In Situ Structural Analysis of Microsomal UDP-glucuronosyltransferases by Radiation Inactivation*

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The structure of the UDP-glucuronosyltransferases in microsomes from guinea pig and rat liver was examined in situ by radiation inactivation analysis. The p-nitrophenol conjugating activity of guinea pig microsomes increased at lower doses of radiation; at higher doses (>36 megarads), activity showed a first order decline yielding a target size of 71 ± 9 kDa. Treating microsomes with Triton X-100 eliminated the activation seen at lower doses of radiation and yielded a simple exponential decrease in activity which gave a larger target size (85 ± 18 kDa). A monoeponential decrease in activity was seen in sonicated microsomes, and the target was close to that for untreated microsomes at >36 megarads. The same response was obtained when the reaction was assayed in the reverse direction. The estrone conjugating activity of guinea pig microsomes was similarly activated at lower doses of radiation and declined at higher doses (>36 megarads), with a target size of 57 ± 11 kDa.

Allosteric activation of the enzyme by UDP-N-acetylglucosamine was eliminated by lower doses of radiation. Thus, activation of the enzyme by radiation, detergent, sonication, and UDP-N-acetylglucosamine appear to be interdependent. These activations are postulated to be due to the existence of the enzyme in an oligomeric form which can be dissociated into monomers with higher activity.

The same biphasic activation-inactivation curves were obtained for p-nitrophenol conjugation in rat liver microsomes. The target sizes were 54 ± 8 kDa (p-nitrophenol in the forward direction) and 66 ± 10 kDa (p-nitrophenol in the reverse direction). Thus, the enzyme appears to be smaller in rat liver as compared with guinea pig liver. Lactoholate glucuronidating activity in rat liver microsomes (at >36 megarads) gave a target size of 74 ± 1 kDa.

The UDP-glucuronosyltransferases represent a family of microsomal membrane-bound enzymes that catalyze the glucuronidation of a wide range of drugs and toxins. A variety of data have led to the suggestion that there are indeed several substrate-specific forms of the enzyme (see Refs. 1-5 for reviews). These data include studies in which UDP-glucuronosyltransferase activity was separated into fractions selectively enriched in activity toward a particular substrate. However, because of overlapping substrate specificities and similar subunit molecular weights, the purity of these fractions is difficult to assess. Thus, it is not known how many different substrate-specific forms of the enzyme there are.

Structural analysis of the UDP-glucuronosyltransferases has also been difficult. Because of the tight association with the microsomal membrane, it has not been possible to purify the enzyme without treatments that dramatically alter the properties of the enzyme and presumably also its structure (1-5). Thus, structural analyses of purified forms of the enzyme most likely do not accurately describe the native enzyme. In addition, the existence of purified preparations of the enzyme in complex aggregate states with detergents precludes the analysis of their structural organization by classical techniques. Because the enzyme has been hypothesized to be an allosteric protein (6), a complex structural organization for the enzyme is expected. A complex organizational structure has also been suggested by the isolation of a hybrid form of UDP-glucuronosyltransferase (7).

We sought an alternative approach for examining the structural organization of UDP-glucuronosyltransferase and for comparing the structure of different substrate-specific forms of the enzyme. The technique of radiation inactivation provides a tool for determining the molecular size of an enzyme. It is based on the principle that ionizing radiation from high energy electrons or γ-rays interacts randomly throughout the mass of exposed matter. In each interaction large amounts of energy are deposited in a discrete packet within one molecule; the subsequent chemical damage disrupts the covalent structure and results in a complete loss of biochemical function (target theory). The mass of the active unit can be calculated from the loss of activity with increasing radiation exposure (8). This technique offers several unique advantages. The enzyme need not be purified and thus can be analyzed in its native microsomal environment. Also, since the technique is based on assays of enzyme activity, the molecular mass obtained gives the size of the functional catalytic unit. Comparison with the subunit molecular weight permits determination of the structural complexity of the enzyme. Previous radiation studies have yielded target sizes that are accurate within 14% (8) or better (36).

MATERIALS AND METHODS

[UH]Inulin was obtained from Du Pont-New England Nuclear. [4,14C]Estrone was obtained from Amersham Corp. [glucuronyl-U-14C]Uridine diphosphate glucuronic acid was obtained from ICN Radiochemicals. Lactoholic acid was obtained from Steraloids Inc., Wilton, NH. Uridine diphosphoglucuronic acid (UDPGA)1 and p-nitrophenol were obtained from Sigma Chemical Co.

1 The abbreviations used are: UDPGA, uridine diphosphoglucuronic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-N,Nbisulfate; SDS, sodium dodecyl sulfate.

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nitrophenol were obtained from Sigma.

The microsomal cell fraction was isolated from fresh guinea pig and rat liver as described previously (9). The final microsomal suspensions (30–40 mg of protein/ml) were placed in 2-ml Kimble No. 12012 glass vials (0.5 ml/vial), sealed with an O2/gas flame, and frozen at −80 °C. For radiation inactivation analysis, the frozen samples were shipped on dry ice and irradiated at −135 °C with 13 MeV electrons from the linear accelerator at the Armed Forces Radiobiology Research Institute (Bethesda, MD). The vials were opened and the microsomes analyzed as described below. The details of radiation exposure, dosimetry, and temperature control are described elsewhere (10). When the loss of enzymatic activity was a simple exponential function of the radiation dose, a least squares analysis of the data permitted calculation of k, the rate of loss of activity. For irradiations conducted at −135 °C, k can be used to calculate the molecular size (in daltons) using the relationship:

\[
\text{Molecular size} = 6.4 \times 10^4 \times S_i \times k
\]

where \(S_i\) is the temperature correction factor (2.8) for irradiations at −135 °C. For graphical presentation the data were normalized to the unirradiated control. Radiation target sizes are reported as the average ± S.D. from three to seven independent experiments.

UDP-glucuronyltransferase activity toward p-nitrophenol was assayed at 0.4 mM p-nitrophenol and 10 mM UDPGA as described previously (11). Activity toward lithocholate was determined radiometrically with [14C]UDPGA employing pBondapak Sep-Pak C18 cartridges to trap the labeled product. The assay was conducted essentially as described by Kirkpatrick et al. (12). The assay contained 0.25 mM lithocholate, 3.0 mM [14C]UDPGA, 2.5 mM MgCl2, and 100 mM HEPES, pH 6.9. In addition, 10 mM NAD+ was added to inhibit nucleotide pyrophosphatase (13) and 5.0 mM saccharo-1,4-lactone was added to inhibit B-glucuronidase (14).

UDP-glucuronyltransferase activity toward estrone was measured radiometrically. A solution containing 10 mM UDPGA, 80 mM potassium phosphate, pH 7.1, and 1–2 mg of microsomal protein was brought to 30 °C. The reaction was initiated by the addition of 10 μl of [14C]estrone (2 mM stock solution in ethanol). A 100-μl aliquot was transferred to 0.25 ml of 0.25 M trichloroacetic acid (adjusted to pH 4.0 with KHPo4) at 0, 1, 2, and 3 min of assay. The samples were extracted twice with 1.5 ml of ethyl ether and the [14C]estrone glucuronide in the aqueous phase quantitated by liquid scintillation counting. Initial rates of reaction were determined from the multiple time points. The permeability of microsomes to [3H]hinulin was determined as described previously (15). Protein was determined by the Biuret procedure (16).

**RESULTS**

The most commonly measured activity for UDP-glucuronyl-transferase is the conjugation of p-nitrophenol by guinea pig liver microsomes. This activity was measured in samples irradiated with high energy electrons. The dose-dependent change in enzyme activity is shown in Fig. 1 in the form of a semi-log plot normalized to the activity of the unirradiated sample. Rather than displaying simple dose-dependent inactivation, p-nitrophenol activity increased after low doses. Only at high doses did the activity show a first order dose-dependent decline in activity. This result was not altogether unexpected, because UDP-glucuronyltransferase is known to possess considerable latency that can be expressed by exposing microsomes to treatments such as detergents, sonication, or phospholipase A digestion (1, 5) which perturb the structure of the membrane.

The question then arose as to whether this dose-dependent increase in activity would be seen in microsomes in which latency had been removed by treatment with detergent. Therefore, microsomes were either irradiated and then treated with Triton X-100 prior to assay or treated with Triton X-100 prior to irradiation. In unirradiated samples, Triton X-100 increased activity by 5.2 ± 0.8-fold. The activity in the Triton-treated microsomes did not show any dose-dependent increase in activity but rather displayed first order inactivation (Fig. 1; the data are normalized to the unirradiated, non-

![Fig. 1. Radiation inactivation analysis of p-nitrophenol conjugating activity in guinea pig liver microsomes. Frozen microsomes from guinea pig liver were exposed at −135 °C to the indicated radiation doses from high energy electrons. The samples were then thawed and assayed for UDP-glucuronyltransferase activity toward p-nitrophenol in the presence (○) or absence (●) of 0.1% (v/v) Triton X-100. The enzymatic activity of the irradiated samples (A) was normalized to that of the non-Triton treated, unirradiated control (Ao). Mrads, megarads.](image-url)
we examined the permeability of microsomes to inulin after various radiation dosages over the range of 0 to 36 megarads. 

No change in the inulin permeability was noted out to 36 megarads.

We were interested in comparing the values obtained with p-nitrophenol as the substrate with those obtained with a substrate representing a different enzyme form. By several criteria (1, 17) the conjugation of estrone is considered to be catalyzed by a different form of the enzyme than that for p-nitrophenol conjugation. We therefore examined the radiation dependence of estrone conjugation by guinea pig liver microsomes (Fig. 3). Estrone conjugation was also characterized by activation at low doses of radiation and a target size of 57 ± 11 for doses >36 megarads. Once again, the addition of Triton X-100 to the assays increased control activity and resulted in a more rapid monoeXponential decline in activity with dose. However, in this case the increase was only 1.5 ± 0.2-fold.

Radiation inactivation analysis was also performed on microsomes from rat liver. UDP-glucuronoltransferase inactivation analysis of p-nitrophenol conjugation by rat microsomes differed in that Triton X-100 did not alter the stability of the enzyme. Addition of Triton X-100 to the assays increased control activity by 4.7 ± 2.1-fold and eliminated the radiation-induced activation (Fig. 4). The target size for the forward direction was 58 ± 12 kDa for assays in Triton and 54 ± 8 kDa for the assays without Triton at >36 megarads. The reverse reaction (p-nitrophenylglucuronide plus UDP) in the presence of Triton X-100 showed a 3.1 ± 0.8-fold increase in control activity and a target size of 66 ± 15 kDa, compared to a target size of 66 ± 10 kDa in the absence of Triton X-100 at >36 megarads.

The glucuronidation of lithocholic acid by rat liver micro-
DISCUSSION

Target analysis of the radiation destruction of biological activity yields the mass of the structures whose integrity is required for the measured function (10). The primary assumption of target theory is that each random interaction of radiation with the functional unit leads to complete loss of activity of that structure. This predicts an exponential loss of activity in samples exposed to increasing radiation doses. However, in the case of UDP-glucuronyltransferases, the effect of lower doses of radiation is to increase activity by inducing change in both $V_{\text{max}}$ and $K_{\text{UDP-GA}}$. Not until higher doses (≥36 megarads) is there an exponential decline in activity. Alternatively, when these samples are assayed in the presence of detergent or after sonication, simple exponential losses are observed.

There are several possible explanations for the complex radiation responses of the UDP-glucuronyltransferases. First, it should be noted that UDP-glucuronyltransferase activity is also increased by physical disruption of the membrane by detergents, phospholipase treatment, sonication, etc. (1, 5). This was interpreted by some investigators to indicate that the enzyme is located behind a permeability barrier to substrates and that activation is associated with increased permeability (18). However, we have found that irradiation at 36 megarads does not render the microsomes permeable to inulin. This result is consistent with the observation that the activation of UDP-glucuronyltransferase by sonication of microsomes is accompanied by pinching off of microsomes into smaller vesicles but not by an increase in membrane permeability (15). Nor does sonication generate inside out vesicles or the release of any UDP-glucuronyltransferase activity (15). On the basis of these observations, and considerable kinetic data (4, 19), it has been alternatively hypothesized that these treatments activate the enzyme by transmitting changes in the physical properties of the lipid phase to the enzyme via lipid-protein interactions (5, 15, 19). However, this conformational model is difficult to invoke as an explanation for the radiation-induced activation because the target size for a phospholipid molecule is so small that at 9 megarads an exceedingly small percentage (<1%) of the phospholipid molecules has been destroyed and yet the activity of the enzyme has more than doubled. Thus, it is hard to envision that radiation induces a change in the physical properties of the bulk lipid phase.

An alternative explanation for radiation-induced activation is the existence of a large molecular weight inhibitor. The size of such a hypothetical inhibitor can be obtained by analyzing data (such as those in Fig. 1) as the difference of two exponentials. For the conjugation of $p$-nitrophenol the value obtained is 150 ± 16 kDa, and for the reverse reaction (UDP-glucuronyltransferase containing both the 50-kDa androsterone conjugating subunit and a 52-kDa testosterone conjugating subunit) the value is 190 ± 23 kDa. The difference in these values could be a result of the insensitivity of the curve peeling procedure or it could be that a single inhibitor model is not appropriate.

Another model that would explain this behavior is an oligomer ⇄ monomer equilibrium in which the subunits are more active as monomers than as part of the oligomer. At lower doses the large oligomer is the predominant target and one hit is sufficient to cause dissociation of the oligomer. By 36 megarads only monomers exist and therefore a simple inactivation curve is obtained above that dose. The monomer/oligomer model can also explain latency of the enzyme. Thus, for the activation of the enzyme by sonication, the reduction in the radius of vesicles due to sonication leads to compression of the inner half of the lipid bilayer (20). This could lead to changes in lipid-protein interactions (34) which prevent oligomer formation. Similarly, detergents could be activating the enzyme by dissociating the oligomeric complex. Consistent with this model is the fact that all three forms of activation (sonication, detergent, and radiation) are similar in producing an increase in the affinity of the enzyme for UDPGA (Ref. 15 and this work).

The existence of oligomeric forms of UDP-glucuronyltransferase is supported by the work of Matsui and Nagai (7). They isolated from rat liver an oligomeric UDP-glucuronyltransferase containing both the 50-kDa androstenedione conjugating subunit and a 52-kDa testosterone conjugating subunit. There is also the suggestion of a monomer/oligomer system from other radiation inactivation studies. Peters et al. (21) irradiated UDP-glucuronyltransferase in detergent-activated, lyophilized preparations of microsomes. Since these studies were conducted in detergent, the radiation-dependent activation of the enzyme was not seen. Interestingly enough, they reported a target size of 175 kDa for the glucuronidation of bilirubin monoglucuronide (to bilirubin diglucuronide) and a target size of only 42 kDa for the glucuronidation of bilirubin. These authors hypothesized a monomer/tetramer equilibrium with the tetramer being the only form capable of glucuronidating bilirubin glucuronide. However, irradiation of lyophilized samples containing the highly denaturing detergent SDS

![Graph](image-url)
is likely to lead to artificially large target sizes (see below).

The complex inactivation curves are more difficult to analyze than the simple exponentials. However, it appears that the data for 36 megarads and above can be fitted with a single exponential. This is supported by the similarity of these slopes to those obtained when latency is removed by sonication. Thus, the target size obtained above 36 megarads is considered to be the measure of a fundamental catalytic unit and is free of complicating latency factors. Values obtained at >36 megarads are used in the comparison of different forms of the enzyme.

Peters et al. (21, 22) determined the target size of UDP-glucuronyltransferase in rat liver microsomes using several aglycones as test substrates. For conjugation of p-nitrophenol they reported a target size of 109 kDa (21). They had previously found a different target size of 48 kDa for the reaction in the reverse direction (i.e. the UDP-dependent cleavage of p-nitrophenylglucuronide) (22). We obtained a target size of 54 ± 8 daltons for p-nitrophenol conjugation in the forward direction and 67 ± 10 kDa for the reverse reaction. The differences between our values and the target values of Peters et al. (21, 22) are probably related to their use of lyophilized samples and a denaturing detergent. A number of reports have appeared recently in which lyophilization versus the frozen state were directly compared (23–26). Samples irradiated in the lyophilized state often yielded target sizes that were much larger than those obtained in the frozen state. Furthermore, values obtained in the frozen state are in close agreement with a monomer or dimer of the polypeptide subunit molecular weights determined by SDS-polyacrylamide gel electrophoresis (27, 28). The explanation for the large target sizes observed in lyophilized samples usually is suggested to be a molecular aggregation phenomenon, but relatively little evidence is presented for this hypothesis. Comparison of the biological activities in frozen samples with fresh preparations before irradiation usually indicates only small losses and relatively little change in intrinsic affinity for substrates, but for lyophilized samples the numbers of surviving units are often quite variable from one system to the next. Since the frozen samples generally retain most of the normal activity and also yield target sizes easily interpreted in terms of subunit structure, it has been suggested that these data are more likely to reflect the in vivo state.

The target sizes we obtained for the different aglycones can be compared. In guinea pig liver microsomes, p-nitrophenol conjugation showed a target size of 71 ± 9 kDa, while estrone conjugation had a target size of 57 ± 11 kDa. In rat liver microsomes, p-nitrophenol conjugation was associated with a target size of 54 ± 8 kDa. Lithocholate glucuroni- cation had a larger size (74 ± 1 kDa). A target size of 54 kDa for the rat liver p-nitrophenol conjugating enzyme is very close to the molecular weight reported by other investigators (29–32). Their values were determined by SDS-polyacrylamide gel electrophoresis and all range from 54,000 to 59,000 for the monomer. The radiation results indicate that the monomer is a functionally active unit. The target size for the p-nitrophenol conjugating enzyme in guinea pig liver (71 ± 9 kDa) appears to be larger. Thus, there may be structural differences in the enzymes from the different species. While the enzyme has not been purified from guinea pig liver, the differences in radiation size are consistent with our findings of major differences in the kinetic properties of the enzyme from rat and guinea pig liver (33).

The target size obtained for the glucuronidation of litho- cholic acid by rat microsomes is uncommonly large (74 ± 1 kDa). Kirkpatrick et al. (12) have purified lithocholate glucuroni- cating activity from rat liver microsomes and obtained a molecular weight of 52,000 by SDS-polyacrylamide gel electrophoresis. The large target size may indicate some degree of aggregation even at the higher doses of irradiation or to interference by the detergent properties of the bile acid substrate. It is clear from our studies with guinea pig liver microsomes (Fig. 1) and other work (35) that the presence of detergent can give rise to abnormally large target sizes.

In conclusion, this study presents a model for the latency of UDP-glucuronyltransferase which provides both a new interpretation for previous data and also explains the results of the current radiation inactivation studies. In addition, comparison of the radiation target sizes has shown that there are differences in the size of p-nitrophenol conjugating forms of glucuronyltransferase between species.

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REFERENCES