Presence of an intermediate synthase form during the conversion of glycogen synthase D into synthase I in rat liver extract

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When synthase D was converted into synthase I in a liver extract, it progressed through a synthase form with activity characteristics that could not be explained by a mixture of the original synthase D and the final product, synthase I. This form was distinguished by an affinity for UDP-glucose, in the absence of glucose 6-phosphate, which was intermediate between those of the two known forms.

Activation of glycogen synthesis is believed to involve the conversion of the inactive synthase D into the active synthase I. Structurally, synthase D is the more phosphorylated form of the enzyme, but the number of phosphate groups involved in the conversion into synthase I is not clear. Various studies indicate that one or more phosphate groups per subunit can be inserted into purified synthase I by various synthase kinases, yielding enzymes with different kinetic properties (Nimmo *et al.*, 1976; Soderling *et al.*, 1977; Brown *et al.*, 1977; Huang *et al.*, 1979; Huang & Huang, 1980; Brown *et al.*, 1980). The ability to generate these multiply phosphorylated forms of synthase *in vitro* leads to the speculation that partially phosphorylated forms exist *in vivo*. However, a method is needed to detect the intermediate synthase forms in crude extract. In this study, a simple method is described which showed the presence of a kinetically different intermediate form of glycogen synthase in rat liver extract during the conversion of synthase D into synthase I.

Materials and methods

The standard assay mixture for synthase contained 6.7 mM-UDP-[¹⁴C]glucose, 6.7 mg of rabbit liver glycogen/ml, 16.7 mM-KF, 13.3 mM-EDTA, 33.3 mM-Tris/HCl (pH 8.5), with or without 7.2 mM-glucose 6-phosphate. One unit of synthase activity is defined as 1 μ mol of UDP-glucose incorporated into glycogen/min at 30°C. Total synthase is the activity measured in the presence of glucose 6-phosphate, and activity ratio is the ratio activity without/activity with glucose 6-phosphate measured by the standard assay.

Male Sprague–Dawley rats weighing 180–250 g

were used. Synthase in the liver of a normal fed rat is essentially all in the D or glucose 6-phosphate-dependent form. A small amount of glucose 6-phosphate-independent activity is sometimes detected, and this can be decreased if the rat is given glucagon (100 μ g/kg) intravenously for 2 min before death.

Results

The kinetic parameters for liver synthase D and synthase I were measured, and the results are shown in Table 1. These values are within the range of kinetic constants determined for the liver enzymes (Hizukuri & Larner, 1964; Mersmann & Segal, 1967) and are comparable with those found for the two forms of the enzyme in other tissues (Rosell-Perez & Larner, 1964; Eichner, 1976; Solling, 1979).

Synthase D was readily converted into synthase I in a liver extract. To determine whether the conversion had occurred by way of an intermediate synthase form, the activity characteristics of the synthase generated at intermediate incubation times were compared with artificial mixtures of the original synthase D with the final product synthase I. Results are shown in Fig. 1. As indicated by the inset, there was a gradual increase in the activity ratio with incubation time, until at 80 min nearly all of the synthase was in the I form. Artificial mixtures of synthase D and synthase I were produced covering this range of activity ratios. As the fraction of synthase I in these mixtures increased, the increase in activity measured at 6.7 mM-UDP-glucose was accompanied by an increase in the activity at 0.5 mM-UDP-glucose, as expected. However, at the intermediate incubation times, where the

Table 1. *Kinetic properties of liver synthase D and synthase I*

A lobe of liver from a fed rat given glucagon was homogenized 1:3 (w/v) in (A) 50 mM-glycylglycine (pH 7.2)/5 mM- Na_2SO_4 /1% glycogen or in (B) 50 mM-glycylglycine (pH 7.2)/5 mM- Na_2SO_4 /1% glycogen/100 mM-KF/10 mM-EDTA. The homogenates were centrifuged at 8000 *g* for 10 min. The extracts obtained were incubated at 25°C for 80 min, at which time they were adjusted to a final dilution of 1:24 (w/v) with 100 mM-KF/100 mM-EDTA/1% glycogen (pH 7.2). Synthase in extract A was now nearly all in the I form, whereas synthase in extract B remained in the D form. The kinetic constants of these two samples were determined. The range of UDP-glucose (UDPG) used was 0.016–15 mM and the range of glucose 6-phosphate (G6P) used was 0–7.2 mM. Numbers in parentheses are the Hill coefficients.

	$s_{0.5}$ for UDPG (mM)		$A_{0.5}$ for G6P (mM)		V at 7.2 mM-G6P
	0 mM-G6P	7.2 mM-G6P	0.5 mM-UDPG	6.7 mM-UDPG	V at 0 mM-G6P
Synthase D	26 (1.5)	0.3 (1.0)	1.2 (1.3)	0.5 (1.3)	6.3
Synthase I	0.5 (1.1)	0.1 (1.0)	0.01 (1.0)	~0	1.0

Fig. 1. Changes in synthase activity measured at two concentrations of UDP-glucose during incubation of liver extract

Extract A containing synthase I and extract B containing synthase D were obtained as described in Table 1. They were mixed in various proportions to give artificial mixtures. In addition, conversion in extract A was stopped at different times during the incubation by dilution with 2 vol. of 100 mM-KF/10 mM-EDTA/1% glycogen (pH 7.2) and the incubation was continued until 80 min. All extracts were adjusted to a final dilution of 1:24 (w/v) with 100 mM-KF/10 mM-EDTA/1% glycogen (pH 7.2). The inset shows the increase in activity ratio with incubation time. The total activity remained constant during the incubation and was 2.7 units/g. Activities at 0.5 mM- and 6.7 mM-UDP-glucose (UDPG) were determined in the absence of glucose 6-phosphate. O, Samples containing artificial mixtures of synthase D and I; ●, samples obtained at different incubation times.

activity at 6.7 mM-UDP-glucose suggested a substantial amount of synthase I, the activity measured at 0.5 mM was much lower than would be expected if the activity was due to synthase I. This indicated

Fig. 2. Changes in the $A_{0.5}$ of synthase for glucose 6-phosphate during incubation of liver extract

Artificial mixtures (O) and samples at different incubation times (●) were obtained as described in Fig. 1. The inset shows the increase in activity ratio with incubation time. The activities were determined at various concentrations of glucose 6-phosphate (G6P) with the concentration of UDP-glucose held constant at 0.5 mM. The $A_{0.5}$, i.e. the amount of glucose 6-phosphate required for half-maximal activation of the synthase samples, was obtained from Hill plots.

that conversion of synthase D into synthase I in liver had occurred by way of another synthase form which was different from synthase D in having substantial activity at 6.7 mM-UDP-glucose and was different from synthase I in having little activity at 0.5 mM-UDP-glucose.

Other unusual forms of synthase reported in the

literature were generally described by their distinctive K_a for glucose 6-phosphate (Saugmann, 1977; Kaslow & Mayer, 1979; Kochan *et al.*, 1979). We next investigated whether the intermediate form of synthase in liver can be distinguished from the classical forms by this parameter. Again enzyme samples taken at intermediate incubation times were compared with artificial mixtures of synthase D and synthase I obtained from the same experiment. Results are shown in Fig. 2. In the artificial mixtures, the increase in the fraction of synthase I, here indicated by the increase in activity ratio at 6.7 mM-UDP-glucose, was accompanied by a decrease in $A_{0.5}$ for glucose 6-phosphate. In the samples obtained at intermediate incubation times, a similar increase in activity ratio resulted in almost the same change in $A_{0.5}$. These data indicate that it would be difficult to distinguish the intermediate form from synthase I by these measurements of $A_{0.5}$ for glucose 6-phosphate.

Since activity of synthase is affected by endogenous metabolites (Mersmann & Segal, 1967; Piras *et al.*, 1968), the same study was repeated in a glycogen-particle preparation, where most of the soluble metabolites had been removed (Gilboe & Nuttall, 1973). Results obtained (not shown) were similar to those of Fig. 1. Therefore, in a glycogen-particle preparation, the intermediate form of glycogen synthase was also detectable.

Discussion

Starting with purified liver synthase I, Jett & Soderling (1979) found that conversion into synthase D was not complete with cyclic AMP-dependent protein kinase until 3 mol of phosphate/mol had been incorporated. With cyclic AMP-independent synthase kinase, only 2 mol of phosphate/mol was required. Similar studies have been reported for muscle synthase I (Nimmo *et al.*, 1976; Soderling *et al.*, 1977; Brown *et al.*, 1977). In addition, it has been found that the partially phosphorylated forms generated had different kinetic properties (Huang *et al.*, 1979; Huang & Huang, 1980; Brown *et al.*, 1980). These results indicate that phosphorylation and dephosphorylation at multiple sites occur in the synthase-D and synthase-I interconversions. However, the nature of the intracellular synthase kinase(s) and synthase phosphatase(s) and the number of enzymes involved are still not clear.

Much effort has been expended searching for kinetically distinct forms of synthase in tissues obtained under various physiological perturbations. A synthase 'X' and a synthase 'Q' have been reported in adipose tissue in addition to the D and I forms (Eichner, 1976). A glycogen synthase 'R', different from the two classical forms, was sug-

gested to be involved in glycogen synthesis in 'un-starved' human leucocytes (Saugmann, 1977). After glycogen depletion by exercise in humans, an intermediate form of synthase was believed to be responsible for the new glycogen synthesized (Kochan *et al.*, 1979). These new forms in general had a low activity ratio, but were different from classical synthase D in having a much lower K_a for glucose 6-phosphate.

It is hazardous to define additional discrete forms of synthase solely on the basis of measured affinities for substrate and modifier, since mixtures of the D and I forms give rise to complex kinetics. In this paper, an independent method was used which demonstrated clearly an intermediate form of synthase in liver extract during the conversion of synthase D into synthase I. This form is characterized by an affinity for UDP-glucose in the absence of glucose 6-phosphate which is high but is intermediate between that of synthase D and synthase I. Whether an additional change in the modifier site, i.e. one resulting in a low K_a for glucose 6-phosphate, has occurred is not clear. The K_a value for glucose 6-phosphate for liver synthase D and synthase I were found to be strongly dependent on the UDP-glucose concentration. Therefore it would be difficult to investigate changes in the glucose 6-phosphate binding site independent of the substrate site unless pure species of the various forms are available.

By using the standard assay with saturating UDP-glucose, liver from a starved rat was found to have higher 'synthase I' activity than did liver from a fed rat (Curnow & Nuttall, 1972). Upon administration of glucose *in vivo*, there was a rapid increase in 'synthase I', followed shortly by a deposition of glycogen in liver (Nuttall *et al.*, 1972). In the light of the present report, it would be interesting to study the nature of the synthase forms found in these animals.

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