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THE IDENTIFICATION OF A UNIQUE *p*-NITROPHENOL CONJUGATING ENZYME IN GUINEA PIG LIVER MICROSOMES

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SUMMARY

Guinea pig liver microsomes catalyze the transfer of a galacturonic acid residue from UDPgalacturonic acid to *p*-nitrophenol. Lineweaver–Burk plots of the rate of the galacturonidation reaction as a function of the concentration of UDPgalacturonic acid, at a fixed concentration of *p*-nitrophenol, are linear for concentrations of UDPgalacturonic acid greater than 4 mM but non-linear below this concentration. This non-linearity is similar to that seen for the synthesis of *p*-nitrophenylglucuronide, and suggests that there is negative cooperativity in the binding of UDPgalacturonic acid to the enzyme. Also compatible with the notion of allosterism, UDP-*N*-acetylglucosamine increases the rate of synthesis of *p*-nitrophenylgalacturonic acid.

Treatment of microsomes with Triton X-100 or phospholipase A increases the rate of synthesis of *p*-nitrophenylgalacturonic acid but to a lesser extent than the conjugation of *p*-nitrophenol with UDPglucuronic acid. In addition, the enzyme catalyzing the synthesis of *p*-nitrophenylgalacturonic acid is activated by mersalyl, maximal activation occurring at 1 mM mersalyl; 5 mM mersalyl inactivates the enzyme completely. The properties of the *p*-nitrophenylgalacturonide and glucuronide synthesizing reactions are, therefore, similar in many respects. On the other hand, UDPgalacturonic acid is not a product inhibitor of the UDP-dependent hydrolysis of *p*-nitrophenylglucuronide, and *p*-nitrophenylglucuronide does not inhibit the reaction of *p*-nitrophenol with UDPgalacturonic acid. It was concluded that the synthesis of *p*-nitrophenylglucuronide and -galacturonide are catalyzed by separate enzymes.

INTRODUCTION

The excretion of a variety of endogenous and exogenous organic compounds is facilitated by their conversion to glucuronides. Recent data indicate that many

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of these organic compounds also can form adducts with sugar moieties other than glucuronic acid. For example, liver microsomes from rabbits can catalyze the transfer of glucose, galactose and *N*-acetylglucosamine from their respective UDPsugars to steroids[1,2] and can also form glucosides of phenolphthalein and various isoflavones[3]. Rat liver microsomes are reported to catalyze the synthesis of xylose and glucose derivatives of bilirubin also utilizing sugar nucleotides as substrates[4,5]. Additionally, a number of bilirubin sugar adducts, other than bilirubin glucuronide, are present in bile from humans and dogs[6–8].

In view of the obvious similarity between the various glycosyltransferase reactions, it is not unreasonable to expect that a single enzyme might be responsible for catalyzing the synthesis of a number of different glycosides of the same aglycone. Yet, it has become apparent recently that there are several aglycone specific forms of UDPglucuronyltransferase, [UDPglucuronate glucuronyltransferase (acceptor unspecific) EC 2.4.1.17][9], and that these different enzymes have great selectivity for aglycones. It is important, therefore, to consider the possibility that the catalysis of sugar transfer might also be selective with respect to the sugar nucleotide. Data presented in this paper demonstrate that microsomes from guinea pig liver synthesize *p*-nitrophenylgalacturonide using UDPgalacturonic acid and *p*-nitrophenol as substrates, but that this reaction is catalyzed by an enzyme separate from the one responsible for the synthesis of *p*-nitrophenylglucuronide.

METHODS

Guinea pig liver microsomes were isolated and stored as previously described[10]. Assays of *p*-nitrophenol conjugation contained 3–5 mg/ml of microsomal protein, 50 mM phosphate buffer, pH 7.5, 0.4 mM *p*-nitrophenol, 5 mM saccharic acid-1,4-lactone and the indicated concentrations of UDPgalacturonic acid. Aliquots were removed from the assays at multiple time points and analyzed for *p*-nitrophenol as described previously[10]. All activities represent initial rates of reaction and are expressed as nmoles of *p*-nitrophenol conjugated per min per mg of protein. The assay for the reverse of the glucuronidation reaction, *i.e.* the UDP-dependent hydrolysis of *p*-nitrophenylglucuronide is described elsewhere[11]. All assays were carried out at 37 °C.

Microsomes were treated with purified phospholipase A from *Naja naja* venom at a ratio of 1 mg of phospholipase A protein per 100 mg of microsomal protein as previously described[10]. EDTA at 5 mM was added to the assays to prevent further phospholipase A action. Modification of the microsomal –SH groups was accomplished by incubating the microsomes at 10 mg protein/ml in 10 mM Tris, pH 7.5, at 25 °C for 5 min in the presence of the indicated concentration of mersalyl.

To characterize the product of the reaction of *p*-nitrophenol and UDPgalacturonic acid, a reaction mixture lacking only saccharic acid-1,4-lactone was incubated at 37 °C until approx. 70% of the free *p*-nitrophenol was reacted. The sample was deproteinized with perchloric acid and the supernatant containing the *p*-nitrophenol conjugate was then spotted on Whatman No. 1 paper and the chromatogram developed in 1 M ammonium acetate (pH 3.6)–ethanol (1:2, v/v)[12]. The spot representing the *p*-nitrophenol conjugate was located with an ultraviolet lamp and eluted with water. The eluted conjugate was treated with 1 mg/ml bovine liver β-glucuronidase

(EC 3.2.1.31) in 50 mM sodium acetate buffer, pH 5, for 90 min at 37 °C. An aliquot was removed and assayed for free *p*-nitrophenol by the standard technique[10]. The remainder of the sample was deproteinized by boiling and the supernatant spotted on Whatman No. 1 paper. The chromatogram was developed in *n*-butanol–acetic acid–water (52:13:35, by vol.) and sprayed with $AgNO_3$[13]. The sample gave one spot with the same mobility as galacturonic acid and glucuronic acid but did not show the advanced lactone spot typical of glucuronic acid in this solvent.

RESULTS AND DISCUSSION

Various UDPsugars were added at 8 mM final concentration to assays containing 3 mg/ml guinea pig liver microsomal protein and 0.4 mM *p*-nitrophenol. No decrease in the concentration of free *p*-nitrophenol was detected in assays containing UDPxylose, UDPgalactose, UDPmannose, UDPglucose or UDP-*N*-acetylglucosamine, in the presence or absence of divalent metals. On the other hand, there was a time-dependent decrease in the concentration of free *p*-nitrophenol in the presence of UDPgalacturonic acid. A *p*-nitrophenol conjugate was isolated from this reaction mixture by paper chromatography. The conjugate could be cleaved by β-glucuronidase with the release of *p*-nitrophenol and a saccharide which is chromatographically identical to galacturonic acid. It can concluded therefore that microsomes from guinea pig liver can catalyze the synthesis of *p*-nitrophenyl-β-D-galacturonide. Preliminary studies indicate that guinea pig liver microsomes can also catalyze the synthesis of galacturonides of additional aglycones, *e.g.* *o*-aminobenzoate and bilirubin.

The rate of synthesis of *p*-nitrophenylgalacturonide as a function of the concentration of UDPgalacturonate is shown in double reciprocal form in Fig. 1. The data yield a linear plot at concentrations of UDPgalacturonate above 4 mM, but below 4 mM UDPgalacturonate the plot is non-linear. Non-linearity in double reciprocal plots, at low concentrations of nucleotide sugar, is seen also for the glucuronidation of *p*-nitrophenol with UDPglucuronic acid as the sugar donor and results from negative cooperativity in the binding of UDPglucuronic acid to the enzyme[12]. The data in Fig. 1 thus suggest that there are cooperative effects in the binding of UDPgalac-

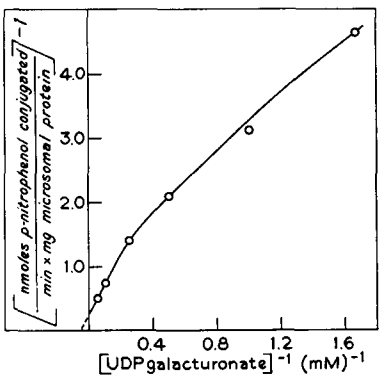

Fig. 1. The rate of synthesis of *p*-nitrophenylgalacturonide as a function of the concentration of UDPgalacturonic acid. Initial rates for the disappearance of *p*-nitrophenol were determined as in Methods in the presence of 0.4 mM *p*-nitrophenol and the indicated concentrations of UDPgalacturonic acid.

TABLE I

EFFECT OF ADDITIONS ON THE RATE OF SYNTHESIS OF *p*-NITROPHENYLGALACTURONIDE

Assays contained 1 mM UDP galacturonic acid and 0.4 mM *p*-nitrophenol. UDP-*N*-acetylglucosamine and Triton X-100 were added directly to the assays. The data for the phospholipase A treated microsomes represent maximal activation. All activities are initial rates of reaction as determined from multiple time point assays as in Methods.

Additions	*p-Nitrophenol conjugated/min per mg microsomal protein (nmole)*
None	0.25
+ 5 mM UDP-*N*-acetylglucosamine	0.46
+ 0.06% Triton X-100	0.40
+ 5 mM EDTA	0.22
Phospholipase A-treated microsomes + EDTA	0.28

turonic acid to the enzyme catalyzing the synthesis of *p*-nitrophenylgalacturonide. This idea is reinforced by the data in Table I which demonstrate that UDP-*N*-acetylglucosamine, an allosteric effector of the glucoronidation of nitrophenol[14], also stimulates the rate of synthesis of *p*-nitrophenylgalacturonide.

In addition to being stimulated by UDP-*N*-acetylglucosamine, the rate of synthesis of *p*-nitrophenylgalacturonide is increased by treatment of microsomes with Triton X-100 and phospholipase A (Table I). Treatment with phospholipase A or Triton had only a relatively small effect on the rate of synthesis of *p*-nitrophenylgalacturonide, however, as compared to their large rate enhancing effect on the rate of synthesis of *p*-nitrophenylglucuronide[15]. The *p*-nitrophenol metabolizing form of UDP-glucuronyltransferase contains distinct sets of –SH groups which are important for catalytic activity[16]. The rate of synthesis of *p*-nitrophenylgalacturonide was also sensitive to –SH group reagents in that concentrations of mersalyl below 1 mM increased the rate of synthesis of this compound. In contrast to the synthesis of *p*-nitrophenylglucuronide[17], however, 5 mM mersalyl inhibited completely the synthesis of *p*-nitrophenylgalacturonide (Fig. 2).

The differences cited above in the properties of the *p*-nitrophenylglucuronide and galacturonide synthesizing reactions suggested that these two compounds are

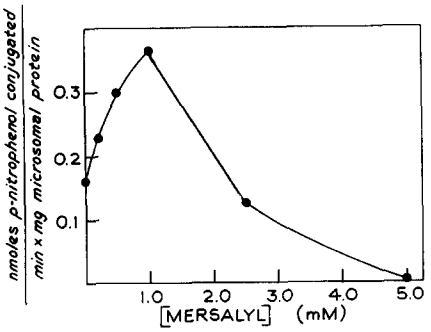

Fig. 2. The effect of treatment with mersalyl on the rate of synthesis of *p*-nitrophenylgalacturonide. Microsomes at a final concentration of 10 mg/ml were treated with the indicated amounts of mersalyl in 10 mM Tris, pH 7.5, at 25 °C for 5 min. At the end of this time, aliquots were removed for determination of enzyme activity as in Fig. 1 with 0.4 mM *p*-nitrophenol and 1 mM UDP-galacturonic acid as substrates.

TABLE II

EFFECT OF PRODUCT INHIBITORS ON THE RATE OF THE UDP-DEPENDENT CLEAVAGE OF *p*-NITROPHENYLGLUCURONIDE

Assays contained 4 mM UDP, 20 mM *p*-nitrophenylglucuronic acid, 50 mM phosphate buffer, pH 7.5, and 10 mM saccharic acid-1,4-lactone. All activities represent initial rates of reaction determined as in Methods.

Additions to assay	*p-Nitrophenol released/min per mg microsomal protein (nmoles)*
None	4.50
+ 5 mM UDPglucuronic acid	2.68
+ 13 mM UDPgalacturonic acid	4.42

synthesized on different enzymes. Product inhibition studies were utilized in order to investigate this possibility since this type of study has proved to be extremely useful in defining the specificity of the aglycone binding sites[9]. Thus, we determined the effect of UDPgalacturonic acid on the UDP-dependent hydrolysis of *p*-nitrophenylglucuronic acid and the effect of *p*-nitrophenylglucuronic acid on the UDPgalacturonic acid dependent disappearance of *p*-nitrophenol. As seen in Table II, UDPgalacturonic acid is not a product inhibitor of the UDPglucuronyltransferase catalyzed hydrolysis of *p*-nitrophenylglucuronide. In contrast, UDPglucuronic acid is an inhibitor of this reaction (Table II and ref. 11). Similarly, *p*-nitrophenylglucuronide is not a product inhibitor of the synthesis of *p*-nitrophenylgalacturonide (Table III), but does inhibit the rate of its own formation from *p*-nitrophenol and UDPglucuronic acid[11]. For reasons which are unclear as yet, concentrations of *p*-nitrophenylglucuronide which inhibit the glucuronidation of *p*-nitrophenol[11], increase the rate of synthesis of *p*-nitrophenylgalacturonide. These product inhibition studies are consistent only with the conclusion that *p*-nitrophenylgalacturonide and -glucuronide are synthesized on separate enzymes.

With regard to increased rates of synthesis of *p*-nitrophenylgalacturonide in the presence of *p*-nitrophenylglucuronide this is not a specific effect of *p*-nitrophenylglucuronide, but rather a general effect characteristic of a number of different glucuronides. Phenylglucuronide, 2-naphthylglucuronide and *p*-nitrophenylglucuronide produce equivalent increases in the rate of synthesis of *p*-nitrophenylgalacturonide and under the conditions of Table III, 4-methylumbelliferylglucuronide increases the rate of synthesis of *p*-nitrophenylgalacturonide 10-fold. Further evidence for

TABLE III

EFFECT OF *p*-NITROPHENYLGLUCURONIDE ON THE RATE OF SYNTHESIS OF *p*-NITROPHENYLGALACTURONIDE

Assays contained 1 mM UDPgalacturonic acid and 0.4 mM *p*-nitrophenol, as in Table I and Methods, in the presence and absence of 5 mM *p*-nitrophenylglucuronide.

Additions	*p-Nitrophenol conjugated/min per mg microsomal protein (nmole)*
None	0.25
p-Nitrophenylglucuronide	0.40

the separateness of the enzymes catalyzing conjugation of *p*-nitrophenol with UDPglucuronic or UDPgalacturonic acids is apparent in the observation that phenyl-, 2-naphthyl- or 4-methylumbelliferylglucuronides have no effect on the rate of conjugation of *p*-nitrophenyl with UDPglucuronic acid as the glycosyl donor. Thus, just as UDPglucuronyltransferases are selective for aglycones, they are selective also for the UDPsugar derivative.

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