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AN ESSENTIAL ARGINYL RESIDUE IN YEAST HEXOKINASE

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Summary

The inactivation of yeast hexokinase A (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) by phenylglyoxal obeys pseudo first-order kinetics. Formation of a reversible enzyme-reagent complex prior to modification is suggested by the observed saturation kinetics. Loss of activity correlates with the incorporation of 1 mol of [^{14}C]phenylglyoxal per mol 50 000 dalton subunit. No significant conformational change occurs concomitantly. Inactivation is attributable to modification of an arginyl residue. The pattern of protection by substrates and analogs favors an interaction of this essential residue with the terminal phosphoryl group of ATP or glucose 6-phosphate.

Introduction

Determination of the nature of amino acid residues involved in substrate binding or in catalysis is fundamental in elucidation of the mechanism of enyzmic reactions. Its interest in the study of hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) is even greater at present as data from X-ray crystallography are becoming available [1] and will have to await confirmation by other techniques of mechanistic investigations.

Only recently has information been provided on active center residues of yeast hexokinases. Evidence have been presented for the location of one thiol [2], one tyrosyl [3], one seryl [4,5] and one glutamyl [6] residue at or near the active center.

Since the introduction of diketones into the chemical modification of enzymes, an increasingly large number of reports have shown that arginyl residues may play an important role in enzymes acting on anionic substrates or cofactors. The presence of one or more arginyl residues in the active center of

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hexokinases may thus be expected. A general survey by means of preliminary modification of ten glycolytic enzymes with 2,3-butanedione in borate buffer supports this expectation [7] which does however deserve confirmation. In the present work, we have undertaken to verify such a hypothesis using phenylglyoxal as the modifying agent. This reagent has been shown to be selective for arginyl residues [8,9] and its reaction is very sensitive to their particular environment in the active center of enzymes [10,11,12]. The results of this study which favor the existence of an essential arginyl residue in yeast hexokinase A is reported herein. A preliminary report of this work has already been given [13].

Materials and Methods

Materials

Yeast hexokinase was purchased from Boehringer Mannheim, and treated by phenylmethylsulfonylfluoride before use [14]. According to its low specific activity on glucose (230 units/mg at 30°C), its high fructose : glucose ratio of 2.6 and its amino acid composition, it may be identified as the P_1 (or A) isozyme, as already pointed out by Colowick [15]. Lyophilized glucose-6-phosphate dehydrogenase from yeast was obtained from Calbiochem.

Phenylglyoxal monohydrate was from Aldrich Chemical Co. [7-^{14}C]Phenylglyoxal prepared from [7-^{14}C]acetophenone (ICN Corp.) by selenium oxidation [16] had a specific activity of 0.035 Ci/mol. Glucose-6-phosphate (sodium salt), ATP (sodium salt) were from Boehringer; ADP (sodium and lithium salts), NADP (sodium salt) were from Calbiochem. Mannose and xylose were from Carlo Erba. Sodium dodecylsulfate from B.D.H. was recrystrallized from ethanol. 5,5'-Dithiobis (2-nitrobenzoate) was a Pierce Chemical reagent. Instagel was obtained from Packard. All other chemicals were reagent grade.

Protein concentration and enzyme assay

Protein concentrations were determined from the absorbance at 280 nm using $A^{1\%}_{1cm} = 9.2$ for hexokinase [17]. A molecular weight of 50 000 per subunit was used in the calculation [15]. Enzyme activity was determined spectrophotometrically using glucose-6-phosphate dehydrogenase as the coupled enzyme [18].

Modifications with phenylglyoxal

Modifications with phenylglyoxal were performed at a protein concentration of 1 mg/ml, in 35 mM veronal buffer pH 7.5, at 25°C or 30°C. In either case, the inactivation follows pseudo first-order kinetics. The concentration of phenylglyoxal used was 2 mM unless otherwise stated. Solutions of the reagent were freshly prepared prior to each experiment.

In samples destined for optical rotatory dispersion studies or differential spectrophotometry, excess reagent was removed either by gel filtration through a column of Sephadex G-25 (medium) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5 at 4°C or ultrafiltration through Diaflo membranes UM 10, at 4°C, using the same buffer.

In order to determine the stoichiometry of the phenylglyoxal incorporation,

the enzyme was incubated with ^{14}C-labeled reagent. Aliquots of the reaction mixture were removed at specified time intervals and assayed for activity and extent of radioactivity incorporation. The modified enzyme was precipitated by addition of an equal volume of 30% trichloroacetic acid to the reaction mixture. The precipitate was then washed 8 times with equal volumes of ethanol and subsequently dissolved in 10 ml Instagel for liquid scintillation counting.

Physical measurements

Ultraviolet difference spectroscopy was performed on a Cary model 118 spectrophotometer, thermostated at 4°C. A matched pair of quartz cells of 0.437 cm light path were used.

Optical rotary dispersion measurements were performed with a Fica type Spectropol I spectropolarimeter. All experiments were carried out in 0.05 M Tris-HCl buffer, pH 7.5, at room temperature using a 1 cm light path cell. Protein concentrations of 0.1—0.2 mg/ml were used.

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

Determination of sulfhydryl groups

Free sulfhydryl groups were estimated with 5,5′-dithiobis (2-nitrobenzoate) in 1% sodium dodecyl sulfate, at pH 8. The enzyme was incubated with phenylglyoxal as described. Aliquots of the reaction mixture were periodically removed and assayed for enzyme activity and sulfhydryl content. Before titration with 5,5′-dithiobis (2-nitrobenzoate), sodium phosphate buffer, pH 8, was added to give a final concentration of 0.1 M. It was verified that the phenylglyoxal present did not cause any release of the nitrothiophenolate anion.

Results

Inactivation of hexokinase by phenylglyoxal

Yeast hexokinase A (1 mg/ml) is progressively inactivated by phenylglyoxal (Fig. 1). This process obeys pseudo first-order kinetics until approximately 80% of the enzymic activity is lost (k_{app} = 0.036 min^{-1} with 2 mM phenylglyoxal at 25°C). The rate of inactivation increases with temperature (k_{app} = 0.140 min^{-1} with 2 mM phenylglyoxal at 30°C) and with reagent concentration. Prolonged incubation with phenylglyoxal results in complete inactivation of the enzyme.

Saturation kinetics are observed when the pseudo first-order rate constant of inactivation is measured as a function of phenylglyoxal concentration (Fig. 1, inset) suggesting that a reversible enzyme-reagent complex forms prior to inactivation:

$$E + I \overset{K_I}{\rightleftharpoons} E - I \overset{k_3}{\rightarrow} E'I$$

A double reciprocal plot of $1/k_{app}$ versus 1/[phenylglyoxal] [19] gives values of 11 mM and 0.21 min^{-1}, at 25°C, for K_I and k_3 respectively.

Stoichiometry of the reaction and nature of the modified residue

A linear relationship is observed between enzyme inactivation and incorpora-

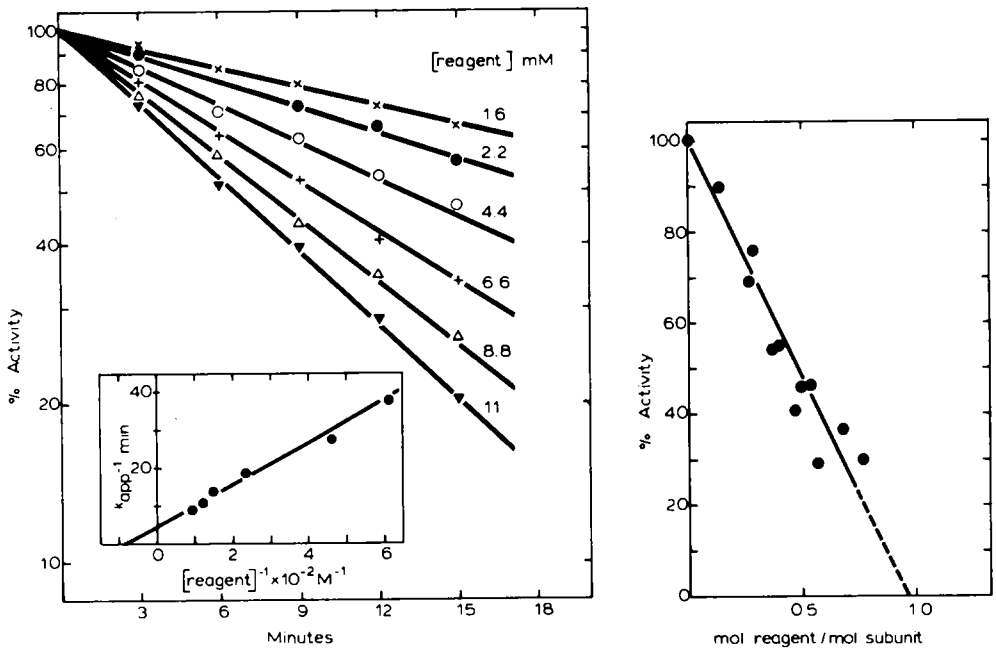

Fig. 1. Inactivation of hexokinase at various phenylglyoxal concentrations. Hexokinase, 20 μM; veronal buffer, 35 mM, pH 7.5, 25°C. Inset: double reciprocal plot of k_{app} versus reagent concentration showing saturation kinetics.

Fig. 2. Incorporation of [^{14}C]phenylglyoxal into hexokinase. Hexokinase, 20 μM; phenylglyoxal, 2 mM; veronal buffer, 35 mM, pH 7.5, 30°C.

tion of [^{14}C]phenylglyoxal (Fig. 2). Extrapolation to zero activity indicates that 1 mol of reagent is incorporated per mol of 50 000 dalton subunit, suggesting that modification of a single residue is responsible for the loss of activity. Phenylglyoxal is known to be highly selective for arginine. In peptides and proteins only the N-terminal α-amino group, ε-amino groups of lysines and sulfhydryl groups may also react with this reagent [8,9]. The reaction of phenylglyoxal with amino groups leads to formation of labile Schiff bases which may be stabilized by borohydride reduction. When partially modified hexokinase is treated with borohydride before dilution and activity assay, no further inactivation is observed. Moreover, a slow partial reactivation of modified hexokinase is observed and this reactivation takes place whether or not the inactivated enzyme has been treated with sodium borohydride. Such lability of phenylglyoxal-modified arginyl residues has been reported previously [8,20,12].

Titrations with 5,5′-dithiobis(2-nitrobenzoic acid) show a loss of about 1.3 sulfhydryl groups per subunit in modified hexokinase. However, this loss is independent of the level of inactivation (from 17 to 71%). Oxidation of the sulfhydryl groups or reaction with phenylglyoxal may have occurred. Schubert [21,22] has shown that cysteinyl residues can give a thiohemiacetal by addition with phenylglyoxal [23]. Such a compound is generally unstable. This may especially be the case in the presence of large excesses of ethanol used in the

purification of the radioactively labeled hexokinase, which could explain why a stoichiometry of only 1 mol of reagent per subunit is found.

Protection by substrates from inactivation

Table I shows the effects of various substrates and analogs on the inactivation of hexokinase by phenylglyoxal. The substrates D-glucose and D-mannose protect efficiently and to about the same extent when added at concentrations that are equal multiples of their K_m values while the protection by xylose, a competitive inhibitor of glucose, is less important under the same conditions. Pyrophosphate as well as Mg—ADP and Mg-ATP alone are also good protectors. Glucose and mannose enhance the effects of pyrophosphate and Mg-ADP, whereas that of Mg-ATP is not augmented by xylose. Glucose 6-phosphate affords the most important protection. Although the reagent used contains a small percentage (0.8%) of contaminant glucose, the almost total protection observed can only be attributed to glucose 6-phosphate itself.

Inactivation of hexokinase in the presence of Mg-ATP or glucose

Considering the affinity of hexokinase for phenylglyoxal, it was interesting

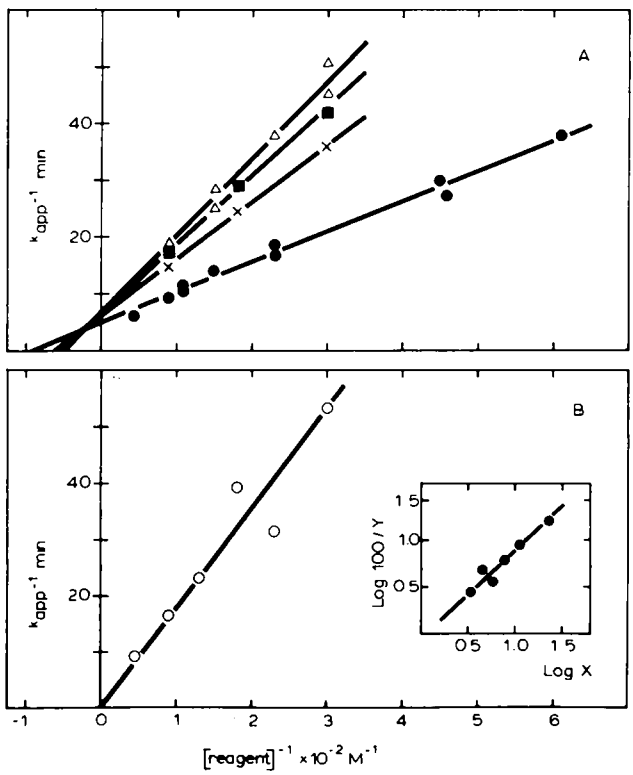

Fig. 3. Effects of substrates on the inactivation of hexokinase by phenylglyoxal. Same reaction conditions as in Fig. 1. (A) Mg-ATP: (●) 0 mM, (X) 2.5 mM, (■) 5 mM, (△) 10 mM. (B) Glucose, 1.0 mM. Inset: determination of the order of reaction with respect to phenylglyoxal concentration, in the presence of glucose. X, phenylglyoxal concentration (mM); Y, half-time of inactivation (min).

TABLE I

PROTECTION OF HEXOKINASE AGAINST INACTIVATION

Same reaction conditions as in Fig. 2. The enzyme was preincubated for 10 min with the ligand before addition of the reagent.

Ligand	K_m (mM)	Conc. (mM)	Protection (%)
Glucose	0.1 *	0.24	55
		1.0	72
		25	85
Mannose	0.05 *	0.125	41
		0.5	73
		25	90
Xylose	10 *	100	42
Pyrophosphate	—	150	61
Mg-ADP	2.8 *	25	61
		100	81
Mg-ATP	4 **	10	59
		100	85
Glucose		0.25	
+			91
Pyrophosphate		150	
Glucose		0.25	
+			80
Mg-ADP		25	
Mannose		0.125	
+			83
Mg-ADP		25	
Xylose		100	
+			61
Mg-ATP		10	
Glucose 6-phosphate	25 *	150	97

* Crane [32].
** Colowick [15].

to examine whether the reagent mimics one of the substrates at the substrate binding sites. Fig. 3 shows that neither Mg-ATP nor glucose are competitive with phenylglyoxal. It is worth noting that, in the presence of glucose, a plot of $1/k_{app}$ versus 1/[phenylglyoxal] passes through the origin, indicating that, under these conditions, no reversible enzyme-phenylglyoxal complex is formed prior to inactivation. The order of inactivation with respect to phenylglyoxal concentration may thus be directly determined according to the method of Levy et al. [24]. A plot of log of the reciprocal of the half-times of inactivation versus log concentration of inactivator gives a straight line with a slope equal to 0.94 (Fig. 3B, inset), indicating that the reaction is first-order with respect to phenylglyoxal concentration.

Properties of inactivated hexokinase

The interaction of substrates with modified hexokinase was studied by difference spectrophotometry. The modified enzyme (inactivated to 70%) fails to produce the difference spectrum characteristic of glucose binding. The interaction with ADP can be visualized spectrophotometrically only when the sugar substrate is already bound to the enzyme. Accordingly, no spectral effect was observed when ADP was added to the modified enzyme in the presence of

glucose. The specific rotations at 233 nm were identical in the native and the modified enzyme, which had lost up to 80% of its original activity. Thus, no significant conformational change had occurred in the modified enzyme.

Discussion

Several lines of evidence lead to the conclusion that the arginyl residue is essential to yeast hexokinase activity. The enzyme is rapidly inactivated by phenylglyoxal, the reaction being of pseudo first-order and complete inactivation can be achieved. Optical rotary dispersion measurements show that modification of the enzyme is not accompanied by any significant conformational change. The stoichiometric incorporation of [^{14}C]phenylglyoxal into the protein suggests that modification of a single residue is responsible for the loss of catalytic activity.

Phenylglyoxal reacts most rapidly with arginyl side chain. Among other side chains, only those of lysyl and cysteinyl residues can react, at a much slower rate [9]. Modification of a lysyl residue is ruled out, as borohydride reduction changes neither the activity nor the stability of modified hexokinase. The loss of about 1.3 SH group — which could a priori be attributed to oxidation or formation of an unstable thiohemiacetal — is not related to enzyme activation. A similar phenomenon has also been noted in the modification of the essential carboxyl group of yeast hexokinase [6] and may indicate the particular sensitivity of non-essential sulfhydryl groups in the enzyme.

Direct evidence for the modification of arginine may normally be obtained by amino acid analyses. However, because of the large number or arginyl residues in hexokinase (18 residues per 50 000 dalton subunit) this method is not sensitive enough in the present case to determine the loss of one residue.

A stoichiometry of 2 : 1 has been demonstrated for the phenylglyoxal adduct when free arginine is involved [8,9]. With active arginyl residues in enzymes, this stoichiometry has been generally admitted or proven for some enzymes [20,25,26]. However, a 1 : 1 stoichiometry has also been observed [27,28,29] and this is clearly valid for yeast hexokinase as well. In agreement with this result is the first order of the reaction with respect to phenylglyoxal concentration, as evidenced by the experiments performed with glucose. The high reactivity of a single arginyl residue out of 18 and the 1 : 1 stoichiometry of the reaction may well be due to a particular microenvironment of the essential arginyl residue involved, as saturation kinetics are observed in the reaction of phenylglyoxal with yeast hexokinase A. Hydrophobic interactions could explain this affinity of the reagent for the enzyme. A similar phenomenon has already been observed in the modification of the enzyme essential glutamyl residue by 1-cyclohexyl-3(2-morpholino ethyl) carbodiimide metho-p-toluene sulfonate and nucleophiles: only aromatic nucleophiles have been incorporated into the enzyme with concomitant inactivation, while other compounds with essentially the same nucleophilicity have no effect [6]. It is well known that in various kinases and dehydrogenases the adenosine moiety of the nucleotide substrates binds in a hydrophobic domain [30,31]; phenylglyoxal may interact with part of this domain. Nevertheless, the non-competitive type of the protection exerted by ATP rules out the precise binding of the reagent

on the nucleotide substrate binding site. On the other hand, the protection by glucose is clearly due to the conformational changes which are known to be induced by this substrate. These extensive alterations apparently suppress the affinity of the enzyme for phenylglyoxal, so that a mere single-step inactivation occurs. Conversely, no difference spectrum characteristic of the binding of glucose was observed when hexokinase had been modified with phenylglyoxal. This could be due to affinity binding of phenylglyoxal with subsequent covalent reaction at the active center near the glucose binding site, thus hampering the induced fit of this sugar substrate. Indeed, the observed interactions between phenylglyoxal and glucose in hexokinase are quite compatible with data on this enzyme now available. Thus, ATP and glucose bindings mutually promote each other, indicating mutual conformational interactions [15]. Inhibitors, like AMP, 1-anilino-8-naphthalene sulfonate, diiodofluorescein and 5-iodosalicylate bind to the enzyme on a small pocket that is adjacent to the sugar site [1,31].Furthermore, AMP binding to that A (or nucleotide active) site and glucose binding are antagonistic [1].

The protection afforded by the substrates or analogs also suggests that the arginyl residue modified by phenylglyoxal is located in the active center. Mg-ADP and Mg-ATP are equally good protectors. Glucose enhances the effect of Mg-ADP, whereas xylose does not change that of Mg-ATP. A difference between the effects of glucose and xylose has been already observed in the phosphorylation of yeast hexokinase induced by Mg-ADP and xylose [4,5]. This is related to a specific conformational change induced only by xylose (and lyxose) in which a serine residue is oriented into the vicinity of the γ-phosphate of ATP. Reactivation by Mg-ADP and xylose (or lyxose) is also specific, and it is inhibited by glucose. Thus, it is probable that the conformation of the active center in the presence of the inhibitor xylose is slightly different from that induced by the sugar substrates. The fact that in our experiments xylose alone protects less effectively than glucose and mannose, under the same conditions, also favors this assumption.

The pattern of the protection afforded by phosphoryl substrates or analogs suggests an interacton of the essential arginyl residue with the transferable phosphate group of the substrate. The almost total protection by glucose-6-phosphate might be attributed to binding of this ligand at the active site with appropriate shielding of the arginyl residue. Furthermore, pyrophosphate alone, which has been found to bind at the sulfate binding site near the 6-hydroxyl of glucose [1], provides an efficient protection and this effect is augmented in the presence of glucose.

Recognition of anionic functional groups of substrates or cofactors has been assigned to essential arginyl residues in a number of enzymes. The present data are consistent with the existence of an essential arginyl residue in yeast hexokinase A and its involvement in interactions with the terminal phosphoryl group of ATP or glucose 6-phosphate.

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