Biochimica et Biophysica Acta, 659 (1981) 23—30
Elsevier/North-Holland Biomedical Press

BBA 69253

LIVER GLYCOGEN SYNTHASE IN THE DEVELOPING FOETAL RAT

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(Received January 19th, 1981)

Key words Glycogen synthase, UDPglucose, Development, (Foetal rat liver)

Summary

Kinetic constants for liver glycogen synthase (UDPglucose: glycogen 4-α-D-glucosyltransferase, EC 2.4.1.11) with respect to UDPglucose have been measured in foetal liver homogenates from samples taken during late gestation (days 17—22) and the first hours after birth The V of the inactive form of glycogen synthase increased markedly in this period and there was a significant increase in V of the active enzyme to a maximum at day 20 of gestation. The K_m for UDPglucose measured in the presence of glucose-6-P (total activity) did not vary greatly, mean values of 0.51 ± 0.04 mM at day 17 to 0.72 ± 0.03 mM after birth being similar to those for adult liver, 0.60 ± 0.04 mM. Values derived for the inactive enzyme were almost identical. In contrast, K_m values for active glycogen synthase in foetal livers during gestation were significantly higher than those for adult liver. Highest values were seen at day 19 of gestation (1.84 ± 0.08 mM) followed by a steady fall to 0.55 ± 0.05 mM in the newborn compared with a mean value of 0.48 ± 0.04 mM for adult liver. Existence of a reduced affinity of active glycogen synthase for UDPglucose must be recognized when assaying the enzyme in foetal liver, particularly when extrapolating values to rates of glycogen synthesis in vivo.

Data were obtained only after removal of an amylase-like contaminant from foetal liver samples which invalidated the radioassay of glycogen synthase.

This work illustrates the care needed in the analysis of foetal tissue and the interpretation of resulting data when utilizing methods developed for adult tissue.

0005—2744/81/0000—0000/$02 50 © Elsevier/North-Holland Biomedical Press

Introduction

In late gestation in the rat there is synthesis and deposition of liver glycogen by the foetus accompanied by the appearance and activation of glycogen synthase (UDPglucose glycogen 4-α-D-glucosyltransferase, EC 2.4.1.11) [1,2]. The question of whether the foetus is able to utilize its liver glycogen hinges on the rates of synthesis of glycogen in vivo calculated from the activity of glycogen synthase measured in vitro. From their measurements of active glycogen synthase and glycogen levels in foetal liver over the last 5 days of gestation, Devos and Hers [1] calculated that the maximal synthetic rate would not allow for simultaneous glycogenolysis. Other workers, however, have postulated that a turnover of foetal liver glycogen is possible [2,3] For these reasons, it is important that conditions for the in vitro assay of glycogen synthase in foetal liver homogenates be optimized to give an accurate measurement of enzyme activity Studies on glycogen synthase in foetal liver so far have involved measuring the enzyme activity in homogenates, using the same techniques and assay conditions as those used for studies on adult liver. Affinity constants of the enzyme for its substrate UDPglucose in the presence or absence of glucose-6-*P* have not been studied in detail in foetal liver, although they have been determined in crude homogenates [4] and partially-purified preparations [5—8] from adult liver In the present study on livers from foetal rats in late gestation and newborn rats, affinity constants of glycogen synthase for UDP-glucose have been measured in the absence (active enzyme) and presence (active + inactive enzyme) of glucose-6-*P*. From these constants values were also derived for the inactive enzyme.

Materials and Methods

Animals All studies were made on rats of a derived Wistar strain. Pregnancy was dated from the presence of a copulation plug after leaving females with a male overnight (day 0), parturition occurred normally on day 22. Non-pregnant females were used to obtained adult liver. Animals were kept in a conventional animal house at 19—25°C and fed a standard diet

Chemicals. UDP[U-^{14}C]glucose and [U-^{14}C]glucose-1-*P* were obtained from the Radiochemical Centre (Amersham, U.K.). UDPglucose, glucose-1-*P*, glucose-6-*P*, oyster glycogen (Type II) and bovine serum albumin were obtained from Sigma Chemical Co. (St Louis, U.S A.). Glycogen solutions were treated with Amberlite monobed resin (BDH, Poole, U.K.) before use

Methods Foetuses were removed by caesarian section after anaesthetizing the mother with pentobarbital (Nembutal, Abbot Lab., New Zealand). Foetal livers were removed with a fine pair of scissors, care being taken to exclude any non-hepatic tissue. In the initial studies livers were frozen immediately in liquid N_2 but in latter studies they were first rinsed by rapid swirling in ice-cold buffer and touch-dried on Whatman No. 52 filter paper. Frozen livers from a litter were pooled for homogenization. Livers from newborn rats were taken 6—18 h after birth under light ether anaesthesia and again livers from newborn in the same litter were pooled Livers from adult rats were removed under pentobarbital anaesthesia and frozen immediately in liquid N_2.

Foetal and adult livers were homogenized in 3 vol. ice-cold 50 mM Tris-HCl (pH 7.8)/5 mM EDTA/100 mM NaF. Homogenates were centrifuged at 3000 × *g* for 15 min at 0°C and the supernatants used for enzyme analysis. In some litters, pancreases were excised from the foetuses after removing the liver. They were homogenized in 9 vol. 50 mM Tris-HCl (pH 7.8) and used immediately.

Glycogen synthase was measured at 30°C by incorporation of [U-^{14}C]glucose from UDP[U-^{14}C]glucose into glycogen and the labelled glycogen counted after adsorption on filter paper [9]. Active glycogen synthase was measured in the presence of 15 mM Na_2SO_4 as described previously [2] but over a range of UDPglucose concentrations (0.17—3 mM final conc.) Total glycogen synthase activity (active + inactive) was measured in the presence of 7 5 mM glucose-6-*P* [2] over the same range of UDPglucose concentrations. A unit of enzyme activity incorporated 1 μmol glucose into glycogen/min.

Glycogen was prelabelled with ^{14}C in the following way. Adult rat liver was homogenized in 3 vol. 200 mM β-glycerophosphate (pH 6.1). 1 ml homogenate supernatant was incubated at 37°C for 1 h with 2 ml 100 mM glucose-1-*P*/200 mM NaF/1% (w/v) glycogen/approx. $4 \cdot 10^6$ cpm from [U-^{14}C]glucose-1-*P* 5 ml 30% (w/v) KOH was then added and the solution boiled for 30 min The glycogen was twice precipitated from the digest with 95% ethanol before being taken up in 1 ml of the appropriate buffer. The preparation contained 48% of the radioactivity originally added.

The amylase activity of liver homogenates was measured in 50 mM Tris-HCl buffer at pH 7.8 by the method of Henry and Chiamori [10].

Protein was measured by the method of Lowry et al. [11] using bovine serum albumin, Fraction V, as standard.

Results

Glycogenolysis by foetal liver homogenates

The activity of glycogen synthase in homogenates of foetal liver frozen immediately in liquid N_2 was found to vary randomly from one gestational age to another and between litters of the same age. The enzyme reaction was non-linear with regard to time and dilution of homogenate. Incubation of foetal and adult liver homogenates with pre-labelled [^{14}C]glycogen under conditions of the synthase assay (Fig. 1) indicated that labelled glycogen in the synthase assay was being degraded when using foetal liver homogenates Amylase activity was also measured in homogenates of foetal and maternal liver (Table I), when higher activities were found in the foetal liver homogenates, these being increased dramatically when the liver was frozen in liquid N_2. The very high amylase activities were associated with greatly reduced synthase activities. Table II shows the effects of deliberately contaminating homogenates of washed foetal liver with a pancreatic homogenate prepared from the same litter and demonstrates that extremely small amounts of pancreatic material can cause inhibitory effects on the glycogen synthase assay.

It was subsequently found that a rapid rinsing of foetal liver in ice-cold buffer before freezing removed the glycogenolytic contamination (Table III) and the enzyme reaction became linear with time. Consequently, all foetal livers were treated this way before carrying out glycogen synthase measurements.

TABLE I

TOTAL GLYCOGEN SYNTHASE AND AMYLASE ACTIVITY IN FRESH AND FROZEN HOMOGENATES FROM FOETAL AND ADULT LIVER

Foetal and adult livers were homogenized in 3 vol. ice-cold 50 mM Tris-HCl (pH 7 8)/5 mM EDTA/100 nM NaF Fresh liver was excised with care, and rinsed in ice-cold buffer before homogenizing Frozen tissue was taken and frozen immediately in liquid N_2 Enzyme activities expressed as munits of synthase and units of amylase/mg protein

	Gestational day		Total glycogen synthase	Amylase
Foetal liver	20	Fresh	36 9	16
		Frozen	12 5	148
	21	Fresh	39 2	46
		Frozen	1 2	801
Maternal liver	20	Fresh	10 5	1 3
		Frozen	10 6	2 3
	21	Fresh	9 3	0 7
		Frozen	9 6	2 3

Kinetic constants for glycogen synthase in foetal liver

The velocity of the glycogen synthase reaction at different UDPglucose concentrations was measured in the presence and absence of 7.5 mM glucose-6-*P* in liver homogenates from litters aged 17—22 days and within the period 6—18 h after birth. The data were plotted as s/v against s and the K_m and V values derived by the statistical method of Wilkinson [12]. Fig. 2 shows the typical plots for day 18 and day 22 foetal livers and for an adult liver. In all the

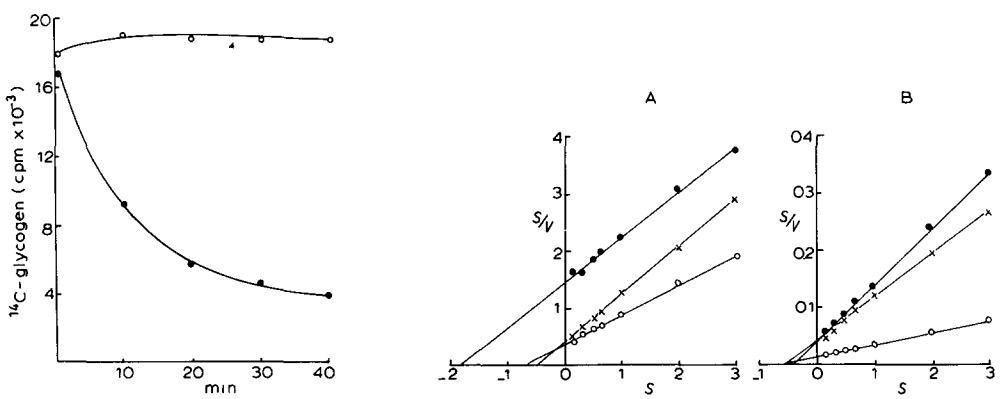

Fig 1 Glycogenolysis in homogenates of foetal liver Foetal and adult livers were frozen immediately in liquid N_2 Supernatants of homogenates of 21-day foetal (●———●) and adult (○———○) liver in 3 vol 50 mM Tris-HCl (pH 7 8)/5 mM EDTA/100 mM NaF were incubated with a solution of ^{14}C-labelled glycogen in the same buffer at 30°C At selected times, 50 µl aliquots were spotted onto filter paper, washed in ethanol as in the glycogen synthase assay and the ^{14}C-labelled glycogen counted

Fig 2 s/v Against s plots for UDPglucose concentration and glycogen synthase activity Liver homogenate supernatants were incubated at 30°C and pH 7 8 with 50 mM Tris-HCl/5 mM EDTA/33 mM NaF/1% glycogen in the absence (A) or presence (B) of 7 5 mM glucose-6-*P* at the UDPglucose concentrations indicated Representative plots are shown for foetal livers at day 18 (●———●) and day 22 (○———○) of gestation and adult liver (X———X)

TABLE II

THE EFFECT OF ADDED FOETAL PANCREATIC EXTRACTS ON THE MEASURABLE GLYCOGEN SYNTHASE OF FOETAL LIVER HOMOGENATES

Homogenates were prepared from washed foetal livers and foetal pancreases Aliquots of serial dilutions of the pancreatic homogenates were added to liver homogenate supernatants to give proportions of pancreas to liver shown Total glycogen synthase was measured and the activity as munits/mg protein calculated from the ^{14}C counts incorporated into glycogen at 15 min, no account being taken of non-linearity

Gestational age	Pancreas liver				
	1 100	1 1000	1 5000	1 10 000	Liver alone
Day 19	0	3 2	10 9	12 7	20 3
Day 21	0 2	4 8	26 9	38 2	53 6
Day 22	3 5	24 5	32 3	36 7	39 1

homogenates studied, these plots were linear over the UDPglucose concentrations studied.

Table IV shows combined data for the kinetic constants of glycogen synthase in liver from litters of foetal and newborn rats and a group of adults. Values for total and active glycogen synthase were obtained from measurements taken in the presence and absence of glucose-6-*P*, while values for the inactive enzyme were derived from the difference in these activities at each substrate concentration. The statistical significance of the data was ascertained by analysis of variance and regression analysis. There was a pronounced increase in the amount of inactive glycogen synthase, expressed as V, produced in the foetal liver from day 17 of gestation through to the period after birth ($P < 0.001$). At birth the V was 3-times greater than that seen for the enzyme in adult liver. There was a trend towards slightly higher K_m values of the inactive enzyme as gestation proceeded ($P < 0.01$) but values were not significantly different from those seen for adult liver. For the active form of glycogen synthase, the V increased significantly from day 17 to a maximum at

TABLE III

THE EFFECT OF WASHING FOETAL LIVER TISSUE BEFORE FREEZING ON MEASURABLE GLYCOGEN SYNTHASE ACTIVITY

For each litter, livers taken from foetuses in one uterine horn were frozen immediately in liquid N_2 (non-washed) while livers from foetuses in the other horn were rinsed in ice-cold buffer and touch-dried before freezing (washed) Homogenates from the washed and non-washed livers were prepared and the total glycogen synthase assayed using two incubation times

Gestational age	Glycogen synthase activity (munits/mg protein)		
Incubation time		5 min	15 min
18 days	Non-washed	7 0	5 5
	Washed	13 3	13 2
19 days	Non-washed	5 2	1 4
	Washed	26 6	26 1
22 days	Non-washed	15 1	3 8
	Washed	29 1	29 1

TABLE IV

KINETIC CONSTANTS FOR GLYCOGEN SYNTHASE AND UDP-GLUCOSE DURING GESTATION OF THE FOETAL RAT

Total glycogen synthase was measured in the presence of 7 5 mM glucose-6-*P*, active glycogen synthase minus glucose-6-*P* and in the presence of 15 mM Na_2SO_4 Data for inactive glycogen synthase were obtained from the difference between the total and active activities at each substrate concentration K_m values are expressed as mM UDPglucose and *V* values as munits/mg protein Results are mean ± S E

Glycogen synthase		Total		Active		Inactive	
Liver	No	K_m	*V*	K_m	*V*	K_m	*V*
Foetal Day 17	5 *	0 51 ± 0 04	10 ± 0 6	1 43 ± 0 10	0 4 ± 0 01	0 50 ± 0 03	9 ± 0 6
Foetal Day 18	5	0 51 ± 0 03	14 ± 1 6	1 69 ± 0 09	1 0 ± 0 17	0 48 ± 0 03	13 ± 1 5
Foetal Day 19	5	0 58 ± 0 03	27 ± 0 9	1 84 ± 0 08	3 2 ± 0 13	0 53 ± 0 04	24 ± 0 8
Foetal Day 20	5	0 59 ± 0 02	33 ± 1 4	1 58 ± 0 10	4 3 ± 0 47	0 53 ± 0 03	30 ± 1 2
Foetal Day 21	5	0 57 ± 0 02	35 ± 2 5	1 34 ± 0 14	3 4 ± 0 40	0 54 ± 0 02	32 ± 2 3
Foetal Day 22	5	0 59 ± 0 03	42 ± 2 5	0 75 ± 0 04	2 4 ± 0 14	0 58 ± 0 03	40 ± 2 7
Newborn	5	0 72 ± 0 03	40 ± 2 4	0 55 ± 0 05	2 4 ± 0 18	0 73 ± 0 03	37 ± 2 6
Adult	10	0 60 ± 0 04	13 ± 0 7	0 48 ± 0 04	1 1 ± 0 08	0 60 ± 0 05	12 ± 0 7

* Number of litters studied for foetal and newborn groups

day 20 ($P < 0.001$). There was then a significant fall in active glycogen synthase towards birth ($P < 0.01$). In contrast to the inactive enzyme, there were significant changes in the affinity of active glycogen synthase for UDPglucose. During gestation K_m values were up to 3-times higher than those seen for adult liver. Values increased slightly from days 17 to 19 ($P < 0.02$), but thereafter there was a steady fall in K_m up to birth ($P < 0.001$) and in the newborn, values were approaching those seen in adult liver.

Discussion

The presence of a contaminant causing glycogenolysis in homogenates of foetal liver must be recognised as a potentially serious hazard in any experiment involving measurement of ^{14}C-labelled glycogen. Fortunately, the contaminant can be removed by rinsing the liver quickly before freezing. The contamination was probably due to amylase in secretions released from the foetal pancreas during removal of the foetus from the uterus and dissecting out the liver. These experiments demonstrate the caution required in working with the small and delicate organs of the foetal rat and the care needed in applying procedures developed for adult tissues directly to the foetal environment.

The precautions required in the handling of foetal liver have not been referred to by previous workers. However, in this context, it is pertinent to note that our results on glycogen synthase are in general much higher than those of two previous studies [1,13] using a radioassay to measure the enzyme but are in good agreement with those of Ballard and Oliver [14], who used an assay based on the production of UDP which would be independent of any glycogenolytic contaminant in the homogenate.

Our previous work has shown that as glycogen is deposited progressively in the liver of the foetal rat from day 17 of gestation, total glycogen synthase activity increases steadily. The present data show that the increase in total

glycogen synthase is primarily due to an increase in V of the inactive enzyme with little variation in the affinity for UDPglucose The K_m values for livers from foetal and newborn animals of 0.48—0.73 mM and 0 60 mM UDPglucose for adult liver compare reasonably with previously reported values for the partially purified enzyme from adult rat liver of 0.5 [6], 0.56 [7] and 0.9 mM [15] Values of 0.79—1.1 mM have been found with crude homogenates [4] All of these were measured in the presence of 4 mM or higher amounts of glucose-6-*P*.

Values reported previously for the K_m for UDPglucose of active glycogen synthase in adult rat liver have varied from 0.74 to 2.5 mM [4,6,8,15] compared with 0.48 mM in the present work. The V of active glycogen synthase increased to day 20 of gestation and then fell towards birth on day 22. This pattern was identical to our previous work [2] where the active form was measured at a substrate concentration of 3 mM UDPglucose which, compared with the present data, measured 85% of the maximal activity in adult and newborn livers, but only 60—65% in foetal livers at days 17 to 20 of gestation. The apparent inhibition of active synthase from day 20 onward, could be due to the high glycogen levels in the liver regulating the rate of glycogen synthesis, as has been postulated recently [16]. We found major differences in the apparent K_m for UDPglucose of active glycogen synthase in foetal and adult liver homogenates. Devos and Hers [1] have stated that there can be no degradation or turnover of liver glycogen by the foetus from days 10 to 21 5 of gestation. They reached this conclusion because their active glycogen synthase values were only just adequate to account for the rate of glycogen accumulation over this period However, they used a UDPglucose substrate concentration of only 0 25 mM and if the K_m was very much higher as in the present work they would greatly underestimate the maximal activity of the active enzyme and invalidate their extrapolation to in vivo synthetic rates. The glycogenolytic contamination could also contribute to an artifical lowering of their enzyme activities Their highest active glycogen synthase value of 80 munits/g wet wt. compares with an equivalent value of 480 munits/g wet wt. calculated from the V in the present work. There are many pitfalls in extrapolating in vitro enzyme activities to synthetic rates in vivo and if calculations were made from our in vitro V values, assuming the active synthase was saturated by its substrate, we would derive a rate of glycogen synthesis may times greater than that obtained by Devos and Hers [1]. However, our K_m values for foetal active synthase and those reported by others for adult liver [4,6,8,15] are much higher than the UDPglucose concentrations measured on whole liver samples of 0.23 [17] and 0.45 [18] pmol/g liver and obviously other effectors could be important in the in vivo situation Despite this, the changes in the affinity of the foetal active synthase for UDPglucose shown here must represent a significant change in properties of the enzyme, although these are difficult to explain. Presumably, an effector which increases the affinity of the active enzyme for UDPglucose could appear or increase in concentration in the liver in late gestation. These could include P_i although we have not detected any major changes in foetal liver homogenates over this period [19]. Others could be glucose-6-*P* [5], or citrate and other tricarboxylic acids [20] which are said to produce conformational changes in active glycogen synthase. Extensive studies will be required to

elucidate the changes that occur in the enzyme and its activation during late gestation.

Acknowledgements

This work was supported by a grant from the Medical Research Council of New Zealand. We would like to thank Dr M Heyworth for the statistical analyses.

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