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SOME PROPERTIES OF A LIVER PROTEIN THAT ACTIVATES GLYCOGEN SYNTHASE b[1]

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SUMMARY: A soluble protein has been identified in rat liver that increases the activity of glycogen synthase without causing synthase b to a conversion. The effect of the activator is to increase synthase b activity in the presence of saturating amounts of UDP-Glc and Glc-6-P. The activator is heat sensitive and does not have protease activity. The effect of the activator is linearly proportional to the amount assayed to a saturable level and its effect is not mimicked by other proteins associated with the control of glycogen metabolism (e.g., phosphorylase).

It is widely accepted, although not definitely established, that glycogen synthesis in vivo is mediated solely by a dephosphorylated form of glycogen synthase (i.e., synthase a) with a high affinity toward substrate and activator (1-3). However, several studies have suggested that synthesis of glycogen can occur where there is no protein phosphatase for the dephosphorylation of synthase b. These include livers of fasted newborn and fed, alloxan diabetic adult rats (4-6), rat adipose tissue (7,8) and choriocarcinoma cells (9). This suggest that glycogen synthesis might be regulated by a variant form of synthase. It has been suggested (10-13) that synthase can exist in vivo in various states of phosphorylation that could give rise to multiple regulatory possibilities resulting from these various phosphorylated forms having different reactivities towards effectors of activity as well as for being better, or poorer, substrates for protein kinase(s) and/or phosphatase(s). However, it has not been established with certainty that the isolated variant forms of synthase b exist as such in vivo, but could represent an artifact of the isolation methods used.

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During an investigation of the properties of liver synthase phosphatase, it was discovered that the phosphatase preparation contained a soluble protein that increased the maximal activity of synthase b without causing either a conversion of synthase b to a or an increased affinity of synthase for Glc-6-P. This report describes some properties of this protein activator of synthase b and suggests that the activator might have a regulatory function for glycogen synthesis.

EXPERIMENTAL PROCEDURES

Isolation of enzymes: Synthase b and phosphorylase a - The isolation procedures used for these enzymes has been described previously (5,14,15), with all buffers supplemented with 0.2 mM PMSF[4], 2.0 mM EGTA, 1 mg/L pepstatin, 10 mg/L TPCK and 2 mg/L aprotinin to inhibit proetolysis (16-18). Synthase a was prepared essentially as described by Jett and Soderling (19), except the buffers used contained the protease inhibitors listed above. Synthase from neonatal rat liver (24 hours postpartum) was prepared as described (14) also in the presence of the protease inhibitors.

Synthase activator - The preparation of activator from livers of adult and neonatal rats is derived from methods used to isolate synthase phosphatase (20,21), except the tissue homogenizing buffer contained 2.0 mM mersalyl, 4 mg/L aprotinin, 2.5 mM PMSF and 1.0 mM EGTA. The $(NH_4)_2SO_4$ precipitated proteins were dissolved in and dialyzed against 50.0 mM glycylglycine, pH 7.4, containing 10% (v/v) glycerol and 5.0 mM each of MgCl and 2-mercaptoethanol. Chromatography on DEAE-cellulose (DE-52, Whatman) for the separation of synthase phosphatase (b to a conversion activity) from the synthase b activator was done using the buffer described above supplemented with a discontinuous NaCl gradient of 0-0.25 mM. Collected fractions were assayed for synthase phosphatase and phosphorylase phosphatase, as described previously (21,22), and for synthase b activator (described below).

Enzyme assays: Synthase and phosphorylase - These enzymes were assayed as described previously (5,14,21) and is based on the filter paper method of Thomas et al. (23). The activity ratio of synthase (-Glc-6-P/+Glc-6-P) was determined in the absence and presence of 10.0 mM Glc-6-P, with 2.0 mM UDP-Glc, and ranged from 0.08-0.1 for the isolated enzyme. Synthase phosphatase increased the activity ratio to 0.85-0.95.

Synthase b activator - The effect of the activator was determined by measuring activator-induced change in the rate of synthase activity in the absence or presence of Glc-6-P. Assay tubes contained (final concentrations) 56.0 mM glycylglycine, pH 7.4, 5.0 mg/ml rabbit liver glycogen (Type III, Sigma), 2.0 mM UDP-Glc (with 50,000 cpm of UDP-[^{14}C]Glc), 10.0 mM of either Glc-6-P (synthase b) or Na_2SO_4 (synthase a), as well as other additions as indicated. The volume was 500 µL after the addition of synthase to initiate the reaction. Incubations were done at 30° and at 10 min intervals, 100 µL aliquots were removed, spotted onto 2 x 2 cm filter paper squares (31-ET, Whatman) and the papers processed for measuring radioactivity as described (23). Control reactions were done in the absence of synthase and/or activator but presence of equivalent protein (bovine serum albumin) to correct for nonspecific [^{14}C] Glc incorporation, which was negligible. Four time points were obtained for the reaction and the data expressed as Glc incorporated into glycogen versus time. At 30° the reaction rate was linear to about 50-60 min; at 37°, the rate was linear to about 30 min.

For the effect of activator on phosphorylase a, the assay tubes contained (final concentration, in a total volume of 500 µL, after addition of phosphorylase) 50.0 mM glycylglycine, pH 6.9, 5 mg/ml glycogen, 5.0 mM 5'-AMP, 40.0 mM KF and 20.0 mM Glc-1-P with 50,000 CPM of [^{14}C]Glc-1-P. Incubations were at 30°and at 5 min

[4]Abbreviations are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloracetic acid; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone.

intervals, 100 µL aliquots were applied to filter paper squares and processed for counting as above. This reaction was linear for 20-25 min.

The specific enzyme activity of synthase was 25-30 units/mg protein (1.0 unit is that amount of that incorporates 1.0 µmol of Glc into glycogen/min) and 2 units of enzyme were used per assay. Phosphorylase had a specific enzyme activity of 40 units/mg protein and 2 units were used per assay. To quantitate the activator, one unit was defined as the amount that increases the rate of the synthase b reaction by 50%. This method of assay was chosen so as to eliminate a preincubation of synthase with the activator as is required for the two-step assay of synthase phosphatase (20,21).

Other assays: Protease was assayed using the procedure of Dayton, et al. (24) with either casein on synthase b as substrate. Proteins were quantitated by the Lowry (25) or Bradford (26) methods using bovine serum albumin as standard. Glycogen was determined by the Anthrone method (27).

Protein-bound phosphate was determined using the method of Hasegawa, et al. (28). Duplicate samples of 50 µg protein with 200 µg of lipid-free bovine serum albumin were precipitated 4 times with 7% TCA (final concentration). Protein pellets from the final precipitation were processed as described for this method.

Preparation of ^{32}P-Labeled Substrates: Synthase a was phosphorylated with the catalytic subunit of cAMP-dependent protein kinase (Sigma) and [γ-^{32}P]ATP (500-1000 cpm/pmol) using methods described by Itarte et al. (29) and Ahmad et al. (30).

Materials: All reagents used were of the best quality available and were purchased from either Sigma Chemical Company or Fisher Scientific. Radioisotopes were obtained from either Amersham Corporation or New England Nuclear.

RESULTS AND DISCUSSION

The separation of synthase activator from synthase phosphatase is shown in Fig. 1. The neonatal rat shows only the presence of the activator (Fig. 1-A), and agrees with the earlier observation of the absence of synthase b to a conversion activity in the liver of newborn, whereas the adult liver shows both activities (Fig. 1-B). For the adult, the fraction applied to the DE-52 column, 26 mg protein/mL, had 43.5 $\pm$ 6.5 (mean $\pm$ S.E.M. from 5 preparations) units of activator/mL representing a specific activity of 1.7 units/mg protein. The activator recovered from the column had a specific activity of 14.2 units/mg protein and represented 40-50% of the total amount applied to the DE-52. The yield and purity of the adult synthase phosphatase was the same as has been described for this procedure (21).

In the presence of the activator, the rate of reaction catalyzed by synthase b in the presence of Glc-6-P was increased, whereas there was little change from the control in the absence of Glc-6-P (Fig. 2-A). Synthase phosphatase (Fig. 1-B) did not increase the rate of reaction of synthase b, but did cause an increase in rate of synthase a reaction (Fig. 2-B). Thus, the activator and phosphatase have separate effects on synthase b and a.

Increasing the amount of added activator in the assay increased synthase b activation in a linear fashion to a saturable level (Fig. 3). There was no increase in

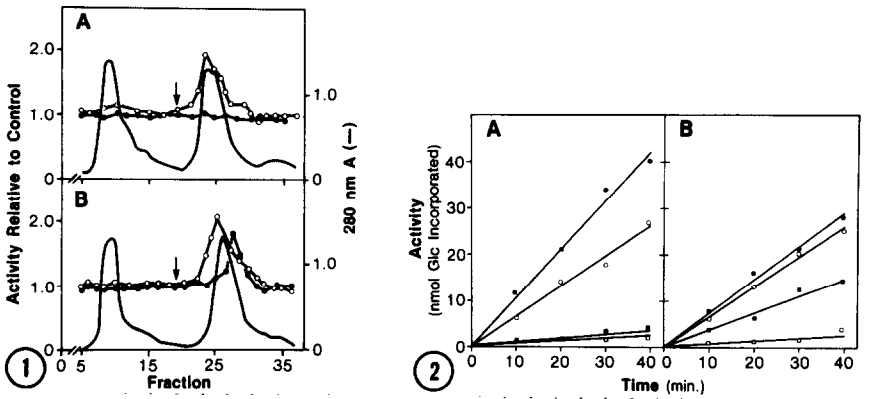

Fig. 1 DEAE-cellulose chromatographic separation of synthase b activator from synthase phosphatase. Two mL of 26 mg protein/mL of resuspended $(NH_4)_2SO_4$ (60% of saturation) precipitated liver proteins of 24-hour newborn (A) and adult (B) rats prepared as described (21), were applied to a DE-52 column (2 x 7 cm). After elution of non-binding proteins, 0.15 M NaCl (arrows, fraction 19) was used to elute the activator. Fractions were assayed for synthase phosphatase in the absence of Glc-6-P (●) and for synthase b activator in the presence of Glc-6-P (O). For ease of comparison, activities are expressed relative to controls (1.0) determined in the absence of activator or synthase phosphatase.

Fig. 2 Comparison of activator and synthase phosphatase activities. Synthase was assayed in the presence of Glc-6-P (O,●) for synthase b or absence of Glc-6-P (□,■) for synthase a. A, effect of activator (●). B, effect of synthase phosphatase (■). In A and B the symbols O, are control values in the presence and absence of Glc-6-P, respectively).

synthase a activity. Usually the maximal activation of synthase b was 1.5-1.8 -fold over the rate of reaction in the absence of the activator and occurs at a low activator to synthase ratio (w/w), inspite of the possible heterogeneity of the activator. This result might suggest that the effect of the activator is caused by a nonspecific protein-protein interaction producing the increase in synthase activity. To examine this

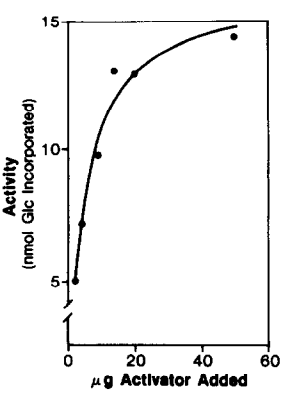

Fig. 3 Activation of synthase b versus amount of activator. Synthase b activity in the presence of Glc-6-P was determined in the presence of an increasing amount of added activator.

Table 1. Activity of Synthase b in the Presence of Added Proteins

Protein Added[a]	% Change in Rate of Synthase Activity[b]
Activator Fraction	+71
Glucagon	-19
Protein Kinase Inhibitor	+11
Phosphorylase Kinase	0
Phosphorylase b	0
Phosphorylase a	0
Insulin	-43
Insulin - beta chain	+3
Protamine-P	-60
Protamine - free base	+12
Bovine Serum Albumin	0

[a]Adult synthase b was assayed with 75 µg of the indicated proteins, in a final volume of 500 µL.

[b]Values are relative to the rate of synthase reaction in the presence of bovine serum albumin alone.

possibility, a number of proteins were tested for activation of adult synthase b (Table 1). Although there are proteins that inhibit the synthase (glucagon and protamine-P), only the activator increased the activity to a significant degree and suggests that there is a direct effect on synthase b.

The $A_{0.5}$ for Glc-6-P of synthase b (at 2.0 mM UDP-Glc) was 0.56 mM and 0.6 mM in the presence and absence of the activator. Vmax increased from 25 to 40 µmol Glc incorporated $min^{-1}mg^{-1}$ in the presence of activator (data not shown).

Proteolysis of synthase b causes an apparent activation of the enzyme (16,31,32). Therefore, the activator was assayed for protease activity against synthase b using the method of Dayton et al. (24), which measures the change in A at 280 nm of the TCA-soluble fraction after acid precipitation of the assay mixture. A 0.05% solution of synthase b, used as substrate for the assay, had an A of 0.8 at 280 nm which favorably compares with the $E^{1\%}_{1cm}$ of 14.5 measured at 278 nm (33). There was no increase in A of the TCA-soluble fraction after incubation (60 min; 30°) with the activator (100 µg). This same result was obtained with 200 µg casein and 60 µg activator. SDS P.A.G.E. (6) of synthase b after incubation with activator did not result in a decrease of synthase

Table 2. Effect of Activator on Synthase a and Phosphorylase a

Enzyme	Relative Activity[a]	
	-Activator	+Activator
Synthase b		
+ Glc-6-P	1.00	1.61
- Glc-6-P	1.00	1.00
Synthase a		
+ Glc-6-P	1.0	1.60
- Glc-6-P	1.0	1.32
Phosphorylase a	1.0	1.04

[a]Reaction rates in the absence of activator are normalized to unity for ease of comparison. Synthase b, a and phosphorylase assays contained 2.0 units of each enzyme with 40 µg of activator, when added.

subunit Mr of 85,000 dalton. Protease inhibitors used in the isolation buffers did not inhibit the activator effect. However, incubation of activator with subtilisn (10:1, w/w, substrate to protease) at 30° for 60 min did inactivate the activator. Heating the activator to 100°, 5 min, destroyed its effect. The activator was stimulated by 30-50% with 2-5 mM Mn^{2+} or Mg^{2+}, but Ca^{2+} at the same concentration was inhibitory. EDTA and EGTA, 5.0 and 2.0 mM, respectively, were without effect.

The specificity of activator was judged from a comparison of effects on synthase a and phosphorylase a (Table 2). The expected stimulation of synthase b was observed and there was no effect on phosphorylase a. However, synthase a, in the presence of Glc-6-P, was activated to the same extent as synthase b and, in the absence of Glc-6-P, was activated to about one-half that in the presence of Glc-6-P. This suggest that the activator is stimulating a partly dephosphorylated form of synthase (10-13), which is a contaminating component of the synthase a preparation. These results do suggest the activator to have specificity toward synthase, relative to phosphorylase, both of which are phosphoproteins.

The total P content of synthase b (12 P/subunit of 85,000 dalton) decreased to 10-8.5 P/subunit after incubation (30°, 40 min) with activator (40 µg) suggesting that the activator has phosphatase activity or is contaminated with protein phosphatase. However, there is no b to a conversion of synthase, typical of protein phosphatase

action, and suggests that the P groups removed do not increase synthase affinity toward substrate. Incubation of ^{32}P-labeled synthase (2 P/subunit) with 40 µg activator (30°, 20 min) results in a decrease to 1.6 P/subunit, whereas synthase phosphatase caused a decrease to 0.8-1.0 P/subunit. These results suggest that the activator does have phosphatase activity; however, it can not be ruled out that a contaminating protein phosphatase, which does not catalyze the b to a conversion, causes the removal of P from synthase. Clarification of this aspect depends upon purification of the activator to homogeneity.

Since the activator has an apparent phosphatase activity, the effect of protein phosphorylation on reversal of the activator action was determined. Analysis of the activator showed that the protein did not have covalent bound P. Also, substituting the activator for the protein kinase in the synthase phosphorylation reaction mixture showed the activator did not have protein kinase activity. These controls were done to determine if the activator was a phosphoprotein that had protein kinase activity. After activation of synthase b (2 units) with the activator (40 µg) to a relative increased synthase reaction rate of 1.8, incubation with the protein phosphorylation system described above, but without [γ^{32}P]ATP, decreased the relative reaction rate of synthase to 1.3 (28% decrease) suggesting a partial reversal of the activator by phosphorylation. The use of protein kinases other than cAMP-dependent protein kinase, might be more effective for complete reversal.

The results of this preliminary study shows the presence of a liver protein with glycogen synthase activator properties independent of synthase b to a conversion. Present studies are directed toward the physiological significance of the activator, which could account for those conditions where glycogen is synthesized in the apparent absence of synthase a (4-9,35), but presence of a variant form of synthase b resulting from activator action.

This activator could be a type of a class of modulators of enzyme action represented by the heat-stable inhibitors of protein phosphatase (3,34) as well as the recently characterized protein activators of muscle and reticulocyte phosphoprotein phosphatases (36,37).

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