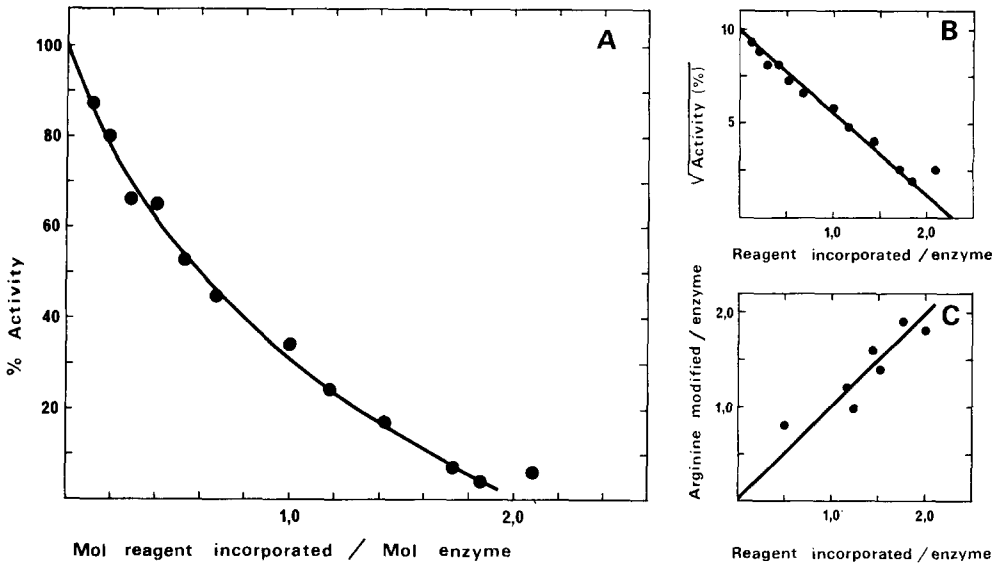

Fig. 3. Correlation of inactivation of 3-phosphoglycerate kinase with the modification of arginyl residues. 3-Phosphoglycerate kinase (48 μM); phenylglyoxal (2 mM); 35 mM veronal buffer, pH 7.5; temperature, 30° C. Plot of respectively the residual activity (A) and the square root of the residual activity (B) versus mol of phenylglyoxal incorporated; (C) correlation of mol of phenylglyoxal incorporated with number of arginyl residues modified (amino acid analysis).

Fig. 2 two classes of arginyl residues are apparently modified. In the beginning of the reaction 2 mol of phenylglyoxal are rapidly incorporated whereas 5—6 mol of reagent are incorporated after long reaction times (20 h). When the contribution of the slowly-reacting arginyl residues is corrected for, the reaction of the first 2 mol of reagent is found to exhibit pseudo-first-order kinetics. The individual rate constants of these 2 mol of reagent which each is equal to the observed overall reaction constant were determined as 0.014 min^{-1} [21]. This value represents half of the observed rate of inactivation in agreement with case I of Ray and Koshland [21].

Stoicheiometry and nature of the modified residue

Fig. 3 illustrates the correlation between the number of mol of phenylglyoxal incorporated and the level of inactivation. As can be seen this relationship is not a linear one. When the data of Fig. 3 are replotted as the square root of the activity versus incorporation of phenylglyoxal a straight line is obtained which can be extrapolated to 2.3 mol of reagent at complete inactivation. The latter result might suggest that two arginyl residues which are both essential for activity are each modified by one molecule of phenylglyoxal [22]. This is confirmed by amino acid analyses which show that the number of arginyl residues lost correspond to the number of mol of phenylglyoxal incorporated.

Effect of the chemical modification on the conformation of 3-phosphoglycerate kinase

The optical rotatory dispersion behaviour of the inactivated enzyme was determined in the far ultraviolet and in the visible range as it was important to

TABLE I

EFFECT OF VARIOUS LIGANDS ON THE INACTIVATION OF 3-PHOSPHOGLYCERATE KINASE

Ligands	Concentrations (mM)	Protection (%)
Mg-ATP	10	88
ADP	20	93
AMP	20	76
3-phosphoglycerate	20	97
3-phosphoglycerate + ADP	20	97
Pyrophosphate	10	93
Tripolyphosphate	10	93

verify whether the modification of the essential residues led to a conformational change. The modified enzyme which has lost 73% of its original activity shows a specific rotation of −4900° at 233 nm whereas the optical parameters obtained were $a_0 = 207°$, $b_0 = -141°$ and $\lambda_c = 253$ nm. The corresponding values for native 3-phosphoglycerate kinase are $[\alpha]_{233nm} = -5000°$, $a_0 = 218°$, $b_0 = -148°$ and $\lambda_c = 256$ nm. Thus, within the limits of experimental error, there is no alteration in the conformation of the arginyl modified enzyme.

Protection against inactivation by phenylglyoxal

The protective effect of several substrates and ligands on the inactivation by phenylglyoxal was tested. The results are listed in Table I. The concentrations of reactants used are saturating (20—100 times the K_M value [23]). As can be seen an almost total protection was afforded by the different substrates as well as by the anions pyrophosphate and tripolyphosphate.

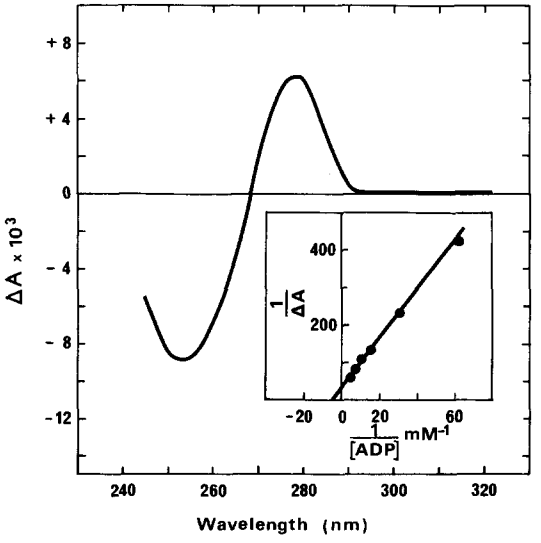

Fig. 4. Interaction of ADP with modified 3-phosphoglycerate kinase. Difference spectra were performed in 0.05 M Tris/acetate buffer, pH 7.5; temperature 20°C; 0.437-cm lightpath cells. To 39 μM modified 3-phosphoglycerate kinase (88% inactivated) was added 0.096 mM ADP. Inset: Lineweaver and Burk plot of ADP binding by modified 3-phosphoglycerate kinase (88% inactivated). Reading at 252 nm.

In a differential labelling experiment 3-phosphoglycerate kinase was incubated with [^{14}C]phenylglyoxal in the absence and the presence of substrate. When either 3-phosphoglycerate or Mg-ATP was present during modification the incorporation of radioactivity was only slight and corresponded to the feable loss of activity. Both of the two arginyl residues are there protected by substrates against the chemical modification.

Binding of substrates to 3-phosphoglycerate kinase after phenylglyoxal inactivation

The ability to bind ADP and Mg-ATP is retained in 3-phosphoglycerate kinase inactivated by phenylglyoxal as determined by ultraviolet difference spectroscopy (Fig. 4). The spectral effect induced by the interaction of the nucleotide substrates with the modified enzyme is similar to that produced by the native enzyme [24]. $\Delta\epsilon_M$ of the minimum peak at 252 nm remains unchanged (ADP, 1700 $cm^{-1} \cdot M^{-1}$; Mg-ATP, 1690 $cm^{-1} \cdot M^{-1}$). Furthermore the constants of dissociation (ADP, 0.21 mM; Mg-ATP, 0.014 mM) are in accord with those previously found for native 3-phosphoglycerate kinase [24].

In contrast the difference spectrum characteristic of 3-phosphoglycerate binding [24] is not observed for the inactivated enzyme.

Discussion

The present paper describes the effect of the modification of yeast 3-phosphoglycerate kinase by phenylglyoxal. Complete inactivation of 3-phosphoglycerate kinase can be achieved by this reagent, the reaction being of pseudo-first-order. The kinetics of inactivation indicate that the reaction of 1 mol of reagent per enzyme active site is sufficient to produce inactivation. The non-linearity of the correlation between loss of enzymic activity and reagent incorporation lends itself to two interpretations. It might be the result of a successive reaction of first one and subsequently a second molecule of phenylglyoxal with a single arginyl residue [20]. Alternatively the kinetics of the phenylglyoxal incorporation could be due to the reaction of two arginyl residues with each molecule of phenylglyoxal [22]. This latter hypothesis is supported by the amino acid analyses which show the loss of two arginyl residues. In addition the kinetics of incorporation of phenylglyoxal as a function of time show that two mol of reagent are incorporated into 3-phosphoglycerate kinase in a rapid initial phase followed by a much slower second reaction. The sum of the constant rates of reaction for these first 2 mol of reagent correspond to the rate of inactivation. We may therefore conclude that two arginyl residues which are both essential and both equally reactive are modified by phenylglyoxal. A similar result has been reported by Werber et al. [25] in the case of porcine carboxypeptidase B.

As the essential arginyl residues react more readily than the remainder and with comparable rates it is likely that they occupy a similar microenvironment. The protection studies which show that ATP as well as 3-phosphoglycerate prevent access of the inhibitor to the essential residues furthermore indicate that these are probably in close proximity to one another. The observed pattern of protection is in agreement with the results reported by Hjelmgren

et al. [9] in their study of the modification of 3-phosphoglycerate kinase by butanedione. In contrast, Rogers and Weber [10] found that 3-phosphoglycerate provides only partial protection whereas Mg-ATP affords complete protection against inactivation by either butanedione or cyclohexanedione. This result leads the authors to suppose that essential arginyl residues could be in the vicinity of the nucleotide binding site.

It is significant that the enzyme modified by phenylglyoxal continues to bind Mg-ATP and ADP with the same spectral effect and with the same affinity as the native enzyme. If one or both of the essential arginyl residues served as recognition site for the negatively charged oligophosphate moiety of the nucleotide a decrease in the affinity of the enzyme for this substrate would be expected [26]. It has previously been observed that the dissociation constant of ATP is affected upon iodination of the essential tyrosine of 3-phosphoglycerate kinase [4]. It is therefore unlikely that the essential arginyl residues modified by phenylglyoxal are situated at the nucleotide binding site. The unaltered binding capacity of the inactivated enzyme for the nucleotide substrates furthermore suggests that no conformational change, in particular at the active site, has taken place concomitantly with the chemical modification as also substantiated by the optical rotatory dispersion measurements.

The inactivated enzyme fails to produce the spectral effect characteristic of 3-phosphoglycerate binding. It would therefore seem that the essential arginyl residues are situated at or near the 3-phosphoglycerate binding site. It is tempting to suggest that the guanidino group interacts with the negative charges on 3-phosphoglycerate.

It is interesting to compare the present data with those obtained by Borders and Wilson [27] on phosphoglycerate mutase, another glycolytic enzyme which possesses 3-phosphoglycerate as substrate and which presents a remarkable structural analogy with 3-phosphoglycerate kinase [1,14,28,29]. Phosphoglycerate mutase also contains two essential arginyl residues. The authors suggest that one might serve as a recognition site for either the carboxylate or the phosphate moiety of the substrates. It might finally be noted that in the case of creatine kinase [26] the modification of an essential arginyl residue abolishes nucleotide binding as determined by ultraviolet difference spectroscopy, contrarily to the present results on 3-phosphoglycerate kinase. The present study emphasizes the general importance of arginyl side chains at enzyme recognition sites for anionic substrates and further demonstrates that the role of this residue may not be the same in different kinases.

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