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DEPHOSPHORYLATION OF RABBIT SKELETAL MUSCLE GLYCOGEN SYNTHASE BY PHOSPHOPROTEIN PHOSPHATASE AND HUMAN PLACENTAL ALKALINE PHOSPHATASE

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SUMMARY: Phosphorylation of rabbit muscle glycogen synthase by cyclic AMP-dependent protein kinase results in the incorporation of ^{32}P into two major tryptic peptides (P-1 and P-2) which are identified by isoelectric focusing on polyacrylamide gel. When ^{32}P-labeled synthase is incubated with rabbit muscle phosphoprotein phosphatase both P-1 and P-2 are hydrolyzed. Incubation of ^{32}P-labeled synthase with human placental alkaline phosphatase results in a specific hydrolysis of P-1. Measurement of the increase in synthase activity ratio accompanied by the dephosphorylation of P-1 with human placental alkaline phosphatase and, subsequently, of P-2 with phosphoprotein phosphatase shows that both P-1 and P-2 affect the glucose-6-P dependency of the synthase.

Phosphorylation of rabbit skeletal muscle glycogen synthase (EC.2.4.1.11) with cyclic AMP-dependent protein kinase results in the incorporation of 1-2 mol PO_4/subunit (1-9). When ^{32}P-labeled synthase is treated with trypsin under non-denaturating conditions approximately 50-80% (4,6,9) of the ^{32}P becomes soluble in 5% trichloroacetic acid. The 5% trichloroacetic acid-soluble and -precipitable fractions are considered to be derived, respectively, from the "trypsin-sensitive" and "trypsin-insensitive" regions of the enzyme (6,10). Soderling and coworkers (6,10) suggested that one phosphorylation site is located in the "trypsin-sensitive" and the other in the "trypsin-insensitive" regions of the synthase molecule. However, on analysis of the ^{32}P-peptides in the "trypsin-sensitive" fraction by column chromatography, Huang and Krebs (8) and Proud and coworkers (9) demonstrated the presence of two major ^{32}P-peptides which were named P-1 and P-2. The functional role of these phosphorylation sites in the determination of the glucose-6-P dependency of the synthase is uncertain. Proud and coworkers (9) reported that the P-2 from the "trypsin-sensitive" region is responsible for the glucose-6-P dependency of the synthase whereas Soderling and coworkers (6,11) claimed that the responsible

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site is in the "trypsin-insensitive" region. In this study we analyze the ^{32}P-labeled tryptic peptides from synthase, which has been completely digested by trypsin under denaturating conditions, by isoelectric focusing on polyacrylamide gel in the presence of urea. This method enables us to analyze all the ^{32}P-labeled peptides from the synthase and avoids the uncertainty concerning the peptide compositions of the "trypsin-sensitive" and "trypsin-insensitive" fractions. Analyzing the tryptic peptides of synthase dephosphorylated by human placental alkaline phosphatase, which hydrolyzes P-1, and by rabbit muscle phosphoprotein phosphatase, which hydrolyzes both P-1 and P-2, we conclude that both P-1 and P-2 affect the glucose-6-P dependency of the synthase.

MATERIALS AND METHODS. The following compounds were obtained from the indicated sources: UDP-glucose, glucose-6-P, rabbit liver glycogen, ATP, and bovine serum albumin from Sigma; TPCK-treated trypsin from Worthington Biochemical Corp.; Ampholine solution from LKB Instrument; acrylamide, N,N'-methylenebisacrylamide from Bio-Rad Laboratories; and UDP-[^{14}C]glucose and [γ-^{32}P]ATP from New England Nuclear Corp. Glycogen synthase activity was determined by measuring the incorporation of glucose from UDP-[^{14}C]glucose into glycogen (12) as previously described except that the concentration of UDP-glucose was 7 mM. The synthase activity ratio is defined as the activity without glucose-6-P divided by the activity with 8 mM glucose-6-P. ^{32}P-Labeled glycogen synthase was prepared by incubation of I-form synthase (0.1 mg/ml) with cyclic AMP-dependent protein kinase (3000 U/ml) (4) in a reaction mixture containing 25 mM Tris-acetate buffer, pH 8.5; 1 mM dithiothreitol; 2 mM magnesium acetate; 10 μM cyclic AMP; 0.12 mM [γ-^{32}P]ATP. Synthase phosphatase activity was measured at 30° in a reaction mixture containing 50 mM Tris-Cl buffer, pH 7.5; 1 mM dithiothreitol; 5 mM $MnCl_2$; 5% glycerol; 0.1 mg/ml ^{32}P-labeled synthase and phosphatase. ^{32}P-Labeled tryptic peptides were prepared from ^{32}P-labeled synthase according to the published method (13). These peptides were analyzed by isoelectric focusing in LKB Multiphor using 1 mm gel polymerized on a cellophane-covered glass plate. Two plates were prepared from 50 ml of the solution containing 12 ml of 29.1% (w/v) acrylamide solution, 12 ml of 0.9% (w/v) N,N'-methylenenbisacrylamide solution, 24 g urea, 2.5 ml Ampholine pH3.5-10 and 1.25 ml of 1% (w/v) ammonium persulfate. Isoelectric focusing was carried out across the width at 10° with power setting of 30W and maximum voltage of 1500V for 90 min. The anode and cathode solutions were 1M H_3PO_4 and 1M NaOH respectively. After the focusing the pH gradient was measured and the gel was dried in a Bio-Rad slab-gel dryer for 90 min. Autoradiography was carried out with intensifying screen using Kodak XR-1 film and the intensity of the radioactive bands was measured in a Quick Scan Jr. densitometer. Rabbit muscle phosphoprotein phosphatase was purified according to the method of Kato and Bishop (14) and human placental alkaline phosphatase was a gift from Dr. J.Y. Chou.

RESULTS AND DISCUSSION

Phosphorylation of rabbit skeletal muscle glycogen synthase by cyclic AMP-dependent protein kinase results in the incorporation of 1.2-1.5 mol PO_4/subunit

and a decrease in the synthase activity ratio from 0.85 to 0.35. Analysis of the ^{32}P-labeled tryptic peptides from the "trypsin-sensitive" fraction according to the methods of Huang and Krebs (8) and Proud and coworkers (9) confirmed the presence of P-1 and P-2. However, when the "trypsin-insensitive" fraction was analyzed we found predominantly P-1. P-1 and P-2 fractions were separated by isoelectric focusing on polyacrylamide gel in the presence of urea. The P-1 fraction contains a single ^{32}P-labeled peptide while P-2 contains one major and two minor ^{32}P-labeled peptides (9). The apparent isoelectric points of P-1 and the major component of the P-2 fraction are 4.5 and 7.2, respectively. Separation of these two peptides by isoelectric focusing greatly simplify the analysis. Since the gel is dried over filter paper immediately after isoelectric focusing without prior staining or destaining, the radioactivity applied to the gel is quantitatively recovered. The radioactivity in each peptide can be calculated by multiplying the total radioactivity applied to the gel by the relative intensity of each band in the autoradiogram.

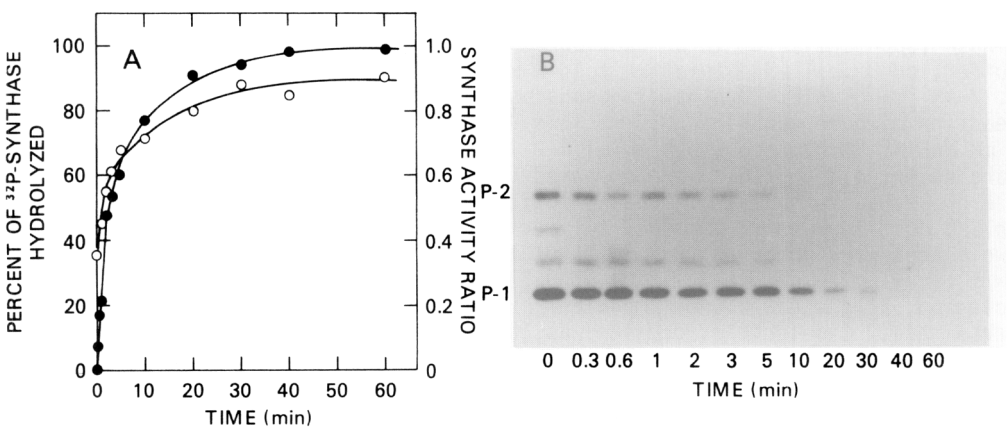


Fig. 1. Dephosphorylation of ^{32}P-labeled synthase by rabbit muscle phosphoprotein phosphatase. Synthase was incubated with phosphatase (0.13 units with phosphorylase a as substrate) in a total reaction mixture of 0.5 ml. At intervals shown samples (2 µl) were taken for the measurement of synthase activity ratio (-O-) in an assay mixture containing 50 mM KF (Fig. A). Other samples (40 ul) were added to the test tubes containing 0.1 ml 10% trichloroacetic acid. Ten ul of bovine serum albumin (10 mg/ml) was added and centrifuged at 9000 X g for 10 min. The percent of ^{32}P-synthase hydrolyzed (-●-) was calculated from the radioactivity present in the trichloroacetic acid-soluble fraction (Fig. A). The trichloroacetic acid-precipitable fraction was washed with 0.1 ml 10% trichloroacetic acid and, subsequently, 0.6 ml ether. It was then incubated with 40 ul of 0.1 M Tris-Cl buffer, pH 8.5, containing 8 M urea and 70 mM dithiothreitol at 37° for 1 hr. Finally, 40 ul water and 10 ul of 4 mg/ml TPCK-treated trypsin were added and the mixture was incubated at room temperature for 24 hr. ^{32}P-Labeled tryptic peptides were analyzed by isoelectric focusing (Fig. B).

Incubation of ^{32}P-labeled synthase with rabbit muscle phosphoprotein phosphatase results in a complete hydrolysis of ^{32}P from the synthase (Fig. 1,A). The dephosphorylation of the synthase is accompanied by an increase in the synthase activity ratio from 0.35 to 0.88. The radioactivities in both P-1 and P-2 are progressively reduced (Fig. 1,B). When ^{32}P-synthase is incubated with human placental alkaline phosphatase, approximately 70% of the total ^{32}P is hydrolyzed (Fig. 2,A). The dephosphorylation is accompanied by an increase in the synthase activity ratio from 0.35 to 0.72. Dephosphorylation of the synthase by this phosphatase takes place predominantly on the P-1 peptide (Fig. 2,B). The hydrolysis of approximately 0.9 mol PO_4/subunit from P-1 is responsible for the increase in the synthase activity ratio from 0.35 to 0.72 (Fig. 3). Addition of rabbit muscle phosphoprotein phosphatase after initial incubation of ^{32}P-labeled synthase with placental alkaline phosphatase results in a further dephosphorylation and an additional increase in the synthase activity ratio (Fig. 4). These results indicate that both P-1 and P-2 affect the glucose-6-P dependency of the synthase. Since the ^{32}P-incorporation into P-1 is approximately 3- to 5-times greater than that into P-2, the dephosphorylation of P-1 is associated with a

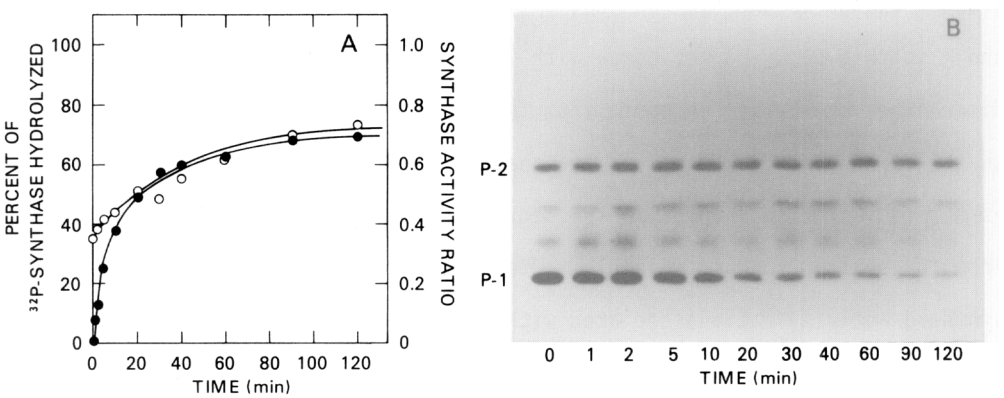
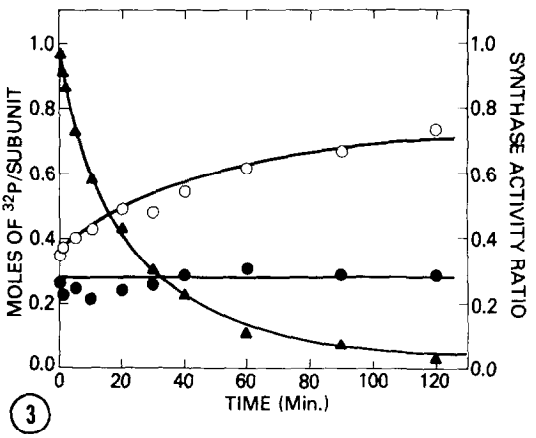


Fig. 2. Dephosphorylation of ^{32}P-labeled synthase by human placental alkaline phosphatase. Synthase was incubated with human placental alkaline phosphatase (80 units with p-nitrophenylphosphate as substrate) under the standard assay condition without $MnCl_2$. The measurement of the change in the synthase activity ratio (-O-) was carried out under the standard assay condition containing 50 mM KF and 36 mM phenylalanine, an inhibitor of placental alkaline phosphatase. Measurement of the percent of ^{32}P-labeled synthase hydrolyzed (-●-) and the analysis of the ^{32}P-labeled tryptic peptides were as described in Fig. 1.



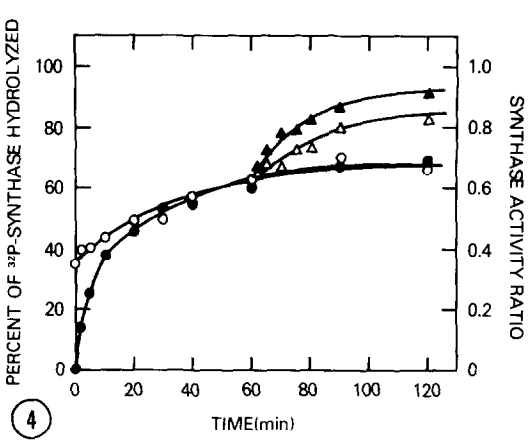


Fig. 3. Measurement of the hydrolysis of ^{32}P from P-1 and P-2 resulted from the incubation of ^{32}P-labeled synthase with human placental alkaline phosphatase. The radioactivities in P-1 (-▲-) and P-2 (-●-) were calculated from the intensity of these two bands in autoradiogram after scanning. The synthase activity ratio (-O-) was obtained as described in Fig. 2.

Fig. 4. Dephosphorylation of ^{32}P-labeled synthase by human placental alkaline phosphatase and, subsequently, by rabbit muscle phosphoprotein phosphatase. Synthase was incubated with alkaline phosphatase (40 units) in a reaction mixture of 0.25 ml under the same conditions described in Fig. 2. After 60 min of incubation an aliquot (0.1 ml) was taken and supplemented with 5 mM $MnCl_2$ and phosphoprotein phosphatase (0.06 units). Samples were taken for the measurements of the hydrolysis of ^{32}P from the synthase and the change in the synthase activity ratio. The symbols are: -●-, percent of synthase phosphate hydrolyzed by alkaline phosphatase alone; -O-, change in the synthase activity ratio by alkaline phosphatase alone; -▲-, percent of synthase hydrolyzed by the combination of two phosphatases; and -△-, the change in the synthase activity ratio by the combination of two phosphatases.

greater change in the synthase activity ratio than that of the P-2. The results shown in this study do not agree with those reported in the literature. The discrepancy may arise, in part, from the differences in the method of analysis. In the current study we analyze the changes in the synthase activity ratio associated with the changes in the degree of phosphorylation of P-1 and P-2 from the entire synthase molecule. Other studies compare the changes in the synthase activity ratio associated with the degree of phosphorylation of peptides derived from the limited trypsinization of the synthase. By employing human placental alkaline phosphatase, which hydrolyzes phosphate on P-1, and phosphoprotein phosphatase, which hydrolyzes phosphates on both P-1 and P-2, the functional roles of P-1 and P-2 in the determination of the glucose-6-P dependency of the synthase are clearly evident. Human placental (15) and E.

coli (16) alkaline phosphatases have been shown to be phosphoprotein phosphatases; however, the substrate specificities of these phosphatases are different. The E. coli alkaline phosphatase, unlike the human placental enzyme, hydrolyzes both P-1 and P-2. The specificity inherent in the human placental alkaline phosphatase should prove useful in defining the functional role of the phosphorylation sites in phosphoproteins.

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