Dephosphorylation of Rabbit Skeletal Muscle Glycogen Synthase (Phosphorylated by Cyclic AMP-independent Synthase Kinase 1) by Phosphatases*

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Phosphorylation of rabbit skeletal muscle glycogen synthase by cyclic AMP-independent synthase kinase 1 results in the incorporation of 4 mol of POJ subunit. Incubation of the phosphorylated synthase with rabbit muscle phosphoprotein phosphatase brings about the hydrolysis of phosphates from all four major tryptic peptides and an increase in the synthase activity ratio from 0.01 to 0.85. Incubation of the phosphorylated synthase with calf intestinal alkaline phosphatase brings about the preferential hydrolysis of phosphates from three of the four major tryptic peptides and a slight increase in the synthase activity ratio from 0.01 to 0.1. The phosphorylation site which is resistant to hydrolysis by calf intestinal alkaline phosphatase can be dephosphorylated by subsequent incubation with rabbit muscle phosphoprotein phosphatase. This dephosphorylation is accompanied by an increase in the synthase activity ratio to approximately 0.9. Measurements of the changes in the kinetic properties of the synthase samples dephosphorylated by alkaline phosphatase reveal that the phosphorylation sites susceptible to hydrolysis by alkaline phosphatase mainly affect the binding of glucose-6-P to the synthase. Comparison of the kinetic properties of the synthase samples dephosphorylated by alkaline phosphatase and by phosphoprotein phosphatase we find that the phosphorylation site resistant to hydrolysis by alkaline phosphatase affects both the binding of UDP-glucose and glucose-6-P to the synthase.

In order to establish whether the multiple phosphorylations of the synthase by multiple kinases have any physiological significance, it is necessary to define the sites phosphorylated by each kinase. This goal can be achieved by the analysis of the amino acid sequence surrounding the phosphorylation sites and the analysis of the change in the state of phosphorylation of these sites on the synthase activity. The identification of the multiple phosphorylation sites by amino acid sequence analysis requires a great effort; nevertheless, some of the sites have been characterized (9-11). In recent studies we found that the multiple sites phosphorylated by cyclic AMP-dependent (12, 13) and -independent (14) kinases can also be characterized by isoelectric focusing of the 32P-labeled tryptic peptides on polyacrylamide gel in the presence of urea. This analytical method, although less precise than the sequence analysis, requires less effort but provides satisfactory identification of the sites phosphorylated by the various kinases.

The phosphorylation of glycogen synthase by cyclic AMP-independent synthase kinase 1 results in the incorporation of approximately 4 mol of POJ subunit (6, 14, 15). By comparing the kinetics of the phosphorylation of these sites with the changes in the properties of the synthase during the phosphorylation reaction, we suggested that one site affects the binding of UDP-glucose, and three sites affect the binding of glucose-6-P to the synthase. This conclusion, however, was not definite because it was difficult to study the effect of phosphorylation of a site on the properties of the synthase without the influence by the other site. In the present study, we find that calf intestinal alkaline phosphatase preferentially dephosphorylates three of the four sites. The site which is resistant to hydrolysis by alkaline phosphatase can be further dephosphorylated by rabbit muscle phosphoprotein phosphatase. Analysis of the changes in the properties of the synthase samples which have been dephosphorylated by these phosphatases will further characterize the effect of phosphorylation of these sites on the properties of the synthase.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following compounds were obtained from the indicated sources: UDP-glucose, glucose-6-P, rabbit liver glycogen, ATP, and bovine serum albumin from Sigma; tosylphenylalanine chloromethyl ketone (TIPCK)-treated trypsin from Worthington Biochemical Corp.; Ampholine solutions from LKB Instruments; acrylamide and N,N'-methylenebisacrylamide from Bio-Rad Laboratories; UDP-[14C]glucose and [γ-32P]ATP from New England Nuclear Corp., and calf intestinal alkaline phosphatase (470 units/mg) from Boehringer Mannheim Biochemicals.

**Methods**—Glycogen synthase activity was determined by measuring the incorporation of glucose from UDP-[14C]glucose into glycogen as described (16). The reaction mixture contained 25 mm glycyglycine buffer (pH 7.5), 8 mm EDTA, 24 mg/ml of rabbit liver glycogen, 0.08 mg/ml of bovine serum albumin, 25 mm UDP-[14C]glucose (400 CPM/
channel of the scintillation counter at a 2-week interval (the half-life of the sample was corrected by counting the synthase assay samples in the I4C ratio is defined as the activity without glucose-6-P divided by the synthase.

accounts for 50% of the total interference caused by "P). The reduction in the counts per min after a two-week period is believed to be caused by the hydrolysis of 1 nmol of phosphate [32P]synthase phosphorylated by synthase kinase, and phosphatase.

rabbit muscle phosphoprotein phosphatase. 32P-labeled synthase was phosphorylated by synthase kinase, and the presence or absence of 40 nM glucose-6-P. Other samples were prepared by incubation of [32P]synthase with calf intestinal alkaline phosphatase. 32P-labeled synthase was phosphorylated by synthase kinase, and phosphatase.

mercaptoethanol 5 mM MnCl2; 5% glycerol; 0.1 to 0.2 mg/ml of [32P]synthase, and alkaline phosphatase. Phosphoprotein phosphatase activity was measured at 30°C in a reaction mixture containing 50 mM Tris-C1 buffer, pH 7.5; 50 mM mercaptoethanol; 5 mM MnCl2; 5% glycerol; 5 mM MgCl2; 0.1 to 0.2 mg/ml of [32P]synthase phosphorylated by synthase kinase, and phosphatase.

The reduction in the counts per min after a two-week period accounts for 50% of the total interference caused by [32P]-synthase.

Phosphoprotein phosphatase activity was measured at 30°C in a reaction mixture containing 50 mM Tris-C1 buffer, pH 7.5; 50 mM mercaptoethanol; 5% glycerol; 5 mM MnCl2; 5% glycerol; 5 mM MgCl2; 0.1 to 0.2 mg/ml of [32P]synthase phosphorylated by synthase kinase, and phosphatase.

One unit of phosphatase is defined as the amount of enzyme which hydrolyzes 1 nmol of phosphate from [32P]-protein/min at 30°C. De-phosphorylation of [32P]synthase by calf intestinal alkaline phosphatase was carried out in a reaction mixture containing 50 mM Tris-C1 buffer, pH 7.5; 1 mM dithiothreitol; 5% glycerol; 5 mM MgCl2; 0.1 to 0.2 mg/ml of [32P]synthase, and alkaline phosphatase.

I-form glycogen synthase from rabbit skeletal muscle was purified as described (17) except that the homogenizing buffer was 50 mM Tris-C1, pH 8.2, containing 1 mM dithiothreitol, 1 mM EDTA, 2 mM ethyleneglycol bis(β-aminetho)ether)N,N,N',N'-tetraacetate, and 0.05 mM phenylmethylsulfonyl fluoride. The purified synthase has a specific activity of approximately 40 units/mg and shows a single protein band upon gel electrophoresis in the presence of sodium dodecyl sulfate. Rabbit muscle phosphoprotein phosphatase was purified according to the method of Kato and Bishop (18).

[32P]-labeled glycogen synthase was prepared by incubation of I-form synthase with cyclic AMP-independent synthase kinase 1 (15) in a total volume of 5 ml under the standard assay conditions (14). After the phosphorolytic reaction, 500 mg of Dowex-1 was added. This mixture was kept at 0°C for 30 min with occasional shaking and was filtered through a disposable pipet fitted with glass wool. The solution was dialyzed extensively against 50 mM Tris-C1 buffer, pH 7.5, containing 1 mM dithiothreitol and 5% glycerol and was concentrated to approximately 0.4 mg/ml. The [32P]-labeled synthase contained approximately 4 mol of PO4/subunit. [32P]-labeled tryptic peptides were analyzed by isoelectric focusing on polyacrylamide gel as previously described (14) except that the concentration of urea in the gel was 6 M and the time of focusing was 2.5 h.

The synthase samples used for the measurement of the kinetic properties were prepared by incubation of synthase, which had been phosphorylated with kinase in the presence of nonradioactive ATP, with phosphatases for the various lengths of time. The phosphorylase reaction was terminated by the addition of 10 mM EDTA and 60 mM KF. The solution was dialyzed against 50 mM Tris-C1 buffer, pH 7.5, containing 1 mM dithiothreitol, 5% glycerol, 5 mM EDTA, and 50 nitrophenylphosphate as substrate) in 0.625 ml of reaction mixture under standard assay conditions. The measurements of the per cent of [32P]synthase hydrolyzed (●●●● and the changes in the synthase activity ratio (○○○○) were the same as described in Fig. 1.

FIG. 3 (right). Hydrolysis of 32P from four major 32P-labeled tryptic peptides by the incubation of [32P]synthase with calf intestinal alkaline phosphatase. The reaction was carried out under the same assay condition described in Fig. 2. The trichloroacetic acid-precipitable fractions were subjected to tryptic digestion and isoelectric focusing (14). Since over 90% of the radioactivity applied was retained in the gel, the distribution of 32P in the various peptides was calculated by multiplying the total 32P incorporation/subunit with the per cent of the total intensity in each band after scanning the autoradiogram. These results represent the average values from two experiments. The symbols are: ●, PLTP-1; A, PLTP-2; ●, PLTP-3 and ○, PLTP-4.

mm KF. The A550 for glucose-6-P and S40 for UDP-glucose were determined as previously described (14).

RESULTS

De-phosphorylation of [32P]Synthase by Rabbit Muscle Phosphophoprotein Phosphatase and Calf Intestinal Alkaline Phosphatase—Phosphorylation of I-form glycogen synthase with cyclic AMP-independent synthase kinase 1 results in the incorporation of approximately 4 mol of PO4/subunit. Incubation of [32P]synthase with rabbit muscle phosphoprotein phosphatase brings about a hydrolysis of over 90% of the 32P from the synthase (Fig. 1). This hydrolysis is accompanied by an increase in the synthase activity ratio from 0.01 to 0.85. Analysis of the 32P-labeled tryptic peptides after the dephosphorylation of [32P]synthase reveals that the phosphates on the four major tryptic peptides are hydrolyzed at a comparable rate. The rate of the increase in the synthase activity ratio is slower than that of the hydrolysis of 32P from the synthase.

Incubation of [32P]synthase with calf intestinal alkaline phosphatase results in a hydrolysis of approximately 70% of the 32P from the synthase and a slight increase in the synthase activity ratio from 0.01 to 0.1 (Fig. 2). The increase in the synthase activity ratio is slower than the hydrolysis of 32P from the synthase.

incubation of [32P]synthase with calf intestinal alkaline phosphatase results in a hydrolysis of approximately 70% of the 32P from the synthase and a slight increase in the synthase activity ratio from 0.01 to 0.1 (Fig. 2). The increase in the synthase activity ratio is slower than the hydrolysis of 32P from the synthase. These results indicate that the phosphorylation site(s) affecting the synthase activity ratio is more resistant to hydrolysis by alkaline phosphatase than the rest of the sites. It has been shown previously that the phosphorylation of glycogen synthase by cyclic AMP-independent synthase kinase 1 results in the incorporation of 32P into four major tryptic peptides named PLTP-1, -2, -3, and -4 (14). Analysis of the hydrolysis of 32P from these peptides after the incubation of [32P]synthase with alkaline phosphatase shows that PLTP-1, -2, and -4 are hydrolyzed at a faster rate than that of PLTP-3 (Fig. 3). After 90 min of incubation, over 90% of the phosphates from PLTP-1, -2, and -4 are hydrolyzed. In contrast, approximately 10 to 15% of the phosphate from PLTP-3 is hydrolyzed during the same period of incubation.

De-phosphorylation of [32P]synthase by rabbit muscle phosphoprotein phosphatase after the initial incubation of the synthase with calf intestinal alkaline phosphatase results in a

The abbreviation used is: PLTP, 32P-labeled tryptic peptides.
Dephosphorylation of Glycogen Synthase

Further hydrolysis of phosphate from the synthase (Fig. 4). The amount of phosphate hydrolyzed by the addition of phosphoprotein phosphatase accounts for approximately 25% of the total 32P in the synthase. This additional dephosphorylation is accompanied by a significant increase in the synthase activity ratio form 0.05 to 0.9. These results indicate that the phosphorylation site (PTLP-3) which is resistant to hydrolysis by calf intestinal alkaline phosphatase but susceptible to subsequent hydrolysis by phosphoprotein phosphatase primarily affects the synthase activity ratio.

Changes in the Kinetic Properties of the Synthase after Dephosphorylation with Calf Intestinal Alkaline Phosphatase and Rabbit Muscle Phosphoprotein Phosphatase—Since the dephosphorylation of the synthase by calf intestinal alkaline phosphatase results in the hydrolysis of phosphates from PTLP-1, -2, and -4, it is possible to determine the collective effect of the phosphorylation of these three sites on the synthase activity. The effect of phosphorylation of PTLP-3 (which is resistant to hydrolysis by alkaline phosphatase) on the activity of the synthase, can be determined by comparing the properties of the synthase samples dephosphorylated by alkaline phosphatase, and by phosphoprotein phosphatase. The extent of dephosphorylation of these samples was determined in parallel experiments using [32P]synthase as substrate. The synthase activity ratio of the phosphorylated enzyme is 0.01 and the A20 (the activator concentration which gives 50% of the maximum activity) for glucose-6-P and S20 (the substrate concentration which gives 50% of the maximum activity) for UDP-glucose in the absence of glucose-6-P are 3.2 mm and 23 mm, respectively (Table I). Incubation of the phosphorylated synthase with calf intestinal alkaline phosphatase for 15 min, which brings about a hydrolysis of approximately 50%, of the total phosphate from the synthase, results in a slight increase in the synthase activity ratio to 0.03 and a decrease in A20 for glucose-6-P to 0.8 mm. The change in the S20 for UDP-glucose either in the presence or absence of glucose-6-P appears to be insignificant. These results indicate that the dephosphorylation of PTLP-1, -2, and -4 by the alkaline phosphatase primarily affects the A20, for glucose-6-P. Further incubation of the synthase with the alkaline phosphatase for 90 min, which brings about a hydrolysis of approximately 70% of the total phosphate from the synthase, results in an increase in the synthase activity ratio to 0.1 and the decreases in A20, for glucose-6-P and S20 for UDP-glucose (without glucose-6-P) to 0.4 mm and 18 mm, respectively. This synthase sample contains phosphate predominantly on PTLP-3. When the synthase is incubated with rabbit muscle phosphoprotein phosphatase for 120 min, the synthase activity ratio is increased to 0.85 and the A20, for glucose-6-P and S20 for UDP-glucose (without glucose-6-P) are decreased to 0.02 mm and 2.3 mm, respectively. These data indicate that the presence of phosphate on PTLP-3 not only affects the synthase activity ratio and S20 for UDP-glucose (without glucose-6-P) but also the A20 for glucose-6-P. The S20 values for UDP-glucose in the presence of glucose-6-P are only slightly influenced by the state of phosphorylation of the synthase. When UDP-glucose concentrations are varied the Hill’s coefficient, n, is not affected by the state of phosphorylation. When

FIG. 4 Dephosphorylation of 32P-labeled synthase by calf intestinal alkaline phosphatase and, subsequently, by rabbit muscle phosphoprotein phosphatase. 32P-labeled synthase was incubated with alkaline phosphatase under the same conditions described in Fig. 2. After 40 min of incubation, an aliquot (0.1 ml) was taken and supplemented with 50 mM MnCl2, 50 mM mercaptoethanol, and phosphoprotein phosphatase (0.05 unit). Samples were taken for the measurements of the hydrolysis of 32P from the synthase and the change in the synthase activity ratio. The symbols are: ●—●, per cent of synthase phosphate hydrolyzed by alkaline phosphatase alone; ○—○, change in the synthase activity ratio by alkaline phosphatase alone; △—△, per cent of synthase phosphate hydrolyzed by the combination of two phosphatases; and Δ—Δ, change in the synthase activity ratio by the combination of two phosphatases.

<table>
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<tr>
<th>Enzyme</th>
<th>Synthase activity ratio</th>
<th>A20 for glucose-6-P</th>
<th>mM</th>
<th>In the presence of 20 mM glucose-6-P</th>
<th>mM</th>
<th>In the presence of glucose-6-P</th>
<th>mM</th>
</tr>
</thead>
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<tr>
<td>Phosphorylated synthase</td>
<td>0.01</td>
<td>3.2</td>
<td>0.85</td>
<td>0.9</td>
<td>0.85</td>
<td>23</td>
<td>0.83</td>
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<tr>
<td>Phosphorylated synthase incubated with calf intestinal alkaline phosphatase for 15 min</td>
<td>0.03</td>
<td>0.8</td>
<td>0.95</td>
<td>0.8</td>
<td>0.87</td>
<td>20</td>
<td>0.86</td>
</tr>
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<td>Phosphorylated synthase incubated with calf intestinal alkaline phosphatase for 90 min</td>
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<td>0.4</td>
<td>1.0</td>
<td>0.6</td>
<td>0.85</td>
<td>18</td>
<td>0.83</td>
</tr>
<tr>
<td>Phosphorylated synthase incubated with phosphoprotein phosphatase for 120 min</td>
<td>0.85</td>
<td>0.02</td>
<td>1.1</td>
<td>0.5</td>
<td>0.92</td>
<td>2.3</td>
<td>0.88</td>
</tr>
</tbody>
</table>

TABLE 1
Changes in the kinetic properties of the synthase after dephosphorylation with calf intestinal alkaline phosphatase and rabbit muscle phosphoprotein phosphatase

The conditions for the dephosphorylation of the synthase by phosphoprotein phosphatase and calf intestinal alkaline phosphatase were the same as those described in Figs. 1 and 2, respectively. The kinetic data were the average of two duplicated measurements which fluctuate within 20% of the averaged values. For the determination of S20, the concentrations of UDP glucose were varied between 0.1 and 10 mm. For the determination of A20, the concentrations of glucose-6-P were varied between 0.01 and 40 mm and the concentration of UDP-glucose was kept at 4 mm. n is the Hill’s coefficient.
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When glucose-6-P concentrations are varied, the n values are slightly increased as the synthase becomes less phosphorylated.

DISCUSSION

Dephosphorylation of rabbit muscle glycogen synthase, which has been phosphorylated by cyclic AMP-independent synthase kinase 1, by calf intestinal alkaline phosphatase results in a preferential hydrolysis of phosphates from sites affecting the binding of glucose-6-P to the synthase. In contrast, rabbit muscle phosphoprotein phosphatase dephosphorylates the sites affecting both the binding of UDP-glucose and glucose-6-P. In comparison of the properties of synthase dephosphorylated by alkaline phosphatase with that dephosphorylated by phosphoprotein phosphatase, we find that PLTP-3 site affects both the binding of UDP-glucose and glucose-6-P. It appears that the phosphorylation of PLTP-3 results in a conformational change which hinders the binding of both glucose-6-P and UDP-glucose. This conformational change also facilitates the phosphorylation of the other three sites (PLTP-1, -2, and -4) by the synthase kinase (14). The changes in the properties of synthase accompanied by the dephosphorylation of PLTP-3 are different from those caused by the phosphorylation of P-1 and P-2 with cyclic AMP-dependent protein kinase (12). The phosphorylation of P-1 and P-2 sites mainly affects the binding of UDP-glucose but only slightly influences the binding of glucose-6-P (A_{o.5} increased from 0.02 mM to 0.05 mM). The collective effect of the dephosphorylation of PLTP-1, -2, and -4 is a decrease in A_{o.5} for glucose-6-P; however, the individual function of these sites remains unsolved.

The substrate specificity inherent in calf intestinal alkaline phosphatase will be useful in determining the functional role of the phosphorylation sites in phosphoproteins. In recent studies, we found that both human placental (13) and calf intestinal (12) alkaline phosphatases selectively hydrolyze phosphate from one of the two sites on glycogen synthase phosphorylated by cyclic AMP-dependent protein kinase. In the present study, we find that calf intestinal alkaline phosphatase preferentially hydrolyzes three of the four sites phosphorylated by cyclic AMP-independent synthase kinase 1. We also observe that this alkaline phosphatase hydrolyzes one of the two major sites on histone H2B phosphorylated by cyclic AMP-dependent protein kinase. It is likely that some kind of specificity can be detected when the other phosphoproteins are dephosphorylated by calf intestinal alkaline phosphatase. The functional role of the phosphorylation sites susceptible and resistant to hydrolysis by alkaline phosphatase can be defined by determination of the changes in the properties of an enzyme dephosphorylated with calf intestinal alkaline phosphatase first and then followed by the dephosphorylation with phosphoprotein phosphatase.

The phosphorylation of glycogen synthase at the P-1 and P-2 sites by cyclic AMP-dependent protein kinase brings about a decrease in the synthase activity ratio from 0.85 to 0.35 and an increase in S_{o.5} for UDP-glucose (assayed without glucose-6-P) from 2 mM to 10 mM (12). However, the phosphorylation of these two sites only slightly affects the A_{o.5} for glucose-6-P (increased from 0.02 mM to 0.05 mM). The phosphorylation of the synthase by cyclic AMP-independent synthase kinase 1 results in the decrease in the synthase activity ratio from 0.85 to 0.01 and the increases in S_{o.5} for UDP-glucose (assayed without glucose-6-P) from 2 mM to 20 mM and A_{o.5} for glucose-6-P from 0.02 mM to 4 mM (14). The most significant difference between synthase samples phosphorylated by these two kinases is the A_{o.5} for glucose-6-P. Therefore, measurement of the kinetic properties of the synthase samples of unknown phosphorylation sites will provide some information concerning the involvement of these two kinases in the phosphorylation of the synthase. Additional information concerning the involvement of these two kinases in the phosphorylation of the synthase can be obtained by measuring the changes in the properties of the synthase samples after the dephosphorylation with calf intestinal alkaline phosphatase. Although glycogen synthase is known to be phosphorylated by multiple kinases in vitro, the physiological significance of these kinases in vivo is unknown. Until all the phosphorylation sites on the synthase are defined, it would be difficult to evaluate the importance of these kinases in the phosphorylation of the synthase in vivo.

REFERENCES


2 Z. Ahmad and K.-P. Huang, unpublished results.