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Effects of High Pressure on the Catalytic and Regulatory Properties of UDP-Glucuronosyltransferase in Intact Microsomes†

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ABSTRACT: The effects of high pressure on the kinetic properties of microsomal UDP-glucuronosyltransferase (assayed with 1-naphthol as aglycon) were studied in the range of 0.001–2.2 kbar to clarify further the basis for regulating this enzyme in untreated microsomes. Activity changed in a discontinuous manner as a function of pressure. Activation occurred at pressure as low as 0.1 kbar, reaching one of two maxima at 0.2 kbar. As pressure was increased above 0.2 kbar, activity decreased, reaching a minimum at about 1.4 kbar followed by a second activation. The pathway for activation at pressure > 1.4 kbar was complex. The immediate effect of 2.2 kbar was nearly complete inhibition of activity. The inhibited state relaxed, however, over about 10 min (at 10 °C), to a state that was activated as compared with enzyme at 0.001 kbar or enzyme at pressures between 1.4 and 2.2 kbar, which was the highest pressure we could test. Examination of the detailed kinetic properties of UDP-glucuronosyltransferase indicated that the effects of pressure were due to selective stabilization of unique functional states of the enzyme at 0.2 and 2.2 kbar. Activation at 0.2 kbar was reversible when pressure was released. This was true as well for activation at pressure > 1.4 kbar, but after prolonged treatment at 2.2 kbar, UDP-glucuronosyltransferase became activated irreversibly on release of pressure. The process by which prolonged treatment at 2.2 kbar led to permanent activation of UDP-glucuronosyltransferase after release of pressure was not reflected, however, by time-dependent changes in the functional state of UDP-glucuronosyltransferase at this pressure. Thus, appearance of the unique functional state of UDP-glucuronosyltransferase at 2.2 kbar occurred within about 10 min after reaching this pressure, and this state of the enzyme persisted for as long as 60 min (longest time studied). By contrast, the distribution of UDP-glucuronosyltransferase between native state and permanently activated state, after release of 2.2 kbar, shifted in favor of the latter with increasing time of treatment at 2.2 kbar. When pressure was released after 60 min at 2.2 kbar, about 80% of UDP-glucuronosyltransferase became permanently activated. We have interpreted this result to mean that treatment at high pressure perturbs interactions between UDP-glucuronosyltransferase and an undefined regulatory factor in microsomes that is important for maintaining the enzyme in its native conformational state.

Prior observations of the effect of high pressure on the function of UDP-glucuronosyltransferase indicated that this technique could be useful for studying the regulation of UDP-glucuronosyltransferase in otherwise untreated microsomes (Dannenberg et al., 1990). Measurements of activity at 2.2 kbar, as a function of temperature (Dannenberg et al., 1990), suggested that high pressure altered the conformation of UDP-glucuronosyltransferase, leading to a functional state different from that at 1 atm, and under some conditions, release of pressure led to irreversible activation of UDP-glucuronosyltransferase. The data for the effects of high pressure on the functional states of UDP-glucuronosyltransferase were compatible with Scheme I (Dannenberg et al., 1990), in which E is the native state of the enzyme, E* is the activated state produced after release of high pressure, and E $\rightleftharpoons$ E′ is a reversible change in the state of the enzyme at 2.2 kbar, 10 °C. A second intermediate (E″) at high pressure was proposed to explain the observations that E → E′ occurred rapidly at high pressure but complete conversion of E to E*, after release of pressure, required 90 min of treatment at 2.2 kbar.

Scheme I

$$E \underset{-P}{\overset{+P}{\rightleftharpoons}} E' \rightarrow E'' \xrightarrow{-P} E^*$$

In the present work, we have extended observations on the response of UDP-glucuronosyltransferase to high pressure, in otherwise untreated microsomes, by measuring activity as a function of pressure from 1 atm (0.001 kbar) to 2.2 kbar. This work shows that UDP-glucuronosyltransferase is sensitive to applied pressures as low as 0.1 kbar and that there are several active states available to UDP-glucuronosyltransferase that are stabilized differentially at pressures above 0.001 kbar. Direct experiments to detect the putative intermediate E″ failed to reveal this form of the enzyme at 2.2 kbar. Instead, it appears that high pressure alters a feature of microsomes that determines whether enzyme at high pressure relaxes to

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the native state E or to E* on release of pressure.

Materials and Methods

Liver microsomes were prepared from 150-day-old male rats as in Zakim and Vessey (1973) and stored at −70 °C until use. Protein concentrations were measured with the biuret method (Gornall et al., 1949). Activities of UDP-glucuronosyltransferase were assayed fluorometrically using 1-naphthol as aglycon as described previously (Dannenberg et al., 1990). Excitation was at 320 nm and emission at 460 nm. All measurements were made on a Greg-PC fluorometer (ISS, Inc., Champaign, IL).

Microsomes and other reagents were deoxygenated by bubbling of N_2. This was essential for limiting the rate of photobleaching of 1-naphthol during assays. These rates were negligible, as compared with rates of glucuronidation, after extensive deoxygenation. Assay cuvettes were pressure-treated in a water-jacketed pressure cell based on the design of Paladini and Weber (1981), as described in Dannenberg et al. (1990). Microsomes were mixed with an otherwise complete assay system (50 mM Hepes, pH 7.5, and 1 mM $MgCl_2$) in the pressure cell within the fluorometer. The concentration of microsomes was 0.12 mg of protein/mL. It is important to note that 1-naphthol partitions extensively into the microsomal membrane (Vessey & Zakim, 1975) and that aglycon within the membrane not in water is likely to be the proximate pool of substrate interacting directly with the active site of UDP-glucuronosyltransferase (Boyer et al., 1983; Noy & Zakim, 1985; Zakim & Vessey, 1977). The ratio of 1-naphthol to microsomal protein and not simply the nominal concentration of 1-naphthol in water thus is an important condition for the assays of enzyme. Exact conditions for assays are given in the figure legends. Unless otherwise noted, the temperature of the pressure cell was 10 °C. Pressurization was achieved by hand-cranking a pump, which required about 3 min to reach 2.2 kbar. Calculated activities in nanomoles of 1-naphthol metabolized per minute were corrected for pressure- and temperature-dependent changes in the quantum yield of 1-naphthol for each set of conditions used. Fluorescence of 1-naphthol was calibrated on a daily basis.

Results

Pressure Titration of UDP-Glucuronosyltransferase in Microsomes. A pressure titration of the activity of UDP-glucuronosyltransferase at 10°C (Figure 1) shows that activity was greater at 0.1 kbar than at 0.001 kbar and reached a pressure-dependent maximum at 0.2 kbar. Activity then declined between 0.2 and about 1.4 kbar. At pressure > 1.4 kbar, however, a second activation occurred. The pressure-dependent maximum for this transition could not be determined because activity increased up to 2.2 kbar, which was the upper safe limit for pressurization in our bomb. Plots of log V versus pressure were linear as activity declined with pressure between 0.2 and 1.4 kbar. We believe, therefore, that this decline reflected a single, pressure-dependent process (inset, Figure 1). The slope of this plot cannot be used to calculate $\Delta V^{\#}$ for UDP-glucuronosyltransferase in the range of 0.2–1.4 kbar because the viscosity of the polymethylene chains of the microsomal bilayer increases with pressure (Dannenberg et al., 1990), and this effect on activity cannot be separated from pressure-dependent effects on activity due to $\Delta V^{\#}$. In addition, the activities in Figure 1 were not measured at V_{max}.

Activities of pressure-treated microsomes in Figure 1 were measured after release of pressure. In accord with results reported earlier (Dannenberg et al., 1990), there was no permanent activation of UDP-glucuronosyltransferase, on

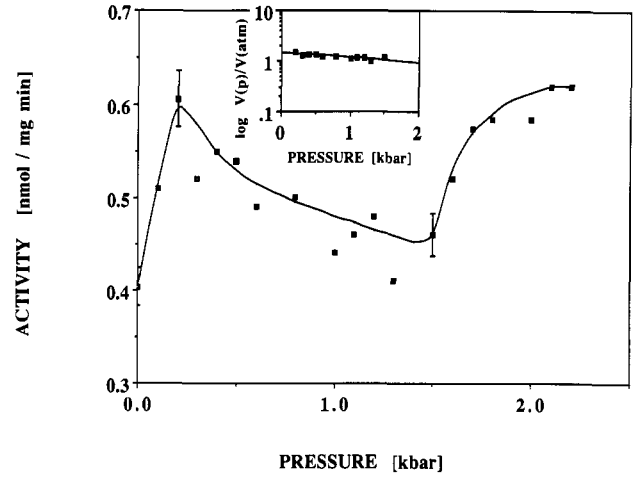


FIGURE 1: Pressure dependence of the activity of UDP-glucuronosyltransferase in microsomes. A complete reaction mixture containing 1 mM UDP-glucuronic acid, 0.02 mM 1-naphthol, and 0.12 mg of microsomal protein was added to a cuvette, which was then inserted immediately into the pressure bomb, which was cooled by a thermostated water jacket to 10 °C. Activities were measured at the indicated pressures after hand-cranking of a pump to the target pressure. The longest interval for reaching the target pressure was about 3 min for 2.2 kbar. Steady-state rates of activity were not attained immediately (see text) on reaching the target pressures, especially at pressures in the range of 2.0 kbar, but all data presented are steady-state rates. Error bars, when present, are standard deviations at the given pressure. V(p) (see inset) is activity at the indicated pressure, and V(atm) is activity at 1 atm.

release of pressure, for treatment at pressures less than about 1.2 kbar; i.e., activities of UDP-glucuronosyltransferase, under this condition, were the same before application of pressure and after release of pressure. Above 1.2 kbar, however, enzyme became permanently activated upon release of pressure. The extent of this activation increased with increases of pressure > 1.2 kbar, and as reported previously (Dannenberg et al., 1990), activation in this setting was time-dependent (data not shown). We found, however, that the extent of activation of UDP-glucuronosyltransferase after release of pressure under the conditions used in Figure 1 was less than reported previously for assays of activity at less than saturating concentrations of 1-naphthol (Dannenberg et al., 1990). This result must reflect that the presence of 1-naphthol altered the response of UDP-glucuronosyltransferase (assayed after release of pressure with either *p*-nitrophenol or 1-naphthol) to pressurization at 2.2 kbar followed by release of pressure. Thus, prior studies have shown that UDP-glucuronic acid does not influence the effect of high pressure on the response of UDP-glucuronosyltransferase to high pressure (Dannenberg et al., 1990). The details of this effect of 1-naphthol will be reported separately.

Mechanism of Pressure-Induced Activation of UDP-Glucuronosyltransferase. For enzymes solvated only by water, pressure effects on activity can be related to changes in the conformational state of the enzyme that are dictated by pressure-dependent changes in the free volume of the system (Heremans, 1982; Jaenicke, 1981; Morild, 1981; Weber & Drickamer, 1983) and independently to volume changes associated with binding of substrates and catalysis per se. Because of compensating effects on packing and H-bonding, application of high pressure does not increase the viscosity of water in the pressure range 0.001–2.2 kbar (Bridgman, 1958). However, the microviscosity of the microsomal membrane will increase with pressure (Dannenberg et al., 1990), and the direct impact of increasing viscosity will be a decrease in the activity of UDP-glucuronosyltransferase. It is apparent from

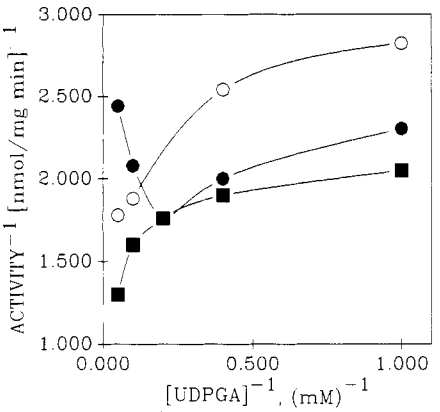


FIGURE 2: Effect of high pressure on interactions between UDP-glucuronosyltransferase and UDP-glucuronic acid. Activities were measured at 0.001 kbar (○), 0.2 kbar (●), and 2.2 kbar (■), as in Figure 1 and Materials and Methods. Data are plotted in double-reciprocal form. The concentrations of protein and 1-naphthol were the same as in Figure 1.

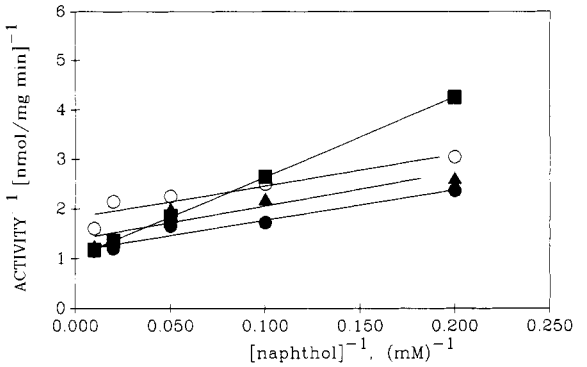


FIGURE 3: Effect of high pressure on interactions between UDP-glucuronosyltransferase and 1-naphthol. Activities were measured as in Figure 1 and Materials and Methods at 0.001 kbar (○), 0.2 kbar (●), 1.5 kbar (▲), and 2.2 kbar (■). Data are plotted in double-reciprocal form. The concentrations of protein and UDP-glucuronic acid were the same as in Figure 1.

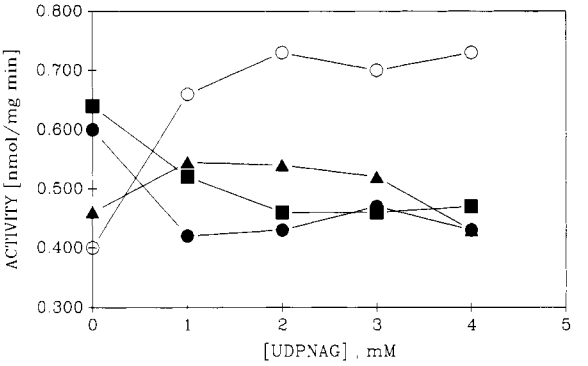


FIGURE 4: Effect of high pressure on modulation of the activity of UDP-glucuronosyltransferase by UDP-N-Ac-glc. Activities were measured as in Figure 1 and Materials and Methods at the indicated concentrations of UDP-N-Ac-glc. Pressures were 0.001 kbar (○), 0.2 kbar (●), 1.5 kbar (▲), and 2.2 kbar (■).

Figure 1, however, that an increase in viscosity of the microsomal membrane was not associated with a monotonous decrease in activity as a function of viscosity. Therefore, some pressure-dependent effect compensated for the expected impact of the change in viscosity of the membrane. There are two possibilities. The less important, from the perspective of regulation of the enzyme, is that pressure-induced activation of UDP-glucuronosyltransferase at 0.2 kbar followed by pressure-dependent inhibition of activity at pressure > 0.2 kbar reflected the balance between volume changes associated with formation of the enzyme–substrate complex and subsequent formation of the activated state of this complex. The most rigorous approach to examining the mechanism of pressure-induced activation of UDP-glucuronosyltransferase is to determine activities at V_{max} and binding constants as a function of pressure. These measurements are extremely tedious and expensive to accomplish. In addition, as shown below, V_{max} cannot be measured accurately at all pressures because of substrate inhibition of UDP-glucuronosyltransferase by UDP-glucuronic acid at 0.2 kbar. Most important, the presence of two discontinuities in the plot of activity versus pressure is difficult to reconcile with any mechanism other than a pressure-induced change in the state of the enzyme. The kinetic measurements in Figures 2–6 support this conclusion.

The data in Figure 2 show the relationship between rates of conjugation and the concentration of UDP-glucuronic acid for assays between 0.001 and 2.2 kbar. As expected for enzyme at ambient pressure, the kinetic pattern was non Michaelis–Menten (Winsnes, 1969; Vessey et al., 1973). This kinetic pattern, which for pure enzyme reflects negatively cooperative binding of UDP-glucuronic acid (Hochman et al., 1983), was not altered at high pressure. However, at 0.2 kbar, there was extensive substrate inhibition at concentrations of UDP-glucuronic acid above 5 mM. By contrast with results at 0.2 kbar, there was no substrate inhibition by UDP-glucuronic acid for assays at 2.2 kbar. Therefore, the details of enzyme–UDP-glucuronic acid interactions were modulated by pressure in a discontinuous fashion. In addition, these data are not compatible with the conclusion that high pressure enhanced the avidity of enzyme for UDP-glucuronic acid. The same conclusion can be drawn from data for the interaction between enzyme and 1-naphthol (Figure 3). As pressure was increased, $K_{naphthol}^{app}$ increased progressively.

The double-reciprocal plots in Figures 2 and 3 show that the effect of pressure on the activity of UDP-glucuronosyltransferase depends on the concentrations of UDP-glucuronic acid and 1-naphthol. Depending on the concentrations of substrates chosen for assay, activity can be shown to increase or decrease as a function of pressure > 0.001 kbar. Activities in Figure 1 were measured at 1.0 mM UDP-glucuronic acid and 0.02 mM 1-naphthol. The data in Figures 2 and 3 show that these conditions will be associated with activation of enzyme at 0.2 and 2.2 kbar versus 0.001 kbar. Different assay conditions would not enhance the activation at 0.2 kbar, but as shown by Figures 2 and 3, the extent of maximum activation of UDP-glucuronosyltransferase at 2.2 versus 0.001 kbar is much greater than depicted by the data in Figure 1 and approaches 10-fold.

The effect of the allosteric activator UDP-N-Ac-glc (Vessey et al., 1973; Winsnes, 1969) on UDP-glucuronosyltransferase was qualitatively different at 0.001 and 0.2 kbar. As expected, there was activation of UDP-glucuronosyltransferase by UDP-N-Ac-glc at ambient pressure (Vessey et al., 1973; Winsnes, 1969); but at 0.2 kbar, UDP-N-Ac-glc inhibited the enzyme (Figure 4). Especially interesting was that inhibition was only partial, even at high concentrations of UDP-N-Ac-glc. For example, inhibition by UDP-N-Ac-glc at 0.2 kbar was maximal at 1.0 mM UDP-N-Ac-glc. This result means that inhibition by UDP-N-Ac-glc cannot be explained by competition between UDP-N-Ac-glc and UDP-glucuronic acid for binding at the active site, which means in turn that inhibition by UDP-N-Ac-glc at 0.2 kbar (and at higher pressures) was an allosteric effect. Thus, the allosteric effect of UDP-N-Ac-glc switched from positive to negative as the pressure of the system increased from ambient to 0.2 kbar.

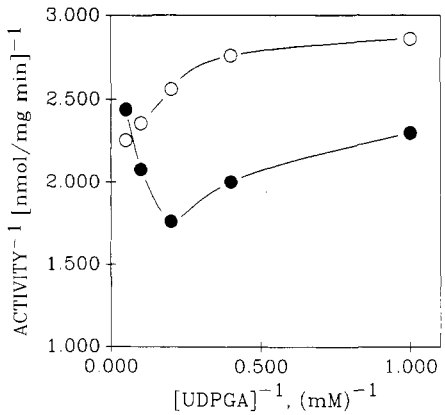


FIGURE 5: Effect of UDP-N-Ac-glc on substrate inhibition by UDP-glucuronic acid at high pressure. Activities were measured as in Figure 1 and Materials and Methods at 0.2 kbar, at the indicated concentrations of UDP-glucuronic acid, in the absence (●) or presence (○) of 1 mM UDP-N-Ac-glc. Data are plotted in double-reciprocal form.

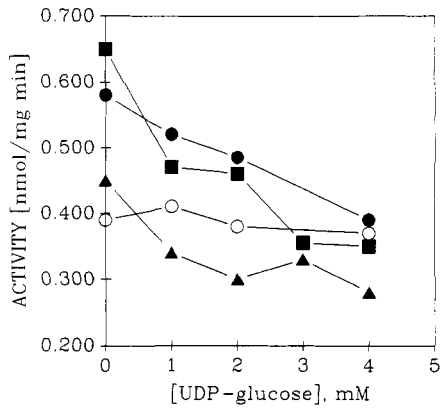


FIGURE 6: Effect of high pressure on the selectivity of binding of UDP-sugars to UDP-glucuronosyltransferase. Activities were measured as in Figure 1 and Materials and Methods at 0.001 kbar (○), 0.2 kbar (●), 1.5 kbar (▲), and 2.2 kbar (■). The concentrations of UDP-glucose are indicated.

This interpretation of the data in Figure 4 is supported by the experiment in Figure 5, which is a double-reciprocal plot of activity (at 0.2 kbar) as a function of the concentration of UDP-glucuronic acid for assays in the absence and presence of 1.0 mM UDP-N-Ac-glc. UDP-N-Ac-glc modulated substrate inhibition by UDP-glucuronic acid, which again indicates that the effects of UDP-N-Ac-glc on UDP-glucuronosyltransferase at 0.2 kbar were not due to binding to the active site.

Although the effects of UDP-N-Ac-glc on the properties of UDP-glucuronosyltransferase were similar at 0.2 and 2.2 kbar (Figure 4), the response to UDP-N-Ac-glc appeared to be different at 1.5 kbar. The apparently discontinuous response of enzyme to UDP-N-Ac-glc at this pressure versus higher and lower pressures was reproducible but its significance is not certain.

UDP-glucuronosyltransferase at 0.001 kbar has high selectivity for binding of UDP-sugars at the active site in that only UDP-glucuronic acid appears to bind at concentrations in the range of 0–5 mM (Zakim et al., 1973). This selectivity was absent from enzyme at pressures above ambient, however. Thus, UDP-glucose was an inhibitor for assays at 0.2, 1.5, or 2.2 kbar (Figure 6). The data in Figure 6 also show that inhibition by UDP-glucose, as compared with that for UDP-N-Ac-glc, did not reach a limiting value.

Activity of UDP-Glucuronosyltransferase at Pressure > 2.0 kbar. Data reported by Dannenberg et al. (1990) showed that

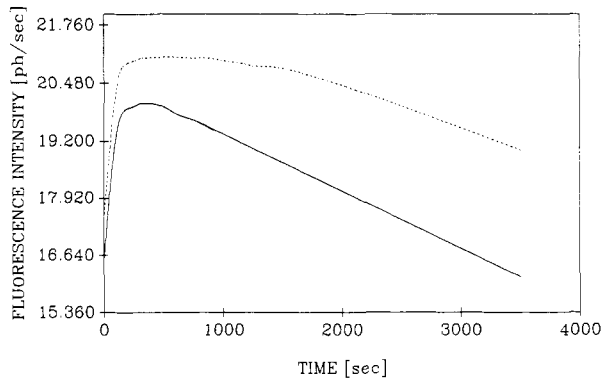


FIGURE 7: Apparent time-dependent relaxation of inelastic effects due to pressurization at 2.2 kbar at 10 °C. Fluoresence measurements were begun as soon as the cuvette was added to the bomb and the latter sealed. Conditions for assay were the same as in Figure 1. Microsomes had been stored at −70 °C for 1 month (—) or for 4 months (---).

the activity of UDP-glucuronosyltransferase at 10 °C, but not at 30 °C, declined when pressure was raised from ambient to 2.2 kbar. These data are contrary to the result in Figure 1. The basis for the discrepancy between the current and prior work was revealed by examining the time course for reaching a steady state rate of glucuronidation after application of 2.2 kbar. Recording of fluorescence in Figure 7 (solid line) was begun at ambient pressure. The immediate effect of 2.2 kbar, reflected by the rate of glucuronidation when 2.2 kbar was reached, was inhibition of UDP-glucuronosyltransferase. A steady-state rate of glucuronidation for enzyme activated at 2.2 kbar versus 0.001 kbar was reached after 10 min at 2.2 kbar. The possibility that this result was an artifact due to the pressure-dependent change in the quantum yield of 1-naphthol (Figure 7) plus accumulation of heat during pressurization was excluded. Thus, accumulation of heat during pressurization did not occur to an extent significant enough to be detected by increasing fluorescence of 1-naphthol after a target pressure of 2.2 kbar was reached in a complete assay system lacking microsomes (data not shown). We concluded, therefore, that the slow approach to a steady-state rate of conjugation in Figure 7 occurred because of a slow relaxation of the immediate effects of high pressure on UDP-glucuronosyltransferase. That pressure of 2.2 kbar had two effects on the functional state of UDP-glucuronosyltransferase, i.e., immediate inhibition followed by a slow rate of activation, was missed in our prior studies (Dannenberg et al., 1990) because the rate of relaxation of inhibited to activated state at 2.2 kbar was a function of the preparation of microsomes. The microsomes used in Figure 1 and for the data shown by the continuous line in Figure 7 were stored at −70 °C for about 1 month prior to use. The microsomes used for the data shown by the dashed line (Figure 7) were stored for about 4 months prior to use. Relaxation of the inhibited state of UDP-glucuronosyltransferase to the active state at 2.2 kbar required about 30 min for the latter microsomes. This result means that enzyme activities at 2.2 kbar reported in Dannenberg at al. (1990) were measured during the relaxation of the pressurized system to its final state and not after attainment of this state. In addition, however, there were fundamental differences in the effect of 2.2 kbar on the function of UDP-glucuronosyltransferase in microsomes stored for 1 or several months before application of high pressure. Microsomes stored for about 4 months prior to pressurization displayed substrate inhibition by 1-naphthol at pressure > 0.001 kbar (Figure 8), and as pressure increased substrate inhibition became apparent at decreasing concentrations of 1-naphthol. By contrast,

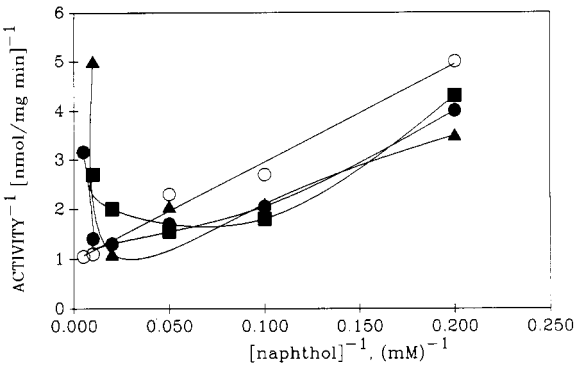


FIGURE 8: Effect of high pressure on interactions between UDP-glucuronosyltransferase and 1-naphthol for microsomes stored at −70 °C for 4 months prior to pressurization. Activities were measured as in Figure 1 and Materials and Methods at 0.001 kbar (○), 0.2 kbar (●), 1.5 kbar (▲), and 2.2 kbar (■). Data are plotted in double-reciprocal form.

substrate inhibition by 1-naphthol was not a property of pressure-treated UDP-glucuronosyltransferase in microsomes stored for only 1 month prior to application of pressure (Figure 3). On the other hand, the properties of UDP-glucuronosyltransferase at 0.001 kbar were not different for microsomes stored more than 4 months or for shorter times.

The data in Figures 7 and 8 for function of UDP-glucuronosyltransferase in microsomes stored for variable periods of time were generated from microsomes prepared on different dates from different rats. The general applicability of these data was validated by "aging" a single preparation of microsomes. These experiments (data not shown) showed that the properties of a single set of microsomes changed, as predicted by the data in Figures 7 and 8, after storage for 3 months.

In view of the importance of the history of a preparation of microsomes for the exact effects of high pressure on the function of UDP-glucuronosyltransferase, we reexamined the relationship between temperature and the effects of pressure on the enzyme. This was done because prior results suggested that permanent activation of UDP-glucuronosyltransferase after release of pressure occurred with maximum rate (duration of pressure treatment needed for complete conversion of native enzyme to an activated state) at 10 °C, with essentially no activation, after release of pressure, for application of pressure at 30 °C (Dannenberg et al., 1990). In addition, it appeared from prior experiments that pressure of 2.2 kbar at 10 °C inhibited UDP-glucuronosyltransferase whereas application of this pressure at 30 °C did not. The previously reported temperature dependence for permanent activation of UDP-glucuronosyltransferase after release of pressure was confirmed for a preparation of microsomes responding to pressure as in Figure 1 (data not shown). However, activation at 2.2 kbar was independent of temperature with this preparation of microsomes (data not shown). It is reasonable to conclude that results in Dannenberg et al. (1990) reflected the temperature dependence of the rate of relaxation of pressure-treated enzyme to its final stable state at 2.2 kbar and not a temperature-dependent response of enzyme to pressure per se.

Discussion

Several physical and chemical perturbations activate UDP-glucuronosyltransferase(s) in untreated microsomes (Leuders & Kuff, 1967; Vessey & Zakim, 1971), indicating that the maximum potential activity of this enzyme is constrained in its native state. There is disagreement, however, as to what accounts for this constraint [see Dutton (1980) and Zakim et al. (1985)]. One explanation (conformation hypothesis) is that UDP-glucuronosyltransferase can exist as different conformational isomers with variable kinetic constants and regulatory properties and that activation of enzyme in microsomes is due to environmentally determined modification of the relative stabilities of different isomers (Dannenberg et al., 1990; Vessey & Zakim, 1971; Zakim & Vessey, 1975, 1982; Zakim et al., 1973). The alternative view (compartmentation hypothesis) is that the active site of UDP-glucuronosyltransferase faces the lumen of microsomes (Berry et al., 1975; Hallinan & DeBrito, 1981; Shepherd et al., 1989). It is proposed that limited access of UDP-glucuronic acid to the active site is via a transport protein and thus that constraint on the activity of UDP-glucuronosyltransferase reflects the barrier to free passage of UDP-glucuronic acid across the microsomal membrane.

Either of the above hypotheses accounts for the increase in specific activity of UDP-glucuronosyltransferase when the native microsomal structure is perturbed. So the event of activation, as defined by a change in specific activity, is not a sufficient basis for choosing between alternative explanations for regulation of enzyme in situ. However, there is considerable evidence from studies of pure enzyme to support the basic assumptions of the conformation hypothesis. Thus, the kinetic constants and regulatory properties of UDP-glucuronosyltransferase are modulated by the chemical and physical characteristics of the lipid matrix into which pure enzyme is inserted (Hochman & Zakim, 1983, 1984; Hochman et al., 1983; Magdalou et al., 1982; Zakim et al., 1988); the relative stabilities of different functional states of pure enzyme depend on the lipid composition of a bilayer (Rotenberg & Zakim, 1989, 1991); and the regulatory properties of UDP-glucuronosyltransferase that change in association with release of constraint on enzyme activity (Vessey et al., 1973; Winsnes, 1972; Zakim & Vessey, 1975, 1982; Zakim et al., 1973) are all modulated by the lipid environment of pure enzyme (Dannenberg et al., 1989; Hochman et al., 1981; Magdalou et al., 1982; Rotenberg & Zakim, 1989). By contrast with the experimental support for the conformation hypothesis, there is no evidence, other than release of constraint, to support the idea that the active site of UDP-glucuronosyltransferase is within the lumen of microsomes or that microsomes contain a transport protein for UDP-glucuronic acid. Most importantly, the kinetic behavior of UDP-glucuronosyltransferase in untreated microsomes is incompatible with the assumptions of the compartmentation hypothesis, which predicts properties the enzyme cannot be demonstrated to possess (Finch et al., 1976; Nuwayhid et al., 1986; Vessey & Zakim, 1978; Zakim & Dannenberg, 1991; Zakim & Vessey, 1975, 1982). Finally, the assumptions of the compartmentation hypothesis do not account for changes in the regulatory properties of UDP-glucuronosyltransferase that accompany release of constraint on specific activity (Dannenberg et al., 1989; Winsnes, 1972; Vessey et al., 1973; Zakim et al., 1973).

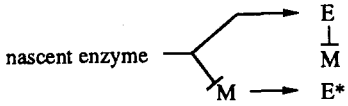
The current data add to the evidence that UDP-glucuronosyltransferase can exist in distinct isomeric states with variable functional parameters. The properties of the different states of UDP-glucuronosyltransferase that are found at 1 atm or higher pressures are summarized in Table I. Comparisons of the properties of the various putative functional states show that each entry in Table I is unique. Moreover, the qualitative differences between E, E*, and enzyme at 0.2 or 2.2 kbar exclude that the latter two states can be mixtures of the former. Finally, in addition to the two activated states of UDP-gluc-

Table I: Comparison of Selected Properties of Different Functional States of UDP-Glucuronosyltransferase at Atmospheric and High Pressure[a]

	property					
state of enzyme	substrate inhibition by UDPGA	allosteric effect of UDPNAG	active-site-directed inhibition by UDPNAG	inhibition by UDP-sugars	kinetic pattern	reversible activation
E	0	+	no	0	n-MM	n/a
E*	0	0	yes	+	MM	no
$E_{0.2}$	+	−	no	+	n-MM	yes
$E_{2.2}$	0	−	no	+	n-MM	yes

[a] E is the native state of the enzyme; E* is the state produced by application of 2.2 kbar for 90 min at 10 °C followed by release of pressure (Dannenberg et al., 1990) or by treating microsomes with detergent (Dannenberg et al., 1989; Vessey & Zakim, 1971). $E_{0.2}$ and $E_{2.2}$ are states of the enzyme at 0.2 and 2.2 kbar. Effects due to UDP-N-Ac-glc (UDPNAG) are +, activation; −, inhibition; 0, no effect. Substrate inhibition by UDP-glucuronic acid or inhibition by other UDP-sugars is designated by +. Michaelis–Menten kinetics are abbreviated MM; n-MM indicates non-Michaelis–Menten kinetics.

Scheme II



uronosyltransferase stabilized at high pressure, high pressure produced at least one other state, which was unstable (Figure 7). The simplest and most direct interpretation of these results is that there are many different functional states accessible to UDP-glucuronosyltransferase and that the relative stabilities of these can be altered by modifications of the environment of the protein. It seems reasonable to propose too that the forms of UDP-glucuronosyltransferase generated at high pressure are substates of the native state of the enzyme, and that pressure alters the equilibrium of enzyme between these substates. On the other hand, E and E* appear to be conformational states with separate energy minima.

Relationship between States of UDP-Glucuronosyltransferase at High Pressure and Regulation of Enzyme at 1 atm. E* formed at 1 atm does not revert to E (Dannenberg et al., 1989, 1990). This result suggests that E* is thermodynamically more stable than E. And, since E* appears to be readily accessible to UDP-glucuronosyltransferase, the data imply that some factor favors generation of state E in preference to E*. In addition, some factor must constrain relaxation of E to E* in untreated microsomes at 1 atm. Scheme II shows a theoretical mechanism for this sort of regulation. M facilitates folding of nascent UDP-glucuronosyltransferase to state E and limits the rate of nascent enzyme → E* as well as preventing relaxation of E to E*. This same scheme appears to apply to the process of relaxation of $E_{2.2}$ (stable state of enzyme at 2.2 kbar) when pressure is released.

Thus, monitoring the rate of glucuronidation of 1-naphthol at 2.2 kbar fails to detect a change in the functional state of UDP-glucuronosyltransferase from onset of a steady-state rate to about 50 min later, which is sufficiently long for about 80% of the maximal amount of E* to be produced on release of pressure. The mechanism proposed in Scheme I seems incorrect because there is no evidence for the proposed intermediate designated E″. Contrary to Scheme I, it appears that $E_{2.2}$ can relax either to E or E* depending on the length of treatment of microsomes at 2.2 kbar. Scheme II accounts for this: whether $E_{2.2}$ relaxes to E or E*, on release of pressure, depends on the status of the regulatory component M. In the presence of M, $E_{2.2}$ relaxes to E. In the absence of M, $E_{2.2}$ relaxes to E*. The fundamental idea for regulation of this sort is not altered if $E_{2.2}$ always relaxes to E but M constrains the subsequent transition E → E*. We propose, therefore, that high pressure alters M, with a pressure threshold of about 1.2 kbar, which is the lowest pressure that leads to state E* on release of pressure (Dannenberg et al., 1990). It is important too, in the context of the threshold pressure, that there is no unique effect of pressure on UDP-glucuronosyltransferase at 1.2 kbar.

The nature of component M is unclear, but we have found recently that brief treatment of microsomes with trypsin followed by trypsin inhibitor also allows enzyme in state E to relax to state E* (Dannenberg and Zakim, in preparation). This effect is not due to limited proteolysis of UDP-glucuronosyltransferase in otherwise untreated microsomes, suggesting that microsomes contain a protein that contributes to constraint on the rate of relaxation of E to E*.

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Registry No. UDP-Glucuronosyltransferase, 9030-08-4; naphthol, 90-15-3; UDP-glucuronic acid, 2616-64-0; UDP-*N*-acetylglucosamine, 528-04-1; UDP-glucose, 133-89-1.

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Regulation of Biological Functions by an Insulin Receptor Monoclonal Antibody in Insulin Receptor β-Subunit Mutants†

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ABSTRACT: We investigated the effects of MA-5, a human-specific monoclonal antibody to the insulin receptor α-subunit, on transmembrane signaling in cell lines transfected with and expressing both normal human insulin receptors and receptors mutated in their β-subunit tyrosine kinase domains. In cell lines expressing normal human insulin receptors, MA-5 stimulated three biological functions: aminoisobutyric acid (AIB) uptake, thymidine incorporation, and S6 kinase activation. Under conditions where these biological functions were stimulated, there was no detectable stimulation of receptor tyrosine kinase. We then combined the use of this monoclonal antibody with cells expressing insulin receptors with mutations in the β-subunit tyrosine kinase domain; two of ATP binding site mutants V1008 (Gly→Val) and M1030 (Lys→Met) and one triple-tyrosine autophosphorylation site mutant F3 (Tyr→Phe at 1158, 1162, and 1163). In cells expressing V1008 receptors, none of the three biological functions of insulin was stimulated. In cells expressing M1030 receptors, AIB uptake was stimulated to a small, but significant, extent whereas the other two functions were not. In cells expressing F3 receptors, AIB uptake and S6 kinase activation, but not thymidine incorporation, were fully stimulated. The data suggest, therefore, that (1) activation of insulin receptor tyrosine kinase may not be a prerequisite for signaling of all the actions of insulin and (2) there may be multiple signal transduction pathways to account for the biological actions of insulin.

Insulin acts upon its receptor to produce a wide range of effects in many cell types, but the mechanism (or mechanisms) whereby the receptor generates transmembrane signals is unknown. The insulin receptor is a tetrameric disulfide-linked glycoprotein consisting of two identical extracellular α-subunits that bind the hormone and two identical transmembrane β-subunits that contain a typical tyrosine kinase motif in their cytoplasmic domains (Goldfine, 1987; Ullrich et al., 1985). The receptor is synthesized as a precursor polypeptide, and is subsequently cleaved into one α- and one β-subunit. When insulin binds to the α-subunit of the receptor, β-subunit tyrosine kinase is activated, tyrosine autophosphorylation of the β-subunit is increased, and various biological functions of

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