A New Variant of Galactosemia: Galactose-1-phosphate Uridylyltransferase Sensitive to Product Inhibition by Glucose 1-phosphate

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Summary

In the erythrocytes of a patient with the clinical symptoms of galactosemia, a galactose-1-phosphate uridylyltransferase with abnormal kinetics was observed. Under standard assay conditions, the uridylyltransferase activity was almost normal initially and became completely inactivated within 30 min. The abnormal kinetics could be ascribed to a product inhibition by glucose 1-phosphate. The inhibition was produced by a variety of sugar phosphates, the most potent of which proved to be glucose 1-phosphate, mannose 1-phosphate, and fructose 6-phosphate. The variant galactose-1-phosphate uridylyltransferase was further characterized by a lowered affinity towards galactose 1-phosphate, non-Michaelis-Menten kinetics towards UDP-glucose, an increased thermal stability, and complete inactivity upon Cellophane electrophoresis.

Speculation

The erythrocyte galactose-1-phosphate uridylyltransferase of the patient was almost completely inhibited at 50 μ M glucose 1-phosphate, which is within the range of the stationary glucose 1-phosphate concentration reported for liver. These findings suggest that the product inhibition of galactose-1-phosphate uridylyltransferase by glucose 1-phosphate is responsible for the impaired galactose-1-phosphate metabolism in the patient.

Classical galactosemia is an autosomal recessive disorder of galactose metabolism characterized by vomiting, diarrhea, jaundice, failure to thrive, progressive mental and motoric retardation, and development of liver cirrhosis and cataracts (for review, see Ref. 12). The basic defect is the inactivity of galactose-1-phosphate uridylyltransferase (UDP-glucose: α -D-galactose-1-phosphate uridylyltransferase, EC 2.7.7.12) (6), which will be referred to as transferase. The transferase deficiency galactosemia has to be distinguished from the galactokinase (EC 2.7.1.6) deficiency galactosemia, which is associated with less severe clinical symptoms (3).

Several variants of the transferase deficiency galactosemia have been reported. The "Negro" variant is characterized by milder expression of the clinical symptoms associated with the absence of transferase activity in erythrocytes and a residual activity of about 10% in liver and intestine (13). The "Rennes" and the "Indiana" variants exhibit the severe symptoms of galactosemia, but their residual activities of transferase in erythrocytes are 7% and up to 45%, respectively (11). The electrophoretic mobility of both variant transferases is reduced, and the Indiana variant is additionally characterized by instability upon storage in heparin and phosphate buffer. The "Chicago" variant is characterized by mild clinical

symptoms associated with 25% residual activity in erythrocytes and fibroblasts. This activity results from the combination of two mutant alleles of the transferase in a genetic compound (2).

The present study describes a new variant of galactosemia in which the transferase of erythrocytes becomes inactivated during incubation. This inactivation is likely to be caused by glucose 1-phosphate, a product of the transferase reaction.

CASE REPORT

A detailed clinical description of the patient, his pedigree, and transferase activities determined with a UDP-glucose consumption test has been reported earlier by Matz *et al.* (9). Within the first two wk, the patient, G. R., now 4 years old, developed jaundice, vomiting, failure to thrive, hepatosplenomegaly, and opisthotonus. Under a galactose-free diet, the symptoms reversed, and the child has shown a normal development up to now.

MATERIALS AND METHODS

MATERIALS

[U-¹⁴C]Galactose-1-phosphate (specific activity, 348 mCi/mmol) and [U-¹⁴C]glucose 1-phosphate (specific activity, 328 mCi/mmol) were obtained from Amersham-Buchler (Braunschweig). UDP-glucose dehydrogenase (EC 1.1.1.22) from bovine liver (specific activity, 0.6 units/mg protein), phosphoglucomutase (EC 2.7.5.1) from rabbit muscle (specific activity, 200 units/mg protein), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) from yeast (specific activity, 140 units/mg protein), and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) from yeast (specific activity, 12 units/mg protein) were purchased from Boehringer Mannheim (Mannheim). NADP and sugar phosphates, all sodium or potassium salts, were obtained from Boehringer Mannheim and Serva (Heidelberg), cellulose acetate gel (Cellophane) was obtained from Chemetron (Milano), DE52 cellulose was obtained from Whatman (Maidstone), and Dowex 1 x 2, 200 to 400 mesh, Cl⁻-form was obtained from Serva.

TRANSFERASE ASSAY

Unless otherwise stated, the transferase activity was determined according to Bertoli and Segal (1) according to the modification of Shin-Bühning *et al.* (14). Heparinized venous blood was centrifuged, and the erythrocyte pellet was washed three times with 0.15 M NaCl and finally suspended in distilled water to yield a hemoglobin concentration of about 70 mg/ml. The hemolysate was frozen once and then incubated for 15 min at 37°C to destroy NAD. Transferase activity was assayed in 100 mM glycine:NaOH, pH 8.7, containing 50 mM cysteine:HCl, 2.95 mM [U-¹⁴C]galac-

tose 1-phosphate (specific activity, 0.254 mCi/mmol), 1.2 mM UDP-glucose, and 50 μ l hemolysate in a final volume of 100 μ l. Blanks were incubated with hemolysate boiled for 5 min.

After incubation for 10 min at 37°C, the mixture was boiled for 3 min. After centrifugation, 50 μ l of the supernatant were loaded on a DE52 cellulose column (0.5 x 4 cm) equilibrated with 20 mM HCl. [U - 14 C]Galactose-1-phosphate was eluted with 8 ml of 20 mM HCl and UDP-[U - 14 C]galactose with 5 ml of 100 mM HCl. The eluants were assayed for 14 C-labeled radioactivity with three volumes of a scintillation medium made of 12 mM PPO and 0.6 mM POPOP in a toluene:Triton X-100 mixture of 1:0.35 (v/v). The counting efficiency was 78 and 76% in the presence of 20 and 100 mM HCl, respectively. Transferase activity was calculated as μ mol UDP-[U - 14 C]galactose formed per g hemoglobin (Hb) and h.

The coupling of the transferase reaction to glucose 1-phosphate-consuming reactions by inclusion of phosphoglucomutase and glucose 6-phosphate dehydrogenase was carried out according to Tedesco (15) and to Pesce *et al.* (10).

ELECTROPHORESIS ON CELLOGEL

Electrophoresis on Cellogel was modified from the starch gel electrophoresis described by Harris and Hopkinson (5). The separation was carried out at 200 V for 2 hr at 4°C in 0.04 M Tris-histidine, pH 8.0. The staining mixture contained 9.84 mM $MgCl_2$, 0.475 mM NADP, 0.02 mM glucose 1,6-diphosphate, 7.7 mM galactose-1-phosphate, 3.5 mM UDP-glucose, 8 units phosphoglucomutase, 2.8 units glucose 6-phosphate dehydrogenase, and 2.4 units 6-phosphogluconate dehydrogenase in a final volume of 2 ml of 0.13 M glycylglycine, pH 8.7. The gel was soaked with the staining mixture and incubated at 37°C for 1 to 2 hr. Transferase activity was visualized as fluorescence under longwave UV light.

RESULTS

The transferase activity in the hemolysate of the patient G. R. was $8.1 \mu\text{mol} \times \text{g Hb}^{-1} \times \text{h}^{-1}$ (mean of six independent determinations with a range of 5.9 to $9.4 \mu\text{mol} \times \text{g Hb}^{-1} \times \text{h}^{-1}$) corresponding to 33% of the activity of controls ($n = 14$). When the time course of UDP-[U - 14 C]galactose formation was followed for up to 2 hr, it became evident that an initially high rate of uridylyl transfer rapidly decreased to zero within 30 min (Fig. 1). The product of the reaction was identified as [14 C]UDP-galactose as follows. The material eluted from DE52 cellulose with 100 mM

HCl was freeze-dried and applied, together with a UDP-galactose standard, onto a Dowex 1 x 2 column. The 14 C-labeled transferase product and the UDP-galactose standard coeluted when a linear NaCl gradient made of 60 ml H_2O and 60 ml 0.5 M NaCl was applied to the column (not shown).

The rapid loss of uridylyl transfer during incubation suggested an increased lability of the enzyme during incubation or inhibition of the enzyme by one of its two products. Compared to controls, the thermal stability of the variant transferase was even increased (Fig. 2). The transferase of patient G. R., however, was sensitive to inhibition by low concentrations of glucose 1-phosphate (Fig. 3), whereas the transferase activity of controls was not inhibited by up to 0.25 mM glucose 1-phosphate. UDP-galactose had no inhibitory effect on the transferase activity of patient G. R. and controls (Fig. 4). A concentration of 40 μ M glucose 1-phosphate, which reduces the variant transferase activity to 50%, is generated under standard assay conditions within less than 3 min. Thus, the 50% reduction of transferase activity in patient G. R. determined after incubation for 5 min (Fig. 1) could well be explained as the result of product inhibition. The transferase activity of patient G. R. is reduced additionally, due to the lowered affinity of the variant enzyme towards galactose-1-phosphate. The K_M for galactose-1-phosphate was repeatedly about 7 times higher than in controls (Fig. 5). Towards UDP-glucose, the patient's transferase showed a non-Michaelis-Menten kinetic whereas the K_M for controls was 0.14 mM (range of four determinations, 0.12 to 0.16 mM). Affinities for glucose 1-phosphate and UDP-galactose could not be determined due to the inhibitory effect of glucose 1-phosphate.

The analysis of the conversion rate of [U - 14 C]glucose 1-phosphate to UDP-[U - 14 C]glucose in the presence of low [U - 14 C]glucose 1-phosphate (below 10 μ M) and high UDP-galactose (2.95 mM) concentrations revealed that under these conditions the rate of UDP-[U - 14 C]glucose formation by the hemolysate of patient G. R. was 45% of that of a control (Table 1). In addition to glucose 1-phosphate, a variety of phosphorylated hexoses and pentoses were found to inhibit the transferase activity of patient G. R. (Table 2). None of these inhibitory sugar phosphates had an effect on the transferase activity of controls.

All attempts failed to prevent the glucose 1-phosphate-mediated inhibition of transferase by inclusion of phosphoglucomutase, glucose 6-phosphate dehydrogenase, and NADP under conditions as described by Tedesco (15) and Pesce *et al.* (10).

In the hemolysates of the parents, transferase activities reduced

Fig. 1. Formation of UDP-[U - 14 C]galactose by hemolysate of the patient (●), control (○), and of the patient's mother (□) as function of incubation time.

Fig. 2. Thermal stability of uridyltransferase activity of patient G. R. (□, ■) and controls (○, ●). The hemolysates were kept for up to 10 min either at 40°C (□, ○) or at 50°C (■, ●).

Fig. 3. Effect of glucose 1-phosphate on the uridyltransferase activity of patient G. R. (●) and of control (○). Activity is expressed as percentage of a glucose 1-phosphate-free control.

to 42 and 45% of that of controls were found. Of 18 relatives investigated, 8 had activities within the patient's parents range (Fig. 6). These results are similar to those previously obtained with an UDP-glucose consumption test (9). When the formation of UDP-[U-¹⁴C]galactose is followed over 120 min, it becomes evident that during the first 20 min the apparent residual activity is relatively lower than after incubation for 60 to 120 min when product formation is impaired in controls by the consumption of up to 30% of the added substrates. Inclusion of 100 μM glucose 1-

Fig. 4. Effect of UDP-galactose on the uridyltransferase activity of patient G. R. (●) and of control (○). Activity is expressed as percentage of a UDP-galactose-free control.

Table 1. UDP-[U-¹⁴C]glucose formation in the presence of low [U-¹⁴C]glucose concentration¹

	[U- ¹⁴ C]Glucose 1-phosphate (μM)	UDP-[U- ¹⁴ C]glucose formation (nmol × 15 min ⁻¹ × g Hb ⁻¹)
Patient G. R.	1	7.6
	10	57
Control	1	16.1
	10	126.5

¹ Concentration of UDP-galactose was 2.95 mM and incubation time 15 min.

phosphate had a slight inhibitory effect on the transferase activity of both parents hemolysate similar to that in controls (inhibition by 18 to 20% in the parents and 10% in the controls). This indicates that the mutant uridyltransferase in the parents hemolysate contributes only little to the formation of UDP-[U-¹⁴C]galactose,

Fig. 5. Effect of galactose-1-phosphate on UDP-[U- 14 C]galactose formation. The UDP-glucose concentration was 1.2 mM. The K_M for galactose-1-phosphate was 7.1 mM for the patient G. R. (●) and 1.02 mM for control (○).

Table 2. Inhibition of transferase activity of patient G. R. by glucose and sugar phosphates¹

Addition (0.25 mM)	Transferase activity	
	($\mu\text{Mol} \times \text{g Hb}^{-1} \times \text{h}^{-1}$)	(% of control)
None	4.00	100
Glucose 1-phosphate	0.30	7
Glucose 6-phosphate	0.88	22
Fructose 1-phosphate	3.84	96
Fructose 6-phosphate	0.33	8
Mannose 1-phosphate	0.23	6
Mannose 6-phosphate	1.24	31
Galactose-6-phosphate	0.84	21
Ribulose 5-phosphate	2.40	60
Glucose	2.52	63
N-Acetylglucosamine	2.88	72

¹ The activity of the control in the presence of these compounds was within 90 and 102% of that of a sugar phosphate-free control.

presumably due to the fact that inhibitory glucose 1-phosphate concentrations are reached more rapidly.

By electrophoresis on Cellogel, the obligate heterozygote parents revealed a normal mobility of the residual transferase activity. Attempts to detect transferase activity in the hemolysate of patient G. R. failed (Fig. 7), although a coupled reaction depleting glucose 1-phosphate was used.

DISCUSSION

The residual transferase activity in the hemolysate of patient G. R. who, within the first two wk after birth, developed the typical symptoms of galactosemia, was characterized by (1) inhibition by glucose 1-phosphate and several other sugar phosphates; (2) reduced affinity towards galactose-1-phosphate; (3) non-Michaelis-Menten kinetics towards UDP-glucose; (4) increased thermal stability; and (5) inactivity upon electrophoresis on Cellogel.

The reduced transferase activity can be attributed in part to the 7-fold increased K_M towards galactose-1-phosphate. The almost complete inactivation of the enzyme within less than 30 min incubation, however, is probably due to the product inhibition by glucose 1-phosphate. It cannot be definitely answered whether the inhibition by glucose 1-phosphate as observed under *in vitro* conditions is responsible for the abnormal galactose metabolism in the patient. The stationary concentrations of glucose 1-phosphate in erythrocytes has been reported to be 500 μM (4). This concentration is sufficient for complete inactivation of the patient's transferase. It is, however, unclear whether similar conditions are met in the liver, which is the main organ for galactose. If the liver transferase has the same characteristics as that of erythrocytes, a marked reduction of transferase activity will be produced by the stationary glucose 1-phosphate concentration in liver, which has been determined to be 60 μM with the freeze-stop technique (7).

The transferase reaction seems to proceed in two half reactions (8).

Fig. 6. Pedigree of patient G. R.'s family with the uridylyltransferase activity ($\mu\text{mol UDP-[U-}^{14}\text{C]galactose} \times \text{g Hb}^{-1} \times \text{h}^{-1}$).

Fig. 7. Mobility of uridylyltransferase of control (1), patient G. R. (2), father (3), and mother (4) on Cellogel.

The backward reaction could only be tested at very low glucose 1-phosphate concentrations. Under such conditions, the rate of UDP-glucose formation was somewhat less than 50% of that of a control. These results indicate that an exchange of glucose 1-phosphate and galactose-1-phosphate at the sugar-binding site of the enzyme (8) is possible, but is inhibited in the presence of high glucose 1-phosphate concentrations. Glucose 1-phosphate might interfere with uridylyl transfer by blocking the interaction of the UDP-galactose and UDP-glucose with the enzyme. Inhibition of uridylyl transfer by glucose 1-phosphate concentrations is also

known for normal transferase, but much higher glucose 1-phosphate concentrations are required. Thus, the transferase activity of adult rat liver is reduced to 50% in the presence of 0.8 mM glucose 1-phosphate (1).

The product inhibition by glucose 1-phosphate could not be reversed by coupling the transferase reaction to glucose 1-phosphate-consuming reactions. This might indicate a high affinity of glucose 1-phosphate for transferase, exceeding that towards phosphoglucomutase and/or an inhibition produced by products generated by the phosphoglucomutase/glucose 6-phosphate dehydrogenase reactions, such as glucose 6-phosphate and 6-phosphogluconate. Summing up, the abnormal product inhibition by glucose 1-phosphate could well explain the development of galactosemia in

the patient. The other abnormal characteristics, such as reduced affinity to galactose-1-phosphate and increased thermostability, seem to be of less significance for the impaired metabolism of galactose-1-phosphate in the patient.

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