Analysis of human hepatic microsomal glucose-6-phosphatase in clinical conditions where the T_2 pyrophosphate/phosphate transport protein is absent

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The availability of a rare set of human hepatic microsomes in which T_2 , a pyrophosphate/phosphate transport protein of the glucose-6-phosphatase system, has been shown immunologically to be completely absent, has permitted further characterization of multicomponent glucose-6-phosphatase (EC 3.1.3.9). Pyrophosphatase activity in intact microsomes was found to be totally absent, but was normal in disrupted microsomes. However, P_i did not accumulate within the lumen of the microsomes when glucose 6-phosphate was the substrate. This was not as predicted if there is only one transport protein in the endoplasmic reticulum capable of transporting P_i , produced by glucose-6-phosphatase, out of the lumen. The results suggest that the pyrophosphate/phosphate transport system of human hepatic endoplasmic reticulum must be more complex than previously thought, as it must comprise at least two protein components.

INTRODUCTION

The microsomal glucose-6-phosphatase enzyme catalyses the final step of hepatic and renal glucose production, and therefore plays an important role in the homeostatic regulation of blood glucose levels [1,2]. The fact that glucose-6-phosphatase is an integral endoplasmic-reticulum membrane protein has greatly hindered both its characterization and the study of its regulation [2,3]. It was originally assumed that glucose-6-phosphatase was a single protein. Later it was proposed, on the basis of kinetic studies, that not only the glucose-6-phosphatase enzyme but also a glucose 6-phosphate transport protein (termed T_1) and a pyrophosphate/phosphate transport protein (termed T_2) were needed for glucose-6-phosphatase activity *in vivo*. This idea was called the 'substrate transport hypothesis' [4,5]. More recently, study of genetic deficiencies that result in impaired glucose-6-phosphatase activity (for recent reviews see [3,6,7]) have shown that glucose-6-phosphatase is more complex, as at least five proteins are needed for normal glucose-6-phosphatase activity *in vivo*. Four of the proteins, the catalytic subunit of glucose-6-phosphatase, a regulatory Ca^{2+} -binding protein, T_2 (a pyrophosphate/phosphate transport protein) and T_3 (or GLUT 7; a glucose transport protein) have been purified [8–12].

The idea of the 'substrate transport model' as originally proposed [4,5] has remained very controversial, as kinetic analysis by many different groups (e.g. see [13] for the most recent paper) do not support all the kinetic predictions of the original analysis [4,5]. In addition, the analysis of the activity of the glucose-6-phosphatase system in the first patient who was described as having type 1c glycogen-storage disease (T_2 deficiency) [14] also did not completely agree with the kinetic predictions of the original substrate transport hypothesis [4,5].

The original analysis of a deficiency of T_2 [14] was based on only one patient, which meant that the results might not be representative of all cases where T_2 is deficient. We have therefore analysed the glucose-6-phosphatase system in two clinical conditions where T_2 is absent, using only liver samples where immuno-

blot analysis demonstrated the complete absence of the T_2 protein [10]. The results obtained suggest that the pyrophosphate/phosphate transport system of glucose-6-phosphatase is not a single polypeptide, as originally thought, but that it must comprise at least two different components, which we have termed $T_{2\alpha}$ and $T_{2\beta}$.

MATERIALS AND METHODS

Sources of chemicals were as in [15]. Liver tissue was obtained from the following sources. (a) Whole liver from fetuses after termination of pregnancy (11–20 weeks gestation). A careful estimate of developmental age was made [16]. Fetuses with malformation or chromosomal anomalies were excluded. All abortuses were refrigerated at 4 °C until collection, within 3 h, for immediate dissection and subcellular fractionation. (b) Premature infants of 28 weeks gestation who died after delivery and as a consequence of immature pulmonary function. (c) Infants and children who in the first 2 years of life had a liver biopsy for investigation of hyperbilirubinaemia or hypoglycaemia. (d) Adult samples were wedge- or needle-biopsy samples obtained for the investigation or treatment of the original condition for which the patient was referred. We assessed and scored adult liver histology as normal (grade 1) to severely diseased (grade 5) and used samples of grade 1 only.

Human liver microsomes were prepared from fresh 'unfrozen' liver samples as described in [17]. Glucose-6-phosphatase, pyrophosphatase and mannose-6-phosphatase activities of glucose-6-phosphatase were measured as previously described [18]. Glucose production by glucose-6-phosphatase was measured by a micro-modification of the method of [19] whenever P_i was added to the assay mixture. Microsomes were disrupted with Histone 2AS [20] or Lubrol-PX. Non-specific hydrolysis of glucose 6-phosphate was assayed and corrected for as described previously [18]. Microsomal intactness was estimated by using both mannose-6-phosphatase activity [21] and UDP-glucuronosyltransferase activity towards 1-naphthol [22]. UDP-glucuronosyltransferase was

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assayed as in [23]. All the 'intact' values that we report have been corrected for any microsomal disruption as in [18], which eliminates the large errors that can occur if even a small proportion of the vesicles are disrupted [21]. All hepatic microsomal preparations used were at least 80% intact. Protein concentrations were measured as in [24]. Direct micro-transport assays using [32 P]PP_i were as previously described [25].

Sheep anti-T₂ antiserum was as described in [10]. SDS/PAGE was carried out in 7–16% acrylamide gels as in [26]. Proteins separated on SDS/polyacrylamide gels were electrophoretically transferred to nitrocellulose [27] in 1% Empigen BB as described in [28]. The Western and dot blots were immunostained with anti-T₂ antibody, and immunoreactive polypeptides were detected by using a biotin-streptavidin-peroxidase-linked detection system with 4-chloro-1-naphthol as the substrate [29] (Western blot) and with an enhanced chemiluminescence detection system (ECL; Amersham) [30] (dot blot).

RESULTS AND DISCUSSION

Microsomes from livers of five clinical specimens of interest, two (A and B) with the classic type 1c glycogenosis [14,15,25,31,32] and three human fetal liver specimens (C, D and E) in which the T₂ component of the microsomal glucose-6-phosphatase system had not yet developed [15], were examined with regard to glucose-6-phosphatase function. The T₂ transport capacity has previously been shown to develop somewhat later in gestation than the other protein components of the glucose-6-phosphatase system [15]. Immunoblot (e.g. Fig. 1a) and dot-blot analysis (e.g. Fig. 1c) with antibody specific for the T₂ pyrophosphate/phosphate transport protein [10] indicated the complete absence of the T₂ protein in samples A, C and D. Similar results were found in samples B and E (results not shown). Direct micro-transport assays with [32 P]PP_i (e.g. Fig. 1b) indicate the absence of T₂ function in transporting PP_i from the medium to the microsomal lumen in sample A and a fetal liver sample. Samples A, B, C and D also demonstrated normal uptake of glucose 6-[32 P]phosphate [25], confirming the presence of T₁ and the failure of 32 P to accumulate in the microsomal lumen.

Kinetic studies of glucose-6-phosphatase with both glucose 6-phosphate and pyrophosphate as substrate, studied with both disrupted and intact microsomes, are summarized in Table 1. With intact microsomes from both the type 1c glycogenosis patients A and B and from fetal livers C, D and E, no activity was detectable with pyrophosphate as substrate, demonstrating lack of pyrophosphate transport by T₂. Hydrolysis of glucose 6-phosphate in intact microsomes was manifest, but somewhat less than in control samples. In contrast, disrupted microsomal preparations from all specimens displayed activity with both glucose 6-phosphate and pyrophosphate as substrates (which were very similar to age-matched control values), indicative of normal functional catalytic subunit of glucose-6-phosphatase [14]. K_m and V_{max} values were obtained for use in the calculations to follow.

With the use of intact microsomes, the strategy here (as in [14]) was to assess the relationship between the amounts of glucose 6-phosphate hydrolysed and the time of reaction, assuming that, in the absence of T₂, the P_i liberated would accumulate in the lumen and inhibit the hydrolysis competitively [33]. A comparison of the time courses of the reaction predicted from kinetic parameters with those observed experimentally would provide a way of testing the original 'substrate transport hypothesis' [4,5].

Studies with microsomes obtained from subject A, a type 1c glycogenosis patient, will serve as an illustrative example of our calculations (Table 2 and Fig. 2). Kinetic parameters for subject

A for glucose-6-phosphatase activity of intact microsomes were used to calculate v values at the various time intervals (0–30 min). These calculated values agree very well with comparable values determined by direct experimental measurement (columns 5 and 2 respectively in Table 2; ■ and ● respectively in Fig. 2), and indicate a linear relationship between time of incubation and hydrolysis of glucose 6-phosphate.

In the absence of T₂, we would expect (if the 'substrate transport model' [4,5] were strictly correct) that P_i, released as glucose 6-phosphate was progressively hydrolysed, would accumulate in the microsomal matrix. This would result in a time-dependent decrease in the hydrolysis of glucose 6-phosphate owing to competitive inhibition by accumulating P_i [14]. Calculations to adjust for inhibition by P_i of glucose 6-phosphate hydrolysis were as follows. The K_m for glucose-6-phosphatase determined with disrupted microsomes (see Table 1), and a K_i value for P_i as a competitive inhibitor [33] of glucose 6-phosphate hydrolysis by the catalytic subunit of 5.6 mM determined in

Fig. 1. Comparison of the lack of the hepatic pyrophosphate/phosphate T₂ protein in patients with type 1c glycogen-storage disease and early in gestation before T₂ has developed

(a) Immunoblot analysis of human hepatic microsomes using an antibody monospecific for the pyrophosphate/phosphate transport protein T₂. Lane 1, microsomes from a patient with type 1c glycogen-storage disease (case A) (50 µg); lane 2, age-matched control human liver microsomes (50 µg); lane 3, microsomes isolated from a human liver of gestational age 28 weeks (50 µg); lane 4, microsomes isolated from a human liver of gestational age 15 weeks (case D) (50 µg). The presence of only one immunodetectable polypeptide indicates that the antibodies used are monospecific. (b) Direct microsomal micro-transport assays showing uptake of [32 P]PP_i versus time. Control adult liver microsomes (□) show normal PP_i uptake. Microsomes isolated from a patient with type 1c glycogen-storage disease, case A (■), and microsomes isolated from human liver of gestational age 11 weeks (○) lack the ability to transport [32 P]PP_i into microsomes. (c) Dot-blot analysis using antibodies monospecific for the T₂ pyrophosphate/phosphate transport protein and an ECL detection system, showing the development of the T₂ protein with gestational age as previously demonstrated [12]. Numbers correspond to the gestational age (weeks) of the human liver microsomal samples used (20 µg): 12 is case C, and control (cont) is a human adult microsomal sample (20 µg). The dot-blot is overdeveloped to demonstrate clearly that the T₂ protein is completely absent in the 12-week sample.

Table 1. Glucose-6-phosphatase activity in human liver microsomes

A and B are cases of type 1c glycogen-storage disease, as shown by the complete lack of activity with pyrophosphate as substrate in intact microsomes. Cases C, D and E are fetal liver samples in which the T_2 protein has not yet developed. Abbreviation: ND = not determinable.

Glucose-6-phosphatase activity with glucose 6-phosphate as substrate				
	Intact microsomes		Disrupted microsomes	
	$V_{\max}$ ($\mu\text{mol}/\text{min per mg}$)	K_m (mM)	$V_{\max}$ ($\mu\text{mol}/\text{min per mg}$)	K_m (mM)
A	0.06	4.5	0.22	0.3
B	0.13	9.0	0.30	1.1
Age-matched controls ($n = 10$)	0.13 ± 0.02	3.1 ± 0.4	0.34 ± 0.05	0.6 ± 0.1
C	0.01	3.8	0.03	1.4
D	0.02	2.2	0.03	0.6
E	0.01	3.8	0.01	0.6
Age-matched controls ($n = 10$)	0.03 ± 0.02	2.4 ± 0.4	0.02 ± 0.0	0.8 ± 0.1

Glucose-6-phosphatase activity with pyrophosphate as substrate				
	Intact microsomes		Disrupted microsomes	
	$V_{\max}$ ($\mu\text{mol}/\text{min per mg}$)	K_m (mM)	$V_{\max}$ ($\mu\text{mol}/\text{min per mg}$)	K_m (mM)
A	0	ND	0.20	1.3
B	0	ND	0.10	0.3
Age-matched controls ($n = 10$)	0.12 ± 0.03	2.7 ± 0.7	0.24 ± 0.3	0.5 ± 0.1
C	0	ND	0.02	0.6
D	0	ND	0.03	0.5
E	0	ND	0.01	0.6
Age-matched controls ($n = 10$)	0.01 ± 0.001	2.0 ± 0.5	0.02 ± 0.01	0.6 ± 0.2

Table 2. Observed and calculated glucose 6-phosphate (Glc-6-P) hydrolysis for a type 1c glycogenosis patient, without and with adjustment for inhibition by P_i presumed to accumulate in the intra-microsomal space in the absence of T_2 function

Incubation time (min) (1)	Observed Glc-6-P hydrolysis ($\mu\text{mol}/\text{mg}$ of protein) (2)	Calc. concn. of P_i in intra-microsomal space (mM) (3)	Conc. of Glc-6-P remaining at indicated time (mM) (4)	Calc. Glc-6-P hydrolysis without P_i inhibition ($\mu\text{mol}/\text{mg}$ of protein) (5)	Calc. inhibition by intra-microsomal P_i of Glc-6-P hydrolysis (%) (6)	Glc-6-P hydrolysis adjusted for inhibition by intra-microsomal P_i ($\mu\text{mol}/\text{mg}$ of protein)	
						Based on observed activity (7)	Based on calculated activity (8)
0	0	0	5.00	0	0	0	0
5	0.153	38.3	4.98	0.158	42.4	0.121	0.124
7.5	0.227	56.8	4.97	0.237	52.3	0.160	0.169
10	0.300	75.0	4.96	0.316	59.1	0.192	0.204
15	0.480	120	4.94	0.474	70.0	0.256	0.260
20	0.609	153	4.92	0.631	74.7	0.292	0.303
25	0.770	193	4.90	0.788	78.9	0.329	0.339
30	0.900	225	4.88	0.944	81.5	0.355	0.370

separate studies with disrupted microsomes from subject A, were employed to calculate the percentage inhibition that would be predicted if all P_i released through intramicrosomal glucose 6-phosphate hydrolysis were to accumulate within the intra-microsomal space. Eqns. (1)–(3) of ref. [34], with P_i in place of 'nucleotide', were used to calculate percentage inhibition values. For simplification, the conservative assumption was made that

the glucose 6-phosphate concentration in the microsomal lumen was the same as that in the medium. According to the substrate transport concept proposed over 10 years ago [4,5], it should be lower in the microsomal matrix than in the medium, and as a consequence percentage inhibition values would be even greater than those presented. It was further assumed that all P_i released accumulated in the intramicrosomal water, which occupied a

Fig. 2. Glucose 6-phosphate hydrolysis of intact hepatic microsomes from type 1c glycogenosis patient (subject A) studied as a function of incubation time

Microsomes were incubated for the indicated time in a reaction mixture containing 5 mM-glucose 6-phosphate and other components as indicated in the Materials and methods section. ●, Glucose 6-phosphate hydrolysis observed at each time interval; ■, hydrolysis values calculated from kinetic parameters in Table 1. Hydrolysis values adjusted on the basis of inhibitions by the concentrations of P_i presumed to accumulate in the intra-microsomal space in the absence of T_2 function are indicated for observed (○) and calculated (□) glucose 6-phosphate hydrolysis values. Data on which the Figure is based are presented in Table 2. Additional details are given in the text.

volume of 4 μ l/mg of microsomal protein [14,35]. Percentage inhibitions predicted at the various time intervals with the indicated combinations of intramicrosomal glucose 6-phosphate and inhibitor P_i are given in Table 2, column 6.

Glucose 6-phosphate hydrolysis values either calculated

(column 5, Table 2) or observed (column 2, Table 2) were adjusted for this presumed inhibition by P_i within the lumen of the endoplasmic reticulum (columns 8 and 7 respectively in Table 2; □ and ○ respectively in Fig. 2). A predicted marked decrease in hydrolysis with time of incubation is apparent. Results of a similar analysis with microsomes from subject A tested with 0.3 mM-, 1.0 mM- and 10 mM-glucose 6-phosphate as substrate (Figs. 3a, 3b and 3c) indicate this same disparity between experimental values and those predicted for inhibition by presumed accumulated P_i . There, ● and ○ symbols respectively indicate observed glucose 6-phosphate hydrolysis and glucose 6-phosphate hydrolysis adjusted for the predicted competitive inhibition by P_i .

A similar analysis was made with microsomes from a second type 1c glycogen-storage-disease patient (subject B, Table 1). Results were comparable with those in Fig. 2. Identical studies were also carried out with microsomes from three fetal liver preparations (from subjects C, D and E, Table 1). Results of a representative experiment (subject D) are presented in Fig. 4. The marked disparity between observed glucose 6-phosphate hydrolysis and glucose 6-phosphate hydrolysis predicted based on the presumption of inhibition by P_i accumulated within the microsomal lumen is apparent with this T_2 -devoid preparation, as with microsomes from type 1c patients. Comparable results (not shown) were obtained with microsomes from fetal preparations C and E. In supplemental experiments, P_i was observed to inhibit glucose 6-phosphate hydrolysis with disrupted microsomes from all specimens.

Conclusions

To date it has been generally assumed that pyrophosphate and phosphate are both transported into and out of the lumen of the endoplasmic reticulum by a single transport protein termed T_2 , as predicted by kinetic studies over 10 years ago [4,5]. A microsomal pyrophosphate/phosphate transport protein was purified and isolated and assumed to be T_2 [10]. This study of five liver samples in which the ' T_2 ' protein was completely absent confirms the original observation of the first case of type 1c glycogen-storage disease [14], and demonstrates that T_2 is not a single protein but two different polypeptides. A number of alternative mechanisms for T_2 function could be proposed, but

Fig. 3. Observed glucose 6-phosphate hydrolysis in intact hepatic microsomes from type 1c glycogenosis patient studied as a function of incubation time with (a) 0.5 mM-, (b) 1.0 mM- and (c) 10 mM-glucose 6-phosphate as substrate

Data presented are observed glucose 6-phosphate hydrolysis values (●) or such observed values adjusted for competitive inhibition by concentrations of P_i presumed to accumulate in the intra-microsomal space in the absence of T_2 (○). Other details are as in Fig. 2.

Fig. 4. Glucose 6-phosphate hydrolysis in intact human microsomes from a fetal liver studied as a function of incubation time

The preparation was from subject D. Observed activities (●) and observed activity adjusted for inhibition by P_i , presumed to accumulate in the intramicrosomal space in the absence of T_2 function (○), are presented. Glucose 6-phosphate concentration was 10 mM; other details are as in Fig. 2.

the simplest is that there is a phosphate transport protein, which for simplicity we have termed $T_{2\alpha}$, and a phosphate/pyrophosphate transport protein, which we have termed $T_{2\beta}$. All cases of type 1c glycogen-storage disease presented to date in the literature are deficiencies of the $T_{2\beta}$, the pyrophosphate/phosphate transport protein [14,15,17,29,31,32,36]. Its deficiency results in a complete inability to transport pyrophosphate into the lumen of the endoplasmic reticulum and a decreased ability to transport phosphate out of the lumen. This results in zero activity in intact microsomes when pyrophosphate is substrate (Table 1) and a higher K_m than normal in intact microsomes when glucose 6-phosphate is the substrate (Table 1), which results in a decreased ability to hydrolyse glucose 6-phosphate *in vivo*, as physiological levels are always much less than 1 mM.

Consistent with this need for further definition of ' T_2 ' are very recent observations [37] in which the effects of the presence of ascites tumours *in situ* on the hepatic glucose-6-phosphatase system indicate strongly the existence of two forms of T_2 , one effectively transporting PP_i or carbamoyl phosphate from the outside into the lumen and a second transporting P_i from the lumen outward.

All of these observations emphasize that, for normal glucose-6-phosphatase activity *in vivo*, several more protein components are needed than were originally hypothesized [4,5]. The substrate transport kinetic hypothesis now needs updating and modifying to take into account the characteristics of the known number of components to provide a more accurate kinetic model of glucose-6-phosphatase structure/function.

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