Properties of membrane-bound bilirubin UDP-glucuronyltransferase in rough and smooth endoplasmic reticulum and in the nuclear envelope from rat liver

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We examined regulatory properties of bilirubin UDP-glucuronyltransferase in sealed RER (rough endoplasmic reticulum)- and SER (smooth endoplasmic reticulum)-enriched microsomes (microsomal fractions), as well as in nuclear envelope from rat liver. Purity of membrane fractions was verified by electron microscopy and marker studies. Intactness of RER and SER vesicles was ascertained by a high degree of latency of the luminal marker mannose-6-phosphatase. No major differences in the stimulation of UDP-glucuronyltransferase by detergent or by the presumed physiological activator, UDPGlcNAc, were observed between total microsomes and RER- or SER-enriched microsomes. Isolated nuclear envelopes were present as a partially disrupted membrane system, with approx. 50 % loss of mannose-6-phosphatase latency. The nuclear transferase had lost its latency to a similar extent, and the enzyme failed to respond to UDPGlcNAc. Our results underscore the necessity to include data on the integrity of the membrane permeability barrier when reporting regulatory properties of UDP-glucuronyltransferase in different membrane preparations.

INTRODUCTION

Microsomal bilirubin UDP-glucuronyltransferase (EC 2.4.1.17) activity is markedly latent in native microsomes (microsomal fractions), but can be fully revealed by treatments resulting in disruption of the structural integrity of the vesicles (Vanstapel & Blanckaert, 1988a). Alternatively, transferase activity can be stimulated by adding UDPGlcNAc to native, sealed, microsomal vesicles. The mechanism responsible for the latency phenomenon has been a subject of speculation and debate. Thus it has been proposed that latency results from confinement by the microsomal membrane of the catalytic site of the enzyme within the lumen of the vesicles (Hallinan & de Brito, 1981). An alternative explanation is that the enzyme may well be directly exposed to its substrates at the cytosolic side of the vesicle, but that the latency reflects presence of a specific enzyme conformer, whose enzymic activity is constrained by a microenvironment that is specific to native microsomes (Zakim & Vessey, 1976). A key issue in the debate appears to be the actual sidedness of the enzyme's catalytic centre with respect to the transverse plane of the ER. Evidence has been obtained for carrier-mediated translocation of UDPGlc and possibly UDPGlcUA into the lumen of microsomal vesicles (Perez & Hirschberg, 1986; Nuwayhid *et al.*, 1986; Vanstapel & Blanckaert, 1988b), where the transferase has direct access to the co-substrate (Vanstapel & Blanckaert, 1987). Consistent with a luminal orientation of the catalytic centre of the enzyme, we found that the latency of bilirubin UDP-

glucuronyltransferase activity is intimately related to preservation of the microsomal-membrane permeability barrier (Vanstapel & Blanckaert, 1988a), which is conveniently assessed by measurement of the latency of mannose-6-phosphatase (Arion *et al.*, 1976; Vanstapel *et al.*, 1986). Not only the extent of stimulation by detergent, but also the activation achieved by adding the presumed physiological activator UDPGlcNAc to native microsomes, depends critically on the degree of intactness of the native vesicles before treatment. Similar observations have been made for microsomal glucuronyltransferase activity with 1-naphthol as the acceptor substrate (Scragg *et al.*, 1985).

Hauser *et al.* (1984) reported on the effects of detergent treatment and of addition of UDPGlcNAc on the activity of bilirubin UDP-glucuronyltransferase in microsomes derived from either the RER or SER. They found that the enzyme is markedly less latent in RER- than in SER-enriched microsomes from rat liver, and they speculated that the regulation of hepatic bilirubin conjugation *in vivo* may be different for RER and SER. They also noticed that the transferase properties observed in the RER resembled the characteristics of the nuclear envelope. The latter two structures are contiguous in the intact cell.

Since membrane integrity was not verified in those studies, we considered the possibility that the reported difference in regulation of transferase activity in the microsomal subfractions reflected different degrees of integrity of the membrane permeability barrier in the various preparations. To test the plausibility of such an

Abbreviations used: Chapso, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate; ER, endoplasmic reticulum; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum.

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alternative explanation, we investigated whether the reported differences in the latency properties of the transferase in RER- and SER-enriched microsomes were also displayed by mannose-6-phosphatase.

EXPERIMENTAL

Materials

Sources of the chemicals have been indicated previously (Vermeir *et al.*, 1984; Vanstapel *et al.*, 1986; Vanstapel & Blanckaert, 1988b).

Preparation and treatment of membrane fractions

Microsomes were prepared from liver of adult male inbred R/A Pfd rats (Leyten *et al.*, 1986), weighing 200–300 g. Non-subfractionated ('total') microsomes were prepared as described previously (Vanstapel *et al.*, 1986). RER- and SER-enriched microsomes were obtained by the procedure of Eriksson *et al.* (1978), as described in detail previously (Vanstapel & Blanckaert, 1988b). Microsomes (approx. 40 mg of protein/ml) were stored at -70°C for subsequent biochemical analysis. Nuclei were obtained as described by Blobel & Potter (1966), and used without freezing.

Detergent treatment of microsomes consisted of pretreatment of the vesicles (2 mg of protein/ml) with 4 mM-Chapso at 0°C for 1 h, as described elsewhere (Vanstapel *et al.*, 1986; Vanstapel & Blanckaert, 1988a). Maximal activation of mannose-6-phosphatase and bilirubin UDP-glucuronyltransferase in isolated nuclei required exposure of the preparation (10 mg of protein/ml) to 3 mM-Chapso for 1 h at 0°C . Partial membrane disruption was obtained by treating the preparations with lower detergent concentrations.

Biochemical analysis of subcellular fractions

Protein was determined by a modified Lowry assay (Markwell *et al.*, 1978), calibrated with bovine serum albumin. RNA was assayed as orcinol-reactive material (Schneider, 1957). UDP-galactosyltransferase (EC 2.4.1.38) was determined by the method of Verdon & Berger (1983), with ovomucoid (17.5 mg/ml) as the acceptor (Bretz & Stäubli, 1977). Galactosyltransferase activity was optimally revealed by pretreating the

microsomes (2 mg of protein/ml) with 12 mM-Chapso for 1 h at 0°C . Latency of mannose-6-phosphatase in the membrane preparations was measured at 20°C , as described by Vanstapel *et al.* (1986). Microsomal bilirubin UDP-glucuronyltransferase was assayed under incubation conditions described previously (Vermeir *et al.*, 1984). Formation of esterified bilirubins was routinely monitored by the standard ethyl anthranilate diazo method (Heirwegh *et al.*, 1972), or activities in nuclei were measured by the radioassay (Vermeir *et al.*, 1984).

Statistics

Results are given as means $\pm$ S.D., with the numbers of observations in parentheses. Significance of differences in properties of total microsomes and RER and SER subfractions were tested by the Wilcoxon matched-pairs signed-ranks test (Siegel, 1956). *P* values were compared with the indicated one-tail significance levels.

RESULTS AND DISCUSSION

Characterization of ER subfractions

The identity of the microsomal subfractions was verified by electron microscopy on Karnovsky-fixed and osmium-impregnated pelleted vesicles. The RER fraction contained more abundant orcinol-positive material ($238 \pm 2 \mu\text{g}/\text{mg}$ of protein; $n = 2$) than the SER fraction ($49 \pm 10 \mu\text{g}/\text{mg}$; $n = 2$). Routine washing of the RER fraction with iso-osmotic sucrose containing 1 mM-EDTA stripped off about 65% of the associated orcinol-reactive RNA. The smooth microsomes had a higher activity of the Golgi marker ovomucoid UDP-galactosyltransferase ($159 \pm 82 \text{ pmol}/\text{min}$ per mg of protein; $n = 10$) than the rough vesicles ($32 \pm 11 \text{ pmol}/\text{min}$ per mg; $n = 16$). The specific activity of this marker in the RER-enriched microsomes was about 60% (range 42–67%) of that displayed by the corresponding total liver homogenates. These relative activities are comparable with previously reported values (Andersson *et al.*, 1978; Bergeron *et al.*, 1982; Hauser *et al.*, 1984).

Total microsomes and RER- and SER-enriched microsomes were mostly sealed, as evidenced by a mannose-6-phosphatase latency exceeding 95% in the native vesicles (see Table 1).

Table 1. Bilirubin UDP-glucuronyltransferase activities in subcellular fractions

The mannose-6-phosphatase activity in native preparations is expressed as a fraction of the activity in optimally detergent-activated preparations. 'Total' enzyme activity was assayed in membrane preparations fully disrupted by pretreatment with detergent. Results are presented as means $\pm$ S.D., with the numbers of observations in parentheses.

Membrane fraction	Mannose-6-phosphatase non-latency (%)	Bilirubin UDP-glucuronyltransferase (pmol/min per mg of protein)		
		Basal	+ UDPGlcNAc (2 mM)	Total
Microsomes				
Total	2.20 ± 0.92 (6)	177 ± 37 (6)	718 ± 122 (6)	1914 ± 442 (6)
RER	3.35 ± 0.64 (14)	283 ± 54 (14)	1109 ± 226 (14)	2467 ± 385 (14)
SER	3.08 ± 0.83 (14)	216 ± 31 (14)*	651 ± 141 (14)*	2151 ± 385 (14)*
Nuclei	57.1 ± 13.7 (3)	196 ± 39 (2)	202 ± 40 (2)	333 ± 127 (2)

* Results significantly different between RER and SER membrane preparations (Wilcoxon matched-pairs signed-ranks test: $P < 0.005$).

Expression of bilirubin UDP-glucuronyltransferase activity in total microsomes and RER- and SER-enriched microsomes

Detergent-activation profiles (Fig. 1a) were investigated in total and subfractionated microsomes (a) to determine the optimal conditions of detergent treatment for measurement of total bilirubin UDP-glucuronyltransferase activity in RER- and SER-enriched microsomes, and (b) to examine whether transferase latency in each membrane subfraction was a direct function of membrane integrity as reflected by mannose-6-phosphatase latency. Chapso-activation profiles were superimposable for total microsomes and RER- and SER-enriched microsomes. The recruitment of transferase activity correlated directly with the loss of mannose-6-phosphatase latency over the whole range of Chapso-induced membrane disruption (Fig. 1b), illustrating that the degree of intactness of the vesicles is a major determinant of the expression of transferase activity in rough as well as in smooth microsomes.

The basal bilirubin UDP-glucuronyltransferase activity in sealed microsomes was about 10% of that in fully disrupted vesicles (Fig. 1a and Table 1), i.e. the total transferase activity expressed by Chapso-disrupted microsomes was 10-fold higher than in the native, sealed, preparations. Consequently, even minor damage to the membrane permeability barrier of native microsomes will result in marked enhancement of enzymic activity relative to the basal activity expressed by well-sealed native vesicles. Therefore, preservation and assessment of vesicle intactness is critical in the study of the properties of microsomal UDP-glucuronyltransferase.

After verifying that the membrane permeability barrier

was equally intact in all native ER membrane preparations, we compared the properties of UDP-glucuronyltransferase in microsomes derived from various regions of the ER (Table 1). Total bilirubin UDP-glucuronyltransferase activity in fully disrupted microsomal subfractions was systematically higher in RER- than in SER-enriched microsomes. Even so, activities in both fractions were of the same order of magnitude, indicating that the RER is not markedly enriched in this enzyme over the SER. Basal enzyme activities in native microsomes, expressed as a percentage of the total activity, were very similar in smooth (mean 10.2%; range 6.8–12.5%) and in rough (mean 11.7%; range 6.9–14.2%) microsomes. Addition of 2 mM-UDPGlcNAc to the native membrane vesicles resulted in an approx. 3.7-fold stimulation of transferase activity in total microsomes, to become 37.5% (range 27–40%) of the total activity. In RER- and SER-derived microsomes, the UDPGlcNAc-treated enzyme displayed respectively 43.8% (range 32.7–54.1%) and 30.0% (range 22.9–40.8%) of the total activity in the corresponding microsomes fully disrupted with Chapso. The enzyme activities expressed by smooth or rough microsomes in the presence of UDPGlcNAc were significantly different, in line with the differences for basal and total transferase activities. The mean stimulation of basal activity in native untreated microsomes by UDPGlcNAc was respectively 3.6- and 3.0-fold for rough and smooth microsomes ($P < 0.005$). This difference does not correlate with a difference in structural intactness of the microsomal vesicles, since both subfractions displayed comparable mannose-6-phosphatase latencies.

Collectively, our findings demonstrate that bilirubin UDP-glucuronyltransferase in SER and RER sub-

Fig. 1. Chapso-activation profiles for microsomal mannose-6-phosphatase and bilirubin UDP-glucuronyltransferase

Microsomes (2 mg of protein/ml) were preincubated for 60 min at 0 °C with the Chapso concentrations indicated on the abscissa of panel (a), after which mannose-6-phosphatase and bilirubin UDP-glucuronyltransferase were assayed. Enzyme activities in Chapso-pre-treated microsomes are normalized by reference to the maximally expressible ('total') activity. Results are shown for (●) total, (○) RER- and (▲) SER-enriched microsomes. Panel (a) shows the gradual recruitment of UDP-glucuronyltransferase activity by pre-treatment of the vesicles with increasing Chapso concentrations. Panel (b) summarizes the effect of gradual disruption of the microsomes by detergent treatment. The activity of mannose-6-phosphatase (abscissa) can be read as a quantitative index of the degree of membrane disruption, and correlates directly with bilirubin UDP-glucuronyltransferase activity.

fractions, in which the membrane permeability barrier remains intact, can be markedly stimulated both by detergent and by UDPGlcNAc. Hence, except for relatively minor quantitative differences, both ER subfractions display comparable regulatory properties, and thus our results do not substantiate the thesis (Hauser *et al.*, 1984) that bilirubin UDP-glucuronyltransferase is regulated differentially in RER and SER. In retrospect, our findings are not unexpected, since it is unlikely that one specific subfraction (RER) would exert properties that would differ profoundly from those of the other fraction (SER) and from those of total unsubfractionated microsomes, as the ER comprises approximately equal proportions of RER and SER (Dallner & DePierre, 1982).

The previously reported (Hauser *et al.*, 1984) decreased stimulation of transferase activity in RER vesicles by detergent may reflect a partially breached permeability barrier. According to the present results and those reported previously (Vanstapel & Blanckaert, 1988a), such breached microsomes will be characterized by a normal 'total' activity but will express relatively elevated 'basal' enzymic activities and a decreased UDPGlcNAc effect. Or, stated otherwise, the stimulatory effect of detergent is decreased.

Hauser *et al.* (1984) assayed the transferase at low bilirubin concentrations (15 μM -bilirubin/7.5 μM -albumin), by using a sensitive radioassay (Vermeir *et al.*, 1984). In the present study we relied on the more convenient ethyl anthranilate diazo method (Heirwegh *et al.*, 1972), in which routinely high bilirubin (100 μM)/albumin (50 μM) substrate concentrations are used. Differences in the bilirubin substrate concentrations appear not to contribute to the discrepancies between our findings and those of Hauser *et al.* (1984). Decreasing the substrate concentration from 100 μM -bilirubin/50 μM -albumin to 6.25 μM -bilirubin/3.13 μM -albumin decreased enzyme activities (in RER membrane fractions) slightly (24 %; mean of two experiments), though without inducing noticeable alterations of the relative proportions between catalytic rates measured in native, UDPGlcNAc-treated or Chapso-disrupted membrane preparations.

The envelope of isolated nuclei as a model of an unperturbed partially permeable membrane system

We verified optimal conditions for the assay in the nuclear envelope of the latency of mannose-6-phosphatase and of bilirubin UDP-glucuronyltransferase (Fig. 2). The latency, expressed as a percentage of maximal 'total' enzyme activity, was 41–65 % ($n = 3$) for mannose-6-phosphatase and 51–67 % ($n = 2$) for bilirubin UDP-glucuronyltransferase in native nuclear preparations (Table 1). Both enzymes responded in parallel to the detergent treatment in terms of the activation that could be achieved (Fig. 2). Thus decreased latency of UDP-glucuronyltransferases appeared to correlate with loss of integrity of the membrane permeability barrier.

Our results reaffirm previous reports on a low degree of latency for UDP-glucuronyltransferases (Wishart & Frye, 1980; Zaleski *et al.*, 1982; Hauser *et al.*, 1984) and for the enzymic activities of the glucose-6-phosphatase system (Gunderson & Nordlie, 1973, 1975; Nordlie *et al.*, 1979; Arion *et al.*, 1983) in isolated nuclei. Arion *et al.* (1983) pointed out that the loss of latency of the glucose-6-phosphatase system in isolated nuclei reflected

Fig. 2. Chapso-activation profile for mannose-6-phosphatase and bilirubin UDP-glucuronyltransferase in isolated nuclei

Isolated nuclei (10 mg of protein/ml) were preincubated for 60 min at 0 °C with the Chapso concentrations indicated on the abscissa, after which mannose-6-phosphatase (○) and bilirubin UDP-glucuronyltransferase (●) were assayed. Because of the relatively low specific transferase activity (see Table 1), a radioassay (Vermeir *et al.*, 1984) was used for measurement of transferase activities (15 μM -bilirubin/7.5 μM -bovine serum albumin). Enzyme activities in Chapso-pretreated preparations are normalized by reference to the maximally expressible activity.

the partial disruption of the nuclear envelope during isolation. The loss of transferase latency in the nuclear envelope might be explained in an identical way.

UDPGlcNAc had no effect (range 1.0–1.1-fold activation; $n = 2$) on nuclear transferase activity (Table 1). Zakim & Vessey (1976) suggested that this possible physiological modulator is a direct allosteric activator of the transferase, and that its effectiveness may depend on a particular conformational status, which may be altered by membrane-perturbing treatments. However, Burchell *et al.* (1983) demonstrated that UDPGlcNAc exerts its effect not directly on the transferase but on a second membrane-bound component, which does not co-purify with the transferase. Hence, absence of the UDPGlcNAc effect in native nuclear membranes does not necessarily reflect conformational perturbation of the transferase. Also, it so happens that conditions which are reputed to abolish the effectiveness of UDPGlcNAc are characterized by a loss of the integrity of the membrane permeability barrier (Hallinan & de Brito, 1981; Vanstapel & Blanckaert, 1988a). The loss of the UDPGlcNAc effect in isolated nuclei may be a mere

reflection of the loss of integrity of the nuclear envelope. Thus the isolated nucleus might be a model for a minimally perturbed membrane system in which, however, membrane permeabilization eliminated the mechanism on which the nucleotide exerts its effect.

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