Is thermostability of glucose-6-phosphatase indeed dependent on a stabilizing protein?

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Partial purification of glucose-6-phosphatase from rat liver microsomes by solubilization of the membranes with the non-ionic detergent Triton X-144 at pH 6.5 and the removal of inactivating detergent by hydrophobic chromatography results in a thermostable enzyme protein which is not dependent on stabilizing phospholipids or proteins. The readdition of low amounts of detergent immediately causes a conversion into a thermo-unstable phosphodydrolase protein. Thus these findings present evidence that heatinstability of partially purified glucose-6-phosphatase derives from traces of inactivating detergent changing the structural properties of the phosphohydrolase rather than from the absence of the postulated specific stabilizing protein.

Glucose-6-phosphatase enzyme purification (Rat liver) Microsomal membrane Thermostability Stabilizing protein

1. INTRODUCTION

Glucose-6-phosphate:phosphohydrolase is an intrinsic component in the metabolic pathways of gluconeogenesis and glycogenolysis. Unlike most of the gluconeogenetic and glycolytic enzymes liver glucose-6-phosphatase is an integral part of the endoplasmic reticulum [1], deeply buried within the hydrophobic domain of the membrane, as our previous studies on microsomal glucose 6-phosphate hydrolysis have shown [2,3]. Despite the existing number of reports regarding the transverse arrangement and the enzymatic parameters of membrane-bound glucose-6-phosphatase the molecular properties of the enzyme are still unknown, since isolation of the glucose-6-phosphatase from microsomal membranes has proved to be difficult. As previously reported, partially purified preparations of glucose-6-phosphatase have been obtained from rabbit liver [4], which, however, were shown to be very heat-unstable without the presence of a stabilizing protein isolated from microsomal membranes [5]. The stabilizing effect of this 21 kDa protein on heat-unstable partially purified glucose-6-phosphatase preparations has been interpreted as a direct specific interaction between the two proteins which in vivo could possibly regulate hepatic glucose 6-phosphate hydrolysis. Here we show that a thermostable glucose-6-phosphatase without stabilizing proteins is obtained using appropriate methods which prevent a rapid inactivation of the enzyme and a further conversion into an unstable enzyme protein.

2. MATERIALS AND METHODS

Glucose 6-phosphate was obtained from Boehringer, Mannheim, imidazole and EDTA from Merck, Darmstadt. Glycerol and Triton X-114 were purchased from Serva, Heidelberg and sarcosyl from Sigma, Deisenhofen. Phenyl-Sepharose CL-4B was obtained from Pharmacia, Heidelberg.

2.1. *Enzyme assay and protein determination*

Glucose-6-phosphatase activity was assayed for

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10 min at 25°C in 0.2 M imidazole-HCl, pH 6.5 [6]. Specific activity is expressed as μmol inorganic phosphorus released/min per mg protein. It should be noted that all glucose-6-phosphatase activities were assayed in the absence of any exogenous proteins or phospholipids. Protein concentrations were determined by the Bradford method [7].

2.2. *Purification procedure*

Microsomes were prepared as in [8] from livers of rats (male Wistar rats AF/Hannover, 250–300 g body wt), fasted for 15 h. Fresh microsomal preparations (2 mg protein/ml 50 mM imidazole-HCl, pH 6.5, 20% glycerol) were treated for 30 min at 0°C with Triton X-114 (Triton X-114/protein ratio, 2, w/w; final Triton concentration, 0.4%, w/v) in 50 mM imidazole-HCl, pH 6.5, 20% glycerol (buffer A) and centrifuged (30000 rpm, 50.2 Ti rotor, 60 min, −2°C). The pellets containing about 20% of the total protein and the major glucose-6-phosphatase activity were resuspended in buffer A, 0.8 M KCl, 5 mM EDTA. This fraction (3.6 mg protein/ml), designated as Triton-extracted microsomes was applied to a phenyl-Sepharose column (8 × 1.5 cm), equilibrated with the same buffer. Glucose-6-phosphatase was obtained in the flow through which contained about 40% of the total protein. Subsequently, the peak fraction (0.8–1.2 mg protein/ml) was treated with 0.15% sarcosyl, w/v, for 5 min at 0°C to allow further separation of glucose-6-phosphatase from non-specific proteins by rechromatography on a phenyl-Sepharose column (4 × 1 cm). The enzyme was obtained in the flow through in buffer A, 0.8 M KCl, 5 mM EDTA. A KCl-soluble form of the partial purified enzyme was obtained when the glucose-6-phosphatase active fractions from the second chromatography were centrifuged at 105000 × g_{max}, although a part of the enzyme displayed a strong tendency to aggregate and sedimented during centrifugation.

2.3. *SDS gel electrophoresis*

Electrophoresis of partially purified glucose-6-phosphatase from the last step of purification was performed in polyacrylamide slab gels in the presence of 0.1% SDS [9].

3. RESULTS AND DISCUSSION

3.1. *Partial purification of glucose-6-phosphatase*

Recently published studies on microsomal glucose 6-phosphate hydrolysis have shown that the activity of glucose-6-phosphatase in detergent-modified and -solubilized microsomal membranes results predominantly from both the release of latent activity and the inactivation of the enzyme [10]. With respect to these findings, optimum conditions had been selected from a series of experiments to minimize the rate of inactivation of glucose-6-phosphatase during solubilization of microsomal membranes by detergent. This detergent-induced inactivation was found to be markedly reduced when membrane solubilization had been performed at slightly acidic conditions,

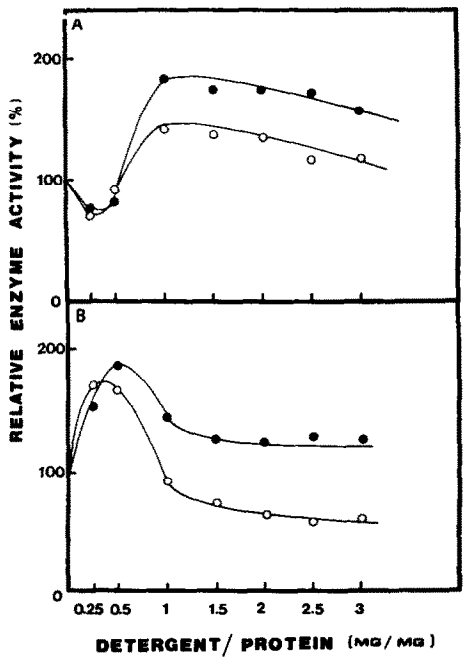

Fig.1. Glucose-6-phosphatase activity after treatment of microsomal membranes with Triton X-114 at different pH. Rat liver microsomes [2 mg protein/ml, 50 mM imidazole-HCl, 20% glycerol, pH 6.5 (●—●) or pH 7.4 (○—○)] were treated with Triton X-114 at 0°C for (A) 30 min or (B) 15 h. Subsequently, glucose-6-phosphatase activity was determined as described in section 2. 100% specific activity corresponds to 0.131 U/mg (pH 6.5) and 0.126 U/mg (pH 7.4) protein, respectively.

near the pH optimum of the enzyme in the presence of the stabilizing agent glycerol. As shown in fig.1A Triton treatment of rat liver microsomes at pH 6.5 for 30 min at 0°C demonstratively increased latent glucose-6-phosphatase activity compared to microsomes treated at pH 7.4. The long-term stabilizing effect of slightly acidic conditions on glucose-6-phosphatase activity in detergent-treated microsomes is presented in fig.1B. Although incubation of microsomal membranes for 15 h at 0°C and pH 7.4 resulted in a loss of enzyme activity at Triton/protein ratios higher than 1, this inactivation was markedly reduced when the membranes had been treated at pH 6.5. More alkaline conditions than pH 7.4 supported the detergent-induced inactivation of glucose-6-phosphatase in solubilized microsomal membranes (not shown) which indicates that slightly acidic pH can give rise to a more stable conformation of the enzyme protein protecting against inactivation during the initial purification step. The addition of NaF which has been proposed to stabilize detergent-soluble glucose-6-phosphatase [11] was omitted, since there was no positive effect of NaF on glucose-6-phosphatase activity during membrane solubilization or further purification steps.

A representative purification scheme is shown in table 1. This particular preparation had a specific activity of 7.0 U/mg protein, the best preparation obtained a specific activity of 14.6 U/mg protein, respectively, representing an 85-fold purification of glucose-6-phosphatase over the activity in microsomes. The SDS-polyacrylamide gel electrophoresis of the glucose-6-phosphatase obtained from the last step of purification showed protein bands in the M_r range 20000, 46000, 55000 and 70000 (not shown) which are comparable with those presented in previously described preparations [4,12].

3.2. *Thermostability*

When partially purified glucose-6-phosphatase had been subjected to heat treatment this fraction did not behave like the thermo-unstable preparations described [5]. As demonstrated in fig.2, glucose-6-phosphatase of detergent-treated and detergent-extracted microsomes is rapidly inactivated during heat treatment at 25°C while partially purified glucose-6-phosphatase remained completely stable over a time period of 20 min. It is unlikely that thermostability of this preparation results from traces of a putative stabilizing protein since glucose-6-phosphatase of the initial steps of purification does not display the same phenomenon. Furthermore, our rat liver microsomes used in purification have been extensively washed with 0.18 M KCl solution at pH 5.4 [8] to prevent contamination with non-specific proteins electrostatically adsorbed to the surface of the vesicles. Since, however, incubation of microsomes at pH 5 was shown to solubilize the putative stabilizing protein from microsomal membranes [5] it again seems very unlikely that our partially

Table 1

Purification of glucose-6-phosphatase from rat liver microsomes

Purification step	Protein (mg)	Activity (U)	Specific activity (U/mg protein)	Purification (-fold)	Yield (%)
Microsomes	80.0	12800	0.160	–	100
Triton-extracted microsomes	12.0	15600	1.3	8	122
Phenyl-Sepharose I	0.44	1144	2.6	16	9
Phenyl-Sepharose II	0.22	704	3.2	20	6
Soluble fraction	0.034	238	7.0	44	2

Rat liver microsomal glucose-6-phosphatase was purified as described in section 2. The data presented were obtained from a typical purification out of 20. Specific activity was assayed at 25°C

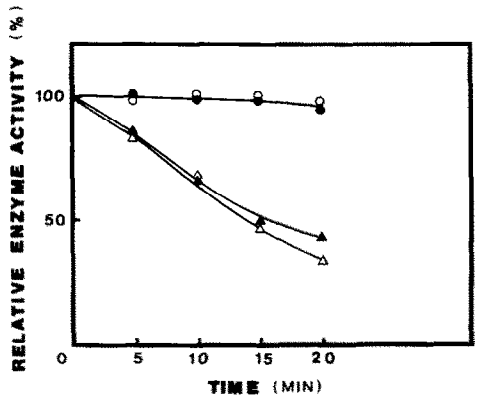

Fig.2. Thermostability of glucose-6-phoshatase at different purification steps. Rat liver glucose-6-phosphatase was purified according to the procedure described in section 2. 1 ml of the corresponding purification step was incubated at 25°C at different time periods and subsequently, glucose-6-phosphatase activity determined as described in section 2. 100% specific activity corresponds to 0.140 U/mg for microsomes (○—○), 0.255 U/mg for Triton-treated microsomes (△—△), 1.2 U/mg for Triton-extracted microsomes (▲—▲) and 2.2 U/mg protein for phenyl-Sepharose I preparation (●—●), respectively.

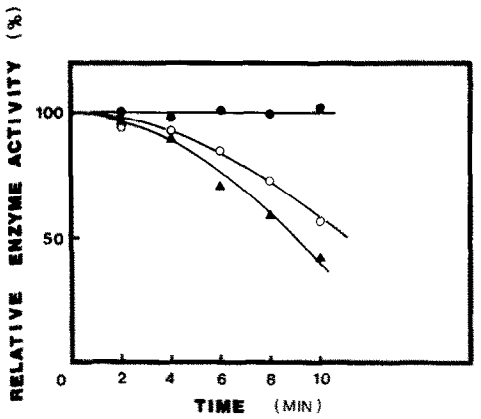

Fig.3. Effect of detergent on partially purified glucose-6-phosphatase. 1 ml of partially purified glucose-6-phosphatase (0.85 mg protein) obtained by chromatography on phenyl-Sepharose I was incubated at 30°C for different time periods in the absence (●—●) and presence of 0.04% (○—○) and 0.06% (▲—▲) Triton X-114, respectively. 100% specific activity corresponds to 2.4 U/mg protein.

purified glucose-6-phosphatase is associated with such a protein. The fact that the putative protein can be easily solubilized from microsomes by changing the pH of the medium while the overwhelming majority of the microsomal proteins remains attached to or within the bilayer leads to the conclusion that this protein must be very loosely adsorbed to the surface of the vesicles. Therefore, it seems very unlikely that such a protein in vivo may display direct specific interactions with the glucose-6-phosphate:phosphohydrolase protein which according to the 'carrier model' is believed to be localized on the opposite luminal surface of the microsomal membrane [13]. Nevertheless, it may be possible to obtain polar interactions of some special peptides and an unstable enzyme protein since proteins of partially purified glucose-6-phosphatase preparations display a strong tendency for aggregation even in the presence of detergents.

Thermal inactivation, however, could be induced by residual amounts of detergent (i.e. Triton, deoxycholate, Lubrol) tightly bound to the partially purified proteins of the preparation. This is supported by the observation that addition of very low amounts of detergent to stable partially purified glucose-6-phosphatase preparations results in a conversion into a heat-unstable form of the enzyme protein (fig.3). Moreover, these findings provide evidence that the native functional and structural properties of microsomal glucose-6-phosphatase in fact are changed in the presence of detergents, as mentioned earlier [10].

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