Biochimica et Biophysica Acta, 735 (1983) 309–313
Elsevier

BBA 71887

EVIDENCE INDICATING THAT UDP-*N*-ACETYLGLUCOSAMINE DOES NOT APPEAR TO STIMULATE HEPATIC MICROSOMAL UDP-GLUCURONOSYLTRANSFERASE BY INTERACTION WITH THE CATALYTIC UNIT OF THE ENZYME

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(Received April 5th, 1983)

Key words: UPDglucuronosyltransferase; Catalytic subunit; UDP-N-acetylglucosamine stimulation; Enzyme regulation; Membrane enzyme; (Rat liver)

The binding studies in this paper indicate that the catalytic unit(s) of microsomal UDP glucuronosyltransferase(s) is not accessible to *N*-ethylmaleimide or UDP-*N*-acetylglucosamine, when the enzyme is in its membrane environment. Thus a separate regulatory factor may exist within the endoplasmic reticulum membrane that mediates the stimulation of UDPglucuronosyltransferase(s) by UDP-*N*-acetylglucosamine. The possible role and the mode of interaction of the putative regulatory factor with the multiple forms of UDPglucuronosyltransferase are discussed.

Introduction

Mammalian hepatic microsomal UDPglucuronosyltransferase (EC 2.4.1.17) catalyses the glucuronidation of endogenous and xenobiotic compounds utilising UDP glucuronic acid as substrate [1]. The glucuronidation of these hydrophobic molecules increases their hydrophilicity thus facilitating their transport in the tissues and excretion from the body.

The activity of UDP-glucuronosyltransferase in intact hepatic microsomes is not fully expressed and can be increased up to 20-fold by detergent disruption of the microsomal vesicles and is therefore considered to be a latent enzyme activity [1].

The latent rates of UDP-glucuronosyltransferase activity towards xenobiotic substrates can be stimulated up to 5-fold by the addition of UDP-*N*-acetylglucosamine (UDP-GlcNAc) to rat or mouse microsomal fractions [2,3]. Recent work has shown that the glucuronidation of bilirubin and steroids by guinea-pig and rat liver microsomal fractions was also stimulated by UDP-GlcNAc [4,5]. Stimulation of the transferase activity was only observed in untreated, intact microsomal fractions. If the integrity of the microsomal membrane was destroyed by treatment with detergent, then the UDPglucuronosyltransferase activity was rendered insensitive to UDP-GlcNAc [3,6].

UDP-GlcNAc has been classified as a physiologicial regulator of UDPglucuronosyltransferase [6–8], since the presence of 'physiological' concentration of UDP-GlcNAc is required during the assay of microsomal UDPglucuronosyltransferase activity to raise the enzyme reaction rate up to 60% of the level observed in the isolated hepatocyte [8].

How does the in vitro addition of UDP-GlcNAc to isolated microsomes restore the glucuronidating capacity up to that of the intact cell? Exposure of microsomal fractions to the thiol-blocking agent, *N*-ethylmaleimide or to diazobenzene sulphonate [9] does not inhibit latent rates of UDPglucuronosyltransferase activity but completely abolishes the

stimulation of transferase activity by UDP-GlcNAc [6]. A possible explanation for all these observations is the presence of a facilitated transport mechanism for carriage of cytosolic UDP-glucuronic acid to the site of glucuronide synthesis by UDPglucuronosyltransferase at the luminal side of the endoplasmic reticulum [10,11]. In contrast to similar transport models for glucose-6-phosphatase [12], the 'translocase' for UDPglucuronic acid transport appears to operate slowly or not at all in the absence of UDP-GlcNAc [8].

UDPglucuronosyltransferase can be purified to apparent homogeneity from Wistar rat liver [13,14] and this has made it possible to further examine the regulation of transferase activity by UDP-GlcNAc. In this paper we have attempted to determine whether UDP-GlcNAc acts directly on UDPglucuronosyltransferase or on another component of the microsomal membrane. The evidence presented suggests that a separate regulator mediates the stimulation of UDPglucuronosyltransferase by UDP-GlcNAc.

A preliminary note of some of this work has been previously reported [15].

Methods

N-Ethyl[2,3-^{14}C]maleimide (spec. act. 8.2 mCi/m mol) was purchased from Amersham International, Amersham, Bucks, U.K. Unlabelled *N*-ethylmaleimide, dithiothreitol, UDPglucuronic acid (triammonium salt), UDP-*N*-acetylglucosamine and bovine serum albumin were all from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey. Coomassie Blue G-250 was from Serva Fine Biochemicals, London, U.K.

UDPglucuronosyltransferase activity using 4-nitrophenol as substrate was assayed as described in Ref. 3 and protein concentrations were determined by the method of Bradford [16] using bovine serum albumin as standard.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was performed as previously described in Ref. 13.

Rat liver microsomal fractions were labelled with *N*-ethyl[2,3-^{14}C]maleimide by resuspending the microsomal pellets obtained from five rat livers [17], by gentle homogenization in 0.25 M sucrose to obtain a syrupy suspension containing 49 mg protein/ml. A final concentration of 2.5 mM *N*-ethyl[2,3-^{14}C]maleimide was added which was sufficient to ensure complete blockade of the stimulation of transferase activity by UDP-GlcNAc (see Results). After immediate and thorough mixing the suspension was incubated for 15 min at 4°C after which the alkylation reaction was stopped by the addition of 25 mM dithithreitol.

UDPglucuronosyltransferase was purified from the labelled microsomal fraction by the methods adequately described elsewhere [13,14,17]. The amount of *N*-ethyl[2,3-^{14}C]maleimide bound to protein in the purification fractions was determined directly by scintillation counting. Aliquots (50 μl) of each purification fraction were added to 6 ml of Aquasol (New England Nuclear, FRG) scintillation fluid and counted at greater than 80% efficiency.

The amount of radioactivity in polyacrylamide gel slices (1 mm) was achieved by immersing the discs in 0.5 ml NCS solubilizer (Searle, Amersham, U.K.) for 2 h at 60°C to solubilize the proteins. Glacial acetic acid (17 μl) followed by 9.5 ml of scintillant (6 g 2,5-diphenyloxazole and 75 mg *p*-bis(2-(5-phenyloxazolyl)benzene per litre of toluene) were added prior to scintillation counting at greater than 70% efficiency.

Results

A previous report [7] has shown that prior exposure of microsomal fractions to *N*-ethylmaleimide prevented the stimulation of UDPglucuronosyltransferase activity by UDP-GlcNAc, although latent rates of transferase activity were not appreciable impaired. The results shown in Table I confirm these observations. The concentration of *N*-ethyl[2,3-^{14}C]maleimide (2.5 mM) which effectively blocks stimulation by UDP-GlcNAc was used in these experiments to label microsomal fractions. This microsomal suspension treated with 2.5 mM radioactive *N*-ethylmaleimide was used for the purification of UDPglucuronosyltransferase. After neutralization of excess *N*-ethylmaleimide with dithiothreitol, UDPglucuronosyltransferase was solubilized and purified to apparent homogeneity (see Methods). The results obtained during the purification of the transferase are shown in Table II. In the experiment reported

TABLE I

BLOCKADE OF UDP-*N*-ACETYLGLUCOSAMINE STIMULATION OF MICROSOMAL UDP-GLUCURONOSYLTRANSFERASE ACTIVITY BY *N*-ETHYLMALEIMIDE

Microsomal fractions were obtained from five Wistar rat livers and resuspended by gentle homogenization in 0.25 M sucrose to give a protein concentration of 50 mg/ml. *N*-Ethyl[2,3-^{14}C]maleimide was added in a final concentration of 2.5 mM. After incubation for 15 min at 4°C, the alkylation reaction was stopped by the addition of dithiothreitol to a final concentration of 2.5 mM. Samples were assayed before and after alkylation in the presence and absence of 2 mM UDP-*N*-acetylglucoasamine. Specific activity units were nmol/min per mg protein.

Treatment	4-Nitrophenol UDPglucuronosyl transferase specific activity
None	0.33
2 mM UDP-*N*-acetylglucosamine	1.32
2.5 mM *N*-ethyl[2,3-^{14}C]maleimide	0.33
2 mM UDP-*N*-acetylglucosamine + 2.5 mM *N*-ethyl[2,3-^{14}C]maleimide	0.32

here 40 μg of the purified enzyme protein contained 0.11 nmol bound *N*-ethyl-maleimide per nmol purified UDPglucuronosyltransferase. An average result of 0.16 nmol bound *N*-ethylmaleimide per nmol purified UDPglucuronosyltransferase was obtained over a series of three experiments (0.11–0.28 nmol bound *N*-ethylmaleimide per nmol purified transferase). Thus much less than 1 mol *N*-ethylmaleimide was bound per mol of purified UDPglucunosyltransferase catalytic subunit. In order to demonstrate that *N*-ethylmaleimide was able to find to other membrane proteins more selectively than UDP-glucuronosyltransferase we have determined the radioactivity of individual proteins present in a highly purified preparation of UDPglucuronosyltransferase.

Fig. 1 shows the SDS-polyacrylamide gel electrophoretic analysis of partially purified UDPglucuronosyltransferase (DEAE-cellulose fraction applied to UDPhexanolamine Sepharose). The majority of the radioactivity (bound *N*-ethylmaleimide) loaded onto the polyacrylamide gels was associated with a polypeptide band of molecular weight 70 000 (peak A) whilst only a negligible amount of radioactivity was associated with the polypeptide staining band of molecular weight 57 000 (peak B). This result supports the data from the analysis of the radioactive content of the final purified preparation in that only a small amount of the radioactivity was associated with UDP-glucuronosyltransferase purified to apparent homogeneity. However, this data does not indicate that the 70 kDa polypeptide is the 'putative regulator'.

TABLE II

PURIFICATION OF UDP-GLUCURONOSYLTRANSFERASE FROM WISTAR RAT LIVER MICROSOMAL FRACTION LABELLED WITH RADIOACTIVE *N*-ETHYLMALEIMIDE

4-Nitrophenol UDPglucuronosyltransferase was purified to apparent homogeneity from *N*-ethyl[2,3-^{14}C]maleimide treated microsomes. Specific activity units are nmol 4-nitrophenol glucuronide formed per min per mg protein.

Purification step	Total protein (mg)	Specific activity (units/mg)	Total radioactivity (cpm)	Specific radioactivity (cpm/mg protein) ($\times 10^{-4}$)
Microsomal fraction	–	–	$4.49 \cdot 10^{10}$	–
Lubrol-soluble supernatant	855	26.1	$3.04 \cdot 10^{8}$	35.5
25–60%-satd. $(NH_4)_2SO_4$ precipitate	430	29.4	$8.29 \cdot 10^{7}$	19.3
DEAE-cellulose eluate	154	35.4	$2.62 \cdot 10^{7}$	17.0
CM-cellulose eluate	27.2	35.5	$4.84 \cdot 10^{6}$	17.8
DEAE-Sephadex eluate	11.2	80.7	$3.57 \cdot 10^{5}$	3.2
UDPglucuronic acid eluate from UDPhexanolamine Sepharose	0.040	1890	$7.95 \cdot 10^{2}$	2.0

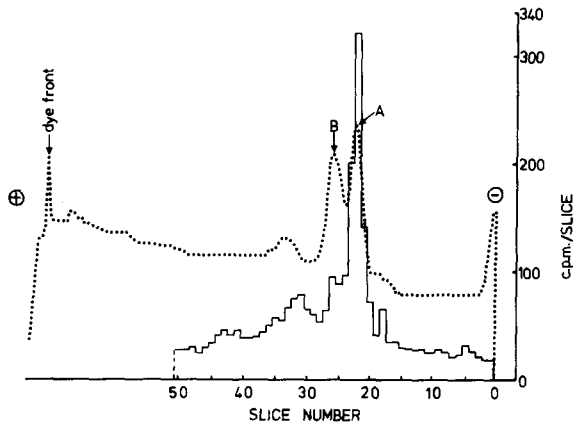

Fig. 1. SDS-polyacrylamide gel electrophoresis of partially purified UDPglucuronosyltransferase preparation from microsomes pretreated with radioactive *N*-ethylmaleimide. Disc electrophoresis was performed using 7.5% cylindrical polyacrylamide gels (7.5 × 0.6 cm) in the presence of 0.1% (w/v) SDS (see Ref. 12). Samples of UDPglucuronosyltransferase (highly purified by passage through DEAE-cellulose and UDPhexanolamine Sepharose 4B), 3 μg protein, were applied to six identical gels. After electrophoresis, staining and destaining, one gel was scanned in a densitometer to record the presence of polypeptides stained by Coomassie blue (·····). The other gels were cut identically into 1 mm slices and the radioactive content determined. The histogram line shows the results obtained from five gels. Peak A is a protein of molecular weight 70000 and peak B is UDPglucuronosyltransferase.

Discussion

We have confirmed that *N*-ethylmaleimide does not inhibit the basal rate of microsomal UDPglucuronosyltransferase activity, but this thiol-blocking reagent does prevent the stimulation of microsomal transferase acitivity by UDP-GlcNAc [6].

These results suggest that both UDP-GlcNAc and *N*-ethylmaleimide exert their effect by acting on the same protein component of the microsomal membrane. Detergent solubilisation of *N*-ethylmaleimide-treated endoplasmic reticulum proteins did not release the bound radioactive (*N*-ethylmaleimide. Thus, purificaton of the transferase from these solubilized membranes should reveal the presence of any bound *N*-ethylmaleimide.

The binding studies reported show that as little as 0.11 nmol *N*-ethylmaleimide was bound per nmol of purified transferase. This small level of labelling can be accounted for since amino acid analysis of purified UDPglucuronosyltransferase [18] shows the presence of an odd number, 11, of half cysteine residues. Thus UDPglucuronosyltransferase can be inferred to have at least one free thiol group available to bind *N*-ethylmaleimide, although detergent solubilized and highly purified UDPglucuronosyltransferase was not inhibited by *N*-ethylmaleimide (data not shown). These data suggest that UDP-GlcNAc does not interact with the UDPglucuronosyltransferase.

Two possible explanations of these results are: (1) The endoplasmic reticulum membrane contains a facilitated transport mechanism for the carriage of substrates from the cytoplasm to the site of glucuronide synthesis [11,19].

(2) UDPglucuronosyltransferase consists of more than one type of subunit, catalytic subunits and regulatory subunits. The regulatory subunit(s) may be situated at the cytoplasmic side of the endoplasmic reticulum as an allosteric regulator of UDPglucuronosyltransferase [7].

It is possible that, during purification of UDPglucuronosyltransferase, proteolytic cleavage has resulted in the removal of a part of the protein that binds *N*-ethylmaleimide and the putative regulatory site. However, a UDPglucuronosyltransferase exhibiting the same physicochemical characteristics can be rapidly purified using immobilised antibodies in the presence of protease inhibitors, benzamidine and *N*-tosyllysine chloromethyl ketone, indicating that loss of bound *N*-ethylmaleimide may not be due to proteolytic degradation of the enzyme (Burchell, B., unpublished work).

Several forms of UDPglucuronosyltransferase have been shown to exist and it has been postulated that the different forms may have evolved to specifically glucuronidate different endogenous substrates (see Ref. 18). The possibility of UDPglucuronosyltransferase being regulated by another factor in the endoplasmic reticulum membrane raises the questions regarding the interaction of this factor with the multiple forms of UDPglucuronosyltransferase.

It is evident that none of the purified forms of UDPglucuronosyltransferase so far isolated can be regarded as complete 'physiological' entities. The regulation of UDPglucuronosyltransferase by UDP-GlcNAc will only be elucidated by identification and isolation of the regulatory factor and reconstitution of UDP-GlcNAc-stimulatable

UDPglucuronosyltransferase activity using the purified components

Acknowledgements

We with to thank Mr. I. Duffy for his excellent technical assistance and the Medical Research Council for grants supporting this work. B.B. is a Wellcome Trust Senior Lecturer.

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