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D-Glyceraldehyde-3-phosphate dehydrogenase purified from rabbit muscle contains phosphotyrosine

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Homogeneous preparations of D-glyceraldehyde-3-phosphate dehydrogenase purified from rabbit muscle were found to contain 0.2–0.7 moles of covalently bound phosphate per mole of the enzyme. With the use of anti-phosphotyrosine antibodies, evidence was obtained that the enzyme is phosphorylated at tyrosine residues.

D-Glyceraldehyde-3-phosphate dehydrogenase; Endogenous phosphate; Phosphotyrosine; Antibody to phosphotyrosine

1. INTRODUCTION

Protein phosphorylation is the most widely occurring post-translational modification used in the control of biological processes [1]; it is believed to constitute one of the mechanisms for the regulation of glycolysis and gluconeogenesis [2]. A number of glycolytic enzymes (pyruvate kinase, 6-phosphofructo-1-kinase, 6-phosphofructo-2-kinase, fructose-1,6-bisphosphatase) were shown to be substrates for various protein kinases [3–7]. Reiss et al. reported in vitro phosphorylation of D-glyceraldehyde-3-phosphate dehydrogenase by epidermal growth factor-receptor tyrosine kinase [8]. The evidence for phosphorylation of this enzyme by Ca^{2+}/calmodulin-dependent protein kinase purified from normal cells was obtained in our laboratory [9]. To estimate the physiological significance of these phenomena, information should be obtained concerning the susceptibility of D-glyceraldehyde-3-phosphate dehydrogenase of phosphorylation in vivo. In this work, the endogenous phosphate content of rabbit muscle enzyme was determined and the evidence is presented that the protein is phosphorylated at tyrosine residues.

2. MATERIALS AND METHODS

Dithiothreitol, EDTA were obtained from Serva, NAD^+ from Boehringer Mannheim, [carbonyl-^{14}C]NAD^+ from Amersham, Protein A-Sepharose, Sephadex G-50 (Fine) and G-100 (Superfine) were purchased from Pharmacia. Mouse monoclonal antibodies C5 (IgG) and G7 (IgM) raised against rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase were kindly provided by Dr. A.G. Katrukha. Mouse monoclonal antibodies to phosphotyrosine were prepared as previously described [10].

Isolation of D-glyceraldehyde-3-phosphate dehydrogenase from rabbit skeletal muscle was performed by the method of Ferdinand [11], recrystallizations being carried out at pH 8.0. Another procedure was also used, which included extraction of the muscle tissue with 50 mM potassium phosphate pH 8.4, 5 mM EDTA, 2 mM dithiothreitol. After centrifugation, the muscle debris was homogenized in the above buffer. Following centrifugation, the two supernatants were combined, pH adjusted to 7.6–7.8 with ammonium solution. Dry ammonium sulfate was added to achieve 60% saturation. After centrifugation, ammonium sulfate was added to the supernatant to 74% saturation. Precipitate was removed by centrifugation, and pH of the supernatant adjusted to 8.2 with concentrated ammonium solution. The mixture was left overnight at 4°C. The precipitate collected by centrifugation was dissolved in 50 mM Tris-HCl, pH 8.4, 10 mM potassium phosphate, 2 mM EDTA, 1 mM dithiothreitol (protein concentration, 15–17 mg/ml) and recrystallized by addition of saturated ammonium sulphate (pH 8.2). Recrystallizations were repeated four times. Preparations obtained by both procedures were homogeneous in electrophoresis performed according to Laemmli [12].

Protein concentration was determined spectrophotometrically from A_{280}/A_{260} ratio [13] or by the method of Bradford [14] with D-glyceraldehyde-3-phosphate dehydrogenase as standard. Before spectrophotometric determinations, the enzyme preparation was incubated with NAD^+ (1–2 mM NAD^+ and 0.03–0.05 mM protein), to displace any ADP-ribose present in coenzyme binding sites [15], with subsequent desalting on Sephadex G-50. Endogenous phosphate content in the enzyme preparation was determined after precipitation of the protein and subsequent two-fold washing of the precipitate with 5–7% trichloroacetic acid. Mineralization of samples and phosphate determination were carried out according to [16]. To determine the amount of NAD^+, bound to the protein after treatment with trichloroacetic acid, the following procedure was used. Native crystalline enzyme was dissolved in 68 mM [carbonyl-^{14}C]NAD^+ (6700 Ci/mmol), the protein concentration being 0.21 mM. After 15 min incubation the protein solution was desalted on Sephadex G-50 (Fine) column. The protein was then precipitated by trichloroacetic acid, the precipitate was washed twice with 5% trichloroacetic acid and then dissolved in 0.5 M KOH (final protein concentration, 7 μM). The NAD^+ content was

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estimated from radioactivity determined by liquid scintillation spectrometry.

Immunoblotting was performed as follows. A series of samples were subjected to electrophoresis in denaturing 7.5–12.5% polyacrylamide gradient slab gels [12]. The proteins were electrophoretically transferred to nitrocellulose filters using the Western blotting technique [17]. Blots were incubated in the solution containing 10 mM Tris-HCl, pH 7.8, 1% bovine serum albumin, 0.1% Tween-20 and anti-phosphotyrosine monoclonal antibodies; phosphotyrosine-containing protein bands were stained by anti-mouse peroxidase-conjugated antibodies (Calbiochem).

Immunoprecipitation was carried out by the procedure developed earlier [19]. Briefly, 1 ml of the immunoprecipitation buffer, containing 1% Triton X-100, 0.1% SDS, 0.4 M NaCl, 10 mM EDTA, 0.1 M potassium phosphate, 50 mM HEPES-NaOH, pH 7.2, and 40 μg of affinity-purified monoclonal antibodies C5 and G7 were added to each sample and incubated overnight at 4°C. After addition of 30 μl of protein A-Sepharose, samples were incubated for 1 h at 30°C, centrifuged, the pellet was washed four times with immunoprecipitation solution. Denaturation mixture was added, samples were boiled and applied onto gel. When G7 monoclonal antibodies (IgM) were used, protein A-Sepharose was additionally preincubated with an excess of rabbit anti-mouse antibodies.

3. RESULTS AND DISCUSSION

Homogeneous preparations of D-glyceraldehyde-3-phosphate dehydrogenase purified from rabbit muscle were found to contain phosphate which could not be removed under denaturing conditions (protein precipitation and subsequent washings with 5–7% trichloroacetic acid). The endogenous phosphate content varied from 0.5 to 1.1 moles per mole of tetramer in different enzyme preparations obtained by two procedures described in section 2. To determine whether this was due to the presence of NAD^+ remaining bound to the denatured protein, an enzyme sample has been pretreated with [carbonyl-^{14}C]NAD^+ and analyzed for the nucleotide as described in section 2. About 0.16 moles of NAD^+ were detected per mole of enzyme. Corrected for this value, the protein-bound phosphate content was estimated to vary from 0.18 to 0.78 moles per mole of enzyme in different preparations.

A series of experiments was then performed using monoclonal antibodies to phosphotyrosine. As shown in Fig. 1, all enzyme preparations tested were able to interact with antibodies. This suggests that at least part of the endogenously bound phosphate was present in the form of phosphotyrosine. To substantiate such a conclusion, we have carried out a competition Western blotting assay. Nitrocellulose sheets containing the enzyme were incubated with anti-phosphotyrosine antibodies in the presence of different phosphoamino acids. As shown in Fig. 2, only phosphotyrosine could block the antigen–antibody interaction.

Further evidence for phosphorylation of D-glyceraldehyde-3-phosphate dehydrogenase at tyrosine residues was obtained in experiments on immunoprecipitation. Taking into account that a lot of proteins having M_r in the range of 34–39 kDa can serve as substrates for

Fig. 1. Immunoblotting of different D-glyceraldehyde-3-phosphate dehydrogenase preparations. Phosphotyrosine-containing protein bands were stained using monoclonal antibodies to phosphotyrosine and anti-mouse antibodies conjugated with horseradish peroxidase and 1-chloro-4-naphthol as a substrate. (Lanes 1, 3–5) Three different protein preparations obtained by the method of Ferdinand [11]; (lane 2) the preparation obtained by the modified procedure (see section 2). Protein content in the lanes 1–5: 3.6, 3.0, 0.8, 1.5, 1.9 μg, respectively.

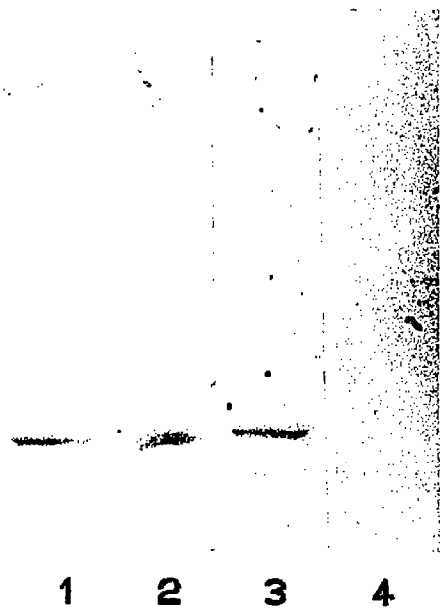

Fig. 2. Effect of phosphoamino acids on the interaction of anti-phosphotyrosine antibodies with D-glyceraldehyde-3-phosphate dehydrogenase. Nitrocellulose filters containing the enzyme (1 μg) were incubated with anti-phosphotyrosine monoclonal antibodies as described in section 2, in the absence of any phosphoamino acid (lane 1), and in the presence of 20 mM phosphoserine (lane 2), 20 mM phosphothreonine (lane 3) or 20 mM phosphotyrosine (lane 4).

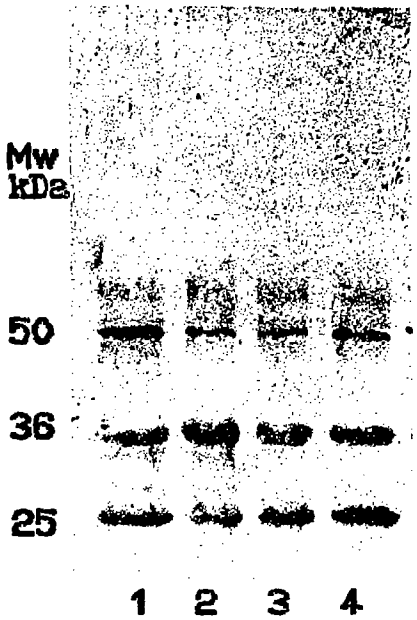

Fig. 3. Immunoblots of protein mixtures obtained after dissociation of D-glyceraldehyde-3-phosphate dehydrogenase–antibody complex. Following immunoprecipitation with monoclonal antibodies (clone G7), the antigen–antibody complex was incubated in the presence of 20 mM Tris-HCl, pH 6.8, 8% SDS, 2.86 M β-mercaptoethanol at 100°C as described in section 2, and subjected to electrophoresis and immunoblotting (see section 2).

tyrosine protein kinases [18], we decided to check whether our highly coloured 36 kDa band could be attributed to D-glyceraldehyde-3-phosphate dehydrogenase and not to some contaminating protein. The immunoprecipitation technique was then used with anti-D-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibodies (see Materials and Methods). The eluates from protein A-Sepharose were subjected to electrophoresis in denaturing 7.5–12.5% polyacrylamide gradient slab gels [12] and transferred to nitrocellulose sheets. After incubation with phosphotyrosine antibodies, protein bands were stained using anti-mouse peroxidase conjugate. As seen in Fig. 3, three protein bands were detected. Two of them represented heavy and light immunoglobulin chains, whereas the third one (36 kDa) could be identified as D-glyceraldehyde-3-phosphate dehydrogenase. According to these data, the enzyme which had been purified by immunoprecipitation retained the capability of interacting with anti-phosphotyrosine monoclonal antibodies. The similarity of results shown in Figs. 1 and 3 indicates that it is D-glyceraldehyde-3-phosphate dehydrogenase (and not a contaminating 36 kDa protein) that is phosphorylated at tyrosine residues.

In conclusion, we have demonstrated that D-glyceraldehyde-3-phosphate dehydrogenase purified from rabbit muscle contains endogenously bound phosphate, part of which belongs to phosphotyrosine. These data are consistent with the idea that phosphorylation of the enzyme at tyrosine residues proceeds in vivo in normal muscle. Further work is needed to clarify the effects of this modification on the properties of the enzyme and on its capability of interacting with other proteins and subcellular structures.

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