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Glycogen Synthesis from the Anomers of Glucose in Rat Diaphragm

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It was examined whether or not a rate-limiting anomerization step is present in the pathway of glycogen synthesis from glucose, in which phosphoglucomutase is known to act specifically on the α anomer of glucose 6-phosphate. Glycogen synthesis from the α and β anomers of D-[U-^{14}C]glucose in rat hemidiaphragms was measured by aerobic incubation in Krebs–Ringer bicarbonate buffer (pH 7.4) at 37 °C for short periods (1 or 3 min) in the presence or absence of insulin (10 mU/ml). There was no statistically significant difference between the rates of glycogen synthesis from the two anomers of glucose under various conditions. These findings suggest that there is no rate-limiting anomerization step in the pathway of glycogen synthesis from glucose in the muscle.

Keywords——glycogen synthesis; glucose anomer; diaphragm; rate-limiting anomerization step; glucose 6-phosphate; rat

We previously reported that the glucose-transport system of rat diaphragm is not preferential for either anomer of glucose under aerobic conditions, whether in the presence or absence of insulin.[1] We also reported that the activity of mutarotase [EC 5.1.3.3], an enzyme catalyzing the mutarotation of glucose, glucose 6-phosphate, and other sugars, in the rat skeletal muscle is negligibly low[2] and that rat muscle hexokinase [EC 2.7.1.1] (type II) is only slightly preferential for the β anomer of glucose (V_{max} for the β anomer/V_{max} for the α anomer = 1.5).[3] These findings suggest that glucose 6-phosphate is produced slightly faster from β-D-glucose than from the α-anomer when rat diaphragm is incubated with either anomer of D-glucose. On the other hand, hexokinase is known to produce α- and β-D-glucose 6-phosphate from α- and β-D-glucose, respectively.[4] Muscle phosphoglucomutase [EC 2.7.5.1] acts specifically on the α anomer of glucose 6-phosphate, yielding α-D-glucose 1-phosphate.[5] In addition, glucose 1-phosphate uridyltransferase [EC 2.7.7.9] catalyzes a reaction between α-D-glucose 1-phosphate and uridine 5′-triphosphate to produce uridine diphosphate glucose and pyrophosphate.

Therefore, there seems to be a possibility that glycogen is synthesized preferentially from the α anomer of glucose if the anomerization of the β anomer of glucose 6-phosphate to the α anomer is the rate-limiting step in glycogen synthesis. It is not yet known, however, whether or not a rate-limiting anomerization step is present in glucose metabolism in tissues including muscle.

We report here that the rates of glycogen synthesis from the α and β anomers of glucose in isolated rat diaphragm muscle are not significantly different, indicating that the anomerization of glucose 6-phosphate is not a rate-limiting step in the pathway of glycogen synthesis in the muscle.

Materials and Methods

Chemicals——Crystalline porcine insulin was obtained from Novo Research Institute. D-[U-^{14}C]Glucose was

supplied by New England Nuclear Corporation. Rabbit liver glycogen was purchased from Nakarai Chemicals, Ltd. Glucose 6-phosphate dehydrogenase [EC 1.1.1.49] (from *Leuconostoc mesenteroides*) and nicotinamide adenine dinucleotide phosphate (NADP) were from Oriental Yeast Co., Ltd. Monophase-40 (scintillation cocktail for aqueous samples) was from Packard Instrument Co. Each anomer of D-[U-^{14}C]glucose was prepared by the same method as used for the preparation of the α and β anomers of D-[1-^{14}C]glucose.[6] The anomeric purity of both anomers of D-[U-^{14}C]glucose was better than 95% as determined by our method for anomer assay[7] using glucose oxidase [EC 1.1.3.4], mutarotase, and an oxygen electrode. Anomers were dissolved in Krebs–Ringer bicarbonate buffer (pH 7.4) just before use. Equilibrated D-[U-^{14}C]glucose was prepared by warming either anomer of the labelled sugar in the bicarbonate medium at 37 °C for 30 min.

Preparation of Rat Hemidiaphragms without Ribs——Male Wistar rats with a body weight of 100—120 g were sacrificed by decapitation. The diaphragm was immediately dissected out, rinsed in cold 0.15 M sodium chloride, and cut into two pieces (90—110 mg each) on filter paper soaked with the saline as described by Goldberg *et al.*[8]

Extraction and Determination of Glucose 6-Phosphate——Hemidiaphragms (3 pieces) were incubated at 37 °C for 30 min under aerobic conditions (95% O_2/5% CO_2) in 100 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) supplemented with insulin (10 mU/ml) in the presence or absence of glucose (5.6 mM). After incubation, the tissues were immediately frozen between a pair of aluminum blocks precooled in liquid nitrogen and pulverized in a porcelain mortar cooled with liquid nitrogen. The frozen powder (300 mg) was added to 1.5 ml of 10% (w/w) perchloric acid containing 1 mM ethylenediaminetetraacetic acid (EDTA), cooled at −10 °C, mixed well until thawed, and centrifuged at 15000 × *g* at 4 °C for 30 min. The supernatant was neutralized to pH 4—6 with ice-cold 30% KOH. The precipitated potassium perchlorate was removed by centrifugation. Glucose 6-phosphate in the clear supernatant was determined by the method of Minakami *et al.* with glucose 6-phosphate dehydrogenase.[9]

Analysis of Incorporation of D-[U-^{14}C]Glucose into Glycogen——Each hemidiaphragm was placed in 30 ml of Krebs–Ringer bicarbonate buffer (pH 7.4), preincubated at 37 °C for 30 min, and transferred to a test tube containing 0.95 ml of the bicarbonate medium. Then α-, β-, or equilibrated D-[U-^{14}C]glucose (specific activity: 0.123 mCi/mmol) was added in a volume of 50 μl, and the test tube was further kept at 37 °C for various periods. The final concentration of glucose and the atmosphere (95% O_2/5% CO_2) were kept constant during preincubation and subsequent incubation. The glucose concentration was specified in the text. In experiments with insulin, the hormone was added at the beginning of preincubation and was present in the incubation medium as well. After incubation, the hemidiaphragm was rinsed quickly (for 2—3 s) in ice-cold bicarbonate medium, blotted on filter paper, placed in 2 ml of hot 30% (w/v) KOH supplemented with 1 mg of carrier glycogen (from rabbit liver), and heated for 20 min at 100 °C. The digest was cooled and 4 ml of ethanol was added. The glycogen was allowed to precipitate at 4 °C for 1 h, then the sample was centrifuged at 1500 × *g* for 15 min. The precipitate was dissolved in 2 ml of water. One ml of the solution was added to 5 ml of Monophase-40 and the radioactivity was measured in a Packard Tri-Carb liquid scintillation counter (model 3255). Statistical significance was determined by the standard Student's *t* test, with *p* less than 0.05 as the criterion of significance.

Results and Discussion

Figure 1 summarizes the results of glycogen synthesis from glucose anomers in

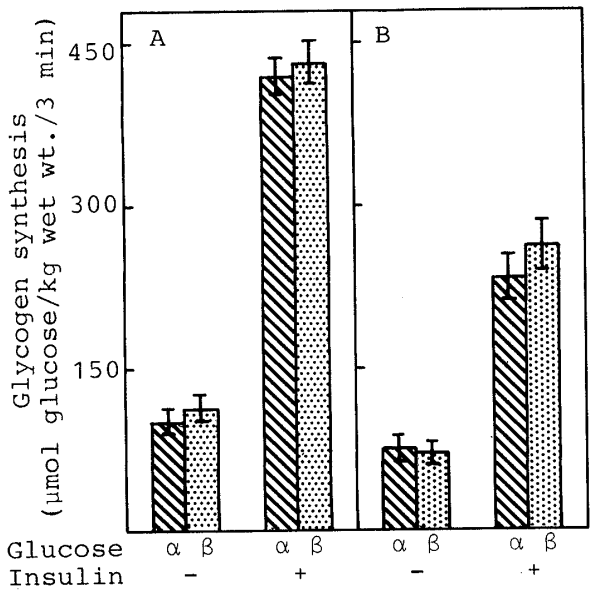

Fig. 1. Glycogen Synthesis from α- and β-D-[U-^{14}C]Glucose in Rat Hemidiaphragms Preincubated in the Presence of Glucose

When insulin was supplemented, the hormone (10 mU/ml) was present throughout both the preincubation and incubation periods. The tissues were incubated at 37 °C in Krebs–Ringer bicarbonate buffer (pH 7.4) containing α- or β-D-[U-^{14}C]glucose under the following conditions. A: incubation time, 3 min; glucose concentration, 5.6 mM. B: incubation time, 3 min; glucose concentration, 2.8 mM. Each value represents the mean of 6 determinations ± S.E.

hemidiaphragms preincubated in the presence of glucose. Whether glycogen synthesis was enhanced by insulin or not, the rates of glycogen synthesis from the α and β anomers of D-[U-^{14}C]glucose in the hemidiaphragms were almost the same when the tissues were incubated for 3 min at 37 °C at a glucose concentration of 5.6 mM (Fig. 1, A). Since the glucose concentration (5.6 mM) was a little higher than that (3.5 mM) which gives a half-maximal incorporation of D-[U-^{14}C]glucose into glycogen (data not shown), it seemed worthwhile to use a lower glucose concentration (2.8 mM) for the purpose of differentiating the rates of glycogen synthesis from glucose anomers. Hemidiaphragms, however, synthesized glycogen from the α and β anomers of D-[U-^{14}C]glucose at almost the same rates when incubated in a medium containing 2.8 mM glucose for 3 min at 37 °C (Fig. 1, B). The rates of glycogen synthesis from the α and β anomers of D-[U-^{14}C]glucose were not significantly different (40.0 ± 4.8 and 38.5 ± 3.2 μmol/kg wet wt./min, respectively) even when the incubation time was shortened from 3 to 1 min in order to decrease mutarotational equilibration of glucose and glucose 6-phosphate. These results show that there is no rate-limiting anomerization step in glycogen synthesis in rat diaphragms.

There appear to be two possible explanations for the lack of anomeric preference in glycogen synthesis from glucose under these conditions. One is that glucose 6-phosphate accumulates in cells to a considerable degree, resulting in complete equilibration of glucose 6-phosphate because of its rapid spontaneous anomerization (half-life at pH 7.8 at 37 °C: 1.5 s[10]). The other is that glucose 6-phosphate is metabolized without any appreciable transient accumulation in spite of the α-stereospecificity of phosphoglucomutase and glucosephosphate isomerase [EC 5.3.1.9] toward glucose 6-phosphate;[5] in other words, glucose 6-phosphate anomerizes rapidly enough to be metabolized to glucose 1-phosphate or to fructose 6-phosphate without any significant delay.

To obtain a clue to the likely explanation, we determined the glucose 6-phosphate content in hemidiaphragms. The concentration of glucose 6-phosphate in hemidiaphragms which were incubated in the presence of 5.6 mM glucose at 37 °C for 30 min was 199 ± 12 μmol/kg wet wt. (mean ± S.E. of 6 separate experiments). This is in good agreement with the result obtained by Beitner *et al.*[11] Hemidiaphragms which were incubated in the absence of glucose at 37 °C for 30 min contained a reduced yet significant level (97.6 ± 17 μmol/kg wet wt.) of glucose 6-phosphate. These data suggest the former explanation to be likely.

We also measured the incorporation of D-[U-^{14}C]glucose into glycogen in hemidiaphragms which were preincubated in the absence of glucose to yield a lower level of intracellular glucose 6-phosphate. No significant difference was observed between the rates of incorporation of the two anomers (2.8 mM) into glycogen (Table I). It appears plausible that the rate of glycogen synthesis (*ca.* 60 μmol glucose/kg wet wt./min) in a medium containing 2.8 mM glucose was too low to overcome the rapid anomerization of glucose 6-phosphate in its

TABLE I. Incorporation of the α and β Anomers of D-[U-^{14}C]Glucose into Glycogen in Rat Hemidiaphragms Preincubated without Glucose

Glucose Concentration (mM)	Anomer	Glycogen synthesis[a] (μmol glucose/kg wet wt./min)
2.8	α	62 ± 4
	β	59 ± 4
16.7	α	242 ± 21
	β	235 ± 18

Insulin (10 mU/ml) was present throughout both the preincubation and incubation periods, and incubation was performed at 37 °C for 1 min. *a*) Mean ± S.E. of 5 determinations.

intracellular pool (97.6 ± 17 μmol/kg wet wt.). To overcome this problem we performed an experiment with a higher glucose concentration (16.7 mM), which also resulted in insignificant differentiation between the two anomers of glucose (Table I).

The anomerization rate of glucose 6-phosphate in cellular fluid is probably higher than the rate of spontaneous anomerization (half-life in 32 mM Tris–HCl buffer, pH 7.8, at 37 °C: 1.5 s) reported by Bailey *et al.*,[10] because muscle glucosephosphate isomerase also has anomerase activity toward glucose 6-phosphate.[12] The extremely rapid anomerization of glucose 6-phosphate in its intracellular pool is likely to be the reason for the lack of a rate-limiting anomerization step in glycogen synthesis from glucose in rat diaphragms.

Incidentally, the data in this study suggest that glycogen originating from the β anomer of glucose in circulating blood is predominant in the muscle compared with that from the α anomer of glucose, since glucose is known to exist as a close-to-equilibrium mixture (37.3% α and 62.7% β) of the two anomers in blood.[13]

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