THE REQUIREMENT FOR MEMBRANE INTEGRITY IN THE INHIBITION OF HEPATIC GLUCOSE 6-PHOSPHATASE BY SULFHYDRYL REAGENTS AND TAUROCHOLATE

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SUMMARY: Exposures of intact rat liver microsomes to various sulfhydryl reagents or to concentrations of taurocholate below 1 mM inhibited glucose 6-phosphate phosphohydrolase activity. The inhibitions were abolished by subsequent disruption of membrane integrity by higher concentrations of taurocholate or 1M NH_4OH. Also the reagents were ineffective when tested directly with disrupted microsomes. Thus, a requirement for an intact membrane structure in the action of these reagents is indicated. Moreover, the involvement of a second component of the endoplasmic reticulum in the catalysis of glucose 6-phosphate hydrolysis is suggested.

Recent studies by Arion et. al. (1) have shown that glucose 6-phosphatase (D-glucose-6-P phosphohydrolase; EC 3.1.3.9) of intact rat liver microsomes is specific for glucose-6-P. In marked contrast, after disruption of microsomes with detergents or high pH, the enzyme utilizes a broad spectrum of phosphate substrates. In an attempt to gain some understanding of the apparent relationship between membrane integrity and the substrate specificity we examined the effects of exposing intact microsomes to a variety of specific reagents. The present report describes the effects of sulfhydryl reagents and the bile salt, sodium taurocholate. Inhibition of glucose-6-P phosphohydrolase by these compounds was found to require an intact microsomal membrane.

MATERIALS AND METHODS: Sources of animals and procedures for preparation of rat liver microsomes were as described previously (1). "Intact microsomes" are microsomes that were diluted with 0.25 M sucrose containing 0.5 mM sodium EDTA and 5 mM Tris acetate, pH 7.4, and assayed without further treatment. The mannose-6-P phosphohydrolase activity of the intact preparations used in the present study was greater than 95% masked (cf. Reference 1). "Disrupted microsomes" were prepared by supplementing 9 vol. of intact microsomes (about

2 mg of protein per ml) with 1 vol. of either 4% sodium taurocholate or 1M NH_4OH. The taurocholate-treated preparations were kept at ice-temperature for 10 min and assayed in a medium containing 10 mg of bovine plasma albumin (cf. Reference 2). Microsomes treated with NH_4OH were heated at 30° for 20 min (3), cooled to ambient temperature and adjusted to pH 7.4 with 1N acetic acid. The ammonium acetate was removed by sedimenting the preparation at 165,000 X g for 30 min. The disrupted preparations were resuspended in the previously described solution of buffered sucrose.

Dithiothreitol and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Calbiochem. N-ethyl maleimide (NEM) was a product of Eastman. Sources of other chemicals and assay procedures for protein and phosphohydrolase activities were as described earlier (1,2). Specific details of other conditions are outlined in the legends to figures and tables. Initial enzymic activities are expressed as units of activity per mg of microsomal protein X 10, where a unit of activity is the amount of enzyme that catalyzed the hydrolysis of 1 micromole of phosphate substrate per min under the conditions specified.

RESULTS AND DISCUSSION: The stimulatory actions of a broad variety of detergents on the various activities catalyzed by glucose 6-phosphatase are well documented (1,2,4-8). Less emphasis has been placed on the activity-discriminating and concentration-dependent effects of these compounds. In studies with liver homogenates Ashmore and Nesbett (9) observed that at low concentrations bile salts inhibited glucose-6-P phosphohydrolase, while higher concentrations enhanced this activity. More recently Stetten and Burnett (10) reported similar biphasic actions of Triton X-100 and deoxycholate. However, in parallel studies these investigators also observed that PP_i hydrolysis and glucose-6-P synthesis from PP_i and D-glucose were activated at all concentrations of these detergents. Nordlie and coworkers (11) also have noted differential effects of long chain fatty acids and their Coenzyme A esters on the synthetase and hydrolase activities of the enzyme. The question of whether or not the inhibition by the detergents required an intact membrane was not determined,

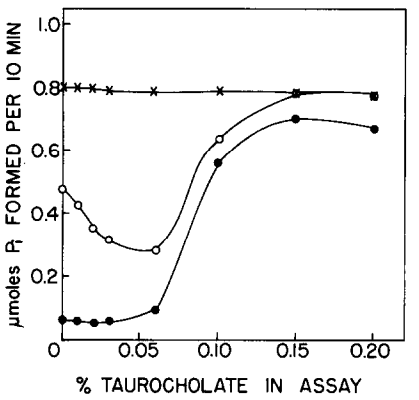

Fig. 1.

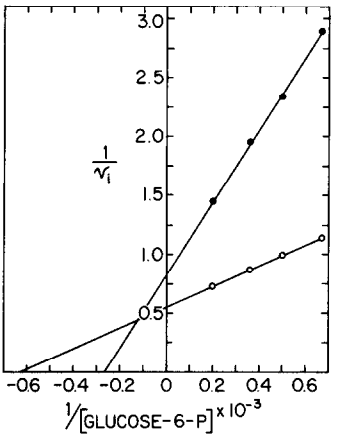

Fig. 2.

Fig. 1 Effects of taurocholate on glucose-6-P phosphohydrolase (O—O) and mannose-6-P phosphohydrolase (●—●) of intact microsomes and glucose-6-P phosphohydrolase of NH_4OH-disrupted microsomes (X—X). Assay media, pH 7.0, contained in 1 ml the indicated amounts of sodium taurocholate, 50 mM Tris-cacodylate buffer (see Ref. 2), 10 mM phosphate substrate and 0.20 mg of microsomal protein.

Fig. 2 Kinetics of taurocholate inhibition of glucose-6-P phosphohydrolase of intact microsomes. Assay media, pH 7.0, contained in 1 ml 50 mM Tris-cacodylate buffer, glucose-6-P from 1.5 to 5 mM, 0.20 mg of microsomal protein and no (O—O) or 0.04% sodium taurocholate (●—●).

nor were the nature of the inhibitions defined kinetically.

In the present study the glucose-6-P phosphohydrolase activities of intact and NH_4OH-disrupted microsomes were compared with respect to the effects of various concentrations of sodium taurocholate. As shown in Fig. 1, glucose-6-P hydrolysis by disrupted microsomes was virtually unaltered throughout the range of taurocholate used. In contrast, glucose-6-P phosphohydrolase of intact microsomes was inhibited below 0.06% (about 1.1 mM) taurocholate and activated at the higher concentrations. Mannose-6-P phosphohydrolase also was monitored, since it provides an excellent index of the degree of intactness of the microsomal membrane (1). Mannose-6-P hydrolysis was essentially unaffected by the lower levels of detergent and markedly stimulated at the higher range. A kinetic description of the inhibition of glucose-6-P phosphohydrolase by

0.04% taurocholate is presented in Fig. 2. The inhibition may be classified as "mixed", since the bile salt depressed the maximal rate and also increased the apparent Michaelis constant for glucose-6-P. Previous studies (2) have shown that taurocholate has little influence on the kinetics of the enzyme of NH_4OH disrupted microsomes.

Preliminary studies revealed that the glucose-6-P phosphohydrolase activity of intact microsomes was sensitive to various sulfhydryl reagents. The nature of the inhibition varied with the particular thiol agent. Representative data for three types of sulfhydryl reagents are presented in Table I. Exposures of intact microsomes to NEM, DTNB or Hg^{+2} increased the apparent Michaelis constant for glucose-6-P, while only the latter two compounds depressed the maximal rate of the reaction. In other experiments intact microsomes were treated with mersalyl, p-mercuribenzoate, or p-mercuriphenyl sulfonate at concentrations between 10^{-5} and 10^{-4}M. The data obtained were virtually identical to that listed in Table I for Hg^{+2}. With the expected exception of NEM, the inhibitory effects of all sulfhydryl reagents were reversed by subsequent exposures of treated microsomes to 1 mM dithiothreitol. Supplementation of poisoned microsomes to 0.4% taurocholate abolished the inhibitory effects of the thiol agents (Table I). In fact, the kinetic constants were identical for disrupted microsomes whether they were prepared before or after the treatment with the sulfhydryl reagents. Supplementary studies revealed that the sulfhydryl reagents had no effects on the enzyme of microsomes previously disrupted with NH_4OH or taurocholate, provided the preparations were treated exactly as described in Table I. In this regard we must emphasize that the treatment with 0.2 mM Hg^{+2} was carried out in the presence of 0.5 mM sodium EDTA. If EDTA was omitted, Hg^{+2} inhibited the enzyme of both intact and disrupted preparations. However, in this case it is interesting that only the activity of the intact structures was restored by treatment with dithiothreitol.

We recently concluded (1) that other than glucose-6-P hydrolysis all activities catalyzed by glucose 6-phosphatase of untreated microsomes (herein

TABLE I. EFFECTS OF SULFHYDRYL REAGENTS ON GLUCOSE 6-PHOSPHATE PHOSPHOHYDROLASE BEFORE AND AFTER DISRUPTION OF MICROSOMES

Reagent Used	Maximal Velocity		Apparent Michaelis Constant	
	Intact Microsomes	Disrupted Microsomes	Intact Microsomes	Disrupted Microsomes
	units per mg protein X 10		mM	
None	1.7	2.4	2.0	0.7
NEM	1.7	2.4	11.0	0.7
DTNB	0.6	2.3	6.7	0.7
Hg^{+2}	0.6	2.4	6.2	0.7

Microsomes were incubated at 0° in a medium, pH 7.5, containing 0.25 M sucrose, 20 mM Tris-HCl, 0.5 mM sodium EDTA and the indicated sulfhydryl reagent. Sulfhydryl reagents and microsomal protein were present at the following concentrations: 0.1 mM NEM and 0.3 mg of protein per ml; 0.05 mM DTNB and 0.3 mg of protein per ml; and 0.2 mM $Hg(NO_3)_2$ and 6 mg of protein per ml. After 20 min, the mixtures were centrifuged at 165,000 X g for 30 min. The pellet was suspended to the original volume of buffered sucrose (see text). The centrifugation was repeated and the microsomes were resuspended in the buffered sucrose. A portion of each preparation was supplemented to 0.4% taurocholate. Assay mixtures, pH 6.5, contained 50 mM Tris-cacodylate, four concentrations of glucose-6-P between 1.5 and 5 mM, 10 mg of bovine plasma albumin, variable NaCl to maintain the ionic strength equal to 0.065 and approximately 0.2 mg of microsomal protein in a final volume of 1.0 ml. Assays were carried out at 30° for 10 min. Kinetic constants were evaluated from Lineweaver-Burk plots (13).

referred to as "intact microsomes") were due to disrupted membranes in the untreated preparations. Additional support for this contention came from the observation during the present study that under the specified conditions the sulfhydryl reagents were without effects on the mannose-6-P and PP_i phosphohydrolase activities of "intact microsomes".

It is clear from the results of this study that the efficacy of inhibition of glucose-6-P phosphohydrolase by low concentrations of detergents or the various sulfhydryl reagents depended on the existence of an intact microsomal

membrane. The nature of the membrane component(s) responsible for this phenomenon remains to be elucidated. It is tempting to speculate that a single component of the membrane is involved in conferring substrate specificity and sensitivity to sulfhydryl poisons. The clear differentiation of the effects of the inhibitors on intact as contrasted with disrupted structures suggests that in addition to the catalytic component a second element of the endoplasmic reticulum is involved in the hydrolysis of glucose-6-P. The present data taken with other observations (12) in our laboratory raises the interesting possibility that the second component may be a glucose-6-P specific permease which translocates the sugar phosphate from its point of biosynthesis in the cytosol to the lumen of the endoplasmic reticulum where the phosphohydrolase is located.

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