Studies on the Mechanism of Glycogen Storage in Ascites Hepatomas

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SUMMARY

Glycogenic and glycogenolytic activities of two rat ascites hepatomas, AH-66F and AH-130, were compared for the purpose of explaining their markedly different capacities for glycogen storage. Glycogen-rich AH-66F differs from glycogen-deficient AH-130 in that, in the former tumor, glycogen is unable to inhibit the phosphatase that converts synthetase D to the I form; hence the suppression of glycogen synthesis. Glycogen synthetase D phosphatase of AH-66F was active even at glycogen levels that would completely inhibit the AH-130 enzyme. When hepatoma cells were incubated without glucose, the glycogen store of AH-130 disappeared readily, whereas that of AH-66F was stable. The difference is not attributable to differences in activity of phosphorylase. These results suggest that the properties as well as levels of the enzyme systems that mediate the molecular conversion of glycogen synthetase and/or phosphorylase are of prime importance for explaining the difference in glycogen storage in the two hepatomas.

INTRODUCTION

More than 70 different strains of rat Yoshida ascites hepatoma are available (6). All these hepatomas grow rapidly and glycolyze highly and, with the exception of AH-66F and AH-13, they are deficient in glycogen while growing in the peritoneal cavity (5). AH-130, the most widely known, also belongs to the glycogen-deficient group. In this laboratory, studies were undertaken to explain the marked difference in glycogen content of AH-66F and AH-130 in biochemical terms (7, 8, 10, 13, 14).

Previous studies demonstrated that even the glycogen-deficient AH-130 hepatoma can convert glucose to glycogen, but its rate is rapidly reduced as glycogen accumulates (8). Since the 2 hepatomas possess identical muscle-type glycogen synthetase (UDP-glucose:α-1,4-glucan α-4-glucosyltransferase, EC 2.4.1.11) in comparable amounts (10, 14), it has been suggested that the feedback inhibition of glycogen synthesis by glycogen may not function efficiently in the AH-66F hepatoma (7).

The present communication deals with a comparison of in vitro glycogen synthesis and breakdown in these 2 hepatoma strains.

MATERIALS AND METHODS

Ascites Hepatoma Cells. Yoshida ascites hepatomas AH-66F and AH-130 were implanted into the peritoneal cavity of male Donryu rats weighing 120 to 150 g, and cells were harvested 4 to 6 days thereafter. The cells were collected by centrifugation and washed twice with cold 0.15 M NaCl solution. The origin and certain biochemical properties of these hepatomas were described elsewhere (5–8, 10, 13, 14).

Synthesis of Glycogen from Glucose. Hepatoma cells were washed once with cold Ca²⁺-free Krebs-Ringer phosphate buffer (pH 7.4) and packed in the same buffer by centrifugation at 600 × g for 2 min. Six ml of the packed cells were mixed with 9 volumes of the above buffer and incubated in a 100-ml Erlenmeyer flask at 37° in the presence of 10 mM glucose (or uniformly labeled glucose-14C) and 10 mM pyruvate while on a shaker oscillating at 60 times per min. The gas phase was oxygen. Pyruvate was used because of its stimulation of glycogen synthesis (13). At the times indicated, 0.5-ml portions were withdrawn and added to 2 ml of 6% trichloroacetic acid.

Consumption of Glycogen Store. The hepatoma cells previously incubated for 60 min with glucose and pyruvate were collected from the incubation mixture by centrifugation, washed twice with cold Ca²⁺-free Krebs-Ringer phosphate buffer (pH 7.4), and suspended in the original volume of the same buffer. The suspension was then incubated under air at 37°.

Analytical Methods. Glycogen was isolated from the acidified incubation mixture, and its amount was estimated by the anthrone reaction. When glucose-14C was used as substrate, the extent of its incorporation into glycogen was also determined. Details of these procedures were described previously (7). The cellular content of glycogen was expressed in terms of μmoles of glucose equivalent/10 mg (dry weight) cells, unless otherwise specified. Lactic acid was determined by the method of Barker and Summerson (1).

Preparation of Hepatoma Cell Homogenate. Hepatoma cells were washed once with 0.4 M sucrose-50 mM Tris-HCl (pH 7.4)-5 mM EDTA, suspended in 5 volumes of the same medium, and sonically disrupted for 2.5 min at 10 kc (Taiyö Co., Tokyo, Japan). The homogenate was centrifuged at 5000 × g for 10 min, and the supernatant was used for enzyme studies. All these procedures were conducted at 0–2°.

Assay of Glycogen Synthetase. Glycogen synthetase was assayed by the method described previously (9). The determination of total (D plus I) activity was carried out by use of an assay mixture that contained 50 mM Tris-maleate...
Kiyomi Sato and Shigeru Tsukita

AH-1301

0 30 60

0 30 60

Incubation time (min)

0 30 60

0 30 60

Glucose (mmoles of glucose equivalent/mg packed cells)

Glucose (mmoles of glucose equivalent/mg packed cells)

Incubation time (min)

Chart 1. Synthesis of glycogen from glucose by ascites hepatoma cells. Cells were incubated with glucose and pyruvate as described in "Materials and Methods." After 60 min, AH-130 cells were collected by centrifugation, washed, resuspended in a new medium containing glucose and pyruvate, and incubated for an additional 60 min. At the times indicated, cellular glycogen was determined by the anthrone reaction.

Chart 2. Relation of cellular glycogen level to glycogen synthetase I activity. Different preparations of hepatoma cells were separately homogenized and each homogenate was assayed for glycogen content and the total and I activities of glycogen synthetase. AH-130 preparations denoted by △ were preincubated with glucose and pyruvate for 60 min prior to homogenization.

RESULTS

Synthesis of Glycogen from Glucose. In general agreement with the results obtained earlier (8), the conversion of glucose to glycogen occurred in a somewhat complicated manner in AH-130 cells (Chart 1). There was a lag period of more than 15 min before the onset of glycogen synthesis. The rate of glycogen synthesis was maximal at 30 to 45 min and was progressively reduced as glycogen accumulated. A change of medium did not prevent this reduction. Throughout these periods, lactate formation occurred almost linearly, thereby excluding the possibility of general impairment of cellular metabolism.

Before the start of incubation, the AH-66F cells, as shown in Chart 1, contained 2.8 μmoles of glucose equivalent of glycogen/10 mg (dry weight) cells, a concentration more than enough to suppress the glycogen synthesis of AH-130 cells. Nevertheless, glycogen synthesis occurred rapidly, and there was no sign of reduction with a further rise in cellular glycogen level.

The data shown in Chart 1 are thus consistent with the view that glycogen synthesis in AH-66F cells is much less sensitive to feedback inhibition by glycogen than that of AH-130 cells.

Relation of Cellular Glycogen Level to Glycogen Synthetase I Activity. Previous studies (8) suggested that the progressive reduction in the rate of glycogen synthesis, observed in AH-130 cells, was a consequence of the I to D conversion of glycogen synthetase. In the present work, different preparations of ascites hepatoma cells were homogenized separately, and the percentage of glycogen synthetase in the I form was determined for each preparation. Chart 2 shows that, for AH-130 cells, an inverse relationship exists between cellular glycogen level and the percentage of synthetase I. Above the glycogen level of 4 μmoles/ml packed cells, the glycogen synthetase of AH-130 was predominantly in the D form.

In AH-66F, however, much higher levels of cellular glycogen were still insufficient to cause an effective I to D conversion of glycogen synthetase. Even at levels of more than 50 μmoles, more than one-half of the synthetase was in the I form. These results readily explain why glycogen synthesis in AH-66F occurs linearly, irrespective of cellular glycogen level.

Effect of Glycogen Level on the Rate of Activation of Glycogen Synthetase. As already reported (10), glycogen synthetase in fresh hepatoma extracts undergoes an activation on incubation at 30°C. This is due to the D to I conversion of the enzyme catalyzed by glycogen synthetase D phosphatase (10). The preparations of AH-130 cells differing markedly from each other in glycogen content were homogenized; the

2 The abbreviation used is: glucose-6-P, glucose 6-phosphate.
homogenates were centrifuged at 5000 × g, and the resulting supernatants were incubated at 30°. As shown in Chart 3, A and B, the rate of activation of glycogen synthetase was much slower in the glycogen-rich extract than in the extract containing little glycogen. It therefore appears that glycogen inhibits the glycogen synthetase D phosphatase of AH-130 as it does the enzyme of muscle (15) or liver (2).

On the other hand, an AH-66F extract containing about 10 times as much glycogen as the glycogen-rich AH-130 extract was still capable of effecting the D to I conversion readily (Chart 3C). This suggested that glycogen hardly inhibited the D phosphatase of AH-66F.

**The Rate of Consumption of Cellular Glycogen.** Although it must be true that glycogen storage in AH-130 cells can be limited by the feedback inhibition of glycogen synthesis by glycogen, the glycogen levels found in vivo are usually below those expected to cause an effective suppression of glycogen synthesis. This prompted us to investigate the glycogenolytic capacity of the 2 hepatomas.

![Chart 3](image)

**Chart 3.** Effect of glycogen on the rate of D to I conversion of glycogen synthetase. Hepatoma cells were homogenized and were centrifuged at 5000 × g. The supernatants were incubated at 30° prior to the assay of glycogen synthetase. A, fresh AH-130 cells; B, AH-130 cells preincubated with glucose and pyruvate for 2 hr; C, fresh AH-66F cells. Extent of D to I conversion at given times was defined as \((\text{increment of I activity/D activity at 0 time}) \times 100\). The values given in µmoles were glucose equivalent of glycogen/10 mg (dry weight) cells.

![Chart 4](image)

**Chart 4.** Consumption of glycogen by AH-130 cells. After incubation with glucose-14C and pyruvate for 60 min in oxygen, the cells were collected, washed, and incubated for 60 min in air without added substrates. Details are given in "Results." At the times indicated, cellular glycogen was determined by radioactivity (○) or by the anthrone reaction (●).

**DISCUSSION**

The glycogen store of AH-66F is markedly stable as compared with the glycogen store of AH-130 (Charts 4 and 5). Since the amount of phosphorylase is rather larger in AH-66F than in AH-130 (14), it is suggested that the activation of phosphorylase may be retarded in AH-66F.

The activation of phosphorylase, *i.e.*, the conversion of inactive phosphorylase *b* to the active *a* form, is mediated by phosphorylase *b* kinase, the activation of which is in turn mediated by cyclic adenosine 3',5'-monophosphate-dependent protein kinase. Since the same protein kinase also catalyzes the conversion of glycogen synthetase I to the D form (11, 12), it may be assumed that the inactivation of glycogen synthetase would also be retarded in AH-66F, and this was precisely what was observed. In this hepatoma, the conversion of glycogen synthetase I to D was not very much accelerated by glycogen (Chart 2), and the cellular level of glycogen continued to rise as long as glucose was provided to the cells (Chart 1). The properties as well as levels of the enzyme systems that mediate the molecular conversion of glycogen synthetase and phosphorylase are thus of prime importance in determining the extent to which glycogen can be stored in ascites hepatomas *in vivo*.

Although no investigations have yet been made on the protein kinases of these hepatomas, we have presented...
Kiyomi Sato and Shigeru Tsuiki

evidence that the glycogen synthetase D phosphatase of AH-66F is either insensitive to inhibition by glycogen or very much less sensitive to inhibition by glycogen than is the enzyme of AH-130 (Chart 3). Hers et al. (3) pointed out that glycogen synthetase D phosphatase from skeletal muscle was inhibited by glycogen much more profoundly than the liver enzyme, and that this difference might explain the difference in the extent to which glycogen accumulated in these 2 glycogenic tissues. Muscle may possess glycogen synthetase D phosphatase differing from the liver enzyme in its regulatory properties. It is similarly possible that the glycogen synthetase D phosphatase and/or the protein kinase of AH-66F differ from those of AH-130 so as to allow the former to retain remarkably high amounts of glycogen while growing in the peritoneal cavity, although the ascites fluids contain little glucose (4).

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REFERENCES

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