Total Conversion of Glycogen Synthase from the I- to the D-Form by a Cyclic AMP-independent Protein Kinase from Rabbit Skeletal Muscle

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A newly discovered cyclic AMP-independent protein kinase, which catalyzes the total conversion of glycogen synthase from the I- to the D-form, has been isolated from rabbit skeletal muscle. This enzyme, designated glycogen synthase kinase, is separable from cyclic AMP-dependent protein kinase by column chromatography on phosphocellulose. Synthase kinase and cyclic AMP-dependent protein kinase are distinct in their specificity for protein substrates, the effects of cyclic AMP and the inhibitor of cyclic AMP-dependent protein kinase on their activities, and the extent to which they phosphorylate I-form glycogen synthase. The phosphorylation of I-form enzyme by synthase kinase results in the incorporation of 4 mol of phosphate/85,000 subunit; however, only two of the phosphate sites seem predominantly to determine glucose-6-P dependence. The resulting multiply phosphorylated enzyme, which is highly dependent on glucose-6-P for activity, has a glucose content comparable to the D-form enzyme isolated from rabbit muscle.

The existence of multiple phosphorylation sites on glycogen synthase was proposed by Larner and co-workers (6, 8) as a result of the chemical analysis of alkali-labile phosphate of purified D-form enzyme. Although the variation ranges between three and six phosphates per 85,000 subunit, these states of phosphorylation cannot be readily achieved by incubation of I-form enzyme with purified cyclic AMP-dependent protein kinase (7), phosphokinase (12), or the combination of these two types of kinases (12). In the present communication we described the isolation of a new cyclic AMP-independent protein kinase which multiply phosphorylates I-form glycogen synthase resulting in a total conversion to the glucose-6-P-dependent form.

MATERIALS AND METHODS

UDP-glucose, glucose-6-P, rabbit liver glycogen, cyclic AMP, type II-A histone (histone mixture), and phosphokinase were obtained from Sigma, vitamin-free casein from Nutritional Biochemicals, and [γ-32P]ATP from New England Nuclear Corp. Glycogen synthase activity was determined by following the incorporation of glucose from UDP-[14C]glucose into glycogen as previously described (14) except that the incubation was at 37°. The ratio of independence, RI, is defined as the activity without glucose-6-P divided by the activity with 8 mM glucose-6-P, the result being multiplied by 100. I-form glycogen synthase from rabbit muscle was purified as previously described (9) except that the D-to-I conversion was carried out at a later stage of purification to prevent proteolysis (15). The heat-stable inhibitor of cyclic AMP-dependent protein kinase was purified through the DEAE-cellulose chromatography step according to the method of Asbhy and Walsh (16).

Protein kinase activity was measured at 37° in a reaction mixture (0.2 ml) containing 25 mM β-glycerol-P, pH 7.0; 1 mM dithiothreitol; 0.5 mM EDTA; 8 mM magnesium acetate; 20 mM cyclic AMP; 0.3 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N ′-tetraacetate; 0.125 mM [γ-32P]ATP (400 to 1000 cpm/pmol); either 800 μg of histone, 800 μg of phosphitin, or the indicated amount of I-form glycogen synthase; and protein kinase. At timed intervals samples (10 μl) were removed, and the resulting 32P-labeled phosphoprotein was separated from [γ-32P]ATP by TLC chromatography (17). One unit of activity is defined as the amount catalyzing the transfer of 1 nmol of phosphate from [γ-32P]ATP to histone or phosphitin per min at 37°. Gel electrophoresis in the presence of sodium dodecyl sulfate was carried out according to the method of Weber and Osborn (18). Protein concentration was determined by the method of Lowry et al. (19).

RESULTS

Separation of Protein Kinases by Column Chromatography on Phosphocellulose—Two hundred grams of fresh rabbit skeletal muscle were homogenized for 1.5 min in a Waring Blender with 600 ml of 50 mM Tris/Cl buffer, pH 7.5, containing 1 mM...
The solution was stirred at 4°C for 20 min and centrifuged at 10,000 × g for 20 min. Rabbit liver glycogen was added to the supernatant fluid to obtain a final concentration of 1 mg/ml. The solution was stirred at 4°C for 20 min and centrifuged at 140,000 × g for 60 min. The supernatant fluid from the high speed centrifugation was adjusted to 60% saturation with solid ammonium sulfate. The precipitate was dissolved in 180 ml of 45 mM Tris/Cl buffer, pH 7.5, containing 1 mM dithiothreitol and 5% glycerol (Buffer A). After extensive dialysis against Buffer A, the solution was applied to a phosphocellulose (Whatman P-11) column (2.5 x 10 cm) previously equilibrated with the same buffer. Several peaks of protein kinase activity were identified upon chromatography on DEAE-cellulose (20) and hydroxylapatite (21). The protein kinases recovered from P-1 were mainly two types (muscle and heart types) of cyclic AMP-dependent protein kinases (22). P-2, which contained both phosvitin and histone kinase activities, was relatively unstable, and no further purification and characterization were attempted. P-3, P-4, and P-5 were three well separated phosvitin kinases which had no detectable histone kinase activity. We suggest the designation glycogen synthase kinase for P-3, which phosphorylates I-form glycogen synthase resulting in a total conversion to D-form. To a small extent, P-4 and P-5 also phosphorylate I-form enzyme. A detailed study of these two enzymes will be reported later.

Comparison of Reactions Catalyzed by Synthase Kinase and Cyclic AMP-dependent Protein Kinase—As judged by assays with phosvitin as substrate in the absence of cyclic AMP, the specific activity of the purified synthase kinase was 1000 times greater than that of the crude extract. With this purified enzyme, the incorporation of 32P into casein or phosvitin progressively increased with the time of incubation (Fig. 2A). The reaction rates were independent of cyclic AMP. Histone was poorly phosphorylated. On the other hand, with cyclic AMP-dependent protein kinase (muscle type) the rate of 32P incorporation into histone was increased nearly 20-fold by the addition of cyclic AMP, and phosvitin and casein were poorly phosphorylated (Fig. 2B). The phosphorylation of histone or I-form glycogen synthase by cyclic AMP-dependent protein kinase was inhibited by the inhibitor of cyclic AMP-dependent protein kinase, but the phosphorylation of phosvitin or glycogen synthase by synthase kinase was unaffected (Fig. 2, C and D). Synthase kinase was also different from phosvitin kinases P-4 or P-5 in many respects (data not shown).

Phosphorylation of I-Form Glycogen Synthase by Synthase Kinase and Cyclic AMP-dependent Protein Kinase—In the presence of cyclic AMP, both kinases catalyzed the incorporation of 32P from [γ-32P]ATP into I-form enzyme (Fig. 2). Incorporation of I-form enzyme without either synthase kinase or cyclic AMP-dependent protein kinase resulted in no 32P incorporation. Incubation of I-form enzyme with both synthase kinase and cyclic AMP-dependent protein kinase resulted in the same level of 32P incorporation obtained with synthase kinase alone. The stoichiometry of 32P incorporation by cyclic AMP-dependent protein kinase averaged 2 mol/mol of 85,000 subunit, and the 32P incorporation by synthase kinase averaged 4 mol/mol of subunit. Cyclic AMP did not affect the incorporation of 32P catalyzed by synthase kinase. A combination of both kinases in the absence of cyclic AMP also gave the same level of 32P incorporation as did the synthase kinase alone. The 32P incorporation into I-form enzyme by cyclic AMP-dependent protein kinase in the absence of cyclic AMP was negligible. Analysis by sodium dodecyl sulfate-gel electrophoresis of the reaction...
Cyclic AMP-independent Glycogen Synthase Kinase

Fig. 3. Phosphorylation of purified I-form glycogen synthase by cyclic AMP-dependent protein kinase and glycogen synthase kinase. A reaction mixture (0.2 ml) containing purified I-form glycogen synthase (0.06 mg/ml) was incubated with: 0.1 unit of glycogen synthase kinase in the absence (○—○) or presence (●—●) of cyclic AMP, 0.1 unit of cyclic AMP-dependent protein kinase in the absence (△—△) or presence (■—■) of cyclic AMP, 0.1 unit of glycogen synthase kinase plus 0.1 unit of cyclic AMP-dependent protein kinase either in the absence (□—□) or presence (■—■) of cyclic AMP. In all experiments, reactions were initiated by the addition of [γ-32P]ATP (600 to 1000 cpm/μmol).

mixture incubated for 2 h in the presence of synthase kinase (Fig. 4) showed that all the 32P incorporated was recovered in the position corresponding to the subunit of glycogen synthase. The control experiment in the absence of I-form enzyme showed no radioactivity in the gel (Fig. 4).

Conversion of Glycogen Synthase I to D by Incubation with Synthase Kinase and Cyclic AMP-dependent Protein Kinase—Changes in glucose-6-P dependence (RI) were determined under the same experimental conditions as described for Fig. 3, with the exception that nonradioactive ATP was used (Fig. 5). Incubation of I-form enzyme with cyclic AMP-dependent protein kinase in the presence of cyclic AMP resulted in a decrease in RI (from 62 to 40), but the reduction in RI by synthase kinase was more rapid and complete (from 62 to 5). When the glycogen synthase activity ratio was obtained by measuring the activity at 30°C in the presence of 10 mM Na2SO4 divided by the activity in the presence of 8 mM glucose-6-P (23), the RI change caused by cyclic AMP-dependent protein kinase was from 102 to 60, and the change caused by synthase kinase was from 102 to 10. The decrease in RI by synthase kinase (Fig. 5) was not parallel to the rate of 32P incorporation (Fig. 3). It was estimated that the incorporation of the first 2 mol of 32P (during 15 min of incubation) produced a rapid decrease in RI (from 62 to 15); the subsequent incorporation of 2 mol of 32P only slightly decreased the RI (from 15 to 5).

Addition of both synthase kinase and cyclic AMP-dependent protein kinase showed the same pattern of decrease in RI as when synthase kinase was added alone (Fig. 5). The control experiment in the absence of added kinase showed no change in RI. In the absence of cyclic AMP, synthase kinase was fully active, but cyclic AMP-dependent protein kinase was virtually inactive.

DISCUSSION

Recently it has been reported that the phosphorylation of glycogen synthase can be achieved by kinase(s) other than cyclic AMP-dependent protein kinase. This has been described for renal medulla (11), renal cortex (13), and rabbit muscle (12). Nevertheless, neither of these kinases alone (7, 12) nor their combination (12) can readily achieve a total I-to-D conversion and a state of phosphorylation similar to that described for the purified D-form enzyme (6, 8).

In this communication we show that multiple forms of rabbit muscle protein kinase can be separated by chromatography on phosphocellulose. One of these kinases, P-3, a cyclic AMP-independent phosphitin kinase, can achieve a total conversion of glycogen synthase I to D. This enzyme, synthase kinase, is distinct from cyclic AMP-dependent protein kinases in its substrate specificity, the effects of cyclic AMP and the inhibi-
tor of protein kinase on their activities, and the extent to which they phosphorylate I-form glycogen synthase. It is unlikely that synthase kinase is derived from cyclic AMP-dependent protein kinases. As indicated by the extent of phosphorylation and degree of conversion obtained, synthase kinase is also different from the previously described phosvitin kinase (12), which corresponds to the P-4 kinase shown in Fig. 1.

The amount of $^{32}$P incorporated by this newly described kinase averages 4 mol/85,000 subunit, which is approximately twice as much as by cyclic AMP-dependent protein kinase. The combination of both kinases does not result in additive $^{32}$P incorporation. It is likely that the phosphorylation sites of cyclic AMP-dependent protein kinase overlap with the sites of synthase kinase. Only two of the four phosphorylation sites greatly influence glucose-6-P dependence. This conclusion is based on the observation that the incorporation of the first 2 mol of $^{32}$P into glycogen synthase is accompanied by a greater change in RI than is the subsequent $^{32}$P incorporation. It is not known whether this disproportionate change in RI is due to a preferential phosphorylation of two specific sites or to a random phosphorylation of any two sites. Although the additional phosphorylation does not contribute to a significant change in RI, these phosphate groups may significantly affect the properties of the resultant enzyme (24, 25). Incubation of I-form enzyme with cyclic AMP-dependent protein kinase results in the incorporation of 2 mol of $^{32}$P per subunit; however, the RI change is only about one-half of that obtained with synthase kinase at the point where the $^{32}$P incorporation is also 2 mol/subunit. Therefore, it is suggested that only one of the phosphorylation sites of cyclic AMP-dependent protein kinase determines glucose-6-P dependence. The extent of phosphorylation and the resulting change in RI achieved with cyclic AMP-dependent protein kinase are comparable to those reported by Roach and co-workers (24). The existence of two phosphorylation sites which predominantly determine glucose-6-P dependence was also demonstrated by the sequential phosphorylation of I-form enzyme by a phosvitin kinase followed by a cyclic AMP-dependent protein kinase (12). Soderling (7), using a relatively large quantity of a preparation of cyclic AMP-independent protein kinase, obtained a virtually complete conversion of synthase I kinase activity while the tissue levels of cyclic AMP remain unchanged (27). These results strongly suggest that cyclic AMP-independent protein kinase may play an important role in vivo. The data in this study as well as those in the literature further indicate the complexity of the regulation of glycogen synthase. Similar complexity may also exist in other regulatory systems where phosphorylation and dephosphorylation reactions are involved.

REFERENCES

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