Histone II-A stimulates glucose-6-phosphatase and reveals mannose-6-phosphatase activities without permeabilization of liver microsomes

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The effect of histone II-A on glucose-6-phosphatase and mannose-6-phosphatase activities was investigated in relation to microsomal membrane permeability. It was found that glucose-6-phosphatase activity in histone II-A-pretreated liver microsomes was stimulated to the same extent as in detergent-permeabilized microsomes, and that the substrate specificity of the enzyme for glucose 6-phosphate was lost in histone II-A-pretreated microsomes, as [U-¹⁴C]glucose 6-phosphate hydrolysis was inhibited by mannose 6-phosphate and [U-¹⁴C]mannose 6-phosphate hydrolysis was increased. The accumulation of [U-¹⁴C]glucose from [U-¹⁴C]glucose 6-phosphate into untreated

microsomes was completely abolished in detergent-treated vesicles, but was increased in histone II-A-treated microsomes, accounting for the increased glucose-6-phosphatase activity, and demonstrating that the microsomal membrane was still intact. The stimulation of glucose-6-phosphatase and mannose-6-phosphatase activities by histone II-A was found to be reversed by EGTA. It is concluded that the effects of histone II-A on glucose-6-phosphatase and mannose-6-phosphatase are not caused by the permeabilization of the microsomal membrane. The measurement of mannose-6-phosphatase latency to evaluate the intactness of the vesicles is therefore inappropriate.

INTRODUCTION

Glucose-6-phosphatase (G6Pase) (EC 3.1.3.9) catalyses the final step of hepatic glucose production and is located at the endoplasmic-reticulum membrane [1]. In intact microsomes G6Pase hydrolyses only glucose 6-phosphate (G6P), whereas in detergent-treated microsomes an increased rate of G6P hydrolysis as well as the hydrolysis of a variety of phosphorylated sugars are observed [1].

The measurement of mannose-6-phosphatase activity (M6Pase) is widely used for the determination of microsomal membrane intactness [2]. This activity is latent in untreated microsomes and is revealed when microsomes are treated with a detergent. Histone II-A has been reported to stimulate G6Pase [3,4] and M6Pase activities [4], and was therefore proposed as an alternative agent to detergent for the permeabilization of the microsomes [4]. In keeping with these findings, we found that G6Pase and M6Pase activities are stimulated in histone II-A-treated microsomes to the same extent as in detergent-treated microsomes, but, unlike the effect of detergents, histone II-A was not found to permeabilize the membrane barrier.

EXPERIMENTAL

Materials

3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate (CHAPSO), EGTA, G6P (monosodium salt), histone II-A and mannose 6-phosphate (M6P) (monosodium salt) were all purchased from Sigma Chemical Co. (St. Louis, MO). Tris base was from Boehringer Mannheim Corp. (Laval, Canada). Sucrose (ultra-pure) and [U-¹⁴C]G6P were from ICN Biomedicals (Montral, Canada). [U-¹⁴C]Mannose was obtained

from NEN-DuPont (Mississauga, Canada). All reagents were of analytical grade.

Preparation of liver microsomes

Liver microsomes were isolated from overnight-fasted male Wistar rats (Charles River, St-Constant, Canada) (200–250 g) as described previously [5], and were resuspended in 0.25 M sucrose/50 mM Tris, pH 7.3, at a protein concentration of approx. 15 mg/ml. Microsomal vesicles were either used the same day or kept at –80 C for later use. Freezing of microsomes, if thawed once at 4 C, did not affect the kinetic properties of G6Pase [6], as also documented by others [7]. Microsomal protein concentration was measured by the method of Bradford, with BSA as standard [8].

Partial purification of G6Pase

A partially purified G6Pase from rat liver microsomes was prepared by a procedure [9] modified from that of Rymsa and De Groot [10]. Briefly, microsomes were solubilized with the non-denaturing zwitterionic detergent CHAPSO (0.8 %, w/v) in the presence of 0.6 M NaCl and centrifuged at 105 000 *g* for 30 min. The resulting supernatant was applied on a hydroxyapatite (HA) column and G6Pase activity was collected in the void volume. The specific activity (with 0.2 mM [U-¹⁴C]G6P as substrate) of the pooled fractions (HA fraction) ranged from 44 to 213 nmol/min per mg of protein for different preparations.

Enzyme assays

G6Pase activity was measured at 30 C in untreated microsomes and in the HA fraction by the production of [U-¹⁴C]glucose from 0.2 mM [U-¹⁴C]G6P (0.4 nCi/nmol), as described previously [5]. M6Pase activity was measured likewise by using 0.2 mM [U-

Abbreviations used: CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate; FSRFA, fast-sampling rapid-filtration apparatus; G6P, glucose 6-phosphate; G6Pase, glucose-6-phosphatase; M6P, mannose 6-phosphate; M6Pase, mannose-6-phosphatase; HA, hydroxyapatite.

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^{14}C]M6P (0.2 nCi/nmol). G6Pase and M6Pase activities were also measured in microsomes previously treated with CHAPSO (0.8 %) for 30 min at 4 °C (0.08 % CHAPSO in the assay medium) as described previously [6] and in microsomes previously mixed with 10 mg/ml histone II-A at 4 °C (1 mg/ml in the assay medium).

[U- ^{14}C]M6P synthesis

[U- ^{14}C]M6P was prepared by the phosphorylation of [U- ^{14}C]mannose (300 mCi/mmol) with ATP by using hexokinase as previously described [11], with the following modifications. The reaction was stopped by the addition of 1 % activated charcoal, followed by centrifugation (1 min, 16000 *g* in an Eppendorf centrifuge). The supernatant was then applied on a Dowex AG 1X8 column (acetate form). [U- ^{14}C]Mannose that had not reacted came out in the flow-through, whereas [U- ^{14}C]M6P was eluted with an acetic acid gradient (0.2–1 M). [U- ^{14}C]M6P was then pooled, freeze-dried (with a Savant SpeedVac concentrator) and resuspended in 4 mM unlabelled M6P (in 50 mM Tris, pH 7.3) to a final specific radioactivity of 2.4 nCi/nmol. The yield of [U- ^{14}C]M6P synthesis was 99 %.

Uptake measurements

[U- ^{14}C]Glucose uptake in microsomes from 0.2 mM [U- ^{14}C]G6P (20 nCi/nmol) was determined by using a fast-sampling rapid-filtration apparatus (FSRFA) as described and validated elsewhere [6,12,13]. Uptake measurements were also performed in microsomes previously treated with 10 mg/ml histone II-A for 2 min at 4 °C (0.4 mg/ml in the uptake medium) and in vesicles previously treated with CHAPSO (0.8 %) for 30 min at 4 °C (0.04 % CHAPSO in the uptake medium).

RESULTS

Table 1 shows that G6Pase activity in liver microsomes is stimulated by about 2-fold and M6Pase by about 6-fold by either histone II-A or CHAPSO. The increased rates of [U- ^{14}C]M6P and [U- ^{14}C]G6P hydrolysis in histone II-A-treated microsomes were not due to the stimulation of non-specific phosphatases, as we verified that the hydrolysis of β -glycerophosphate (20 mM)

Table 1 Stimulation by histone II-A of G6Pase and M6Pase activities from rat liver microsomes

[U- ^{14}C]Glucose production from 0.2 mM [U- ^{14}C]G6P was measured in liver microsomes and in a partially purified G6Pase preparation (HA fraction) under different conditions. [U- ^{14}C]Mannose production from 0.2 mM [U- ^{14}C]M6P was measured in liver microsomes. Data are the means $\pm$ S.E.M. for *n* experiments: **P* < 0.05 compared with the respective untreated values by an unpaired Student's *t* test, and ***P* < 0.01 and ****P* < 0.05 compared with the untreated value by a paired Student's *t* test.

	Liver microsomes		HA fraction	
	G6Pase (nmol/min per mg) (<i>n</i> = 5)	M6Pase (nmol/min per mg) (<i>n</i> = 4)	G6Pase (%) (<i>n</i> = 9)	M6Pase (%) (<i>n</i> = 5)
Untreated	12.8 $\pm$ 1.2	3.3 $\pm$ 0.6	100	100
Histone II-A- treated (1 mg/ml)	22.1 $\pm$ 3.7*	17.2 $\pm$ 3.1*	123 $\pm$ 5**	119 $\pm$ 5***
CHAPSO-treated (0.08%)	23.5 $\pm$ 1.4*	21.9 $\pm$ 2.2*	—	—

Figure 1 Inhibition by M6P of the production of [U- ^{14}C]glucose from 0.2 mM [U- ^{14}C]G6P

[U- ^{14}C]G6P (0.2 mM) hydrolysis was measured in the presence of the indicated concentrations of unlabelled M6P in untreated microsomes (○), histone II-A-treated microsomes (●), CHAPSO-treated microsomes (△), and in microsomes previously treated with both CHAPSO and histone II-A (▲). The results are expressed as the percentage of the enzymic activities measured in the absence of M6P (100% represents 9.6, 24.9, 31.3 and 31.6 nmol/min per mg for untreated, CHAPSO-, histone II-A- and CHAPSO + histone II-A-treated microsomes respectively).

was unchanged. The effect of other polycationic proteins (1 mM spermine, spermidine and putrescine) was tested on G6Pase activity, and we observed results (not shown) similar to those reported by Nordlie et al. [14]. We have also tested the effect of histone II-A on G6Pase and M6Pase activities in a partially purified preparation (HA fraction). Since the purification factor differed from one preparation to another, the specific activities of G6Pase and M6Pase in the HA fraction were expressed as percentages of the control value for each experiment. A slight but significant stimulation of G6Pase and M6Pase activities was observed when the partially purified enzyme was treated with histone II-A (Table 1).

The results shown in Figures 1–3 are illustrative of a series of experiments on different microsomal preparations which gave consistently the same type of results. The specificity of G6Pase for [U- ^{14}C]G6P was further studied by competition with unlabelled M6P. Figure 1 shows that M6P had little effect on G6Pase activity in untreated microsomes, but almost completely inhibited G6Pase activity either in detergent-treated microsomes, as reported previously [15,16], or in microsomes treated with histone II-A or with CHAPSO and histone II-A together. The *K_i* values of M6P towards [U- ^{14}C]G6P hydrolysis are 0.85, 0.89 and 0.90 mM in CHAPSO-, histone II-A- and CHAPSO + histone II-A-treated microsomes respectively.

The stimulation of G6Pase activity by histone II-A herein reported was observed previously by other investigators, who suggested that this effect was due to the disruption of the microsomal membrane [4]. To verify this possibility, we compared the effect of the detergent CHAPSO and histone II-A on [U- ^{14}C]G6P uptake and hydrolysis (Figure 2). As expected, the accumulation of [U- ^{14}C]glucose was precluded by the treatment of vesicles with detergent, but, in contrast, was increased by histone II-A pretreatment, thus indicating that histones do not permeabilize microsomes in these experimental conditions. The same plateau of [U- ^{14}C]glucose accumulation was observed whether histone II-A was added together with [U- ^{14}C]G6P or 2 min after preincubation of microsomes with the latter. The higher accumulation of [U- ^{14}C]glucose in histone II-A-treated microsomes compared with control microsomes is proportional

Figure 2 Rapid kinetics of $[U-^{14}C]$ glucose accumulation from $[U-^{14}C]$ G6P into microsomes

The accumulation of $[U-^{14}C]$ glucose from 0.2 mM $[U-^{14}C]$ G6P in untreated ($\circ$), histone II-A-treated (0.4 mg/ml in the uptake medium) ($\bullet$) or CHAPSO-treated ($\triangle$) microsomes was measured for up to 240 s at 30 °C with the FSRFA as described in the Experimental section. Alternatively, histone II-A (0.4 mg/ml) ($\blacksquare$) or CHAPSO (0.04%) ($\blacktriangle$) was added to microsomes previously incubated for 2 min in the presence of 0.2 mM $[U-^{14}C]$ G6P.

Figure 3 Effect of EGTA on G6Pase and M6Pase activities in histone II-A-treated microsomes

G6Pase activity was measured in the presence of increasing concentrations of EGTA in 50 mM Tris buffer, pH 7.3, in untreated ($\circ$) and histone II-A-treated (1 mg/ml in the assay medium) ($\bullet$) microsomes. M6Pase activity was measured in the same conditions in untreated ($\triangle$) and histone II-A-treated ($\blacktriangle$) microsomes.

to the higher G6Pase activity observed in the same conditions. Prolonged exposure (up to 15 min) of microsomes to histone II-A at 30 °C did not lead to the permeabilization of the microsomal membrane (results not shown). Figure 2 also shows that CHAPSO added after 2 min of incubation readily permeabilized the microsomes, as indicated by the immediate leakage of $[U-^{14}C]$ glucose from the vesicles. The possibility that the presence of histone II-A in the incubation medium leads to an increased non-specific binding of $[U-^{14}C]$ G6P either to the cellulose nitrate filters of the filtration apparatus or to the microsomal membrane

is unlikely, since the $t_{1/2}$ of $[U-^{14}C]$ G6P uptake was similar in the absence (38 s) or in the presence (26 s) of histone II-A, and was proportional to G6Pase activity. We have observed no accumulation of $[U-^{14}C]$ mannose from $[U-^{14}C]$ M6P in untreated or histone II-A-treated microsomes (results not shown). Presumably, mannose produced from M6P by histone II-A-treated microsomes is released directly in the extravesicular medium.

Figure 3 shows that increasing concentrations of EGTA (0.1–25 mM) have no effect on G6Pase or M6Pase activities in untreated microsomes, as we have shown previously [17,18], but almost completely reversed the stimulatory effect of histone II-A on both G6Pase and M6Pase. Similar results were obtained when EDTA was used instead of EGTA (not shown).

DISCUSSION

The stimulation of G6Pase by treatment of microsomes with polycationic proteins has been well documented [3,4,14,19,20]. This effect was attributed to the removal of the microsomal membrane barrier and the free access of substrates to the catalytic site [4]. However, the mechanism by which polycationic proteins disrupt membranes is unknown. It was suggested, based on ion fluxes and membrane conductivity experiments, that electrostatic interactions between eosinophil cationic proteins and phospholipids lead to membrane damage and formation of trans-membrane pores [21]. Other reports showed that cytosolic protein kinase and lactate dehydrogenase activities leaked from histone-treated cultured cells [22,23]. Another group reported that poly-L-lysine, protamine or histone treatment of gall-bladder epithelium caused loss of structural integrity of the microvilli [24]. However, all the effects of polycationic proteins on membrane permeability were observed after exposure of the cells at 25 °C or 37 °C for times ranging from 5 min to 1 h. Maximal haemolysis induced by eosinophil cationic proteins was observed at 37 °C, was decreased several-fold at 28 °C and was virtually not observed at 4 °C [21]. We found, however, that even prolonged exposure of liver microsomes with histone II-A at 30 °C (up to 15 min) did not permeabilize the vesicles. The discrepancy between the effects of polycationic proteins on plasma membranes and of histone II-A on microsomal membranes could be explained by the different lipid compositions of these membranes. This possibility is consistent with a recent report that spermine and other related polyamines inhibited the InsP_3 -induced Ca^{2+} efflux from rat cerebellar microsomes by direct binding to the microsomal InsP_3 receptor channel [25]. If polyamines were responsible for the permeabilization of the microsomal membranes, these authors would, however, have observed an increased rather than a decreased release of Ca^{2+} from the vesicles.

The stimulation of G6Pase activity and loss of specificity for G6P after detergent treatment of the microsomal vesicles have been attributed to the liberation of the catalytic site of the enzyme from its lipidic environment. However, the increased G6Pase activity in histone II-A-treated microsomes cannot be due to membrane disruption, since these vesicles are able to accumulate $[U-^{14}C]$ glucose from $[U-^{14}C]$ G6P proportionally to G6Pase activity. Histone II-A probably induces a conformational change in the G6Pase protein similar to that induced in detergent-treated microsomes, resulting in the loss of substrate specificity, as indicated by similar K_i values for competitive inhibition of G6Pase by M6P. The reversibility of the effect of histone II-A on G6Pase and M6Pase activities by EGTA and EDTA suggests that these chelators act by neutralizing the polycation charge. More importantly, this reversibility would be unlikely to be observed if histone II-A had damaged and permeabilized the membrane. The mechanism by which histone II-A affects G6Pase

and M6Pase is unknown and beyond the scope of this study. Among the possible explanations are an interaction of histone II-A with membrane phospholipids, affecting the enzyme, or a direct interaction with the protein itself, as a slight stimulation is still retained in purified preparations.

In the present paper, we report that (1) G6Pase and M6Pase activities are both increased upon treatment of the microsomes with histone II-A without permeabilization of the vesicles, (2) the vesicles (treated or not with histone II-A) never accumulate mannose, and (3) therefore, mannose produced from M6P by histone II-A-treated microsomes is presumably released directly into the extramicrosomal medium. Taken together, these three observations indicate that the topology of G6Pase would not be that proposed by the substrate-transport model.

In summary, histone II-A cannot be used as a permeabilizing agent of microsomes, since histones do not affect the property of these vesicles to accumulate products. Also, the measurement of M6Pase latency cannot be used to evaluate the microsomal membrane intactness, since M6Pase can be revealed to the same extent by detergent and histone II-A, but by the latter without permeabilization of the vesicles. It is likely that M6Pase activity in untreated microsomes is the reflection of a small amount of G6Pase in a conformation capable of M6P hydrolysis.

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