ESSENTIAL CARBOXYL GROUPS IN YEAST HEXOKINASE

Dang Ba PHO, Claude ROUSTAN, Gisèle DESVAGES, Louise-Anne PRADEL and Nguyen van THOAI
Laboratoire de Biochimie Cellulaire, Collège de France, Place Marcellin Berthelot, 75231 Paris Cedex 05, France

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1. Introduction

Although a great deal of kinetic data are available on yeast hexokinase and several schemes have been proposed, the mechanism of its action is far from being understood [1,2]. It is generally agreed, however, that the transphosphorylation step requires the formation of a ternary complex between the enzyme and its substrates, ATP-Mg and D-glucose. Although the pH dependence of the enzyme activity suggests that at least one ionisable group, such as the imidazole of histidine or the γ-carboxyl group of aspartic or glutamic acids, takes part in the catalytic process [3, 4], the nature of the amino acid residues involved in enzymatic catalysis or substrate binding remains to be determined.

According to the results of Grouselle et al. [5], as well as our own unpublished results, the histidine residues do not appear to be associated either with the catalytic step or with the enzyme–substrate interactions. Thus, the inactivation observed when the enzyme is carboethoxylated is caused by a conformational change in the enzyme protein rather than by the modification of an essential histidine residue.

It seemed, therefore, important to investigate the effect of specific carboxyl group reagents upon the enzyme activity. Water soluble carbodiimides have been shown to be good reagents for this purpose [6].

This paper reports some preliminary results obtained by chemical modification of essential carboxyl groups in yeast hexokinase.

2. Materials and methods

The Boehringer yeast enzyme was further purified by column chromatography on carboxymethyl-Sephadex as previously described [7] and the preparation thus obtained had a specific activity of 230 μmoles of glucose-6-phosphate produced per min per mg enzyme at 30°C and pH 7.5, as determined by spectrophotometric method [8]. –SH groups are titrated by DTNB [9] in 6 M urea, pH 8.2. Amino acid analysis were carried out on a TSM 1 Technicon autoanalyser.

3. Results

Hexokinase was incubated with 1-cyclohexyl-3-(-2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate (CMC), at pH 6. Fig. 1 illustrates the inhibition obtained with increasing concentrations of the reagent. CMC (0.05 M) alone produced a partial inactivation which increased with time. This effect was markedly enhanced by the presence of a nucleophile such as nitrotyrosine ethyl ester (fig. 2). This latter reagent alone had no effect on enzyme activity. These results are indicative of a specific reaction of the nucleophile, in the presence of the carbodiimide, which leads to the formation of an amide bond with a free carboxyl group of the enzyme [6].

To follow the progress of this incorporation with enzyme inactivation, the amount of bound nitrotyrosine was determined on autoanalyser after total acid hydrolysis (110°C, 6 N HCl) of the modified protein. The data presented in fig. 3 show that 2 molecules of nitrotyrosine are incorporated per 50 000 dalton subunit [10] concomitant with complete loss of enzyme activity.

To test for protection against inactivation, various

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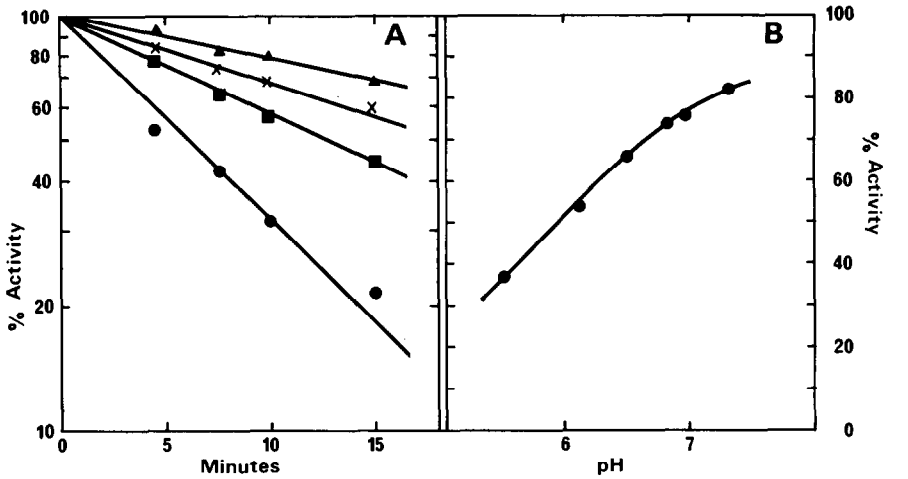

Fig. 1. Inhibition of hexokinase by CMC: A) Effect of CMC concentration. Hexokinase 36 μM, phosphate buffer pH 6, 20°C. CMC (▲) 0.015 M; (X) 0.03 M; (■) 0.05 M; (●) 0.1 M; B) Effect of pH. Hexokinase 34 μM, phosphate buffer at the pH values indicated.

substrates or substrate analogues were added before the addition of carbodiimide (fig. 2). D-Glucose and D-mannose, which are good substrates for hexokinase, afforded a partial protection. Furthermore, ADP, which forms an abortive complex with the enzyme and its acceptor substrate, significantly enhanced this protection. In contrast, xylose which is a competitive inhibitor towards glucose [11], did not protect at all. A similar result was obtained with the nucleotide substrate (ATP-Mg) alone. The binding of both ATP-Mg and xylose to hexokinase gave a slight but significant protection.

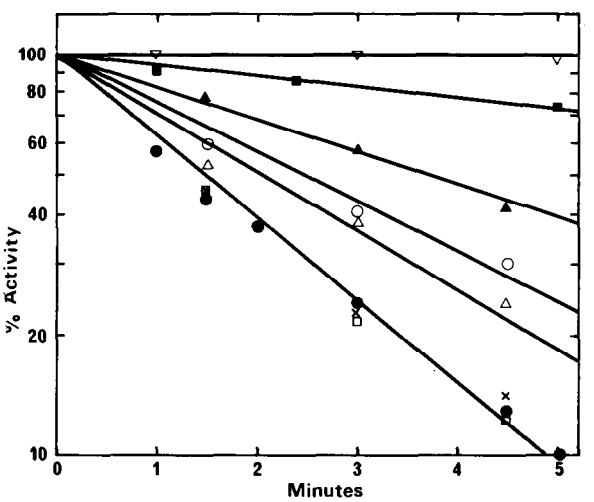

Fig. 2. Inhibition of hexokinase by 50 mM CMC and 30 mM of nitrotyrosine ethyl ester, in the presence of various ligands. (●) No ligand; (X) 10 mM ATP-Mg; (□) 100 mM xylose; (△) 100 mM xylose + 10 mM ATP-Mg; (○) 25 mM glucose; (▲) 25 mM glucose + 2.5 mM ADP; (■) CMC alone; (▽) nitrotyrosine ethyl ester alone.

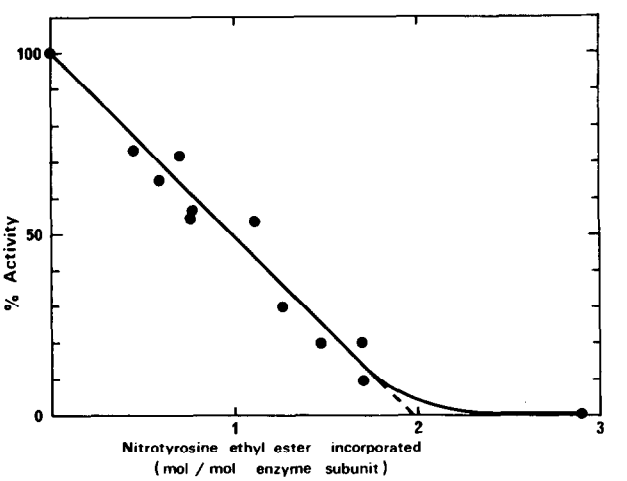

Fig. 3. Effect of nitrotyrosine ethyl ester incorporation on hexokinase activity.

Apart from the carboxyl group, different functional groups of the enzyme such as cysteine, lysine, histidine, tyrosine [12,13] may react with carbodiimide on its own. Thus, we have found that two of the four sulphydryl groups per 50 000 dalton subunit are readily modified by CMC with or without nucleophile. However, this took place whatever the inactivation observed (from 9 to 90%). This is consistent with the existence of two more reactive and non-essential sulphydryl groups in yeast hexokinase [1]. Lysine modification is unlikely at the pH employed. The decrease in enzyme inactivation with increasing pH is incompatible with histidine substitution (fig. 1). Moreover, neither histidine nor tyrosine is lost as judged by amino acid analysis.

4. Discussion

The inactivation of hexokinase by carbodiimide in the presence of nitrotyrosine ethyl ester provides evidence for the involvement of carboxyl groups at the active site. Complete inactivation correlates with the substitution of only two residues out of the total of about 110 carboxyl groups of the protein [1]. In order to determine whether one or both of these residues is located in the active site, further studies are in progress. However, the pattern of protection afforded by different substrates clearly emphasizes their implication in the catalytic process. It is interesting to point out that our results are in accord with those previously obtained from binding studies [7,14]. In fact, these data strongly suggest that the enzymatic reaction is essentially ordered, the sugar binding first. Thus, if ATP-Mg forms a binary complex with the enzyme, its dissociation constant would be high [7,15]. This accounts for its ineffectiveness in protection against inactivation. Similarly, there is no protection with xylose alone, which binds to hexokinase without inducing any significant conformational change as detected by difference spectrophotometry. However, its presence induces a normal binding of ATP-Mg and a small microenvironmental rearrangement of the binding sites. This may be correlated with the slight protection obtained by the simultaneous binding of ATP-Mg and xylose. The conformational change is more important with the true substrates [1,15], and the protection is more effective with glucose alone and greatest with glucose and ADP.

Thus, a carboxyl group could take part in the transphosphorylation catalysed by yeast hexokinase. It may function in the reaction in the same way as the carboxyl groups of 3-phosphoglycerate kinase [16,17] or acetate kinase [18]. So, one could imagine the occurrence of similar transient steps in the catalysis promoted by these kinases [19], particularly the formation of more or less stable covalent phosphoryl enzymes. Further investigation, including partial isotopic exchange studies, will aim at determining the exact function of essential carboxyl groups in yeast hexokinase.

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