A Comparison of the Kinetic Properties of Two Different Forms of Microsomal UDPglucuronyltransferase*

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Two forms of UDPglucuronyltransferase (EC 2.4.1.17) have been purified from microsomes of pig liver. One form is free of phospholipids and the other contains a small amount of residual phospholipids. Each form, however, is responsive to activation on addition of purified phospholipids. Comparisons of kinetic properties of these enzymes, after reconstitution with identical phospholipid environments, indicate that these are unique functional forms of UDPglucuronyltransferase. The two differ by as much as 100-fold in their rates of conjugation at \( V_m \) for \( p \)-nitrophenol. Relative rates of glucuronidation of a variety of phenolic aglycones are different for the two enzymes, which suggests different reaction mechanisms. The energetic basis for binding of UDP-glucuronic acid to the active sites is different for the two forms of UDPglucuronyltransferase. Moreover, one form, but not the other, binds \( \text{Mn}^{2+} \), which leads to modulation of kinetic properties.

Studies of the developmental pattern of glucuronidating activities in liver suggest that there are several forms of hepatic microsomal UDPglucuronyltransferase (EC 2.4.1.17) (1-3). Recent work seems to confirm this idea in that each of three laboratories has reported the purification of at least two different forms of these enzymes, based on the observation that different, apparently homogeneous fractions of microsomal protein have variable relative activities when assayed with different aglycones (4-6). A fourth laboratory has reported recently (7) the purification of a form of UDPglucuronyltransferase that does not metabolize aglycones conjugated by other enzymes. There are no data, however, on the variability of the kinetic properties of different forms of UDPglucuronyltransferase, or on the extent to which the putative distinct enzymes are related and/or different. This is, we believe, a potentially important aspect of the function of these enzymes which has not been considered until now. Instead, interest in the multiplicity of UDPglucuronyltransferases has focused on the idea that different forms of these enzymes are different principally because they have variable catalytic specificities for aglycones (4-7). Other differences in the properties of UDPglucuronyltransferases have not been explored. The data presented in this paper indicate that the differences between forms of UDPglucuronyltransferase involve significant aspects of function other than their specificities for aglycones. Two purified forms obtained from pig liver microsomes that seem primarily to catalyze the glucuronidation of phenols have differences in activities at \( V_m \) on the order of 100-fold with \( p \)-nitrophenol as aglycone, different reaction mechanisms, and different mechanisms for interacting with UDP-glucuronic acid. In addition, the function of one of these enzymes, but not the other, is affected by \( \text{Mn}^{2+} \).

MATERIALS AND METHODS

LPC\(^1\) was prepared from egg yolk by treating purified lecithin from eggs with phospholipase \( \text{A}_\text{s} \), as in Ref. 8. This LPC was used only for purification procedures. Phospholipids used in reconstitution experiments were purchased from P-L Biochemicals, Milwaukee, WI. They were used without further purification. All other chemicals used were the best available commercial grades. All solutions were prepared with deionized, double distilled water.

Protein concentration was measured by the biuret method (9) except when glycerol was present. In this instance, protein was determined by the method of Lowry et al. (10) after precipitation of the protein with trichloroacetic acid (11). Phospholipid phosphorus was determined after digestion of the CHCl\(_3\)/CH\(_3\)OH extracts of the different forms of UDPglucuronyltransferase (12).

Purification of UDPglucuronyltransferase—The details of the purification of GT\(\text{IP} \) and GT\(\text{IP} \) are given in Ref. 13. Partially purified GT\(\text{IP} \) (2-3 mg) was applied to a hydroxylapatite column (Bio-Rad Laboratories, Bio-Gel HT), (1.5 x 15 cm) equilibrated with 5 mm Tris, pH 8 (0 °C), 0.1 mm dithioerythritol, 2 mm EDTA, 1.1% (w/v) cholate, and 10% (v/v) glycerol. The column was washed with 80 ml of 150 mm phosphate in the same buffer. GT\(\text{IP} \) was dialyzed extensively as in Ref. 13 in order to reduce the concentration of cholate and phosphate. The enzyme solutions were frozen at -20 °C and could be stored for several months without appreciable loss of activity.

Based on activities at \( V_m \) and after correction for detergent-induced activations, the purification procedures described in Ref. 13 yield enzyme preparations that are enriched 1350-fold for GT\(\text{IP} \) and 20-fold for GT\(\text{IP} \), as compared with the rate of conjugation of \( p \)-nitrophenol in intact microsomes. The figure for the enrichment of GT\(\text{IP} \) versus intact microsomes has to be interpreted cautiously because GT\(\text{IP} \), as compared with GT\(\text{IP} \), has 100-fold greater activity with \( p \)-nitrophenol (see below) and probably represents the major enzyme in microsomes for glucuronidation of this substrate. Hence, one cannot compare the specific activity of pure GT\(\text{IP} \) with that of GT\(\text{IP} \) per se in intact microsomes. The yield of enzyme as a fraction of microsomal protein in the final preparation is less than 0.1%. Activities at \( V_m \) and other relevant kinetic constants were determined as in Refs. 12 and 13, for

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\(^{1}\) The abbreviations used are: LPC, lysophosphatidylcholine; GT\(\text{IP} \), the form of UDPglucuronyltransferase eluted from hydroxylapatite at 90 mm \( P \); GT\(\text{IP} \), the form of UDPglucuronyltransferase eluted from hydroxylapatite at a concentration of \( P \) greater than 90 mm; PC, phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; the subscripts UDPGA and pNP refer to UDP-glucuronic acid and \( p \)-nitrophenol.
an enzyme with a rapid equilibrium, random order mechanism (14, 15).

Reconstitution of GTlp and GTlp—Enzyme was mixed with the indicated lipid in a ratio of 1:10 (w/w) when enzyme was reconstituted with LPC and in a ratio of 1:6 when enzyme was reconstituted with PC. These ratios of protein to lipid yield maximal activity. Mixtures of enzyme and lipid were kept at 0 °C under a stream of argon until aliquots were removed for assay. The activities of the enzyme-lipid mixtures were stable for at least 8 h, which was the longest interval tested. Further details of reconstitution are given in Refs. 12 and 13.

Dispersion of Phospholipids—LPC was dispersed in water. PC was microdispersed in 10 mM Tris, pH 7.5 (30 °C), 1-2 ml, by ultrasonication with a microtip (Heat Systems-Ultrasonication, Plainview, NY, model W185F) for 10 min. The sonicated mixture was centrifuged at 10,000 × g in order to remove multilamellar structures.

RESULTS

Comparison of activities at Vm of GTlp and GTlp—GTlp, when assayed with p-nitrophenol as aglycone, has no measurable activity in the absence of added phospholipids. This is true even though the preparation of GTlp contains a small amount of residual phospholipid phosphorus, which we were unable to remove by washing lyophilized enzyme with cold dry acetone or CHCl₃/CH₃OH. The reasons for the strong apparent avidity of GTlp for a small amount of phospholipid for GTlp reconstituted with a broader range of LPC than GTlp is far larger than that for GTlp, when activity was measured with p-nitrophenol as aglycone (Table I). The other hand, to the extent that the relationship was examined, the length and unsaturation of the acyl chain of LPC had similar quantitative effects on the activities at Vm for GTlp and GTlp. Activities at Vm were not determined for GTlp in lipid environments other than those in Table I. Activities were measured, however, at a single set of substrate concentrations for GTlp reconstituted with a broader range of LPC than listed in Table I. These data were in agreement with the idea that longer chain length and unsaturation of the acyl chain of LPC are associated with greater activation of GTlp as compared with LPC containing shorter and saturated acyl chains (data not shown). Also, as for GTlp, LPC was a better activator of GTlp than was PC. Despite these similarities, interactions between GTlp and a given lipid must have functional consequences that differ from those due to interactions between GTlp and the same lipid. For example, the ratio of activities at Vm for GTlp to activity at Vm for GTlp was different as the lipid environment of the reconstituted enzymes were varied.

Comparison of Rates of Glucuronidation at Vm of Substituted Phenols—Recent studies of the function of GTlp and GTlp indicate that the relative rates of glucuronidation at Vm of substituted phenols, catalyzed by enzyme reconstituted with oleoyl-LPC, depend only on the pKₐ of the phenolic aglycone (13). These data are compatible with the conclusion that glucuronidation catalyzed by GTlp reconstituted with oleoyl-LPC proceeds via a mechanism in which the rate-determining step is nucleophilic attack of phenolate ion on the bond between UDP and C-1 of glucuronic acid (12, 13). The large differences between GTlp and GTlp (for enzymes reconstituted with oleoyl-LPC) in rates of glucuronidation of p-nitrophenol, could be due to a kinetic mechanism for GTlp in which the rate-determining step differs from that observed for catalysis by GTlp. Another possibility is that the active sites of GTlp and GTlp have differential effects on the nucleophilicity of the phenolate form of phenolic aglycones. These possibilities were investigated by determining for GTlp the rate of glucuronidation at Vm with a series of para-substituted phenols. The data are shown in the form of log Vm versus the Hammett function for para-substituted phenols. Rates of conjugation catalyzed by GTlp reconstituted with oleoyl-LPC vary with the acidity of the phenolic aglycones, but the slope of the plot is 0.58. The theoretical slope for this plot is 2.23 (16). Hence, the rate of glucuronidation of phenols catalyzed by GTlp as compared with GTlp is dependent only partially on the concentration of phenolate ion. There are several possible explanations for the differences, but these cannot be evaluated by the present data, nor is it possible to draw conclusions about the nucleophilicity of phenols within the active sites of GTlp and GTlp. It seems clear, nevertheless, that the mechanism of the conjugation reaction catalyzed by GTlp (reconstituted with oleoyl-LPC) is different from the mechanism of the reaction catalyzed by GTlp (reconstituted with oleoyl-LPC). Another important aspect of the data in Fig. 1 is that the rates at which GTlp catalyzes the glucuronidation of p-aminophenol are 10-fold greater than the rate at which GTlp catalyzes the conjugation of this aglycone (13). Therefore, whereas GTlp

![Fig. 1. Activities at Vm of GTlp reconstituted with oleoyl-LPC as a function of the acidity of phenolic aglycones.](http://www.jbc.org/)
versus GT\textsubscript{IP} seems to be especially suited for catalyzing the glucuronidation of acidic phenols, GT\textsubscript{IP} may be a more efficient enzyme for catalyzing the glucuronidation of weakly acidic phenols, at least under the condition that each enzyme is reconstituted with oleoyl-LPC.

We also determined whether GT\textsubscript{IP} has high catalytic specificity for functional groups other than phenols. This does not seem to be the case. GT\textsubscript{IP} has lower specific activity for conjugation of COO\textsuperscript{-} and SH compared with rates of conjugation of p-nitrophenol; and it does not catalyze formation of N- and C-glucuronides.

**Comparison of the Binding of UDP glucuronic Acid to GT\textsubscript{IP} and GT\textsubscript{TP}**—In addition to the large difference in activities at V\textsubscript{m}, GT\textsubscript{IP} and GT\textsubscript{TP} reconstituted with oleoyl-LPC differ in their affinities for substrates, as reflected by the kinetically determined values of K\textsubscript{UDP\textsubscript{G}} and K\textsubscript{SNP}. The kinetic constants of GT\textsubscript{IP} and GT\textsubscript{TP} after reconstitution of enzyme in oleoyl-LPC and other lipids are shown in Table II. It has not been confirmed by kinetic isotope effects (17) that these enzymes have truly rapid equilibrium mechanisms, but patterns of product inhibition and initial rate data are consistent with a rapid equilibrium, random order kinetic mechanism (14). We think it is reasonable, therefore, to use the kinetically determined binding constants in Table II as reflections of the affinity of GT\textsubscript{IP} and GT\textsubscript{TP} for ligands. The data show a small difference in the value of K\textsubscript{UDP\textsubscript{G}} for GT\textsubscript{IP} versus GT\textsubscript{TP}. The values of other K terms depend on the phospholipid environment of each form of UDPglucuronotransferase. Of interest are the differential effects of variable phospholipid environments on the ratios K\textsubscript{UDP\textsubscript{G}}/K\textsubscript{UDP\textsubscript{G}} (or K\textsubscript{SNP}/K\textsubscript{SNP}) for GT\textsubscript{IP} versus GT\textsubscript{TP} which reflect, most likely, ligand-induced changes in the affinity of the enzyme for other ligands. For example, for GT\textsubscript{TP} reconstituted with myristoyl-LPC, the data in Table II suggest, based on the presumed kinetic mechanism, that UDP-glucuronic acid binds to free GT\textsubscript{TP} with less avidity as compared with the affinity of the complex GT\textsubscript{TP}-p-nitrophenol for UDP-glucuronic acid. The ligand-induced alteration in affinity of free enzyme for the second ligand depends on the lipid environment since it is different for GT\textsubscript{IP} in each of the lipid environments tested. It is always different for GT\textsubscript{IP}, however, as compared with the same effect in GT\textsubscript{TP}.

Although the differences between GT\textsubscript{IP} and GT\textsubscript{TP} in their affinities for UDP-glucuronic acid appear to be small, the data in Table III indicate that there are major differences in the manner in which each of these enzymes (reconstituted with oleoyl-LPC) interacts with UDP-glucuronic acid. GT\textsubscript{IP} has high affinity for UDP and poor affinity for glucuronic acid. Also, the binding of UDP-glucuronic acid to GT\textsubscript{IP} is as strong as the binding of UDP. This suggests that interactions between UDP and GT\textsubscript{IP} provide nearly all the binding energy for interactions between enzyme and UDP-glucuronic acid. By contrast, GT\textsubscript{TP} has an appreciable affinity for binding for glucuronic acid. GT\textsubscript{TP}, as compared with GT\textsubscript{IP}, also has a higher affinity for UDP. As pointed out previously, however, the binding of UDP-glucuronic acid to GT\textsubscript{TP} must be associated with a considerable loss of inherent binding energy from interactions between UDP and GT\textsubscript{TP} and glucuronic acid and GT\textsubscript{TP} (12). This loss of inherent binding energy does not appear to occur when UDP-glucuronic acid binds to GT\textsubscript{IP}, as evidenced by a close correspondence of K\textsubscript{UDP\textsubscript{G}} and K\textsubscript{UDP\textsubscript{G}} for GT\textsubscript{IP} (Table III).

**Differential Effects of Mn\textsuperscript{2+} on the Properties of GT\textsubscript{IP} and GT\textsubscript{TP}**—The activity of UDPglucuronotransferase in intact microsomes is stimulated by divalent cations (18). Divalent cations, however, have no effect on the activity of GT\textsubscript{TP} (12).

We observed, however, that Mn\textsuperscript{2+} and other divalent cations activated GT\textsubscript{IP} (Table IV). The kinetic mechanism of this activation was enhancement of activity at V\textsubscript{m} and of affinity of enzyme for substrates. The data in Table IV are for GT\textsubscript{IP} reconstituted with oleoyl-LPC; but the effects of Mn\textsuperscript{2+} on GT\textsubscript{IP} were independent of the type of lipid used to reconstitute delipidated GT\textsubscript{IP}. Since Mn\textsuperscript{2+} enhances the affinity of GT\textsubscript{IP} for UDP-glucuronic acid, we determined the effects on Mn\textsuperscript{2+} affinity of GT\textsubscript{IP} for the UDP and glucuronic acid regions of this substrate. Addition of Mn\textsuperscript{2+} (1.5 mm) to GT\textsubscript{IP} enhanced the affinity of GT\textsubscript{IP} for each of these moieties (Table IV). This concentration of Mn\textsuperscript{2+} gave maximal enhancement of activity. At the concentrations of Mn\textsuperscript{2+} and UDP present in the assays, virtually all the UDP existed as a UDP-Mn\textsuperscript{2+} chelate (19). It is not possible with these data to decide whether GT\textsubscript{IP} has a higher affinity for UDP-Mn\textsuperscript{2+} versus UDP, or whether binding of Mn\textsuperscript{2+} to GT\textsubscript{IP} enhances affinity for UDP by a mechanism.
not involving formation of UDP-Mn^{2+}. The data for binding of the UDP-Mn^{2+} to GT_{IP} are significant, nevertheless, because, as shown in Table V, UDP-Mn^{2+} does not bind to GT_{SP}. The data, therefore, show that there must be substantial differences between GT_{IP} and GT_{SP} in the regions of these enzymes that interact with UDP.

**Discussion**

We have purified from pig liver two forms of UDPglucuronyltransferase, for which phenols appear to be the best substrates. These two enzymes, however, appear to have different rate-limiting catalytic steps, widely variable catalytic activities with the same substrates, and a different energetic basis for the binding of UDP-glucuronic acid to the active site of each enzyme; and one, but not the other, is responsive to activation by divalent cations. These two forms of UDPglucuronyltransferase, therefore, would appear to contain significant differences in their primary structures; and, in fact, the only homology of function between them is that they catalyze the glucuronidation of the same substrates. Because of the similarity of substrates metabolized in vitro by GT_{IP} and GT_{SP} and the low specific activity of the former versus the latter, the physiological significance of the GT_{IP} is not completely clear. It is of interest, however, that whereas GT_{SP} as compared with GT_{IP} has higher activity with relatively strongly acidic phenols, GT_{IP} versus GT_{SP} has significantly higher activity with relatively weakly acidic phenols, such as p-aminophenol. Possibly, the best substrate for GT_{IP} has not been established by the above experiments. It is possible, too, that its physiologic lipid environment has an effect on the activity of GT_{IP} that cannot be discerned from these experiments. These uncertainties do not affect, however, the conclusions about the marked differences in the kinetic properties of GT_{IP} versus GT_{SP}.

There is reason to believe that the uniqueness of different forms of UDPglucuronyltransferase cannot be established firmly by comparing the relative rates of glucuronidation of multiple aglycones catalyzed by different functions of microsomal proteins. The basis for this idea is the demonstration that the relative rates of glucuronidation at V_{m} of phenolic aglycones catalyzed by a single pure form of UDPglucuron-

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Additions and Corrections

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Oxygen toxicity in *Streptococcus sanguis*. The relative importance of superoxide and hydroxyl radicals.

*James DiGuiseppe and Irwin Fridovich*

Pages 4046 and 4050: Each occurrence of the compound ethylenediamine-$N,N'$-diacetate should be replaced by ethylenediamine-di(o-hydroxyphenyl acetic acid).

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Page 4146, second column, line 3: The correct reference number should be "13" (not "7").