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Phosphorylation of D-glyceraldehyde-3-phosphate dehydrogenase by Ca^{2+}/calmodulin-dependent protein kinase II

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Rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase was shown to serve as a substrate for Ca^{2+}/calmodulin-dependent protein kinase II with a K_m of 0.33 μM and a V_{max} of 2.63 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ at pH 7.5 and 30°C. In the absence of calmodulin, the V_{max} was halved and K_m unchanged. 0.99 mol of phosphate was incorporated per tetrameric molecule of D-glyceraldehyde-3-phosphate dehydrogenase under the experimental conditions employed.

Phosphorylation; D-Glyceraldehyde-3-phosphate dehydrogenase; Ca^{2+}/calmodulin-dependent protein kinase II

1. INTRODUCTION

A considerably body of evidence accumulated in recent years indicates that phosphorylation catalyzed by specific protein kinases can constitute one of the mechanisms for the regulation of glycolysis and gluconeogenesis [1]. Several glycolytic enzymes were shown to undergo covalent modification by phosphorylation-dephosphorylation. Pyruvate kinase, 6-phosphofructo-1-kinase, 6-phosphofructo-2-kinase and fructose 1,6-bisphosphatase were most intensely studied in this respect and demonstrated to be substrates for various protein kinases [2–6]. Little is known, however, about covalent modification of other enzymes catalyzing reactions which are not considered to be rate-limiting steps of glycolysis. Among these, D-glyceraldehyde-3-phosphate dehydrogenase is of special interest, since, due to the high cellular concentration of this enzyme and to the fact that an appreciable fraction of its active sites are acylated in vivo, it can function as a warehouse for rapidly convertible energy [7,8]. Reiss et al. [9] reported D-glyceraldehyde-3-phosphate dehydrogenase to be one of the substrates for epidermal growth factor-receptor tyrosine kinase, but no information is yet available about phosphorylation of this enzyme by protein kinases operating in normal cells. The aim of the present work was to test several protein kinases for their ability to phosphorylate rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase. As will be shown, this enzyme appears to be a rather good substrate for Ca^{2+}/calmodulin-dependent protein kinase II.

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2. MATERIALS AND METHODS

Tris, ATP, dithiothreitol, NAD^+, glyceraldehyde-3-phosphate, phosphorylase *b*, histone H1, phosphatidylserine, cAMP and phosvitin were obtained from Sigma, EDTA and glycine from Reanal, $CaCl_2$, acrylamide and EGTA were purchased from Serva, [γ-^{32}P]ATP was a product of Amersham. The following enzymes were prepared according to the literature cited: Ca^{2+}/calmodulin-dependent protein kinase II from rat brain [10], the catalytic subunit of cAMP-dependent protein kinase from pig brain [11], phosphorylase kinase from rabbit skeletal muscle [12], protein kinase C from rat brain [13]. Casein kinases 1 and 2 purified from rabbit skeletal muscle were a gift from Dr N.B. Gusev. Calmodulin and synapsin from

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bovine brain were prepared by the methods of Anderson [14] and Ueda [15] respectively. D-Glyceraldehyde-3-phosphate dehydrogenase was isolated from rabbit skeletal muscle according to Scoups [16] with some modifications. Its activity was assayed in 0.1 M glycine buffer, pH 8.9, 5 mM sodium arsenate, 5 mM EDTA, 0.5 mM glyceraldehyde-3-phosphate, 0.5 mM NAD^+. Protein concentration was determined by the method of Bradford [17].

Phosphorylation of rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase with Ca^{2+}/calmodulin-dependent protein kinase II was carried out at 30°C, pH 7.5, in 50 mM Tris-HCl, 5 mM dithiothreitol, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1.0 mM calmodulin, 0.03 mg/ml protein kinase, 0.5 mM [γ-^{32}P]ATP (5×10^{-5} cpm); the concentrations of D-glyceraldehyde-3-phosphate dehydrogenase are indicated in the legends to figures. At the end of incubation, aliquots of the reaction mixture were applied on Whatman 3 MM filters, washed with 5% trichloroacetic acid and dried. Radioactivity was determined by liquid scintillation spectrometry. Control samples contained no D-glyceraldehyde-3-phosphate dehydrogenase; very low levels of radioactivity due to autophosphorylation of the protein kinase were detected in these samples.

3. RESULTS AND DISCUSSION

Rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase was tested as a potential substrate for cAMP-dependent protein kinase, phosphorylase kinase, I and II types casein kinases, protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II. As shown in table 1, only type I casein kinase and Ca^{2+}/calmodulin-dependent protein kinase II were found to be capable of phosphorylating the enzyme. The level of phosphorylation was considerably higher with Ca^{2+}/calmodulin-dependent protein kinase II and corresponded to incorporation of 0.99 mol phosphate per mol of tetrameric D-glyceraldehyde-3-phosphate dehydrogenase. A more detailed study of this process was then undertaken. The results presented in fig.1 demonstrate that the radioactive phosphate was incorporated in D-glyceraldehyde-3-phosphate dehydrogenase subunits (M_r 36000), lanes 1 and 2; phosphorylation is Ca^{2+}-dependent (compare lanes 1 and 3) and is stimulated by calmodulin (lane 2). It is also seen that no measurable autophosphorylation of protein kinase could be detected in these experiments (lane 5). The time-course of the reaction is shown in fig.2, which also demonstrates the effect of calmodulin. As follows from the data of fig.3, calmodulin causes a 2-fold acceleration of the reaction by increasing the V_{max} without affecting the K_m of the process.

Further work is needed to evaluate the role of the protein kinase catalyzed enzyme phosphorylation in its functioning. According to our preliminary data, incorporation of 1 mol phosphate per tetrameric molecule of D-glyceraldehyde-3-phos-

Table 1

Phosphorylation of D-glyceraldehyde-3-phosphate dehydrogenase by different protein kinases

Enzyme:	cAMP-dependent protein kinase[a]		Kinase phosphorylase		Casein kinase I		Casein kinase II		Protein kinase C[b]		Ca^{2+}/calmodulin-dependent protein kinase[c]	
Substrate:	GPDH	Synapsin (20 mg/ml)	GPDH	Phosphorylase *b* (5 mg/ml)	GPDH	Phosvitin (0.3 mg/ml)	GPDH	Phosvitin (0.3 mg/ml)	GPDH	Histone H1 (10 mg/ml)	GPDH	Synapsin (0.1 mg/ml)
Phosphate incorporation mol/mol of substrate	0.021	1.0	0.009	0.72	0.093	0.85	0.008	1.03	0.019	1.9	0.99	2.1

[a] 0.5 mM cAMP was present
[b] 1 μg/ml phosphatidylserine was present
[c] 1.0 μM calmodulin was present

Incubation was carried out in 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM $MgCl_2$, 50 μM ATP, 0.5 mM $CaCl_2$, 0.5 mg/ml of D-glyceraldehyde-3-phosphate dehydrogenase (GPDH), 0.03 mg/ml of a protein kinase

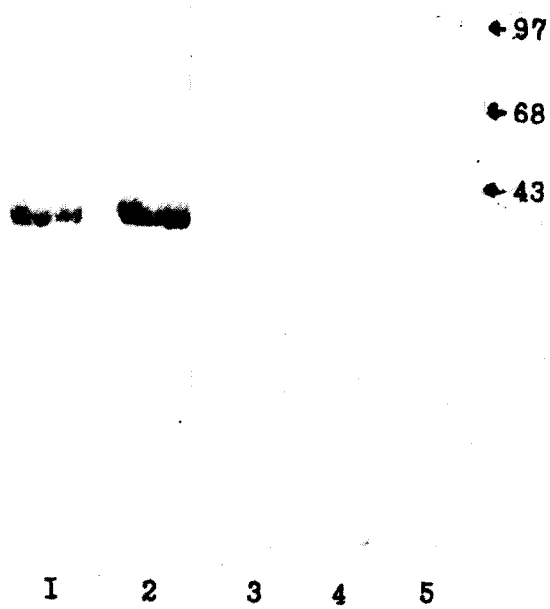

Fig.1. Phosphorylation of rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase with Ca^{2+}/calmodulin-dependent protein kinase II. The reaction was carried out as described in section 2 for 15 min with 0.5 mg/ml D-glyceraldehyde-3-phosphate dehydrogenase (lane 2). Lane 4, protein kinase was absent; lane 3, $CaCl_2$ was absent; lane 1, calmodulin was absent; lane 5, D-glyceraldehyde-3-phosphate dehydrogenase was absent. The reaction was stopped by 5% trichloroacetic acid. The precipitate collected by centrifugation was dissolved in 0.1% SDS. SDS-polyacrylamide gel electrophoresis was then performed according to Laemmli [18] with the use of 9 × 12 × 0.04 cm plates of 10% gel in presence of 0.1% SDS. Protein bands were detected by Coomassie blue R 250 staining and autoradiography. M_r markers (values on the right) were phosphorylase, bovine serum albumin and ovalbumin.

phate dehydrogenase results in a 1.5-fold activation of the enzyme. Given the low stoichiometry of phosphate incorporation achieved in these experiments, we may expect that the activatory effect could be higher under conditions favoring phosphorylation of more than one subunit in the tetramer. No attempts to obtain the maximum degree of phosphorylation have been made thus far. Another consequence of D-glyceraldehyde-3-phosphate dehydrogenase phosphorylation was an increased stability (incubation in 10 mM Tris-HCl, pH 7.5, at 4°C and 0.5 mg/ml protein concentration resulted in a loss of activity of an

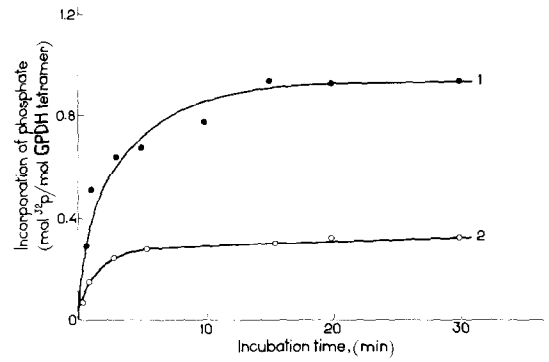

Fig.2. Time-course and stoichiometry for the phosphorylation of rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase with Ca^{2+}/calmodulin-dependent protein kinase. The reaction conditions are described in section 2. 1, calmodulin was present (1.0 mM); 2, no calmodulin was added. At indicated time intervals aliquots were taken for radioactivity determination.

unmodified enzyme after 24 h, whereas a phosphorylated one remained stable for at least 72 h). This suggests that the covalent modification of the enzyme brings about some conformational alterations of its molecule. We may speculate that this can be reflected in the ability of the enzyme to form complexes with other proteins and to associate with intracellular structures. We are planning to examine such possibilities in our future studies.

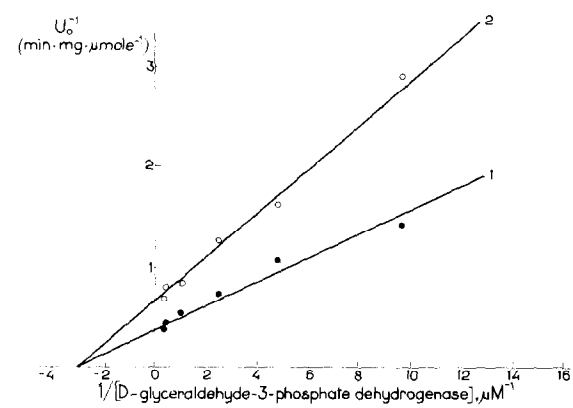

Fig.3. Double-reciprocal plots for the phosphorylation of D-glyceraldehyde-3-phosphate dehydrogenase by Ca^{2+}/calmodulin-dependent protein kinase. The reaction was carried out with 1.0 mM calmodulin (1) or with no calmodulin (2). The following kinetic parameters were estimated from the plots: K_m = 0.33 μM; V_{max} = 1.44 μmol·min^{-1}·mg^{-1} (1) and 2.63 μmol·min^{-1}·mg^{-1} (2).

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