Transverse topology of glucose-6-phosphatase in rat hepatic endoplasmic reticulum

Ian D. WADDELL* and Ann BURCHELL

Centre for Research into Human Development, University of Dundee Medical School, Ninewells Hospital, Dundee DD1 9SY, Scotland, U.K.

Antibodies raised against purified components of glucose-6-phosphatase were used to study the transmembrane orientation of the complex. Measurements of glucose-6-phosphatase activities and immunoblot analysis of sealed microsomes and detergent-solubilized microsomes after treatment with proteases suggested that most of the catalytic subunit resides within the lumen of the endoplasmic reticulum. In contrast, other components of glucose-6-phosphatase are accessible to the cytoplasm. Treatment of the partially purified glucose-6-phosphatase enzyme with glycopeptide *N*-glycosidase indicated that the catalytic subunit of the enzyme was a glycoprotein.

INTRODUCTION

Hepatic microsomal glucose-6-phosphatase (EC 3.1.3.9) is the terminal step of both gluconeogenesis and glycogenolysis, and is a key step in the regulation of blood glucose concentrations [1,2]. Early attempts to investigate the topology of glucose-6-phosphatase were based on histochemical staining [3,4]. This served to demonstrate that glucose-6-phosphatase activity was within the lumen of the endoplasmic reticulum (ER). It has also been recognized since the 1950s that glucose-6-phosphatase is more active in the test tube in disrupted microsomal vesicles than in intact microsomes (for reviews see [1,2,5,6]). The enzyme activity is therefore described as latent. Recent genetic evidence has proved that at least five polypeptide subunits are required for the physiological functioning of the glucose-6-phosphatase enzyme complex: the catalytic subunit (a 36.5 kDa polypeptide), a 21 kDa Ca^{2+} -binding regulatory protein, and three transport proteins termed T_1 , T_2 and T_3 , which respectively allow glucose 6-phosphate, phosphate (and pyrophosphate) and glucose to cross the ER membrane [6–12].

The membrane topology of glucose-6-phosphatase has been the subject of much controversy for many years. For example, the most recent study of transmembrane topology in 1989 was surprising [13]. A photoreactive derivative of DIDS (4,4'-diisothiocyanostilbene-2,2'-disulphonic acid) was used to study the accessibility of the glucose-6-phosphatase enzyme in a variety of conditions. DIDS binds to a large number of ER proteins and inhibits both T_1 and the catalytic subunit of the glucose-6-phosphatase enzyme (for a review, see [6]). The authors, however, concluded that glucose-6-phosphatase is a single integral channel protein embedded within the lipid phase of microsomes and is accessible to the inhibitor from the cytoplasmic surface [13]. This conclusion ignores most of the recent glucose-6-phosphatase literature [6–12,14–23].

The most illuminating previous report on the glucose-6-phosphatase enzyme's topology involved the use of diazo-benzenesulphonate and proteases on intact and disrupted microsomes [24]. This work suggested that T_1 , the glucose 6-phosphate transport protein and the glucose-6-phosphatase enzyme were separate entities, and provided strong evidence in favour of the substrate-transport model of the glucose-6-phosphatase system explaining the latency of glucose-6-phosphatase [25]. Unfortunately this work was carried out before specific probes for

the individual components of the system had been isolated. Thus the information provided by that study was limited by its inability to consider the system as a whole.

We have therefore studied the transmembrane topology of the individual proteins of the hepatic microsomal glucose-6-phosphatase complex. The evidence presented here indicates that most of the glucose-6-phosphatase catalytic subunit, a glycoprotein, is located on the luminal side of the ER. In contrast, transport protein T_1 and T_2 and the Ca^{2+} -binding regulating protein are all accessible to the cytosol.

MATERIALS AND METHODS

Materials

Glucose 6-phosphate (disodium salt), mannose 6-phosphate (monosodium salt), sodium deoxycholate, phenylmethanesulphonyl fluoride, 1,10-*o*-phenanthroline, subtilisin BPN, trypsin inhibitor, 4-chloro-1-naphthol and Triton X-100 were all obtained from Sigma (Poole, Dorset, U.K.). Peptide *N*-glycosidase F (EC 3.2.2.18; PNGase F) and trypsin were from Boehringer Mannheim (Lewes, Sussex, U.K.). Proteinase K was from Merck (BDH, Glasgow, U.K.), Lubrol 12A9 from ICI (Manchester, U.K.), and Schleicher & Schuell nitrocellulose from Anderman (London S.E.1, U.K.). All other chemicals where available were analytical-reagent grade.

Glucose-6-phosphatase assays

Glucose-6-phosphatase, pyrophosphatase and mannose-6-phosphatase activities were assayed and calculated as in [7] and expressed as μmol of P_i released/min per mg of microsomal protein. Microsomes isolated from liver homogenates are a mixture of intact and disrupted microsomes. The proportion of intact microsomes was determined by assays of mannose-6-phosphatase activity, which is only expressed in disrupted structures, as previously described [7,26]. All activity values given in this paper are V_{max} values, which were calculated by using a BBC computer program of non-linear multiple regression analysis based on [27].

Partial purification

Liver microsomes were prepared as previously described from Wistar rats which had been starved for 16 h [16]. Microsomes were partially purified by centrifugation through a 12–62 %

Abbreviations used: ER, endoplasmic reticulum; PNGase F, peptide *N*-glycosidase F.

* To whom all correspondence should be addressed.

linear (w/v) sucrose gradient containing 0.2% deoxycholate at 105000 *g* for 17 h. The peak containing glucose-6-phosphatase activity was then further purified by passage through an affinity column consisting of anti-(glucose-6-phosphatase catalytic subunit) antiserum coupled to Sepharose 4B beads, resulting in four bands on an SDS/polyacrylamide gel. The partially purified preparation was electrophoresed on a preparative polyacrylamide gel [28]. The glucose-6-phosphatase catalytic subunit was made visible by reversible copper staining [29] and excised. Protein was then electroeluted from the excised gel [30] and dialysed against double-distilled deionized water.

Deglycosylation of purified glucose-6-phosphatase with PNGase F

Samples of purified glucose-6-phosphatase were incubated with 2 units of PNGase F as previously described [31] in the presence of 0.1 M-sodium phosphate, 1% (w/v) Triton X-100, 0.1% SDS, 1% 2-mercaptoethanol and 10 mM-1,10-*o*-phenanthroline, pH 7, for 18 h at 37 °C. Some samples of the purified enzyme were also incubated with 5 units of PNGase F in a non-denaturing modified buffer system (20 mM-K₂HPO₄, 20 mM-EDTA, 143 mM-2-mercaptoethanol, 120 μ M-deoxycholate, pH 7.5) for 3 h at room temperature.

Proteolysis of glucose-6-phosphatase in hepatic microsomal fractions

Freshly prepared hepatic microsome suspensions (2 mg of protein in 0.25 M-sucrose/5 mM-Hepes, pH 7.4) from 4-week-old rats were incubated with 0.2–5 mg of detergent/mg of protein in 0.25 M-sucrose/5 mM-Hepes, pH 7.4, containing 5 mM-MgCl₂ for 30 min at 20 °C (solubilized microsomes). Controls were incubated with buffer alone (sealed microsomes). Proteases were added in the same buffer and the incubation mixtures were made up to 1800 μ l. Proteolysis was performed for 30 min at 20 °C, and then 200 μ l of a solution containing 2 mM-phenylmethanesulphonyl fluoride and 4 mM-1,10-*o*-phenanthroline in 40% (w/v) ethanol was added to inhibit subtilisin BPN and proteinase K; 20 μ g of trypsin inhibitor in 200 μ l of water was added to inhibit trypsin. The incubation mixtures were assayed for glucose-6-phosphatase activity. Samples (200 μ l) were removed, and an additional 20 μ l of protease inhibitors (20 mM-phenylmethanesulphonyl fluoride and 100 mM-1,10-*o*-phenanthroline) was added to prevent proteolysis during denaturation in SDS before gel electrophoresis.

Immunoblot analysis of untreated detergent-solubilized and protease-treated hepatic microsomes

Samples of the various treated or untreated microsomes (50 μ g of protein) were mixed with 25 μ l of 0.23 M-sucrose/2.8 M-2-mercaptoethanol/0.28 M-SDS/0.5 M-Tris buffer, pH 6.8, and incubated at 100 °C for 3 min and the microsomal proteins were separated by 7–16%-polyacrylamide-gel electrophoresis in the presence of SDS [32]. The separated proteins were transferred from the gel to nitrocellulose as described by Towbin *et al.* [33]. Blotted proteins were then specifically examined by using the appropriate antibody as previously described [17]. Three antisera were used in this study: an anti-(glucose-6-phosphatase catalytic subunit) antibody [8], anti-(transport protein T₂) antibody [12], and finally an antibody raised against the Ca²⁺-binding stabilizing protein [9].

RESULTS AND DISCUSSION

Glycosylation of rat liver microsomal glucose-6-phosphatase

The catalytic subunit of the glucose-6-phosphatase enzyme runs as a doublet on SDS/PAGE [6,8]. When either partially purified glucose-6-phosphatase enzyme or starved rat liver micro-

somes were incubated with 2 units of PNGase F and subjected to immunoblot analysis as described in the Materials and methods section, the upper band of the immunostained doublet was removed, with a concomitant increase in intensity of the lower band (Fig. 1a).

Incubation of starved rat liver microsomes with PNGase F under non-denaturing conditions also resulted in the disap-

Fig. 1. Deglycosylation of the glucose-6-phosphatase enzyme protein

(a) Samples (80 μ g) of starved-rat liver microsomes or of affinity-purified catalytic subunit were incubated with or without PNGase F as described in the text. Samples were electrophoresed on a 7–16%-polyacrylamide gel and electrophoretically transferred to nitrocellulose. Bands were made visible by immunoblotting with an anti-(catalytic subunit) antibody and a biotin/streptavidin detection system. Lanes: 1, starved-rat liver microsomes (20 μ g); 2, affinity-purified protein (5 μ g); 3, lanes 2 and 4 mixed before electrophoresis (25 μ g total); 4, affinity-purified protein incubated with PNGase F; 5, starved-rat liver microsomes incubated with PNGase F. (b) A sample (20 μ g) of labelled protein was run on a 7–16%-polyacrylamide gel before being dried down and autoradiographed against Kodak X-OMAT film for 72 h.

Table 1. Effect of proteases on glucose-6-phosphatase activity in untreated and deoxycholate-treated (0.4 mg/ml) rat liver microsomes

All protease treatments were 30 min incubations at 20 °C with 50 (or 10*) μ g of protease/mg of microsomal protein. Values shown are means $\pm$ S.E.M. of at least five experiments.

Protease treatment	Glucose-6-phosphatase activity (glucose 6-phosphate as substrate) (μ mol of P _i released/min per mg)		
	Intact microsomes	Microsomes disrupted after proteolysis	Microsomes disrupted before proteolysis
None	0.17 $\pm$ 0.01	0.57 $\pm$ 0.04	0.51 $\pm$ 0.04
Proteinase K	0.03 $\pm$ 0.01	0.65 $\pm$ 0.05	0.25 $\pm$ 0.03
Trypsin*	0.05 $\pm$ 0.03	0.60 $\pm$ 0.09	0.31 $\pm$ 0.05
Subtilisin	0.03 $\pm$ 0.02	0.60 $\pm$ 0.05	0.11 $\pm$ 0.06

Fig. 2. Immunoblot analysis of microsomes after proteolytic treatment (glucose-6-phosphatase)

Microsomes were diluted and treated with deoxycholate (DOC; 0.4 mg/ml, where added) and/or proteases [P, proteinase K (50 μ g); T, trypsin (10 μ g); S, subtilisin (50 μ g)] as indicated above and in the text. Samples were subjected to SDS/PAGE, then immunoblotted with an anti-(glucose-6-phosphatase catalytic subunit) antibody.

pearance of the upper band of the enzyme doublet. Unfortunately the incubation conditions, even in the absence of PNGase F, resulted in the total loss of glucose-6-phosphatase enzyme activity. For this reason it was impossible to estimate directly the importance of glycosylation on the activity of the catalytic subunit, but both bands of the doublet were radiolabelled by the substrate [32 P]pyrophosphate (Fig. 1b). This indicates that substrate binding to the active site of the glucose-6-phosphatase catalytic subunit can be demonstrated in both bands of the doublet.

These results suggest that the upper band of the catalytic subunit doublet is glycosylated and that both bands of the subunit are active.

Previous studies demonstrate that the regulatory Ca^{2+} binding protein ('SP'), which is essential for glucose-6-phosphatase activity *in vivo*, is also a doublet on SDS/PAGE and that the upper band is also glycosylated [6,18]. Although the data strongly suggest that both subunits of glucose-6-phosphatase exist in a glycosylated form, definite proof of the glycosylation of both proteins must await a chemical analysis of the individual purified polypeptides.

Effects of proteolytic treatment on the activity of the glucose-6-phosphatase enzyme

Rat hepatic microsomes were incubated with various proteases in the absence and presence of detergents. The microsomal preparations were assayed for glucose-6-phosphatase activity with mannose 6-phosphate as substrate [26,34], to assess the integrity of the sealed or intact microsomes before and after protease treatment. This crucial assessment indicated the sidedness of the proteolytic attack. In intact sealed vesicles mild proteolysis should result only in degradation of proteins exposed on the cytoplasmic membrane surface; with detergent-dispersed membranes this treatment should degrade proteins which were located at both surfaces [35,36].

Glucose-6-phosphatase activity in fully disrupted microsomes is a measure of the activity of the catalytic subunit of glucose-6-phosphatase alone, without the rate limitations imposed by the

Table 2. Effect of proteases on pyrophosphatase activity in untreated and deoxycholate-treated (0.4 mg/ml) rat liver microsomes

Experimental conditions were as described in the legend to Table 1.

Protease treatment	Glucose-6-phosphatase activity (pyrophosphate as substrate) (μ mol of P_i released/min per mg)		
	Intact microsomes	Microsomes disrupted after proteolysis	Microsomes disrupted before proteolysis
None	0.06 ± 0.01	0.46 ± 0.04	0.23 ± 0.05
Proteinase K	0.01 ± 0.01	0.39 ± 0.03	0.12 ± 0.07
Trypsin*	0.02 ± 0.02	0.42 ± 0.07	0.17 ± 0.08
Subtilisin	0.01 ± 0.01	0.44 ± 0.03	0.09 ± 0.10

transport proteins. This rate limitation is illustrated (in Table 1) by the much higher activity in disrupted microsomes than in intact microsomes. Intact starved-rat liver microsomes were incubated with a variety of proteases and then, immediately after the incubation was stopped, the microsomes were disrupted with detergent to allow assay of the catalytic subunit of glucose-6-phosphatase with either glucose 6-phosphate (Table 1) or pyrophosphate (Table 2) as substrate. The proteases had no effect on glucose-6-phosphatase enzyme activity. In contrast, disruption of microsomes before protease treatment resulted in considerable loss of enzyme activity (Tables 1 and 2). These results indicate that the catalytic subunit of glucose-6-phosphatase is not readily susceptible to proteolysis at the cytoplasmic surface of microsomes.

Fig. 3. Immunoblot analysis of microsomes after proteolytic treatment (T_2 and Ca^{2+} -binding protein)

Microsomes were diluted and treated with deoxycholate (DOC; 0.4 mg/ml) and/or proteases as indicated above and in the text. Samples were subjected to SDS gel electrophoresis, then immunoblotted using (a) anti-(transport protein T_2) antisera; and (b) anti-(calcium binding protein) antisera (P = proteinase K, 50 μ g, T = trypsin, 10 μ g, and S = subtilisin, 50 μ g).

Fig. 4. Putative transmembrane orientation of the rat hepatic microsomal glucose-6-phosphatase system

Key: G-6-Pase, the catalytic subunit of the glucose-6-phosphatase enzyme; SP, stabilizing protein; T₁, the glucose 6-phosphate transport protein; T₂, the phosphate/pyrophosphate transport protein; T₃, the glucose transport protein.

Effects of proteolytic treatment on glucose-6-phosphatase activity in intact microsomes

Glucose-6-phosphatase activity in intact microsomes when glucose 6-phosphate is substrate is a measure of the combined rates of the three transport proteins and the glucose-6-phosphatase enzyme. It has been shown [25] that the process facilitated by T₁ is the rate-limiting step of hydrolysis of glucose 6-phosphate. Table 1 shows that a variety of proteases all decrease glucose-6-phosphatase in intact microsomes, suggesting that T₁ is accessible to the cytoplasmic surface. The protease treatment did not alter the intactness of the microsomal membrane; the mean intactness of the microsomes after incubation with buffer, proteinase K, trypsin and subtilisin was 98%, 97%, 97% and 95% respectively.

Glucose-6-phosphatase activity in intact microsomes with pyrophosphate as substrate is a measure of the combined rates of T₂ and the glucose-6-phosphatase enzyme. Table 2 shows that activity in intact microsomes is greatly decreased by proteases, suggesting that T₂ is also accessible to the cytoplasmic surface of microsomes.

Immunoblot analysis of microsomal samples

Intact and detergent-treated microsomes were analysed by immunoblotting with the anti-(glucose-6-phosphatase) antibody after treatment with proteases. Figs. 2(a) and 2(b) show the results of these experiments. Lanes 2–5 (Fig. 2a) show that in intact microsomes the proteases do not affect the glucose-6-phosphatase enzyme protein, as we expected from the results in Table 1. This reinforces the conclusion that the decrease in activity in intact microsomes must be due to proteolytic damage of the translocase T₁. Lanes 7–9 (Fig. 2b) show the degradation of the enzyme by all three proteases tested in fully disrupted microsomes. Treatment, even under conditions of optimal enzyme activation, did not lead to the total degradation of the glucose-6-phosphatase protein, suggesting that many susceptible peptide bonds are inaccessible to proteolysis and raising the possibility that much of the protein may be within the membrane and protected from proteolytic attack. In proteinase-K- and subtilisin-treated microsomes (lanes 7 and 9 respectively) only the lower band in the doublet remains visible, suggesting that a trace of a deglycosylating impurity is present in the commercially obtained proteases. Both bands of the 36.5 kDa doublet are faintly visible in the lane treated with trypsin. In each case only one immunoreactive peptide was produced by protease treat-

ment, with molecular masses of 30 kDa (proteinase K), 21 kDa (trypsin) and 26 kDa (subtilisin) respectively.

Immunoblot analysis was also carried out for another two components of the multiprotein glucose-6-phosphatase system. Fig. 3(a) demonstrates the susceptibility of transport protein T₂ to external protease attack, confirming the data presented in Table 2. Similarly the 21 kDa Ca²⁺-binding regulatory protein is extensively located on the cytosolic side of the ER membrane (Fig. 3b).

A model of the transverse organization of glucose-6-phosphatase in the ER

The results suggest that none of the glucose-6-phosphatase enzyme protein is exposed to the cytoplasmic surface and that most of the protein resides within the ER membrane, with a small portion of the protein on the luminal surface of the ER. This is in contrast with the other components of the glucose-6-phosphatase system, which were extensively exposed to cytosolic protease attack. Fig. 4 represents a simple model of glucose-6-phosphatase based on the evidence presented here together with the known interactions of the individual components with each other [6,9,22,37].

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