Stabilization of glucose-6-phosphatase activity by a 21 000-dalton hepatic microsomal protein

Ann BURCHELL and Brian BURCHELL
Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, Scotland, U.K.

and Madeline MONACO, Heather E. WALLS and William J. ARION
Division of Nutritional Sciences and Section of Biochemistry, Molecular and Cell Biology of the Division of Biological Sciences, Savage Hall, Cornell University, Ithaca, NY 14853, U.S.A.

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1. Hepatic microsomal glucose-6-phosphatase activity was rendered extremely unstable by a variety of techniques: (a) incubation at pH 5.0; (b) extraction of the microsomal fraction in the presence of 1% Lubrol; (c) various purification procedures. 2. These techniques all result in the removal of a 21 kDa polypeptide from the fraction containing glucose-6-phosphatase activity. 3. The 21 kDa protein was purified to apparent homogeneity by solubilization in the detergent Lubrol 12A-9 and chromatography on Fractogel TSK DEAE-650(S) and centrifugation at 105 000g. 4. The 21 kDa protein stabilizes glucose-6-phosphatase activity, whereas other purified hepatic microsomal proteins do not. 5. The 21 kDa protein appears to be a potential regulator of glucose-6-phosphatase activity.

Hepatic microsomal glucose-6-phosphatase (EC 3.1.3.9) catalyses a key step in the homoeostatic regulation of blood glucose concentrations (Ashmore & Weber, 1959; Nordlie, 1971; Newgard et al., 1984). Substantial kinetic (Arion et al., 1975, 1976a, 1980) and genetic (Lange et al., 1980; Nordlie et al., 1983) evidence indicates that glucose 6-phosphate hydrolysis in the glucogenic tissues is catalysed by a multicomponent system. It has been proposed (Arion et al., 1975) that the active site of glucose-6-phosphatase is situated at the luminal membrane surface of the endoplasmic reticulum and that a specific translocase (T₁) mediates entry of glucose 6-phosphate. P₁ released at the luminal surface is believed to equilibrate via a second translocase, T₂ (Arion et al., 1980).

The hepatic microsomal glucose-6-phosphatase enzyme was partially purified to a preparation containing five major polypeptides (Burchell & Burchell, 1982). One of the polypeptides could be purified by heating microsomes (microsomal preparations) at 100°C. Antiserum raised against the heat-extractable protein of approx. 21 kDa immunoprecipitated glucose-6-phosphatase activity from solubilized microsomal fractions (Burchell & Burchell, 1982). The immunoprecipitation experiments suggested that the 21 kDa protein might be the phosphohydrolase component of the glucose-6-phosphatase enzyme.

Here we show that the 21 kDa protein is not the catalytic subunit of the glucose-6-phosphatase enzyme, but is possibly a novel regulatory protein which stabilizes hepatic microsomal glucose-6-phosphatase activity.

Materials and methods

Glucose-6-phosphate was purchased from Sigma Chemical Co., Poole, Dorset, U.K. Egg phosphatidylcholine was purchased from Lipid Products, South Nutfield, Surrey, U.K. Lubrol 12A-9 (a condensate of dodecyl alcohol with approx. 9.5 mol of ethylene oxide/mol) was from ICI Organics Division, Manchester, U.K. Fractogel TSK DEAE-650(S) 'Merck' was purchased from BDH Chemicals, Poole, Dorset, U.K. Protein standards used for SDS/polyacrylamide-gel electrophoresis were purchased from Boehringer, Mannheim, Germany, and Sigma. All other chemicals were analytical-reagent grade. Staphylo-
**coccus aureus** V8 protease was purchased from Sigma, St. Louis, MO, U.S.A., and bovine chymotrypsin was purchased from Worthington, Freehold, NJ, U.S.A.

**Enzyme assays**

Glucose-6-phosphatase activity was assayed at 30°C and at pH 6.5 in a 0.1 ml reaction mixture as described by Bickerstaff & Burchell (1980). All microsomal subfractions were diluted at least 50-fold in the assay to ensure that the glucose-6-phosphatase assay was kinetically sound and was not affected by buffers at other pH values or containing potentially inhibitory detergents. The final concentration of deoxycholate in the assays reported in Table 1 was 0.002%, and was not inhibitory. All other assays described in this paper did not contain deoxycholate. One unit of activity represents 1.0 μmol of P<sub>i</sub> released/min. Nonspecific hydrolysis of glucose-6-phosphate was assayed as described by Burchell & Burchell (1980). It averaged less than 3% of the total glucose-6-phosphatase activity in microsomal fractions.

Protein concentrations were determined by the method of Bradford (1976). Phosphatidylcholine liposomes were prepared as described by Cater et al. (1975) and added to enzyme assays of all partially purified fractions at a final concentration of 0.1 mg/ml.

**Gel electrophoresis**

Slab gradients (polyacrylamide-gel electrophoresis) were preformed at 20°C in the presence of 0.1% SDS as described by Laemmli (1970). The M<sub>r</sub> of the purified stabilizing protein was determined by comparison with the mobility of bovine serum albumin (66000), ovalbumin (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000), soya-bean trypsin inhibitor (24100) and lactalbumin (14200).

**Purification of heat-extractable proteins**

The heat-extractable protein was prepared from rat and rabbit liver as previously described (Burchell & Burchell, 1982) and resuspended in 0.25 M-sucrose/5 mM-Hepes, pH 7.4.

**Conventional purification of the stabilizing protein**

Lubrol-solubilized microsomes were prepared from 20 g of fresh liver of adult female New Zealand White rabbits and of starved Wistar rats as previously described (Burchell, 1985). The Lubrol supernatant was applied to a Fractogel TSK DEAE-650(S) column (45 cm × 2 cm) previously equilibrated in 20% (v/v) glycerol/5 mM-Hepes/20 mM-NaF/0.05% (w/v) Lubrol, pH 7.4 (buffer A). The column was washed with buffer A until the A<sub>280</sub> of the eluate reached zero. The column was then washed with buffer A + 70 mM-NH<sub>4</sub>SO<sub>4</sub>. No proteins are precipitated by use of this buffer. The fractions containing the 21 kDa polypeptide were identified by electrophoresis in the presence of 0.1% SDS, pooled and centrifuged at 105000 g for 1 h. The purified 21 kDa protein in the 105000 g pellet was resuspended in 0.25 M-sucrose/5 mM-Hepes, pH 7.4, to give a clear 'solution'. This solution was dialysed against 0.25 M-sucrose/5 mM-Hepes for 16 h at 4°C. This preparation contains a protein which is easily dissolved in buffer, and which can be sedimented by centrifugation at 105000 g for 1 h.

**Preparation of antisera against the heat-extractable protein**

Antisera against the heat-extractable protein were raised in White Leghorn hens by the method described by Cohen et al. (1976).

**Purification of cytochrome b<sub>5</sub> and epoxide hydrolase**

Rat liver microsomal cytochrome b<sub>5</sub> was purified as described by Burchell (1985). Rat liver epoxide hydrolase was purified as described by Knowles & Burchell (1977).

**Ouchterlony double diffusion in 1% agar**

This was carried out as described by Ouchterlony (1949).

**Results and discussion**

Hepatic microsomal glucose-6-phosphatase has proved difficult to purify, owing to its extreme lability, especially as the purity of the enzyme increases. Therefore we have investigated whether the instability might be caused by the dissociation of stabilizing factors from the glucose-6-phosphatase enzyme.

**Instability of microsomal glucose-6-phosphatase at pH 5.0**

Glucose-6-phosphatase activity becomes very unstable at pH 5.0 (de Duve et al., 1949; Hers & Van Hoof, 1966; Arion et al., 1976b; Walls, 1978; Arion & Walls, 1979). Rabbit liver microsomes were incubated at pH 5.0 for 15 min at 30°C in 0.25 M-sucrose/20 mM-sodium cacodylate buffer, and the suspension was then centrifuged for 2 min at 15000 g. The microsomal pellet was resuspended in 0.25 M-sucrose/20 mM-sodium cacodylate, pH 7.0, and the supernatant adjusted to pH 7.0.
Stabilization of glucose-6-phosphatase activity

Table 1. Separation of glucose-6-phosphatase activity from the 21 kDa polypeptides

<table>
<thead>
<tr>
<th>Microsomes before treatment</th>
<th>Rabbit</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 5.0 supernatant</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>pH 5.0 pellet</td>
<td>97 ± 4</td>
<td>93 ± 2</td>
</tr>
</tbody>
</table>

Immediately before assay, the fractions were diluted 50-fold in pH 6.5 assay buffer and assayed for glucose-6-phosphatase activity as described in the Materials and methods section. Up to 97% of the glucose-6-phosphatase activity was inactivated in the microsomes by this procedure, and none was recovered in the pH 5 supernatant (de Duve et al., 1949; Walls, 1978). However, when the microsomes were exposed to pH 5.0 for 15 min at 0°C, all the activity was recovered in the membrane pellet fraction (see Table 1), provided that the assays were done immediately, as exposure to low pH causes the glucose-6-phosphatase activity to become extremely labile (Walls, 1978; Arion & Walls, 1979). Similar results were obtained with rat liver microsomes (Table 1).

Isolation of the major polypeptides in the pH 5.0 supernatant

Rabbit liver microsomes were incubated at pH 5.0 at both 0°C and 30°C as described above, the suspension was then centrifuged at 15000g and the supernatants were analysed by SDS/polyacrylamide-gel electrophoresis. Two prominent polypeptide bands of approx. 21 kDa were observed. The polypeptides were further purified by centrifugation at 105000g for 90 min. The proteins in the 105000g pellets were dissolved in 0.25M-sucrose/5mM-Hepes, pH 7.4, and appeared to be purified to homogeneity as determined by SDS/polyacrylamide-gel electrophoresis (Fig. 1).

When rat liver microsomes were incubated at pH 5 and then centrifuged as described above, only one polypeptide band of approx. 21 kDa was purified to homogeneity.

Identity of the polypeptides in the pH 5 supernatant with purified microsomal heat-extractable proteins

The rabbit liver microsomal polypeptides purified from the pH 5.0 supernatant appeared to be very similar to the heat-extractable proteins that we had previously isolated from rabbit liver microsomes (Burchell & Burchell, 1982). Antibody raised against the heat-extractable proteins immunoprecipitates approx. 90% of the glucose-6-phosphatase activity in solubilized hepatic microsomes (Burchell & Burchell, 1982). Since this immunological evidence indicated a close association between heat-extractable protein and glucose-6-phosphatase activity (Burchell & Burchell, 1982), we have compared the heat-extractable proteins with the polypeptides in the pH 5.0 supernatant.

The polypeptides isolated from the pH 5.0 supernatant co-migrated on SDS/polyacrylamide-gel electrophoresis with a heat-extractable protein doublet which had previously been obtained from rabbit microsomes (Burchell & Burchell, 1982; see Fig. 1). The $M_r$ values of the polypeptides were determined to be 21 300 and 22 400 by comparison with protein standards of known $M_r$ on a SDS/11%polyacrylamide gel (results not shown). The $M_r$ values are slightly higher than previously reported for the heat-extractable protein (Burchell & Burchell, 1982), as the previous determinations were done in less accurate gradient gels.

Identical peptide maps were obtained from both the purified rabbit liver microsomal heat-extractable protein and the purified rabbit liver microsomal pH 5.0-extractable protein (see Fig. 2). Both proteins from rabbit liver microsomes gave lines of identity on Ouchterlony double-diffusion experiments against antibody raised in either hens or sheep (results not shown) against rabbit microsomal heat-extractable protein (see Fig. 3).

Thus the rabbit liver proteins obtained by pH 5 extraction or heat treatment of microsomes appear to be identical. Antibodies raised against the 21 and 22 kDa polypeptides precipitate 90% of glucose-6-phosphatase activity from solubilized microsomes, and we have previously proposed that the 21 or 22 kDa polypeptides might be the phosphohydrolase component of glucose-6-phos-
phatase (Burchell & Burchell, 1982). However, as described above, 100% of the glucose-6-phosphatase activity remains in the pH 5.0 pellet after removal of the 21 and 22 kDa polypeptides at 0°C, which indicates that these polypeptides are not the catalytic subunit of glucose-6-phosphatase. The immunochemical studies (Burchell & Burchell, 1982) suggested a close association between glucose-6-phosphatase and the 21 kDa polypeptides. Thus, in an effort to determine their role in the glucose-6-phosphatase system, we have purified the 21 kDa proteins from rat and rabbit liver microsomes by conventional procedures, which should maintain their biological activity.

Conventional purification of 21 kDa protein from hepatic microsomes

The proteins were purified to homogeneity from rabbit liver microsomes by Lubrol solubilization, followed by chromatographic isolation on Fractogel TSK DEAE-650(S) and centrifugation at 105000 g (see the Materials and methods section). The purified preparation contained two polypeptides (Fig. 1) which co-migrate during electro-
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Fig. 3. Ouchterlony double diffusion in 1% agar
Wells: centre, hen antiserum raised against rabbit heat-extractable protein as described in Burchell & Burchell (1982); 1, rabbit heat-extractable protein; 2, rabbit pH 5.0-extractable protein extracted at 30°C; 3, rabbit pH 5.0-extractable protein extracted at 0°C; 4, conventionally purified rabbit stabilizing protein; 5, conventionally purified rat stabilizing protein; 6, sucrose/Hepes buffer.

...phoresis in the presence of SDS to positions identical with those of the two proteins that can be extracted by heat treatment or pH 5.0 extraction of rabbit liver microsomes.

All three preparations exhibited lines of identity in Ouchterlony double diffusion analysis (Fig. 3).

This purification procedure also yielded a single 21 kDa polypeptide from rat liver microsomes similar to the pH 5.0-extractable protein purified from rat liver microsomes. The apparently homogeneous rat polypeptide co-migrates to a position identical with that of the purified rabbit liver microsomal 21 kDa polypeptide during electrophoresis (Fig. 4), and these rat and rabbit 21 kDa liver microsomal proteins also showed a single sharp line of identity on cross-reaction with antibody in Ouchterlony double-diffusion experiments (see Fig. 3).

We have determined that the preparation from rat liver microsomes contained only the 21 kDa polypeptide and is apparently identical with the rabbit protein; we have used the homogeneously purified rat protein to try to determine its role and interaction with glucose-6-phosphatase.

Stabilization of microsomal glucose-6-phosphatase by a 21 kDa protein

Various preparations of rat liver microsomal glucose-6-phosphatase depleted of the 21 kDa protein by treatment with 1% Lubrol were incubated with the conventionally purified rat liver microsomal 21 kDa protein to see whether the protein affected glucose-6-phosphatase activity.

When rat liver microsomes were partially solubilized by 1% Lubrol, 100% of the glucose-6-phosphatase activity was recovered in a 105000 g pellet, whereas the 21 kDa polypeptide was found in the 105000 g supernatant. The glucose-6-phosphatase activity in the 105000 g pellet was unstable (see Table 2); 60% of the activity was lost during incubation for 20 min at 20°C. The inactivation...
could be prevented by the addition of conventionally purified rat 21 kDa protein. The ability of the pH 5.0-extracted protein to stabilize glucose-6-phosphatase varied from preparation to preparation, and similarly treating conventionally purified rat 21 kDa protein at pH 5.0 lowered its ability to stabilize glucose-6-phosphatase activity (Table 2). Glucose-6-phosphatase activity could not be stabilized by the addition of the 21 kDa protein extracted by heating at 100°C, or by the addition of purified rat liver microsomal cytochrome b5 or purified rat liver microsomal epoxide hydrolase, or by addition of purified carbonic anhydrase (Table 2). Therefore the stabilization of glucose-6-phosphatase by the purified 21 kDa polypeptide seems to be via specific interaction between the two microsomal components. The stabilization of glucose-6-phosphatase activity requires interaction with biologically active 21 kDa protein, as the heat-extracted 21 kDa polypeptide does not stabilize the activity of glucose-6-phosphatase and even the relatively mild treatment at pH 5.0 decreases the ability of the 21 kDa protein to stabilize glucose-6-phosphatase (Table 2).

Essentially the same results were obtained with the rabbit 21 kDa proteins (results not shown).

Table 2. Prevention of the inactivation of rat liver microsomal glucose-6-phosphatase at 20°C by various purified rat microsomal protein preparations

<table>
<thead>
<tr>
<th>Addition</th>
<th>10min</th>
<th>20min</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (0°C)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>None (20°C)</td>
<td>64 (66-62)</td>
<td>38 (39-35)</td>
</tr>
<tr>
<td>Rat 21 kDa protein (0.04mg)</td>
<td>100 (101-97)</td>
<td>82 (84-79)</td>
</tr>
<tr>
<td>Rat 21 kDa protein* (0.04mg)</td>
<td>92 (93-90)</td>
<td>76 (77-79)</td>
</tr>
<tr>
<td>Rat 21 kDa protein† (0.04mg)</td>
<td>72 (75-70)</td>
<td>48 (49-47)</td>
</tr>
<tr>
<td>Rat heat-extracted protein (0.05mg)</td>
<td>62 (64-61)</td>
<td>37 (38-34)</td>
</tr>
<tr>
<td>Rat pH 5.0-extracted protein (0.05mg)</td>
<td>74 (85-63)</td>
<td>52 (65-39)</td>
</tr>
<tr>
<td>Carbonic anhydrase (0.2mg)</td>
<td>51 (52-47)</td>
<td>33 (33-32)</td>
</tr>
<tr>
<td>Epoxide hydrolase (0.03mg)</td>
<td>31 (33-30)</td>
<td>18 (19-17)</td>
</tr>
<tr>
<td>Cytochrome b5 (0.03mg)</td>
<td>62 (64-61)</td>
<td>36 (38-35)</td>
</tr>
</tbody>
</table>

* Rat 21 kDa protein after extensive dialysis against sucrose/Hepes, pH 7.4.
† Conventional purified rat 21 kDa protein after exposure to pH 5.0 at 0°C for 15 min, followed by centrifugation at 105000g and resuspension in sucrose/Hepes, pH 7.4.

which may indicate that the 21 kDa protein slowly loses its conformation during the incubation or that perhaps some other factor (e.g. a metal ion) is missing from the incubation. We have not yet examined whether addition of other factors might improve stability of the interaction between the 21 kDa protein and glucose-6-phosphatase.
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