A Study on Thermostability of Immobilized Uridine Diphosphate-Glucuronyltransferase

Toshio NANBO,* Shin-ichi NINOMIYA and Masao YONEYAMA

Tokai Research Laboratories, Daiichi Pure Chemicals Co., Ltd., 2117 Muramatsu, Tokai-mura, Naka-gun, Ibaraki 319-11, Japan.
Received September 5, 1991

Uridine diphosphate-glucuronyltransferase (UDPGT) in the hepatic microsome of the rat was solubilized with Emulgen 911 and then immobilized on agarose. The thermostability of immobilized UDPGT was compared with solubilized UDPGT and was found to be higher at temperatures above 52.5 °C. The activation energy (E_a) for thermoinactivation was lowered by immobilization. The fluorescence polarization of tryptophanyl residue of immobilized protein showed little change compared with solubilized protein when heated to 54 °C. The influence of immobilization on thermostability of UDPGT is discussed.

Keywords immobilization; microsome solubilization; uridine diphosphate-glucuronyltransferase; thermostability; fluorescence polarization; thermodynamic parameter

Several researchers[1,2] have reported the effect of immobilization on the thermostability of enzyme. This effect suggests that products are obtained in good yield by incubation at high temperature. However, since little is known about the thermostability of drug metabolism enzyme by immobilization,[3] we studied the effect of immobilization on the thermostability of uridine diphosphate-glucuronyltransferase (UDPGT) thermodynamically. UDPGT, catalyzing glucuronidation of various drugs, is located in the microsomal membrane. In this work, we investigated the best conditions for solubilization of UDPGT and its immobilization.

Experimental

Animals Male Wistar rats weighing 260—280 g were used.

Chemicals CNBr-activated Sepharose 4B and Sephadex G-75 were purchased from Pharmacia. Emulgen 911 was supplied from Kao Chemicals (Japan). ^{14}C-*p*-Nitrophenol was synthesized by Daiichi Pure Chemicals.

Solubilization of Microsomal Protein Rat liver was homogenized in 4 volumes of 0.25 M sucrose, and the microsomal fraction was prepared by centrifugation as described by Bock and White.[4] Unless otherwise noted, the microsomal pellet (25 mg protein) was resuspended in 5 ml of 0.1 M phosphate buffer (pH 7.5) containing 3 mg of Emulgen 911 and 1 g of glycerol, and then was stirred at 4 °C for 40 min. The solubilized UDPGT was obtained by centrifugation at 100000 *g* for 1 h.

Standard Immobilization Solubilized microsomal proteins (125 mg protein) were coupled to CNBr-activated Sepharose 4B (5 g) at 4 °C for 6 h in 0.5 M NaCl/0.1 M sodium bicarbonate buffer (pH 8.3). The remaining binding groups on beads were deactivated with the above buffer containing 0.2 M glycine and beads were washed with the coupling buffer. The amount of bound protein was determined from difference in the total protein amount before and after coupling. Protein was assayed by the method of Lowry *et al.*[5]

Enzyme Assay In general, incubations were performed with ^{14}C-*p*-nitrophenol (0.1 mM), uridine diphosphate (UDP)-glucuronic acid (0.3 mM), $MgCl_2$ (2 mM) and 0.28 mg protein in 0.1 M phosphate buffer (pH 7.5) at a total volume of 0.5 ml. The incubation mixture of immobilized UDPGT contained 10% by volume of agarose beads. The mixtures were incubated at 37 °C for 20 min and the product was then assayed in the manner described previously.[6]

Heat Stability Solubilized and immobilized proteins (3 mg) were added to 0.5 ml of 0.1 M phosphate buffer (pH 7.5). The mixture was incubated at various temperatures over 10 min, and then cooled in ice water.

Fluorescence Measurement Solubilized microsomes (0.1 mg protein) were added to 5 mM phosphate buffer (pH 7.4). Immobilized microsomes (0.1 mg protein) were mixed with Sephadex G-75 in 5 mM phosphate buffer (pH 7.4). Fluorescence was measured at 395 nm using an exciting light at 290 nm, and polarization was determined according to the method of Shinitzky and Inbar.[7]

Results and Discussion

To immobilize UDPGT on agarose, we attempted to solubilize this enzyme with Emulgen 911. Figure 1 shows influences of concentrations of microsomal protein and of detergent on this solubilization. The highest specific activity was obtained at 0.5 mg/ml of the detergent when 3.3 mg/ml

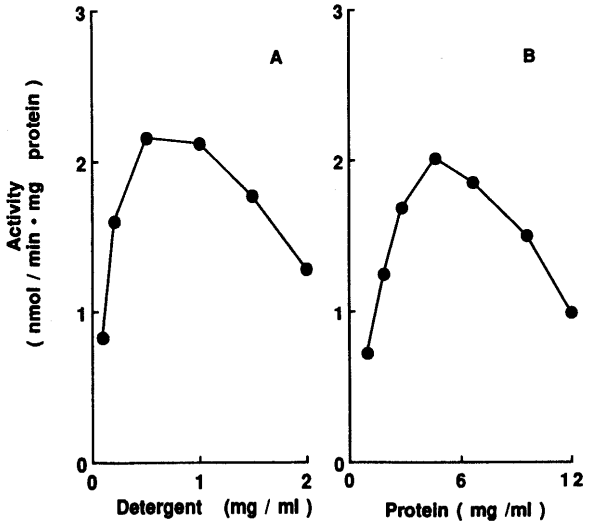

Fig. 1. Influence of Detergent Concentration (A) and Microsomal Protein Concentration (B) on Solubilization of UDPGT

Each point represents the mean of five experiments. A; protein concentration, 3.3 mg/ml. B; detergent concentration, 0.63 mg/ml.

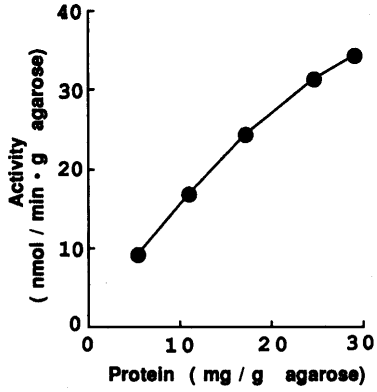

Fig. 2. Relationship between Protein Concentration and Immobilized UDPGT

Each point represents the mean of five experiments.

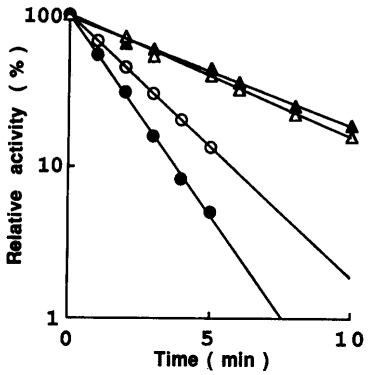

Fig. 3. Time Course of Thermoinactivation of Solubilized and Immobilized UDPGT

The values are the mean of five experiments. ▲, solubilized UDPGT at 48 °C; ●, solubilized UDPGT at 54 °C; △, immobilized UDPGT at 48 °C; ○, immobilized UDPGT at 54 °C.

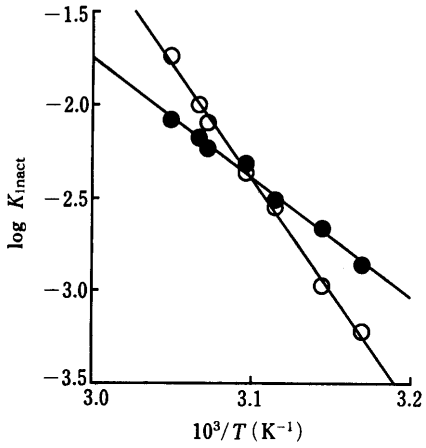

Fig. 4. Arrhenius Plot for Thermoinactivation of Solubilized and Immobilized UDPGT

The values are the mean of five experiments. ○, solubilized UDPGT; ●, immobilized UDPGT.

TABLE I. Effect of Heating on Fluorescence Polarization of Solubilized and Immobilized Microsomes[a]

	Polarization	
	Before heating	After heating
Solubilized	0.147±0.006	0.209±0.008[b] (42.2%)[c]
Immobilized	0.189±0.005	0.213±0.007[b] (12.7%)[c]

Data are means±S.E. of five experiments. *a*) Fluorescence polarization of each sample was measured before and after heating at 54 °C for 15 min. *b*) Significantly different from the value before heating ($p<0.05$). *c*) Increase in ratio.

of protein was used, and at 5 mg/ml of protein when 0.63 mg/ml of the detergent was used. From these results, microsomal protein (4 mg/ml) was solubilized with the detergent as a concentration of 0.63 mg/ml in the standard experiment, since UDPGT in solubilized protein bound effectively to agarose under this condition. Figure 2 shows the relationship between the added amount of protein and the activity of immobilized UDPGT. The activity of UDPGT coupled with agarose increased with the increase in protein amount. For the stability studies, solubilized protein was applied to agarose at 25 mg protein/g agarose; the resulting beads were found to contain 19.5 mg/g beads.

Figure 3 shows the time course of thermal inactivation of solubilized and immobilized UDPGT at 48 °C and 54 °C. The inactivation processes of both enzymes appeared to follow apparent first-order kinetics. No remarkable difference in the profile of residual activity between the two types of UDPGT was observed at 48 °C, whereas the residual activity in immobilized UDPGT decreased more slowly than that in solubilized UDPGT at 54 °C; the effect of thermostability by immobilization is thought to appear at high temperature.

Inactivation of enzyme by heating has been reported[8,9]; thermal inactivation of enzyme was apparently due to conformational change *via* unfolding of protein. Thus we attempted to examine the thermal influence on conformation of protein by fluorescence polarization measurement. As shown in Table I, polarization of the solubilized microsomes was much more increased by heat than were the immobilized microsomes. This increase in polarization indicates the decrease of rotational freedom of tryptophanyl residues due to the environmental change[10]; thus, the change of the polarization in Table I suggests that the conformation of the solubilized was changed more easily than that of the immobilized microsomes. Considering the difference in the thermostability at 54 °C between the two types of UDPGT, the conformational changes of both would relate to that of the corresponding microsomes.

To analyze the thermoinactivation thermodynamically, inactivation rate constants at various temperatures were plotted against Arrhenius plots (Fig. 4). The slope of the line shows the apparent activation energies (E_a) of solubilized and immobilized UDPGT to be 56.1 kcal/mol and 29.7 kcal/mol, respectively. E_a can be interpreted as the energy which is required to change the conformation of UDPGT; therefore, the low E_a value of the immobilized UDPGT suggests that the degree of conformational change was lower than that of the solubilized UDPGT, since the denaturation process involving breakage of the hydrogen bridge is common to both types. We determined the entropy of activation ($\Delta S^{\neq}$) from Fig. 4 using the transition state theory.[11] The values of $\Delta S^{\neq}$ of the solubilized and immobilized UDPGTs were 104 e.u. and 22.4 e.u. at 55 °C, respectively. The increase of $\Delta S^{\neq}$ by denaturation is apparently due to exposure of the hydrophobic chains during the unfolding of protein; this results in destruction of ordered structures of protein and solvent.[12] Thus, the low value of $\Delta S^{\neq}$ in the immobilized UDPGT suggests that the extent of protein unfolding by heat was reduced by immobilization. The enthalpy of activation was 55.5 kcal/mol in solubilized UDPGT and 29.1 kcal/mol in immobilized UDPGT, showing temperature independence; thus neither type of UDPGT changed heat capacity within the temperature range employed in the experiment.

The presence of UDPGT isozymes is well known.[13] This work used microsomes since these offer glucuronides of various drugs, and focused on the thermodynamic mechanism of thermostability by using *p*-nitrophenol which has often been used for UDPGT assay. It is thereofore necessary to keep in mind that present results indicate the thermodynamic characters of some of the isozymes catalyzing *p*-nitrophenol.

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