STABILIZATION OF PARTIALLY-PURIFIED GLUCOSE 6-PHOSPHATASE BY FLUORIDE

Is enzyme inactivation caused by dephosphorylation?

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

Received 16 July 1980

1. Introduction

Hepatic microsomal glucose 6-phosphatase is an important enzyme which plays a key role in the maintenance of blood glucose concentration within a narrow range [1]. The enzyme activity can be increased by administration of steroids, by starvation and in some diabetic conditions and those increased activities have often been attributed to changes in protein turnover [2].

Stadtman [3] has pointed out that regulation of glucose 6-phosphatase is 'mandatory'. However, the molecular mechanisms involved in the regulation of this enzyme are unknown. Zakim has proposed that this enzyme is completely controlled by the membrane phospholipid environment [4], whereas Cater et al. [5] concluded that glucose 6-phosphatase was only partially phospholipid-dependent.

The extreme lability of 'solubilised' glucose 6-phosphatase [6,7] is undoubtedly the major reason for repeated failure to purify the enzyme and has also confused attempts to study the mechanisms controlling this enzyme.

Here we demonstrate that the rapid time-dependent inactivation of partially purified glucose 6-phosphatase is completely abolished by fluoride, molybdate and glutathione disulphide. Glucose 6-phosphatase can also be slightly reactivated in the presence of ATP-Mg^{2+}. The possible regulation of glucose 6-phosphatase by a phosphorylation/dephosphorylation mechanism is discussed.

2. Materials and methods

Glucose 6-phosphate, sodium cholate, octyl glucoside, dithiothreitol; glutathione, glutathione disulphide and phenyl methylsulphonylfluoride were from Sigma (London). Benzamidine hydrochloride was obtained from Aldrich Chemical Co. and egg lecithin, grade 1, was purchased from Lipid Products, South Nutfield, Surrey. Polyethylene glycol 6000, sodium fluoride, sodium molybdate and ATP were from BDH Ltd, Poole, Dorset. Purified *B. cereus* phospholipase C was a kind gift from Dr R. Zwaal, Department of Biochemistry, University of Maastricht.

2.1. *Assays*

Glucose 6-phosphatase activity was assayed at 37°C in a 0.1 ml reaction mixture as in [8]. One unit of activity represents 1.0 μmol P_i released/min. Non-specific hydrolysis of glucose 6-phosphate by acid phosphatases other than glucose 6-phosphatase was assayed by preincubating enzyme preparations for 10 min at 37°C and pH 5 prior to addition of 30 mM glucose 6-phosphate in cacodylate buffer (pH 6.5) [5,9]. It averaged <3% of total glucose 6-phosphatase activity in either enzyme preparation. Phosphorylase phosphatase was assayed using γ-^{32}P-labelled phosphorylase *a* by the method in [10]. Protein concentrations were determined as in [11].

2.2. *Partial purification of glucose 6-phosphatase*

Glucose 6-phosphatase was partially purified from fresh adult New Zealand white rabbit liver microsomal fraction by solubilisation with 1% sodium cholate and polyethylene glycol fractionation [8].

3. Results and discussion

3.1. *Prevention of time-dependent inactivation of glucose 6-phosphatase by fluoride, molybdate and glutathione disulphide*

Glucose 6-phosphatase activity in freshly prepared hepatic microsomal suspensions in 0.1 M Tris–acetate, 20% glycerol (pH 7.0) buffer (buffer A) was completely stable during incubation at 25°C for ≥90 min, as in [5,9]. However, after solubilisation of glucose 6-phosphatase with 1% cholate in buffer A (see section 2), the enzyme can be rapidly inactivated by incubation at 25°C prior to assay (fig.1). This inactivation of glucose 6-phosphatase occurs slightly more rapidly after partial purification of the enzyme by polyethylene glycol fractionation (fig.1). The rate of inactivation of glucose 6-phosphatase can be increased by the presence of 10 mM EDTA during preincubation of either cholate soluble or polyethylene glycol preparations.

It should be noted here that the preincubation mixtures were diluted 125-fold in the assay of glucose 6-phosphatase activity. After dilution no further inactivation of the glucose 6-phosphatase occurred and hydrolysis of glucose 6-phosphate was linear for ≥20 min at 37°C in control and experimental samples.

The time-dependent inactivation of glucose 6-phosphatase can be completely prevented by the presence of 50 mM sodium fluoride or 10 mM sodium molybdate during preincubation (fig.1). Sodium fluoride (50 mM) and 10 mM sodium molybdate would in fact inhibit the assay of glucose 6-phosphatase [9]

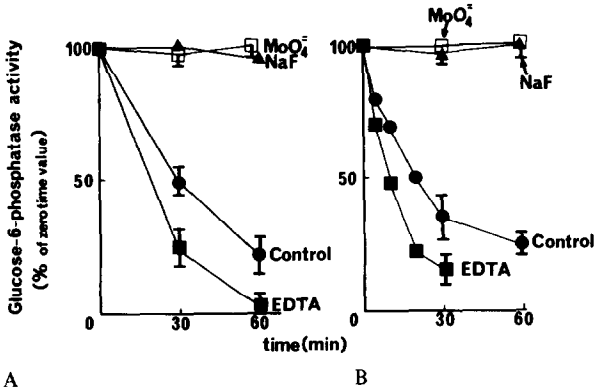

Fig.1. Prevention of the inactivation of partially purified glucose 6-phosphatase by fluoride and molybdate. (A) Rat liver microsomal cholate-soluble fraction (13 mg protein/ml) or (B) polyethylene glycol fractions (15 mg protein/ml) were incubated at 25°C in the presence of 10 mM sodium molybdate (□), 50 mM sodium fluoride (▲), 10 mM EDTA (■) or buffer alone (●). Results shown are av. 5 expt ± SEM (where bars are indicated) or ≥3 expt (without bars). Glucose 6-phosphatase activity was: (A) 0.29 ± 0.05 units/mg protein; (B) 0.68 ± 0.1 units/mg protein.

but both the preincubation medium and inhibitory ions are diluted 125-fold prior to assay of glucose 6-phosphatase and thus they do not inhibit enzyme activity during assay.

Molybdate inhibits the action of a variety of phosphatases [12] and Leach et al. [13] have proposed that molybdate prevents inactivation of glucocorticoid receptors by inhibition of a protein phosphatase. Fluoride is also a well known inhibitor of protein phosphatases [14]. Thus our results suggest that 20–50 mM sodium fluroride or 10 mM molybdate may inhibit a protein phosphatase and thereby prevent inactivation of glucose 6-phosphatase by dephosphorylation of the enzyme. A single large dose of fluoride injected into rats caused a 3–4-fold increase of hepatic microsomal glucose 6-phosphatase activity [15]. This increase of glucose 6-phosphatase activity is not likely to be caused by the presence of fluroride in the microsomal preparations because fluoride inhibits the hydrolysis of glucose 6-phosphate [5,15].

These changes in glucose 6-phosphatase may be due to increased synthesis of glucose 6-phosphatase [15] but attempts to suppress increases of glucose 6-phosphatase by simultaneous administration of cycloheximide or actinomycin D were not effective [15].

Glutathione disulphide (100 mM GSSG) also prevents the inactivation of glucose 6-phosphatase (table 1) and does not inhibit assay of glucose 6-phosphatase. GSSG has been shown to be an inhibitor of phosphorylase phosphatase [16].

Fluroride (50 mM), molybdate (10 mM) and GSSG (100 mM) all completely inhibited the endogeneous phosphorylase phosphatase activity present in cholate solubilised microsomal preparations. Thus our results imply that glucose 6-phosphatase may exist as an active phosphorylated form or as an inactive dephosphorylated form.

3.2. *Search for non-specific inactivation of glucose 6-phosphatase*

As inactivation of glucose 6-phosphatase does not occur until after solubilisation of the enzyme by 1% cholate in buffer A, we have considered the possibility that this effect may be due to the cholate alone or a contaminant present within the cholate preparation.

Microsomal fraction (13.5 mg protein/ml) was treated with either 1% cholate or 1% octylglucoside and the inactivation of glucose 6-phosphatase in the treated microsomes was compared after incubation

Table 1
Inactivation of glucose 6-phosphatase in the presence of salts, protease inhibitors, reducing agents and other compounds at 25°C

Additions[a]	Glucose 6-phosphatase activity remaining[b] (%)
None, 0°C	100
None, 25°C	30
Benzamidine (0.1 mM)	31
Phenylmethylsulphonyl fluroride (0.1 mM)	28
Cysteine (50 mM)	20
Dithiothreitol (50 mM)	28
Glutathione (20 mM)	14
Glutathione disulphide (100 mM)	90
Sodium fluoride (10 mM)	91
Sodium fluoride (20 mM)	100
Sodium chloride (50 mm)	28
Calcium chloride (10 mM)	32
Manganese chloride (5 mM)	27
Magnesium acetate (10 mM)	31
EGTA (10 mM)	32
Lecithin (1 mg phospholipid/mg protein)	31

[a] Incubation for 30 min at 0°C does not inactivate glucose 6-phosphatase. Glucose 6-phosphatase in the polyethylene glycol fractions was spec. act. 0.68 ± 0.1 units/mg protein

[b] A variety of compounds were added to polyethylene glycol fractions (15–18 mg protein/ml) at concentrations indicated. These mixtures were then incubated for 30 min at 25°C. The results shown are av. ⩾3 expt and are expressed as % of enzyme activity remaining after this incubation

at 25°C for 60 min. In both cases 93% of glucose 6-phosphatase activity was destroyed after the preincubation period. Thus enzyme inactivation can also occur after octylglucoside treatment of microsomes, and is not an artifact due to cholate alone.

It is also important to show that this inactivation is not an artefact common to all detergents, thus we have used a completely different treatment to obtain a thermally unstable form of glucose 6-phosphatase, using phospholipase C. Pre-treatment of microsomes with 10 units/ml of pure *B. cereus* phospholipase C for 60 min at 5°C hydrolysed up to 97% of the phosphatidylcholine present and thereby reduced glucose 6-phosphatase activity by 80% [5]. The remaining glucose 6-phosphatase activity could be further inactivated by incubation at 20°C for 60 min [5]. Our work has confirmed these results, but in addition we have observed that the inactivation by incubation at 25°C can be prevented by the presence of 50 mM sodium fluoride or 10–20 mM sodium molybdate in the pre-assay incubation mixture. This result indicates that glucose 6-phosphatase is also unstable after microsomes have been severely phospholipid depleted and that this inactivation is not an artefactual property of detergents.

3.3. *A search for other agents which might prevent inactivation of glucose 6-phosphatase*

Solubilised and partially-purified preparations of glucose 6-phosphatase may still be contaminated by degradative enzymes and thus a number of control experiments were done in an attempt to exclude the following possibilities:

(i) Non specific proteolytic inactivation of glucose 6-phosphatase: This problem cannot be easily studied until proteolysis of the purified enzyme has been examined. However, we have determined that addition of two commonly used protease inhibitors, 0.1 mM benzamidine and 0.1 mM phenylmethylsulphonylfluoride (sufficient to inactivate serine proteases [17]) did not affect the rate of inactivation of glucose 6-phosphatase (table 1).

(ii) Inactivation of glucose 6-phosphatase by thiol–disulphide interchange: The activity of a number of enzymes are now known to be altered by thiol–disulphide interchange catalysed by microsomal [18,19] and cytosolic [20] thiol–protein disulphide oxidoreductases.

Glucose 6-phosphatase may be inactivated by oxidation of exposed thiol groups. However, the inactivation of glucose 6-phosphatase could not be prevented by preincubation with 50 mM dithiothreitol, 50 mM cysteine or 20 mM glutathione (table 1). Further, glucose 6-phosphatase cannot be reactivated by dithiothreitol after removal of cholate.

Glucose 6-phosphatase may be inactivated by reduction of an exposed disulphide group. GSSG (100 mM) does prevent inactivation of glucose 6-phosphatase (table 1), although 100 mM GSSG does not reactivate pre-inactivated glucose 6-phosphatase.

Thus it seems unlikely that glucose 6-phosphatase is inactivated by thiol–disulphide interchange,

although we cannot judge from the literature whether the relevant oxido–reductase might be activated or inhibited by the presence of 1% (w/v) cholate [18,19].

It is important to note here that we have confirmed that the solubilised partially purified glucose 6-phosphatase activity is not inhibited or stabilised by 10 mM *N*-ethylmaleimide indicating that free thiol groups are not important enzyme-active site components [21].

(iii) Phospholipid hydrolysis: Treatment of microsomes with detergents may promote breakdown of phospholipids by endogeneous phospholipases resulting in reduced glucose 6-phosphatase activity. However, addition of phosphatidylcholine (1 mg/1 mg protein) did not decrease the rate of inactivation of glucose 6-phosphatase (table 1). Further, the presence of 10 mM Ca^{2+} an activator, or alternatively 10 mM EGTA, an inhibitor of phospholipase [5] did not affect the rate of glucose 6-phosphatase inactivation (table 1). Even after phospholipase C treatment of microomes, further inactivation of glucose 6-phosphatase was observed at 25°C after inhibition of the added phospholipase C (see section 3.2).

(iv) The effect of salts and metal ions: Table 1 shows that 10 mM sodium fluoride almost completely prevented enzyme inactivation and 20–50 mM sodium fluoride completely protect against time-dependent inactivation of glucose 6-phosphatase. Sodium chloride (50 mM) or 10 mM calcium chloride did not prevent glucose 6-phosphatase inactivation. Manganese chloride (5 mM) and magnesium acetate (10 mM) also did not affect the rate of enzyme inactivation. Thus the stabilisation of partially purified glucose 6-phosphatase does appear to require specific elements.

Glucose 6-phosphatase does not appear to be inactivated by proteolysis, thiol–disulphide interchange, or phospholipid hydrolysis, although none of these possibilities can be completely dismissed.

3.4. *Reactivation of glucose 6-phosphatase activity by protein phosphorylation*

In order to prove that glucose 6-phosphatase is regulated by a phosphorylation/dephosphorylation mechanism it is necessary to show its inactivation can be reversed by a protein kinase.

Addition of 1 mM ATP–10 mM Mg^{2+} to cholate soluble or polyethylene glycol fractions did not prevent the inactivation of glucose 6-phosphatase during preincubation at 25°C. However, if inactivated glucose 6-phosphatase was incubated with 1 mM ATP–10 mM Mg^{2+} in the presence of 20 mM NaF a 10% (5–20%) reactivation was observed. Addition of liver cytosol did not improve this recovery (even if cyclic AMP was also added). A major problem is that a membrane bound protein kinase(s) may be essential and if these are already present they may be inactivated by 1% (w/v) sodium cholate which is also present [22].

Thus the possible regulation of glucose 6-phosphatase activity by a phosphorylation/dephosphorylation mechanism is far from proven. We must realise that similar observations have been recorded during studies of muscle phosphofructokinase [23] and although the purified enzyme contains phosphate [24] regulation of muscle phosphofructokinase by phosphorylation has not been proven, but the corresponding liver enzyme activity is modulated by this type of regulatory mechanism [25,26].

Acknowledgements

We thank Dr P. Cohen for his advice and stimulating discussions. We also thank the Medical Research Council for a grant supporting this work. B. B. is the repicient of a Wellcome Special Research Leave Fellowship.

References

[1] Cori, G. T. and Cori, C. F. (1952) J. Biol. Chem. 199, 661–667.
[2] Nordlie, R. C. (1971) in: The Enzymes (Boyer, P. D. ed) vol. 4, pp. 543–561, Academic Press, London, New York.
[3] Stadtman, E. R. (1966) Adv. Enzymol 28, 41–154.
[4] Zakim, D. (1970) J. Biol. Chem. 245, 4953–4961.
[5] Cater, B. R., Trivedi, P. and Hallinan, T. (1975) Biochem. J. 148, 279–294.
[6] Beaufay, H. and De Duve, C. (1954) Bull. Soc. Chim. Biol. 36, 1551–1568.
[7] Garland, R. C., Cori, C. F. and Chang, H. W. (1974) Proc. Natl. Acad. Sci. USA 71, 3805–3809.
[8] Bickerstaff, G. F. and Burchell, B. (1980) Biochem. Soc. Trans in press.
[9] Beaufay, H., Hers, H. G., Berthet, J. and De Duve, C. (1954) Bull. Soc. Chim. Biol. 36, 1539–1550.
[10] Antoniw, J. F., Nimmo, H. G., Yeaman, S. J. and Cohen, P. (1977) Biochem. J. 162, 324–433.
[11] Bradford, M. M. (1976) Anal. Biochem. 72, 248–254.

[12] Hollander, V. P. (1971) in: The Enzymes (Boyer, P. D. ed) vol. 4, pp. 449–498, Academic Press, New York.
[13] Leach, K. L., Dahmer, M. K., Hammond, N. D., Sando, J. J. and Pratt, W. B. (1979) J. Biol. Chem. 254, 11884–11890.
[14] Cohen, P., Nimmo, G. A., Burchell, A. and Antoniw, J. F. (1978) Adv. Enz. Regul. 16, 97–119.
[15] Suketa, Y. and Sato, M. (1980) Toxicol. App. Pharmacol. 52, 386–390.
[16] Shimazu, T., Tokutake, S. and Usami, M. (1978) J. Biol. Chem. 253, 7376–7382.
[17] Walsh, K. A. (1970) Methods Enzymol. 19, 41–63.
[18] Hawkins, H. C. and Freedman, R. B. (1976) Biochem. J. 159, 385–393.
[19] Francis, G. L. and Ballard, F. J. (1980) Biochem. J. 186, 571–579.
[20] Mannervik, B. and Axelsson, K. (1975) Biochem. J. 149, 785–788.
[21] Garland, R. C., Cori, C. F. and Chang, H.-W. (1976) Mol. Cell. Biochem. 12, 23–31.
[22] Sommarin, M. and Jergil, B. (1978) Eur. J. Biochem. 88, 49–60.
[23] Parmeggiani, A., Luft, J. H., Love, D. S. and Krebs, E. G., (1966) J. Biol. Chem. 241, 4625–4637.
[24] Hofer, H. W. and Fürst, M. (1976) FEBS Lett. 62, 118–122.
[25] Brand, I. A. and Söling, H. D. (1975) FEBS Lett. 57, 163–168.
[26] Brand, I. A., Müller, M. K., Unger, C. and Söling, H. D. (1976) FEBS Lett. 68, 271–274.