Organization of Microsomal UDP-Glucuronosyltransferase. Activation by Treatment at High Pressure†

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ABSTRACT: Treatment of microsomes at pressures as high as 2.25 kbar led to an apparent irreversible activation of UDP-glucuronylsyltransferase when pressure was released. The response of the enzyme to pressure, as reflected by activity measured after release of pressure, appeared to be discontinuous in that no activation was seen for any preparation at pressures less than 1.2 kbar. In addition, activation was temperature dependent. Maximum activation at 2.25 kbar occurred at about 12 °C; the extent of activation in 10 min was less for either higher or lower temperatures. Activation was also time dependent. Maximum activation at 2.25 kbar and 9 °C required 90 min of pressure treatment. Activation appeared to occur more slowly at lower pressure. Pressure-induced activation was associated with a loss of sensitivity of the enzyme to allosteric activation by UDP-*N*-Ac-Glc and a conversion of the kinetic pattern from non-Michaelis–Menten to Michaelis–Menten. Pressure did not activate enzyme that had previously been activated maximally by adding detergent to microsomes. Pressure also did not activate pure UDP-glucuronosyltransferse reconstituted into unilamellar vesicles of dioleoylphosphatidylcholine. Pressure treatment did not release UDP-glucuronosyltransferase from microsomes into water. Pressure had a continuous effect on the polarization and excimer/monomer formation of fluorescent probes incorporated into microsomes, and the properties returned essentially to their values at 1 atm when pressure was released. Measurements of activity at 2.2 kbar showed that pressure-induced activation of UDP-glucuronosyltransferase in microsomes occurred via two intermediates that were inactive and that the activated state of the enzyme was generated during/after release of pressure. These experiments also showed that the temperature dependence of pressure-induced activation was due primarily to stabilization of the native state of UDP-glucuronosyltransferase at $T > 20$ °C.

The activity at V_{max} (Hochman et al., 1981; Magdalou et al., 1982), catalytic specificity (Magdalou et al., 1982), allosterism (Hochman & Zakim, 1983; Hochman et al., 1983), and thermal stability (Rotenberg & Zakim, 1989) of the microsomal enzyme UDP-glucuronosyltransferase (EC 2.4.1.108) depend on the nature of the phospholipid in which pure, delipidated enzyme is embedded. Almost all the properties displayed by enzyme in untreated microsomes can be modulated, in fact, by the lipids used to reconstitute pure enzyme (Hochman & Zakim, 1983; Hochman et al., 1983; Rotenberg & Zakim, 1989; Zakim & Vessey, 1982). These data suggest that the regulation of UDP-glucuronosyltransferase by the microsomal environment is a reasonably straightforward problem. We have found, however, that no lipid or combination of lipids, including lipids extracted from microsomes, reconstitutes UDP-glucuronosyltransferase in a state that displays simultaneously the exact properties of enzyme in microsomes (Dannenberg et al., 1989). Bilayers of phospholipids in a gel phase, for example, reconstitute the allosteric properties (Hochman & Zakim, 1983; Hochman et al., 1983) but not the thermotropic properties of enzyme in untreated microsomes (Dannenberg et al., 1989; Rotenberg & Zakim, 1989). Bilayers of 1-stearoyl-2-oleoylphosphatidylcholine reconstitute enzyme with thermotropic properties like those of enzyme in microsomes but not with the same allosteric properties (Kelley et al., 1983; Rotenberg & Zakim, 1989). Possible explanations for failure to reconstitute pure enzyme with all the properties of enzyme in microsomes are irreversible changes in the enzyme during purification, lipid–protein interactions do not account completely for regulation of the functional state of the enzyme in microsomes, or perhaps the enzyme in microsomes is in an organized domain of lipids and proteins that does not form spontaneously in reconstituted systems. These possibilities are not mutually exclusive

Treatment of microsomes at high pressure is a potentially useful tool for probing the possible organization of UDP-glucuronosyltransferase in a domain in microsomes, either with other proteins or with lipids. This is so because the volume of an organized structure, as for example an aggregate of proteins, will be larger than the total volume of the individual monomers in the aggregate. Upon application of pressure, aggregates of protein will dissociate due to more efficient packing of solvent in void volumes (Heremans, 1982; Jaenicke, 1981; Paladini & Weber, 1981; Penniston, 1977; Weber & Drickamer, 1983), replacement of nonpolar interactions with more tightly packed polar ones, and an increase in electrostriction caused by breaking of salt bonds (Kauzman, 1959). Thus, pressure in the range of 1.5–2.5 kbar, which does not appear to alter the tertiary structure of proteins (Heremans, 1982; Weber & Drickamer, 1983), will dissociate oligomeric proteins (Heremans, 1982; Jaenicke & Lauffer, 1969; Jaenicke, 1981; King & Weber, 1986; Paladini & Weber, 1981; Payens & Heremans, 1969; Penniston, 1977; Ruan & Weber, 1988). This leads to changes in the functional states of aqueous globular proteins that are not reversed immediately when pressure is released (King & Weber, 1986; Ruan & Weber, 1988; Silva et al., 1986). If the properties of UDP-glucuronosyltransferse in untreated microsomes depend on organized interactions with other microsomal components, it is likely that treatment of microsomes at high pressure would disrupt these interactions. We therefore examined the effect

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of high pressure on the functional state of UDP-glucuronosyltransferse in microsomes. The data presented below show that application of high presure caused apparently irreversible activation of UDP-glucuronosyltransferase and changed the regulatory properties of the enzyme, which became like those of pure enzyme reconstituted into bilayers in a liquid-crystalline phase (Hochman & Zakim, 1983; Hochman et al., 1983; Dannenberg et al., 1989).

Materials and Methods

Microsomes were prepared from rat liver as in Zakim and Vessey (1973) and stored at −70 °C until use. Pure enzyme was prepared as in Magdalou et al. (1982). Pure enzyme was reconstituted into bilayers of dioleoylphosphatidylcholine as in Rotenberg and Zakim (1989). 3-Palmitoyl-2-(10-pyrenedecanoyl)phosphatidylcholine (10pyPC)[1] and 11-(9-anthroyloxy)undecanoic acid (11AU) were purchased from Molecular Probes (Eugene, OR) and used without further purification. Enzyme activities were assayed by using *p*-nitrophenol as aglycon as described previously (Hochman et al., 1981). Glucuronidation of 1-naphthol was measured fluorometrically (Vaisanen et al., 1983). Excitation was at 320 nm; emission was measured at 460 nm. Exact conditions for assays are given in the tables and legends to figures. Protein was measured with the biuret method (Gornall et al., 1949).

Microsomes were pressure treated in a water-jacketed pressure cell based on the design of Paladini and Weber (1981). Temperatures for pressurization in different experiments are indicated in the text and legends. The pressure gauge used to monitor pressure in the bomb was accurate to ±0.01%. Pressurization was achieved by hand-cranking a pump, which required about 3 min to reach 2.25 kbar (highest pressure achieved). The concentration of microsomes during treatment at high pressure was 4–5 mg of microsomal protein/mL in 50 mM Hepes, pH 7.0. After release of pressure, microsomes were kept on ice until assay of UDP-glucuronosyltransferase. Assays usually were performed within minutes of release of pressure, but neither the interval between release of pressure and assay nor the temperature at which microsomes were stored during this interval affected results.

Fluorescence measurements were made on a Greg-PC spectrofluorometer (I. S. S., Inc., Champaign, IL) equipped with polarizers. For microsomes containing 11AU, excitation was at 381 nm, and polarized emission was collected through a GG 400 filter (Schott Glass Co.) and a monochromator set at 460 nm. Scattered light accounted for less than 1% of the total signal. For microsomes labeled with 10pyPC, excitation was at 340 nm, and emission intensity was collected at 381 and 480 nm for the monomer and excimer peaks respectively. Microsomes (0.35 mg of protein/mL) in 50 mM Hepes, pH 7.0, and 250 mM sucrose were labeled with 11AU (final concentration of 0.38 μg/mL) by adding a small volume of probe dissolved in ethanol. Labeling with 10pyPC was done in the same way. The concentrations of microsomes and probe in these experiments were 1.4 mg/mL and 3 μg/mL, respectively.

Results

High Pressure Activates UDP-Glucuronosyltransferase in Intact Microsomes. The data in Figure 1 illustrate the effect of high pressure on the activity of UDP-glucuronosyltransferase, which was assayed after release of pressure. The data are for treatment for 10 min at 9 °C for each pressure. Activity was unchanged in microsomes treated at 9 °C for 10 min at 1 atm. Several important features of the system are apparent in Figure 1. Treatment of microsomes at high pressure activated UDP-glucuronosyltransferase, but there appeared to be a threshold effect in that no activation was seen after release of pressure for microsomes treated at less than about 1.2 kbar. The extent of activation increased smoothly at pressures higher than 1.2 kbar. The significance of the threshold effect for activation is unclear. It could be related to properties of the enzyme per se, as, for example, cooperativity in activation, or it could reflect that UDP-glucuronosyltransferase does not experience high lateral pressure within the membrane until pressure exceeds 1.2 kbar. Pressure-induced activation was not reversed to a detectable extent over 18 h when microsomes treated at 2.25 kbar were allowed to stand at 0–4 °C on ice or at 25 °C (see below). The question of the irreversibility of the pressure-induced activation is considered under Discussion.

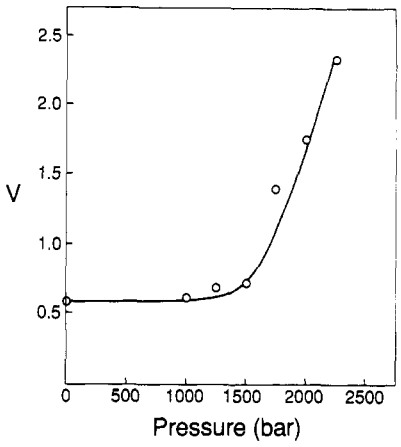

FIGURE 1: Effect of pressure on the activity of UDP-glucuronosyltransferase in microsomes. Microsomes were treated at the indicated pressures at 9 °C for 10 min. After release of pressure, activities were measured at 30 °C as described under Materials and Methods using 1.0 mM UDP-glucuronic acid and 0.05 mM *p*-nitrophenol as substrates. Activities are in nanomoles per minute per milligram of protein.

The extent of pressure-induced activation depended on the pressure applied up to 2.25 kbar, which was the upper limit that could be achieved in our cell. Pressure-dependent activation also depended on the duration of pressurization at any applied pressure (Figure 2). Activation was maximal at 2.25 kbar only after 90 min of treatment (see below). For any time studied, activity was greater at 2.2 kbar than at 1.5 kbar for pressurization at 9 °C. In fact, it seemed that the rate of activation was greater at 2.25 kbar than at 1.5 kbar. We examined whether pressure at 2.25 kbar, applied at 9 °C, led to release of enzyme from microsomes into the water phase. Microsomes treated under these conditions were centrifuged in a Beckman 40 rotor for 60 min at 40000 at 4 °C. UDP-Glucuronosyltransferase activity was measured in the supernatant. Negligible rates of glucuronidation were detected in these supernatants. Thus, it appears that enzyme was not released from microsomes by treatment at high pressure.

The activity of UDP-glucuronosyltransferase is stimulated by treating microsomes with detergents (Leuders & Kuff, 1967; Vessey & Zakim, 1971). A question of interest, therefore, was the relationship between pressure-induced and detergent-induced activation of enzyme. This was tested by treating microsomes with palmitoyllysophosphatidylcholine, which activates UDP-glucuronosyltransferase in microsomes

[1] Abbreviations: 11AU, 11-(9-anthroyloxy)undecanoic acid; 10pyPC, 3-palmitoyl-2-(10-pyrenedecanoyl)phosphatidylcholine.

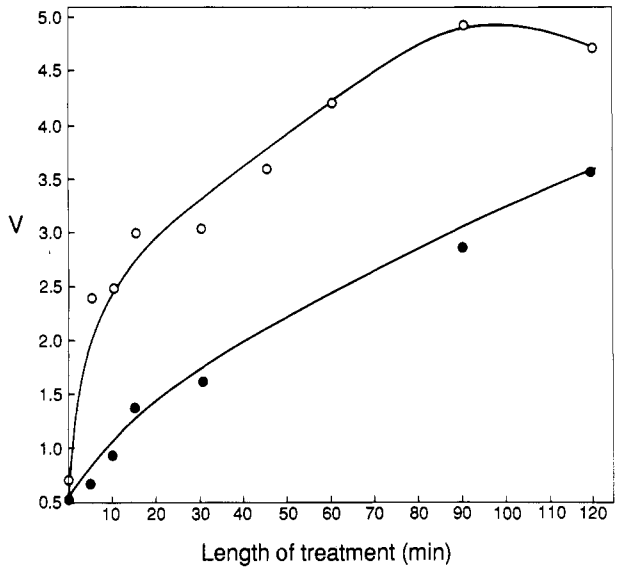

FIGURE 2: Time-dependent activation of UDP-glucuronosyltransferase at high pressure. Microsomes were treated at 1.5 kbar (●) or 2.25 kbar (○) at 9 °C for the times indicated. Enzyme activity was measured as in Figure 1 after release of pressure. Each time point is for a separately treated preparation of microsomes.

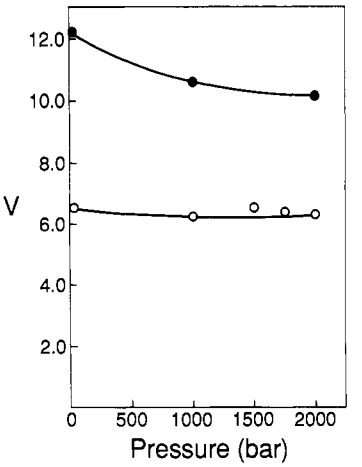

FIGURE 3: Effect of high pressure on the activity of UDP-glucuronosyltransferase in microsomes pretreated with detergent or on pure enzyme. Microsomes represented by the open circles (○) were treated with cholate at 0 °C at a ratio of 1/1 (w/w) cholate/microsomal protein, which gave maximal activation of the enzyme. The cholate-treated microsomes were then treated at the indicated pressures, at 9 °C for 10 min. The data represented by the closed circles (●) are for pure enzyme reconstituted into unilamellar vesicles of dioleoylphosphatidylcholine and treated at pressure in the same way as cholate-treated microsomes. Activities were assayed as in Figure 1 after release of pressure.

(Zakim et al., 1975) and reconstitutes the activity of delipidated enzyme (Hochman et al., 1981). Microsomes were treated at 2.25 kbar for 10 min at 12 °C. Palmitoyllysophosphatidylcholine then was added to treated and untreated microsomes and activity measured as described under Materials and Methods at 30 °C, in the presence of 1 mM UDP-glucuronic acid. Activities for enzyme in microsomes not treated at high pressure were 1.97 and 17.9 nmol min^{-1} (mg of protein)$^{-1}$ for microsomes in the absence and presence of detergent. Values for pressure-treated microsomes were 6.57 and 16.2 nmol min^{-1} (mg of protein)$^{-1}$. These results indicate that the effects of palmitoyllysophosphatidylcholine and pressure were not additive. These activities are higher than for the microsomes used to generate the data in the figures. This was so because microsomes treated with lysophosphatidylcholine, with and without prior pressurization, were from younger rats than microsomes used to generate other data. Enzyme activity is higher in younger versus older rats (Borghoff & Birnbaum, 1985). It is important to point out, however, that the age of rats from which microsomes were collected did not affect the response of UDP-glucuronosyltransferase to pressure or detergents.

On the basis of the response to added palmitoyllysophosphatidylcholine, pressure treatment of microsomes at 2.25 kbar for 90 min, as in Figure 2, appeared to produce maximum activation of UDP-glucuronosyltransferse to the same extent as the lysophosphatide. Addition of palmitoyllysophosphatidylcholine to microsomes treated this way did not lead to further activation of the enzyme, but activity in microsomes treated at 2.2 kbar, 10 °C, for less than 90 min was enhanced by adding palmitoyllysophosphatidylcholine (data not shown). Application of 2.25 kbar to microsomes treated previously with an amount of detergent that activated UDP-glucuronosyltransferase maximally did not cause further activation of the enzyme (Figure 3). In addition, pressure did not activate pure enzyme that was embedded in unilamellar bilayers of dioleoylphosphatidylcholine (Figure 3). In fact, activity measured after release of pressure was lower for pure enzyme than activity prior to pressurization.

Effect of Temperature on Pressure-Induced Activation of UDP-Glucuronosyltransferase. The data in Table I show the

Table I: Effect of Temperature on Pressure-Induced Activation of UDP-Glucuronosyltransferase[a]

	activity [nmol min^{-1} (mg of protein)$^{-1}$]	
T (°C)	pressure	pressure + detergent
−8	2.74	9.48
2	3.79	11.39
12	4.16	11.73
23	1.92	11.67
34	2.06	9.19

[a] Microsomes were treated for 10 min at 2.25 kbar at the indicated temperatures. After release of pressure, activity was assayed as described under Materials and Methods at 30 °C with 1.0 mM UDP-glucuronic acid and 0.05 mM *p*-nitrophenol. Palmitoyllysophosphatidylcholine when present was added to a ratio of 1/5 (w/w), which gave maximum activation of UDP-glucuronosyltransferase for both pressure-treated and untreated microsomes. Activity in microsomes not treated at 2.25 kbar was 1.23 nmol min^{-1} mg of protein)$^{-1}$ and increased to 14.0 nmol min^{-1} mg of protein)$^{-1}$ after addition of detergent.

effects of temperature on pressure-induced activation of the enzyme at 2.25 kbar for 10 min. Maximum activation was achieved at 12 °C. The extent of activation was less at either lower or higher temperatures. The second column of activities in Table I is for assays of pressure-treated microsomes after addition of an amount of palmitoyllysophosphatidylcholine that gave maximum activation of the preparation. The activities after addition of lysophosphatidylcholine indicate that the limited pressure-induced activation of enzyme at some temperatures, 23 °C, 2.2 kbar for example, did not reflect combined effects of pressure-induced activation and thermal inactivation of a modified form of UDP-glucuronosyltransferase. A pressure titration of microsomes at 25 °C is shown in Figure 4. These data show no pressure-induced activation of enzyme until pressure exceeded 1.5 kbar. However, if microsomes were pressurized at 9 °C and then warmed to 25 °C after release of pressure, UDP-glucuronosyltransferase remained activated for at least 18 h (longest time studied).

Effect of Pressure on the Allosteric Properties of UDP-Glucuronosyltransferase. Activation of UDP-glucuronosyltransferase in microsomes, by any method reported to date,

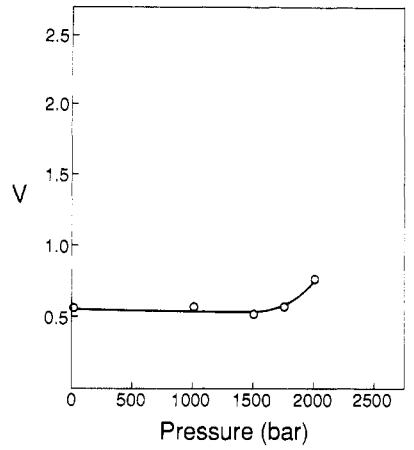

FIGURE 4: Pressure–titration of UDP-glucuronosyltransferase in microsomes at 25 °C. Microsomes were treated at the indicated pressures for 10 min at 25 °C. Activities of UDP-glucuronosyltransferase were assayed after release of pressure as in Figure 1.

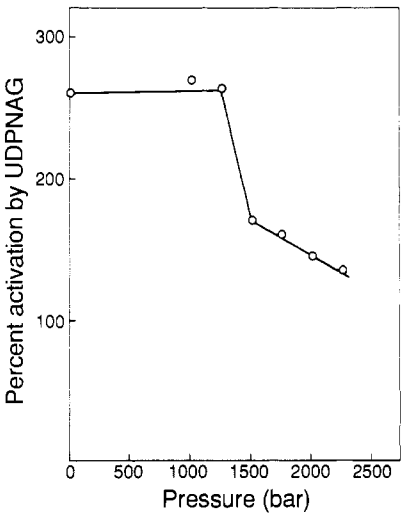

FIGURE 5: Effect of pressure on activation of UDP-glucuronosyltransferase in microsomes by UDP-*N*-Ac-Glc. Microsomes were treated at the indicated pressures at 9 °C for 10 min. After release of pressure, enzyme activity was assayed as in Figure 1 in the presence 1.0 mM UDP-*N*-Ac-Glc. The data are given as percent activation by UDP-*N*-Ac-Glc for each pressure.

is associated with a loss of sensitivity to activation of the enzyme by UDP-*N*-Ac-Glc (Vessey et al., 1973; Winsnes, 1971; Zakim et al., 1973) and conversion of the non-Michaelis–Menten kinetics displayed by enzyme in untreated microsomes to Michaelis–Menten kinetics (Zakim et al., 1973). Pressure treatment had similar effects on these regulatory characteristics of the enzyme. There was, for example, a correspondence to the pressure-induced activation of the enzyme (Figure 1) and loss of sensitivity to activation by UDP-*N*-Ac-Glc (Figure 5). The data in Figure 6 show that pressure-induced activation changed the shape of the double-reciprocal plot for activity as a function of the concentration of UDP-glucuronic acid. The plot was more linear for pressure-treated versus untreated microsomes. Hill plots of the data in Figure 6 gave slopes of 0.54 for untreated and 0.82 for pressure-treated microsomes. Note, however, that despite the pressure-induced changes that UDP-*N*-Ac-Glc did activate pressure-treated enzyme to a small extent (Figure 5) and the double-reciprocal plot for pressure-treated enzyme in Figure 6 was not linear. These effects are expected in that the pressure treatments in Figures 5 and 6 did not fully activate UDP-glucuronosyltransferase. So the microsomes used in Figures 5 and 6 contained mixtures of UDP-glucuronosyltransferase in the native state and in the state induced by high pressure.

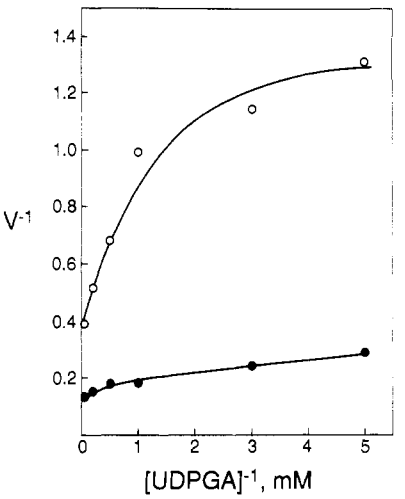

FIGURE 6: Effect of treatment at high pressure on the kinetic pattern of UDP-glucuronosyltransferase in microsomes. Microsomes were treated at 2.25 kbar, 12 °C, for 15 min (●). Control microsomes were kept at 1 atm (○). Activities were measured after release of pressure, as in Figure 1 at the indicated concentrations of UDP-glucuronic acid. Data are plotted in double-reciprocal form.

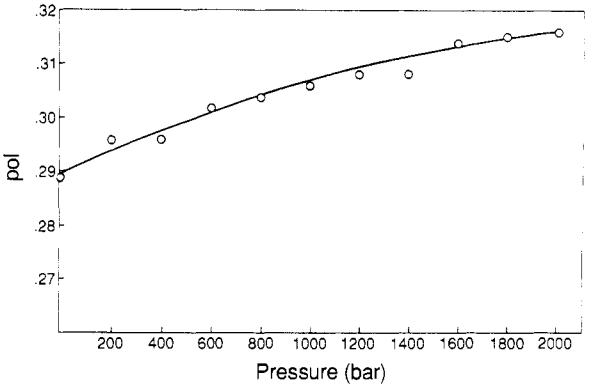

FIGURE 7: Polarization of 11AU in pressure-treated microsomes at 9 °C. All data are corrected for pressure-induced window birefringence (Paladini & Weber, 1981) and have an average error of ±0.002.

Effects of Pressure on the Properties of the Bulk-Phase Lipids in Microsomes. The anisotropy of 11AU-labeled microsomes increased smoothly and reversibly with pressure (Figure 7) due to pressure-induced increases in viscosity. As compared with the effects of pressure on the activity of UDP-glucuronosyltransferase, there was no apparent threshold effect for the onset of changes of anisotropy. The intensity of fluorescence remained essentially constant over the entire range of pressure studied in Figure 7. Therefore, we believe that the change in anisotropy cannot be attributed to changes in the fluorescence lifetime of the probe. The values of the anisotropy are higher in these experiments than those reported in fluid model membranes [e.g., see Thulborn and Sawyer (1978)], which indicates that the probe molecules in our experiments occupied more rigid regions of the microsome and/or were subject to a quencher. We also measured the amount of pyrene energy transfer with pressure, using 10pyPC, since this parameter is known to be sensitive to membrane fluidity (Eisinger et al., 1986). The excimer/monomer ratio decreased smoothly with pressure (data not shown), corroborating the results from the measurements of anisotropy. In addition, the excimer/monomer ratio returned to within 10% of the starting value on release of pressure, the ratio being smaller after release of pressure as compared with untreated microsomes.

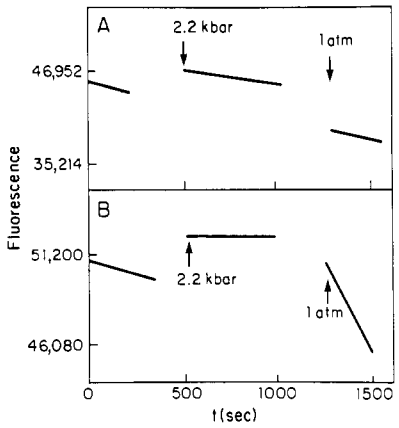

FIGURE 8: Activity of UDP-glucuronosyltransferase at high pressure. The pressure cell contained 0.02 mM 1-naphthol, 1.0 mM UDP-glucuronic acid, and 50.0 mM Hepes, pH 7.5, in a final volume of 1.4 mL. Reaction was started by adding 0.045 mg of microsomal protein in (A) and 0.09 mg of microsomal protein in (B). Temperature was 36 °C in (A) and 11 °C in (B). Excitation was at 320 nm, and emission was measured at 460 nm as described under Materials and Methods. Initial conditions were 1 atm in (A) and (B). Arrows indicate the times at which the noted conditions were reached. Data were redrawn from the real-time record of fluorescence. The intervals between data points reflect the times needed to effect the noted difference in conditions.

Activity of UDP-Glucuronosyltransferase at 2.2 kbar. We do not have an absorption spectrophotometer that accommodates the pressure cell. For this reason, UDP-glucuronosyltransferase was assayed at 2.2 kbar with a fluorescent aglycon, 1-naphthol, which is conjugated by the same pure isoform of UDP-glucuronosyltransferase that conjugates *p*-nitrophenol (Hochman & Zakim, 1983; Magdalou et al., 1982). This result does not ensure that 1-naphthol and *p*-nitrophenol are conjugated exclusively by the same isoforms in microsomes, but the data indicate similar responses to the pressure of activities measured with either substrate.

Microsomes were mixed with a complete assay system in the pressure cell within the fluorometer. The cell was thermostated at the temperatures indicated in Figure 8A,B. Pressure was increased to 2.2 kbar, which took 3–4 min. When pressure was applied at 36 °C, activity at 2.2 kbar was less than at 1 atm (Figure 8A). When pressure was released, the activity of UDP-glucuronosyltransferase returned to the control value. The system behaved differently when pressurization was at 11 °C (Figure 8B). There was no conjugation of 1-naphthol at 2.2 kbar, 11 °C, and when pressure was released at this temperature, the enzyme was activated as compared with untreated enzyme. Since the reaction pathway for glucuronidation must be the same at 11 and 36 °C, ΔV^* for this reaction will be the same at these temperatures. It follows, therefore, that 2.2 kbar applied at 11 °C effected a transition of native GT to a pressure-modified form with no activity, e.g., E → E′. This reaction did not appear to occur at 36 °C, as reflected by the small effect of pressure at this temperature on the activity of UDP-glucuronosyltransferase. However, E′, when formed at 2.2 kbar, 11 °C, reverted to E when temperature was increased to 20 °C and then 30 °C while pressure was maintained at 2.2 kbar (Figure 9). Therefore, E ↔ E′ is reversible, and clearly high temperature stabilizes E versus E′, which explains the lack of activation of UDP-glucuronosyltransferase when pressure is applied at relatively high temperature.

Comparison of the results in Figures 2 and 8B indicates that E′ is not the activated state of UDP-glucuronosyltransferase or even the immediate precursor of this state. Thus, the data

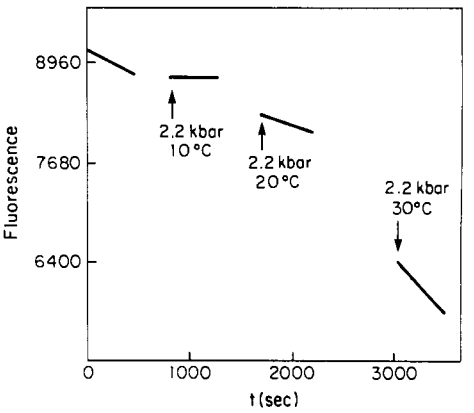

FIGURE 9: Reversibility of pressure-induced inactivation of UDP-glucuronosyltransferase at 2.2 kbar. The rate of glucuronidation of 1-naphthol was measured as in Figure 8. The concentration of microsomes was 0.046 mg of protein/mL. Initial conditions were 10 °C at 1 atm. Arrows indicate the times at which the noted conditions were reached. The intervals between data points have the same meaning as in Figure 8.

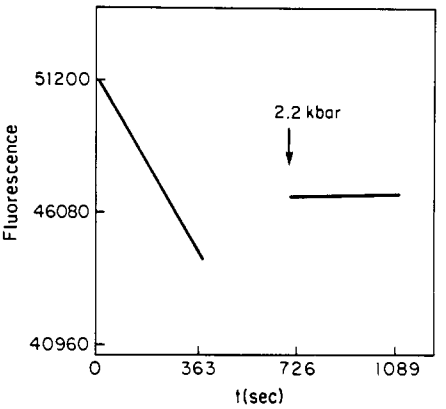

FIGURE 10: Pressure-induced inactivation of the activated state of UDP-glucuronosyltransferase. Microsomes were treated at 2.2 kbar, 11 °C for 12 min. Pressure was then released to 1 atm. The rate of glucuronidation was measured in the pressure cell as in Figure 8. The temperature was kept at 11 °C and the pressure increased to 2.2 kbar, which was reached at the arrow.

in Figure 8B show that the conversion of E to E′ was complete in less than 4 min, but the data in Figure 2 show that 90 min at 2.2 kbar, 9 °C was required for maximum activation of the enzyme. Therefore, E′ must convert (at ~10 °C, 2.2 kbar) to another form of UDP-glucuronosyltransferase, which we designate E″, in a reaction that occurs slowly even at 2.2 kbar. We found in separate experiments that no rate of conjugation of 1-naphthol was detected after prolonged treatment of microsomes at 2.2 kbar, 10 °C, which are the conditions that generate E″. Therefore, the E″ state of UDP-glucuronosyltransferase is inactive, and the activated state seen after release of pressure must be generated by release of pressure. E′ → E″ could not be reversed by application of pressure at 2.2 kbar, 11 °C, and then raising the temperature to 30 °C while maintaining pressure. Release of pressure at 30 °C in this experiment led to activation of UDP-glucuronosyltransferase (data not shown). On the other hand, the generation of the activated state of UDP-glucuronosyltransferase, designated as E*, was reversed by pressure (Figure 10). These data are compatible with reaction 1 as the pathway for pressure-induced

$$E \underset{-P}{\overset{+P}{\rightleftharpoons}} E' \rightarrow E'' \underset{+P}{\overset{-P}{\rightleftharpoons}} E^* \qquad (1)$$

activation of UDP-glucuronosyltransferase, although it is not certain that E″ is the immediate precursor of E*. An obvious and potentially important difference between the experiments

with *p*-nitrophenol and 1-naphthol is that pressure was applied in the latter case in the presence of substrates, which could alter the response of UDP-glucuronosyltransferase to pressure (Varga et al., 1986). The effect of pressure on UDP-glucuronosyltransferase in microsomes was examined, therefore, in the presence of UDP-glucuronic acid. Enzyme was assayed in these experiments with *p*-nitrophenol after release of pressure. We found no significant effect of UDP-glucuronic acid on the pressure-induced activation of UDP-glucuronosyltransferase (data not shown).

Discussion

Purification of UDP-Glucuronosyltransferase Appears To Alter the Enzyme Irreversibly. Irrespective of the mechanism by which pressure activates UDP-glucuronosyltransferase, the data for the effects of pressure provide an explanation for why pure enzyme cannot be reconstituted with the exact properties of enzyme in untreated microsomes. For example, treatment of microsomes at 2.2 kbar, 11 °C, leads to a change in the functional state of UDP-glucuronosyltransferse: E → E′. Although this initial change is reversible, either by lowering pressure or by raising temperature, E′, the modified state of the enzyme formed at high pressure, undergoes a second change in state (E → E″) that is irreversible, and E″ relaxes only to E* when pressure is released. It appears, therefore, that E″ is trapped in a deep potential well from which states E′ and E are not accessible. This explains the "permanence" of pressure-induced activation on the time scale of the experiments reported above, i.e., hours to days. Moreover, it appears that the state E* is accessible to E even at 1 atm in that the kinetic properties of UDP-glucuronosyltransferase are the same in microsomes treated with pressure, detergent, or alkaline pH or for pure enzyme reconstituted into lipid bilayers in a liquid-crystalline phase (Dannenberg et al., 1989, 1990; Hochman & Zakim, 1983; Hochman et al., 1983; Leuders & Kuff, 1967; Magdalou et al., 1982; Rotenberg & Zakim, 1989; Vessey & Zakim, 1971) and just as pressure-induced activation appears permanent, activations due to these other treatments are irreversible when the perturbing agent is removed (Dannenberg et al., 1990; Vessey & Zakim, 1971; Zakim, unpublished data). This explains the failure to reconstitute pure enzyme will all the properties of enzyme in untreated microsomes because the E* state is generated when UDP-glucuronosyltransferase is extracted from microsomes in the first step of purification.

Mechanism of Pressure-Induced Activation of UDP-Glucuronosyltransferase in Microsomes. Pressure in the range used above does not alter the tertiary structure of water-soluble proteins (Heremans, 1982; Jaenicke, 1981). Infrared studies of UDP-glucuronosyltransferase reconstituted into bilayers of dimyristoylphosphatidylcholine indicate that the secondary and tertiary structure of the enzyme in this environment is less perturbed at pressures up to 25 kbar than water-soluble enzymes (Zakim and Wong, unpublished data). In addition, interactions with phospholipids stabilize the structure of polylysine against pressure-induced changes (Carrier et al., 1990). It seems reasonable to conclude that membrane-bound enzymes in general and UDP-glucuronosyltransferase in particular are not more susceptible to pressure-induced changes in tertiary structure than are water-soluble enzymes, which implies that pressure-induced activation of UDP-glucuronosyltransferase was due to alterations in the quaternary structure of the enzyme (Heremans, 1982; Jaenicke, 1981; Weber & Drickamer, 1983).

There are several possible types of pressure-induced perturbations of the structure of UDP-glucuronosyltransferase within the microsome: organization with self in an oligomer of identical subunits, interactions with other proteins, or selective organization of UDP-glucuronosyltransferase with membrane lipids. The pressure data alone cannot define the organized structure disrupted by it. There are, however, interesting similarities between the effects of pressure on UDP-glucuronosyltransferase and oligomeric enzymes in water. Application of high pressure to both systems alters their functional states, and the pressure-induced changes are not reversed immediately when pressure is released (Jaenicke, 1981; King & Weber, 1986; Ruan & Weber, 1988; Weber, 1986). Moreover, the effects of pressure, in both systems, comprise several steps, only one of which may represent the direct effect of pressure on the enzyme. For example, pressure dissociates oligomers in water, which can be written as E ↔ E′, but as a consequence of dissociation, E′ is not subject to the same forces as E, and E′ can "drift" to new conformational states (Jaenicke, 1981; King & Weber, 1986; Penniston, 1971; Ruan & Weber, 1988; Weber, 1986), as E′ ↔ E″. An oligomer will re-form on release of pressure, but it will be composed of subunits that are different from those in the original, e.g., E″ ↔ E*, which accounts for the persistence of the effects of pressure after release of pressure. Of interest too is that higher temperature (e.g., 30 vs 10 °C) stabilized the native state of UDP-glucuronosyltransferase, which is similar to the effects of temperature on the stability of the native oligomeric state of aqueous soluble proteins (King & Weber, 1986; Ruan & Weber, 1988). Most important is the considerable evidence that UDP-glucuronosyltransferase is an oligomer, as for example negatively cooperative binding of UDP-glucuronic acid (Hochman et al., 1983) and abrupt changes in the effects of pressure (Figure 1) and temperature (Rotenberg & Zakim, 1989) on the properties of the enzyme. Recently published data provide excellent evidence that the functional state of UDP-glucuronosyltransferase is constrained by protein–protein interactions. Vessey and Kempner (1989) have shown that UDP-glucuronosyltransferase can be activated by radiation of microsomes with a neutron beam at an intensity incompatible with destruction of significant amounts of membrane lipids, which implies that the neutron beam modified protein–protein not lipid–protein interactions (Vessey & Kempner, 1989). Vessey and Kempner concluded that UDP-glucuronosyltransferase was regulated by subunit interactions within an oligomer.

We think it is important to be cautious in applying data for water-soluble oligomers to membrane systems in an exact manner, even though Verjovski-Almeida et al. (1986) have shown that high pressure dissociated dimeric Ca-ATPase in detergent micelles. Micelles have a different structure from bilayers, and it has not been demonstrated that oligomeric enzymes integral to the apolar portion of a membrane dissociate at high pressure. High pressure might cause integral membrane proteins to be squeezed out of "solution" in a bilayer in that they appear not to be solvated by lipids in a gel phase (Heyn et al., 1981a,b). On the other hand, pressure could cause remarkable changes in subunit interactions within an oligomer in the absence of dissociation.

Implications of the Effects of Pressures on the Organization of Microsomes. Independent of the exact structural basis for the organization of UDP-glucuronosyltransferase in microsomes, it seems that the E* conformational state of UDP-glucuronosyltransferase is more stable than the state of the enzyme in intact, untreated microsomes. One could argue that E* is a metastable state that would relax to E eventually. We think this possibility can be excluded, however. Thus, as

pointed out already, E* seems to be the state of pure enzyme, which does not relax to E even after days of storage as a complex with lipid bilayers.

Whether or not the stability of E* as compared with E (native enzyme) depends on kinetic or thermodynamic parameters, it appears that this state is accessible to UDP-glucuronosyltransferase at 1 atm (see above). There hence must be a mechanism in microsomes that prevents UDP-glucuronosyltransferase from relaxing to state E*. Assume that the mechanism constraining UDP-glucuronosyltransferase to the native state is another protein and that pressure, as well as other activators, dissociates this protein from UDP-glucuronosyltransferase. One would expect reassociation to occur between this protein and UDP-glucuronosyltransferase on release of pressure, leading to regeneration of enzyme in its original state. The latter was not observed. Thus, the organization of UDP-glucuronosyltransferase in untreated microsomes may depend on two mechanisms. One of these may direct the enzyme to state E as it assembles into endoplasmic reticulum, and the other might act to prevent the enzyme in microsomes from reaching state E*. The former component is absent from microsomes. It appears, therefore, that the regulation of UDP-glucuronosyltransferase in microsomes is extremely complex and dependent on several factors. Studies at high pressure together with other physical techniques should serve to delineate these factors.

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