The Phospholipid-Dependence of Uridine Diphosphate Glucuronyltransferase

TEMPERATURE-DEPENDENCE OF MICROSOMAL ENZYME ACTIVITY (REVERSE REACTION)

Jeffrey CUMMINGS, Allan B. GRAHAM and Geoffrey C. WOOD

Drug Metabolism Research Unit, Department of Pharmaceutical Chemistry, University of Strathclyde, Glasgow G1 1XW, Scotland, U.K.

(Received 3 August 1979)

Arrhenius plots of the non-latent UDP-glucuronyltransferase reverse reaction (*p*-nitrophenyl glucuronide donor) activity of guinea-pig microsomal membranes prepared with 154 mM-KCl were linear from 5 to 40°C. These plots for other preparations from guinea-pig and rat liver (i.e. preparations that show transferase latency) exhibited two linear regions intersecting at a transition point near 19–21°C. This discontinuity was abolished when latency was removed by treating the membranes with perturbants of phospholipid-bilayer structure. Thus the temperature-dependences of the reverse reaction catalysed by the enzymes of these various preparations are similar to those of the corresponding forward reactions [Pechey, Graham & Wood (1978) *Biochem. J.* 175, 115–124]. Perturbants activated the enzyme of KCl-prepared guinea-pig microsomal membranes only slightly and caused no significant alteration to Arrhenius plots of its forward or reverse reaction activities. These results support the 'compartmentation' theory of UDP-glucuronyltransferase latency.

UDP-glucuronyltransferase (EC 2.4.1.17) catalyses the reversible transfer of glucuronic acid from UDP-glucuronate to a variety of acceptor molecules. The liver microsomal enzyme is modulated by the intact phospholipid structure of native microsomal membranes (see Dutton & Burchell, 1977; Wood *et al.*, 1978; Graham *et al.*, 1979). In most microsomal fractions, especially those isolated with 0.25 M-sucrose, the enzyme is latent and membrane phospholipids contribute to restrict activity. Mild membrane perturbation activates latent enzyme, and the resulting highly active form(s) of the transferase requires intact phospholipid for activity (Jansen & Arias, 1975; Berry *et al.*, 1976). Transferase activity is less latent when microsomal membranes are isolated and washed with 154 mM-KCl, and in this type of preparation from guinea-pig liver the enzyme is almost free from latency (see Pechey *et al.*, 1978) and appears, unlike latent enzyme forms, to be located near the external surface of the microsomal membrane (Graham *et al.*, 1979). This highly active transferase form requires phosphatidylcholine for full activity (Graham *et al.*, 1977; Wood *et al.*, 1978).

Two theories of UDP-glucuronyltransferase latency have emerged. One view is that transferase

molecules are located deep within the microsomal membranes, so that phospholipids act as part of a hydrophobic barrier to substrates (see Wood *et al.*, 1978; Hallinan, 1978; Graham *et al.*, 1979). It is envisaged that mild perturbation activates by increasing membrane permeability to substrates. Zakim & Vessey (1976) have proposed an alternative model in which latency is due to intact membrane phospholipid constraining the enzyme in a low-activity conformation. According to this theory, phospholipid perturbants activate by altering protein conformation, producing a variety of enzyme conformers (see Vessey & Zakim, 1978). Part of the evidence used to formulate this theory was that perturbants differentially affected the kinetic parameters of the forward (glucuronidation) and reverse reactions (see Vessey & Zakim, 1978).

We have studied the temperature-dependence of *p*-nitrophenol glucuronidation in intact and perturbed liver microsomal membranes (Pechey *et al.*, 1978; Graham *et al.*, 1978). Arrhenius plots of the activity of guinea-pig microsomal membranes were linear from 5 to 40°C, whereas those for membranes showing transferase latency exhibited two linear regions intersecting at a sharp transition point near 20–25°C. This discontinuity was abolished by

perturbing membrane phospholipids. We detected a thermotropic change in the fluidity of the phospholipid acyl chains of all the microsomal preparations studied, at temperatures close to those of the Arrhenius-plot transitions, and we concluded that the thermotropic change in lipid-bilayer structure affected membrane permeability to transferase substrates. To study membrane modulation of the enzyme further, we now extend this work by investigating the influence of temperature on the reverse reaction both before and after membrane perturbation. We also present Arrhenius plots of transferase activity in perturbant-treated guinea-pig microsomal membranes prepared with 154 mM-KCl. The data are discussed in relation to current ideas of transferase latency.

Materials and Methods

Materials

UDP and glucaro-1,4-lactone were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. *p*-Nitrophenyl β -D-glucuronide (free acid) was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Male rats (Wistar strain, 200–250 g) were from Animal Supply Ltd., Welwyn, Herts., U.K. Preparation of microsomal fractions, their storage and all other materials were as described by Pechey *et al.* (1978).

UDP-glucuronyltransferase assays

Forward reaction. Transferase activities (nmol of *p*-nitrophenol glucuronidated/min per mg of microsomal protein) were determined with 0.6 mM-*p*-nitrophenol and 4 mM-UDP-glucuronate (Pechey *et al.*, 1978) and were calculated from initial reaction velocities.

Reverse reaction. Activities were measured with two sets of substrate concentrations. At the higher set of concentrations 5 mM-UDP and 10 mM-*p*-nitrophenyl glucuronide were used (Zakim & Vessey, 1973), and at the lower concentrations, 2 mM-UDP and 4 mM-*p*-nitrophenyl glucuronide (Berry *et al.*, 1975; Graham *et al.*, 1979). In both determinations the reaction mixtures (final vol. 0.8 ml) also contained 50 mM-KH₂PO₄/KOH buffer (pH 7.1), 4 mM-EDTA and 10 mM-glucaro-1,4-lactone. After addition of microsomal protein, portions (0.1 ml) were removed at six appropriate times and added to 2 ml of cold 0.1 M-trichloroacetic acid (Graham *et al.*, 1979). Activities (nmol of *p*-nitrophenol liberated/min per mg of microsomal protein) were calculated from initial velocities.

For Arrhenius plots, activities were determined at various temperatures between 5 and 40°C with these amounts of microsomal protein in the reaction mixtures: forward reaction, 0.5–8 mg; reverse reaction, 1–20 mg. The concentrations of KCl or sucrose were

constant over the entire temperature range (Pechey *et al.*, 1978); the variation of buffer pH with temperature and its influence on UDP-glucuronyltransferase activity are described by Pechey *et al.* (1978).

Perturbation methods

Microsomal membranes (final concn. 5 or 10 mg of protein/ml) were treated with phospholipase A (EC 3.1.1.4), Triton X-100, lysophosphatidylcholine or linoleic acid essentially as described by Pechey *et al.* (1978). The following conditions were shown to produce maximum activation of the transferase reverse reaction by using rat microsomal fraction prepared with sucrose: phospholipase A, 10 μ g/mg of microsomal protein for 10 min at 20°C; Triton X-100, 0.2%; lysophosphatidylcholine, 0.3 μ mol/mg of microsomal protein; linoleic acid, 0.5 μ mol/mg of microsomal protein for 10 min at 20°C.

Results and Discussion

Arrhenius plots for the activity of the UDP-glucuronyltransferase reverse reaction in liver microsomal membranes from guinea-pig and rat prepared with 154 mM-KCl or 0.25 mM-sucrose are shown in Fig. 1. The transferase of the KCl-prepared guinea-pig membranes, which is almost free from latency in both forward (Pechey *et al.*, 1978; see also Fig. 3 of the present paper) and reverse (Graham *et al.*, 1979; see also Fig. 3 of the present paper) reactions, exhibited linear plots from 5 to 40°C (Fig. 1a), with values of activation energy (E_a) of 76–105 kJ/mol (Table 1). The remaining microsomal fractions, which show different degrees of transferase latency of both reactions (Pechey *et al.*, 1978; Graham *et al.*, 1979; see also Table 2 and Fig. 2), gave Arrhenius plots (Figs. 1b–1d) with two linear portions intersecting at a transition point (T_c). Although values of E_a varied between microsomal preparations, in all these preparations E_a above T_c (83–107 kJ/mol) was greater than below this temperature (43–61 kJ/mol, Table 1). Thus the responses of the transferase reverse reaction in all of these membrane preparations are very similar to those reported for the forward reaction (Pechey *et al.*, 1978; Graham *et al.*, 1978). T_c for latent-enzyme reverse reaction was 19–21°C (Table 1), whereas the value for the forward reaction was in the range 20–25°C. With latent transferase in both forward and reverse reactions, values of E_a above T_c were greater than below T_c , but it was observed that values of E_a for the reverse reaction in all four microsomal preparations were higher than those for the forward reaction. As with the forward reaction (Pechey *et al.*, 1978), different substrate concentrations during transferase assay did not significantly affect the enzyme's response to temperature (Fig. 1a).

Fig. 1. Arrhenius plots of UDP-glucuronyltransferase (reverse reaction) activity of native microsomal membranes. Transferase activity was determined with (■) 2 mM-UDP and 4 mM-*p*-nitrophenyl glucuronide or (□) 5 mM-UDP and 10 mM-*p*-nitrophenyl glucuronide. (a) Guinea-pig membranes prepared with 154 mM-KCl; (b) guinea-pig membranes prepared with 0.25 M-sucrose; (c) rat membranes prepared with 154 mM-KCl; (d) rat membranes prepared with 0.25 M-sucrose.

Table 1. Arrhenius-plot transition parameters of microsomal UDP-glucuronyltransferase (reverse reaction) activity. Apparent activation energies (E_a) and transition temperatures (T_c) were estimated from Arrhenius plots of transferase reverse-reaction activity of the various microsomal fractions (see Fig. 1). Activities were determined with (a) 5 mM-UDP and 10 mM-*p*-nitrophenyl glucuronide or (b) 2 mM-UDP and 4 mM-glucuronide.

Animal	Membranes prepared with	Concn. of substrates	$T_c(^{\circ}\text{C})$	$E_a(\text{kJ/mol})$		All values of T measured
				$T < T_c$	$T > T_c$	
Guinea pig	154 mM-KCl	a	—	—	—	76–105
		b	—	—	—	79–84
Guinea pig	0.25 M-sucrose	a	19–20	49–55	88–102	—
Rat	154 mM-KCl	a	19	44–53	86–96	—
Rat	0.25 M-sucrose	a	20–21	43–61	83–107	—

Table 2. Arrhenius-plot transition parameters of microsomal UDP-glucuronyltransferase (reverse reaction) activity after removal of latency with membrane perturbants

Rat microsomal membranes prepared with 0.25 M-sucrose were treated with the various perturbants as described in the Materials and Methods section. Transferase activity was determined with 5 mM-UDP and 10 mM-*p*-nitrophenyl glucuronide. The extent of perturbant activation is assessed as the percentage increase in activity measured at 37°C. See also Fig. 2 for extent of activation. The data obtained with linoleic acid came from two experiments. In one experiment (a) a transition was observed with slightly different values of E_a above and below T_c . In the other (b) the Arrhenius plot was linear.

Perturbant	Activation (%)	$T_c(^{\circ}\text{C})$	$E_a(\text{kJ/mol})$		All values of T measured
			$T < T_c$	$T > T_c$	
Phospholipase A	400–500	—	—	—	62–69
Linoleic acid	280–350	23 ^(a)	65 ^(a)	72 ^(a)	58 ^(b)
Lysophosphatidylcholine	280–500	21–23	47–51	57–70	—
Triton X-100	230–350	—	—	—	63–64

Transferase latency is overcome by modifying membrane phospholipids with phospholipase A or by treating membranes with detergents (see Pechey *et al.*, 1978). Activation of latent enzyme by phospholipase A is due to surface-active reaction products, and lysophosphatidylcholine and linoleic acid

are powerful activators (Graham & Wood, 1974). Treatment of sucrose-prepared rat microsomal membranes with these agents (under conditions producing maximum activation) strongly activated the reverse reaction over the entire temperature range studied (Fig. 2; Table 2). Phospholipase A and

Fig. 2. Arrhenius plots of UDP-glucuronyltransferase (reverse reaction) activity of rat microsomal membranes treated with perturbants

Membranes prepared with 0.25M-sucrose were treated with the various perturbants as described in the Materials and Methods section. Transferase activity was determined with 5mM-UDP and 10mM-*p*-nitrophenyl glucuronide after perturbation (■) and at 37°C before perturbation (□). (a) Treated with phospholipase A; (b) treated with linoleic acid; (c) treated with lysophosphatidylcholine; (d) treated with Triton X-100. See Table 2 for the parameters estimated.

Triton X-100 abolished the biphasic nature of the Arrhenius plot by lowering E_a above T_c (Figs. 2a, 2d; Table 2). Lysophosphatidylcholine did not remove the transition completely (T_c , 21–23°C), but substantially decreased E_a above T_c to 57–70 kJ/mol (Fig. 2c; Table 2), and linoleic acid in one experiment abolished the transition (Fig. 2b), and in another decreased E_a above T_c to 72 kJ/mol with T_c at 23°C (Table 2). Thus perturbants have very similar effects on the temperature-dependence of the transferase's forward and reverse reactions. In both reactions E_a above T_c is decreased by treating latent enzyme with perturbants; however, it is observed again that the values of E_a for the activated enzyme's reverse reaction (47–72 kJ/mol) are higher than those for its forward reaction (23–41 kJ/mol; Pechey *et al.*, 1978).

From kinetic studies of UDP-glucuronyltransferase (see Zakim & Vessey, 1976; Vessey & Zakim, 1978) it has been proposed that removal of latency by different perturbants produces a variety of high-activity conformers which possess quite different kinetic parameters. Their data also suggest that a single perturbant can affect the kinetic parameters of the transferase forward and reverse reactions in completely different ways. For example, Triton X-100 increased the affinity of transferase for UDP-glucuronate and UDP. However, the detergent had no effect on forward-reaction maximum velocity, but it increased this parameter for the reverse direction (Vessey & Zakim, 1978). If 'conformational constraint' is responsible for transferase latency, it would seem unlikely that the activity of the transferase forms generated by the different perturbants would all respond to temperature in the same way. It

would even be less likely for these different conformers to yield similar Arrhenius plots in both the forward and reverse reactions.

Our data show that the thermotropic alteration in phospholipid-bilayer structure which occurs in all these microsomal membranes near 20–25°C (Pechey *et al.*, 1978) affects both the forward and reverse transferase reactions in the same manner. They also show that the characteristics of the activated enzyme form(s) do not depend on the perturbant used to remove latency. According to the 'compartmentation' theory of latency (see the introduction), membrane perturbants do not alter enzyme properties specifically, but interact with membrane phospholipids, increase membrane permeability and remove latency by facilitating access of substrates to deeply located intramembrane catalytic sites. Our findings are consistent with this hypothesis, and they support our earlier conclusion (Pechey *et al.*, 1978) that the thermotropic change in the membrane lipid bilayer affects permeability by increasing the fluidity of the latent enzyme's phospholipid environment.

This conclusion is also supported by our data on the temperature-dependence of the non-latent transferase form in guinea-pig membranes prepared with KCl. This enzyme form appears to be located at or near the surface of the microsomal membrane, where it would not be constrained by a permeability barrier (Graham *et al.*, 1979). It displays linear Arrhenius plots for both forward (Pechey *et al.*, 1978) and reverse (Fig. 1a) reactions. If permeation of substrates does determine the response of latent enzyme to perturbants, it would be expected that treatment of guinea-pig KCl-prepared membranes with these agents should produce very little activa-

Fig. 3. Arrhenius plots of UDP-glucuronyltransferase activity of guinea-pig microsomal membranes treated with perturbants

Membranes prepared with 154 mM-KCl were treated with the perturbants as described in the Materials and Methods section. Transferase activity was determined after perturbation (■) and at 37°C before perturbation (□). Reverse-reaction activity was determined with 5 mM-UDP and 10 mM-*p*-nitrophenyl glucuronide. (a) Treated with lysophosphatidylcholine, forward reaction; (b) treated with Triton X-100, forward reaction; (c) treated with lysophosphatidylcholine, reverse reaction; (d) treated with Triton X-100, reverse reaction. See Table 3 for the parameters estimated.

Table 3. Arrhenius-plot transition parameters of UDP-glucuronyltransferase activity in perturbant-treated guinea-pig microsomal membranes prepared with 154 mM-KCl

Microsomal membranes were treated with the perturbants as described in the Materials and Methods section. Reverse-reaction transferase activity was determined with 5 mM-UDP and 10 mM-*p*-nitrophenyl glucuronide. The extent of perturbant activation is assessed as the percentage increase in activity measured at 37°C. See also Fig. 3 for extent of activation.

Perturbant	Transferase reaction	Activation (%)	E_a (kJ/mol) at all values of T measured
Lysophosphatidylcholine	Forward	40	63
	Reverse	48	75
Triton X-100	Forward	50–60	68–69
	Reverse	10–60	78–80

tion of the transferase and should not alter values of E_a for either its forward or reverse reactions. Fig. 3 shows the response of the transferase to temperature after treatment with lysophosphatidylcholine or Triton X-100 (under the same conditions producing full activation of the latent enzyme form). Very little increase of enzyme activity was observed (at 37°C) in either catalytic reaction (Table 3). The perturbants did not affect the linearity of the Arrhenius plots and caused no significant change in their slopes. The untreated guinea-pig enzyme had values of E_a for its forward and reverse reactions of 56–63 kJ/mol (Pechey *et al.*, 1978) and 76–105 kJ/mol (Table 1) respectively; after perturbant treatment, values of 63–69 kJ/mol and 75–80 kJ/mol were recorded for these reactions (Table 3).

J. C. thanks the Glasgow Educational Trust for financial support.

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