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Immobilization of solubilized UDP-glucuronosyltransferase from rat liver microsomes to Sepharose 4B

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Abstract—A method for the covalent binding of rat liver UDP-glucuronosyltransferase to a cyanogen bromide-activated agarose matrix is described. The rat liver microsomal fraction was solubilized with 8 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); 90% of the microsomal protein was solubilized. Some 50–60% of this protein became bound covalently to the activated agarose matrix. The immobilized UPD-glucuronosyltransferase remained completely active for 50 days when stored at 4° in a 20% (v/v) glycerol buffer (pH 7.4). The immobilized enzyme has a temperature optimum around 37°, and a broad pH optimum (pH 5–7.4). The enzyme displayed linear kinetics over a period of 1 hr; it conjugates a large variety of substrates.

UDP-glucuronosyltransferase (UDPGT*) (EC 2.4.1.17) represents a family of enzymes present in the endoplasmic reticulum of many organs which conjugates a wide variety of xenobiotic and endogenous compounds with glucuronic acid [1]. Some purified UDPGTs have been characterized with respect to their ability to use xenobiotics and endogenous compounds as substrates. Substrate specificity of several of these enzyme forms specific for steroids or bile acids was relatively narrow [2, 3]; however, most enzyme forms have overlapping substrate specificities.

Purification of these enzymes has presented a major difficulty, because they are labile after solubilization from the endoplasmic reticulum. Immobilization of solubilized enzymes onto a solid support often stabilizes the solubilized enzymes. Immobilization of rabbit [4–7], rhesus monkey and man [8, 9] liver UDPGTs to Sepharose has been described. Since the rat is the most commonly used animal in studies on drug metabolism, we have developed a method for the covalent binding of solubilized rat liver UDPGTs on a cyanogen bromide activated agarose matrix. Furthermore, we have investigated the substrate specificity of this immobilized UDPGT system.

Materials and Methods

Chemicals. Sepharose 4B was purchased from Pharmacia (Woerden, The Netherlands). UDP-Glucuronic acid (UDPGA; disodium salt), pyruvate kinase from rabbit muscle (500 U/mg), lactate dehydrogenase from beef muscle (450 U/mg), NADH, phosphoenolpyruvate (trisodium salt) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were from Boehringer (Mannheim, Germany). All other chemicals were of analytical reagent grade and used without further purification.

Solubilization of microsomal proteins. All steps (agarose activation, solubilization of the microsomal proteins and immobilization) were performed at 0–4°. Buffers containing glycerol were set to the required pH before the glycerol was added.

Male SPF Wistar rats (250 g) of the Sylvius Laboratories, University of Leiden, were decapitated. The livers were homogenized for 90 sec at 1200 rpm in 3 volumes of a 0.15 M KCl in a Potter–Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged in a Beckman LC5 ultracentrifuge for 60 min at 105,000 *g* at 4°. The microsomal pellet was resuspended for 10 sec at 1200 rpm with the homogenizer in 50 mL 0.15 M KCl. This microsomal suspension was solubilized at 4° by adding 50 mL of 0.25 M sucrose buffer containing 0.1 M potassium phosphate (pH 7.4) and 20% glycerol, and 65 mL of solubilization buffer (0.15 M potassium citrate, 0.15 M KCl, 2 mM dithiothreitol, 0.12 mM Na_2EDTA, 20 mM CHAPS (pH 7.0) and 50% glycerol). This mixture was sonicated in a Branson Automatic cleaner four times for 30 sec with 1 min intervals between every sonication step. The mixture was stirred for 30 min in an ice-salt-water bath at 0–4°. The solubilized protein mixture was centrifuged for 60 min at 105,000 *g* at 4°. The supernatant was immediately added to the activated agarose matrix.

Enzyme immobilization. Sepharose 4B (250 mL; 80 g) was washed with 2.5 L of distilled water, and suspended in 250 mL distilled water. Subsequently 250 mL of a 2 M Na_2CO_3 solution (room temperature) was added, under constant stirring on ice. Cyanogen bromide (25 g) was dissolved in 50 mL acetonitrile, and added to the agarose. The activation reaction was allowed to proceed for 2–3 min, under constant stirring in an ice-salt-water bath at 0–4°. The activated agarose was decanted, and washed with 250 mL 0.1 M Na_2CO_3 (room temperature), followed by 2 L of distilled water and 500 mL of a 0.25 M sucrose buffer containing 0.1 M potassium phosphate (pH 7.7). The washed agarose was resuspended in 250 mL of the sucrose–phosphate buffer. The solubilized enzyme mixture was added and incubated under gentle shaking at 4° for 15 hr to immobilize the enzyme on the activated agarose matrix. Then the agarose was washed with 250 mL 0.1 M glycine–NaOH buffer containing 0.1 mM Na_2EDTA, 0.4 mM dithiothreitol, 0.2 mM NaN_3 (pH 6.9) and 50% glycerol. The washed agarose was resuspended in this buffer and shaken gently at 4° for another 15 hr. The agarose was finally washed with a 0.1 M Tris–HCl buffer containing 2 mM dithiothreitol, 0.1 mM Na_2EDTA, 0.2 mM NaN_3 (pH 7.4) and 20% glycerol, and resuspended in this buffer. The agarose beads with the immobilized enzyme were stored in this buffer at 4°.

Enzyme assays. In order to assay UDPGT activity, 4-nitrophenol [10] was used; UDPGT activity towards other substrates was measured with the NAD^+–NADH linked assay [11]. Protein concentrations were measured by the method of Lowry *et al.* [12] with bovine serum albumin as standard. Apparent K_m and V_{max} values were obtained by nonlinear least-squares fitting of initial rate data using the HYPER program developed by Cleland [13].

* Abbreviations: UDPGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

Table 1. Glucuronidation of some aglycones using immobilized rat liver UDPGT and Triton X-100-activated microsomes

Substrate	Concn (mM)	Glucuronidation rate (nmol/min/mg)	
		Immobilized UDPGT	Microsomes
Phenols			
4-Nitrophenol	0.5	46	38
3-Methyl-2-nitrophenol	0.5	128	144
2-Chlorophenol	0.5	120	72
Naphthols			
1-Naphthol	0.5	31	30
2-Naphthol	0.5	35	41
5,6,7,8-Tetrahydro-2-naphthol	0.5	28	41
(*R*)-(−)-1,2,3,4-Tetrahydro-1-naphthol	0.6	ND	ND
(*S*)-(+)-1,2,3,4-Tetrahydro-1-naphthol	0.6	ND	ND
Coumarins			
7-Hydroxycoumarin	0.05	2.4	ND
4-Methyl-7-hydroxycoumarin	0.05	4.0	1.5
Steroids			
Androsterone	0.05	ND	ND
Testosterone	0.05	ND	5.3

Glucuronidation rates were measured at 37°. Protein concentration was 75 μg/mL of immobilized enzyme and 280 μg/mL of Triton X-100-activated microsomal preparation, respectively. The substrates were dissolved in ethanol/water; the final concentration of ethanol in the incubation buffer was 4% (v/v).

ND means that glucuronidation activity could not be detected.

Table 2. K_m and V_{max} values for the immobilized UDPGT

Substrate	Concn range (mM)	K_m (mM)	V_{max} (nmol/min/mg)
4-Nitrophenol	0.05–1.0	0.8	135
1-Naphthol	0.01–1.0	0.1	35
4-Methyl-7-hydroxycoumarin	0.01–1.0	0.7	95

Kinetic constants were determined with the NAD^+–NADH linked assay. Initial UDPGA concentration was 1.5 mM.

Results and Discussion

Since the rat is the most commonly used and readily available experimental animal, we investigated whether we could bind rat liver UDPGT covalently to a solid support, as described for rabbit [4], rhesus monkey and human [8, 9] liver UDPGT.

UDPGTs are located in the endoplasmic reticulum and they have to be solubilized before they can be covalently bound to a solid support. When rat liver microsomes were solubilized with sodium cholate or Triton X-100, as described for rabbit liver UDPGT solubilization [4–6], and subsequently bound to a solid support, all UDPGT activity towards 4-nitrophenol was lost. However, the zwitterionic detergent CHAPS [8, 9, 14] at 8 mM solubilized 90% of the microsomal proteins, while UDPGT was still active after binding. Our results on rat liver UDPGT are in good agreement with the solubilization of mouse liver UDPGT activity [14]: in both cases 90% of the total protein was solubilized. At this detergent concentration, glucuronidation of 4-nitrophenol by the solubilized UDPGTs was also maximal. Of the CHAPS solubilized proteins, 55–65% was covalently bound to the matrix. The protein amount, covalently bound to the matrix, was between 2 and 3 mg/g activated Sepharose beads.

The enzyme was stable when immobilized on the agarose matrix: it remained fully active for 50 days when stored at 0–4° in the Tris–glycerol buffer; thereafter, activity declined. Activity of solubilized non-immobilized UDPGTs declined within 24 hr by 80% when stored at 0–4° in the Tris–glycerol buffer.

The immoblized enzyme exhibited a linear time course of glucuronidation over a period of 1 hr. Glucuronidation rate was linear with protein concentration, over the range 0–125 μg/mL. The immobilized rat liver UDPGTs show a rather broad pH optimum between pH 5 and 7.4. The optimum temperature was around 37°. The ethanol content, used for dissolving the substrates, was less than 4% (v/v); at higher ethanol concentrations UDPGT activity declined.

Characterization of the substrate specificity of the covalently bound UDPGT activity was done with a variety of aglycones (Tables 1 and 2). Since purified UDPGT isozymes have an overlapping substrate specificity [1] this does not enable which enzyme forms were bound to be determined. The fact that Triton X-100-activated

microsomes are able to conjugate a larger variety of aglycones than the immobilized rat liver UDPGT suggests that not all isozymes were covalently bound or solubilized.

The lack of stability of UDPGTs seems to be the main problem in purification of these membrane-bound enzymes, which may be explained by the loss of protection offered by the lipid bilayer. Immobilization of partially or fully purified rat liver UDPGT enzymes to Sepharose prolongs their stability activities as seen with rabbit liver [4]. Therefore, the immobilization of rat liver UDPGT potentially provides a versatile method for studying drug metabolism *in vitro* and for synthesizing UDPGA-conjugated metabolites, and offers an alternative to the rabbit preparation.

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REFERENCES

1. Mulder GJ, Coughtrie MWH and Burchell B, Glucuronidation. In: *Conjugation Reactions in Drug Metabolism* (Ed. Mulder GJ), pp. 51–105. Taylor & Francis, New York, 1991.
2. Falany CN and Tephly TR, Separation, purification and characterization of three isoenzymes of UDP-glucuronsyltransferase from rat liver microsomes. *Arch Biochem Biophys* **227**: 248–258, 1983.
3. Kirkpatrick RB, Falany CN and Tephly TR, Glucuronidation of bile acids by rat liver 3-OH Androgen UDP-glucuronsyltransferase. *J Biol Chem* **259**: 6176–6180, 1984.
4. Parikh I, MacGlashan DW and Fenselau C, Immobilized glucuronosyltransferase for the synthesis of conjugates. *J. Med Chem* **19**: 296–299, 1976.
5. Fenselau C, Pallante SL and Parikh I, Solid-phase synthesis of drug glucuronides by immobilized glucuronsyltransferase. *J Med Chem* **19**: 679–683, 1976.
6. Lehman JP, Ferrin I, Fenselau C and Yost GS, Simultaneous immobilization of cytochrome P450 and glucuronsyltransferase for synthesis of drug metabolites. *Drug Metab Dispos* **9**: 15–18, 1981.
7. Pallante SL, Lisek CA, Dulik DM and Fenselau C, Glutathione conjugates. Immobilized enzyme synthesis and characterization by fast atom bombardment mass spectrometry. *Drug Metab Dispos* **14**: 313–318, 1986.
8. Dulik DM and Fenselau C, Species-dependent glucuronidation of drugs by immobilized rabbit, rhesus monkey, and human UDP-glucuronosyltransferases. *Drug Metab Dispos* **15**: 473–477, 1987.
9. El Mouelhi M, Ruelius HW, Fenselau C and Dulik DM, Species-dependent enantioselective glucuronidation of three 2-arylpropionic acids. *Drug Metab Dispos* **15**: 767–772, 1987.
10. Burchell B and Weatherhill P, 4-Nitrophenol UDP-glucuronsyltransferase (rat liver). *Methods Enzymol* **77**: 161–177, 1981.
11. Mulder GJ and van Doorn ABD, A rapid NAD^+-linked assay for microsomal uridine diphosphate glucuronosyltransferase of rat liver and some observations on substrate specificity of the enzyme. *Biochem J* **151**: 131–140, 1975.
12. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J. Biol Chem* **193**: 265–275, 1951.
13. Cleland WW, The statistical analysis of enzyme kinetic data. In: *Advances in Enzymology* (Ed. Nord FF), pp. 1–32. Interscience, New York, 1967.
14. MacKenzie PI, Lang MA and Owens IS, Effect of different detergent systems on the molecular size of glucuronosyltransferase and other microsomal drug-metabolizing enzymes. *Membrane Biochem* **5**: 193–207, 1984.

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Effects of chronic ethanol feeding on rat liver mitochondrial energy metabolism

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Abstract—Chronic alcohol consumption is known to decrease hepatic mitochondrial respiration rate. It was shown here that the proton leak through the mitochondrial inner membrane was unaffected by chronic ethanol treatment. This indicates that changes in proton leak are not responsible for the alterations in respiration found in mitochondria isolated from ethanol-treated rats. Therefore, the lowered coupled respiration rate is solely due to a decrease in the activity of the electron transport chain. However, this alteration was only evident in coupled respiration (i.e. state 4) and was not apparent in uncoupled respiration. Thus, chronic ethanol treatment decreases the activity of the mitochondrial electron transport chain components which have control over coupled, but not uncoupled, respiration. Mitochondrial energy metabolism is regulated by thyroid hormone status. It was shown that the chronic alcohol treatment did not affect the circulating levels of thyroxine. Furthermore, the activity of mitochondrial α-glycerophosphate dehydrogenase, which is strongly affected by thyroid hormones, was unaltered by alcohol treatment. Thus, the effects of ethanol treatment on mitochondria occur independently of changes in circulating thyroid hormone levels.