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TOPOLOGY OF GLUTATHIONE-INSULIN TRANSHYDROGENASE IN RAT LIVER MICROSOMES *

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Glutathione-insulin transhydrogenase (EC 1.8.4.2) catalyzes the inactivation of insulin through scission of the disulfide bonds to form insulin A and B chains. In the liver, the transhydrogenase occurs primarily in the microsomal fraction where most of the enzyme is present in a latent ('inactive') state. We have isolated rat hepatic microsomes with latent transhydrogenase activity being an integral part of the vesicles. We have used these vesicles to study the topological location of glutathione-insulin transhydrogenase by investigating the effects of detergents (Triton X-100 and sodium deoxycholate), phospholipase A_2 and proteinases (trypsin and thermolysin) on the latent enzyme activity. Treatment of intact vesicles with variable concentrations of detergents and phospholipase A_2 resulted in the unmasking of latent transhydrogenase activity. The extent of unmasking of transhydrogenase activity is dependent upon the concentration of detergent or phospholipase used and is accompanied by a parallel release of the enzyme into the soluble fraction. Activation of the transhydrogenase by phospholipase A_2 is partially inhibited by bovine serum albumin and the extent of inhibition is inversely proportional to the phospholipase concentration. In intact vesicles, latent transhydrogenase activity is resistant to proteolytic inactivation by both trypsin and thermolysin, while in semipermeable and permeable vesicles these proteases inactivate 60 and 25% of the total transhydrogenase activity, respectively. Together these results indicate that in microsomes transhydrogenase is probably weakly bound to membrane phospholipid components and that most of the enzyme is present on the cisternal surface (i.e., the luminal surface of the endoplasmic reticulum) of microsomes. Each detergent and phospholipase apparently unmasks glutathione-insulin transhydrogenase activity through disruption of the phospholipid-enzyme interaction followed by translocation of the enzyme to the soluble (cytoplasmic) fraction and not through increases in substrate availability.

Introduction

Glutathione-insulin transhydrogenase (protein-disulfide reductase (glutathione), EC 1.8.4.2), hereafter referred to as transhydrogenase, catalyzes, in the presence of a thiol compound such as reduced glutathione [1], the inactivation of insulin by cleavage of the disulfide bonds, thereby producing A and B chains. This reaction is the initial and rate-controlling step in the sequential degradation of insulin [2–7]. The mechanism of action of the enzyme has recently been reviewed [8]. Studies from this [9–12] and other [13–15] laboratories have shown that hepatic transhydrogenase activity is under feedback control by circulating insulin levels. This feedback control primarily results from insulin's action as a positive regulator of de novo enzyme synthesis [16] apparently at the transcription level [10]. This regulation of de novo

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enzyme synthesis by circulating insulin occurs over an extended period of time [12,16] and is, therefore, a form of chronic regulation. Although a number of mechanisms for the acute regulation of hepatic transhydrogenase activity are possible (see Ref. 17 for a review), several lines of evidence suggest that the intracellular organelle, the microsomes, may be an important regulatory site for transhydrogenase activity.

In rat liver, while glutathione-insulin transhydrogenase is found in all subcellular fractions [18,19], the majority of the enzyme is located in the microsomes [16,18–20] predominantly in a latent ('inactive') state [18,19]. In hypoinsulinemic diabetic rats, non-latent ('active') transhydrogenase predominates [12], whereas in hyperinsulinemic obese mice (characterized by insulin resistance), the latent ('inactive') form predominates [11]. Since both animal models are characterized by hyperglycemia, a direct or indirect influence of glucose upon the latency of the enzyme appears to be unlikely. In isolated preparations of plasma membranes, all the enzyme occurs in the non-latent form [21]. Based upon these observations it has been suggested that microsomal transhydrogenase in the latent state may represent a storage form of the enzyme which, in response to altered physiological needs, may be rapidly mobilized for cell function [20,21].

In the present communication the topology of glutathione-insulin transhydrogenase in microsomes of rat liver is explored. The data indicate that hepatic microsomal transhydrogenase is mainly located on the cisternal surface (the luminal surface of the endoplasmic reticulum). The enzyme seems to be associated via the membrane phospholipids [19]. The unmasking of latent microsomal transhydrogenase by detergents and phospholipase proceeds through the disruption of membrane proteins and subsequent translocation of the enzyme to the soluble (cytoplasmic) fraction.

Materials and Methods

Materials

Bovine serum albumin, phospholipase A_2 from bee venom, sodium deoxycholate, thermolysin and Triton X-100 were purchased from the Sigma Chemical Company (St. Louis, MO). Bovine pancreatic trypsin and lima bean trypsin inhibitor (1 mg inhibits 2.8 mg trypsin) were purchased from Worthington Enzymes and Biochemicals (Freehold, NJ). The animals used were adult male Sprague-Dawley rats obtained from Harlan Industries (Cumberland, IN) and maintained on standard laboratory chow and water, ad libitum.

Isolation of hepatic microsomes

Rats were fasted overnight and killed by decapitation. Livers were rapidly removed and minced and a 20% (w/v) homogenate was prepared in 250 mM sucrose/50 mM Tris (pH 7.5) at 4°C. The homogenization buffer when supplemented with 5 mM EDTA is noted in the figure legends. Microsomes were isolated from the homogenate by differential centrifugation at 4°C as previously described [19]. Briefly, the homogenate was centrifuged at $750 \times g$ for 10 min, $5100 \times g$ for 10 min and $10000 \times g$ for 20 min to remove the nuclear, mitochondrial and intermediate fractions, respectively. Microsomes were then separated from the cytoplasm by centrifugation at $160000 \times g$ for 60 min. The microsomal pellet was rinsed twice and suspended by gentle homogenization (Duall with a Teflon pestle) using sucrose/Tris buffer to yield 13–15 mg microsomal protein per ml.

Microsomal suspensions were divided into aliquots of approx. 1.0 ml and rapidly frozen at −54°C using an ethyl alcohol bath and then stored at −75°C until use. Since repeated freeze-thaw cycles alter transhydrogenase latency [19], microsomal suspensions were thawed only once and used immediately for the experiments reported.

Microsome treatment with various effectors

The characteristics of the latency of microsomal transhydrogenase were studied using 3–4 mg protein per ml of microsomal suspensions in sucrose/Tris buffer. Solutions of various effectors and inhibitors were freshly prepared daily in sucrose/Tris buffer. The permeability of microsomal vesicles was modified by treatment with Triton X-100 or deoxycholate for 30 min at 25°C or phospholipase for 20 min at 4°C. Treatment of microsomes with trypsin or thermolysin was carried out for 30 min at 25°C. Trypsin inhibitor was used at 160 μg per ml to inhibit or terminate the action of trypsin [19]. EDTA was used at 5 mM

[22] and 15 mM [23] to inhibit or terminate the action of phospholipase and thermolysin, respectively. Concentrations of detergents and enzymes used are given in the legends. Following incubation, the reaction tubes were placed on ice, rapidly diluted and assayed for transhydrogenase activity as described.

Glutathione-insulin transhydrogenase assay

Glutathione-insulin transhydrogenase was measured by a modification of the trichloroacetic acid precipitation method previously described [24]. Briefly, samples of treated or untreated microsomes were assayed in a system composed of two parts homogenization buffer containing the enzyme and eight parts 100 mM potassium phosphate buffer (pH 7.5). The final reaction volume was 1.0 ml containing, at final concentrations, 5 mM EDTA, 1 mM glutathione, 3 mg/ml bovine serum albumin, 1 μM insulin and trace amounts (30000–40000 cpm) of ^{125}I-insulin. The reaction was carried out for 5 min at 37°C and was terminated by the addition of an equal volume of 10% trichloroacetic acid. One unit of activity is that amount of enzyme which solubilized 1% of the total radioactivity in 5 min at 37°C. All activities were corrected for non-enzymatic insulin degradation, which was determined in parallel reactions containing glutathione and insulin in the absence of enzyme. Total transhydrogenase activity (i.e., non-latent plus latent) refers to the activity found following treatment with 0.08–0.2% (v/v) Triton X-100, 0.05% (w/v) deoxycholate or 0.5 μg per ml of phospholipase. Non-latent transhydrogenase activity refers to the activity measurable in microsomes treated in buffer without detergents or phospholipase. Percent non-latent transhydrogenase activity = (non-latent/total) × 100. Latent transhydrogenase activity is calculated as total minus non-latent activity.

Other analytical procedures

Protein was measured by the Folins-phenol method of Lowry et al. [25] using crystalline bovine serum albumin as the standard.

Sample distribution was determined by an analysis of the sample variances and, unless noted otherwise, the appropriate Student's two-tailed *t*-test for unpaired observations was then applied to determine the statistical significance (*P* value).

Results

Experiments were carried out to determine the conditions under which latent glutathione-insulin transhydrogenase activity in isolated microsomes would remain stable during prolonged storage. Isolated microsomes were suspended in buffers of different composition and stored at different temperatures, and, at the times indicated, the percentage of non-latent microsomal transhydrogenase activity was determined (Fig. 1). Storage of microsomes at 4°C for 4 days results in a 2.8-fold increase in the percentage of non-latent transhydrogenase activity in the absence of EDTA and a 1.7-fold increase following storage in 5 mM EDTA. Hence, EDTA helps to stabilize insulin transhy-

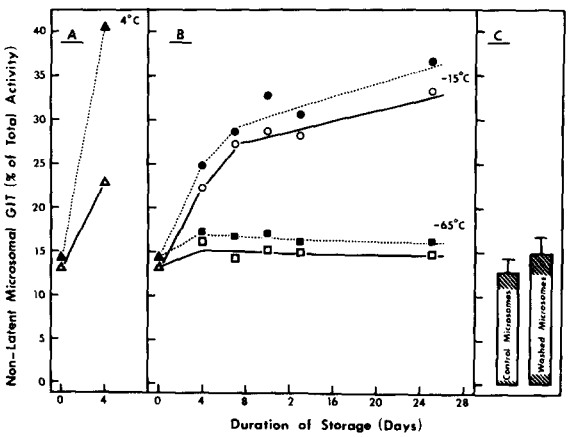

Fig. 1. Effect of storage under different conditions and of washing of microsomes on the latent form of glutathione-insulin transhydrogenase activity. Panels A and B: microsomes were isolated in sucrose/Tris buffer in the absence (closed symbols) or presence (open symbols) of EDTA and stored in the same buffers at 4°C (panel A), −15°C (panel B) or −65°C (panel B). Panel C: microsomes were isolated and suspended in sucrose/Tris buffer in the absence of EDTA (control microsomes, left-hand column). Portions of control microsomes were diluted 3- to 17-fold with buffer and the microsomes were resedimented by a second high-speed centrifugation. The pellets were then carefully resuspended in fresh sucrose/Tris buffer (washed microsomes, right-hand column). At the indicated times following storage (panels A and B) or after washing (panel C), aliquots of the microsome suspensions were assayed for transhydrogenase activity following treatment with buffer (non-latent transhydrogenase activity) and following treatment with 0.2% Triton X-100 (total transhydrogenase activity) and the percentage of non-latent transhydrogenase was calculated. See text for other details. GIT, glutathione-insulin transhydrogenase.

drogenase in the latent state. At −15°C, the production of non-latent transhydrogenase activity is slower than that at 4°C, with the percentage of non-latent transhydrogenase doubling after 8 days storage and continuing to increase slowly with additional storage time. At −15°C, the inclusion of EDTA results in a consistent, but minor, stabilization of latent transhydrogenase activity. However, when microsomes are stored at −65°C (Fig. 1, panel B, lower curves), the percentage of microsomal transhydrogenase found in the nonlatent state remains constant for up to 24 days regardless of the presence or absence of EDTA. Likewise, non-latent transhydrogenase activity remained unchanged in microsomes stored at −75°C (data not shown).

Microsomes were therefore suspended in sucrose/Tris buffer and stored at −75°C for use in the further studies reported here.

Removal of adsorbed protein from isolated microsomes by dilution and washing had no significant effect on the percentage of non-latent transhydrogenase found in the vesicles (Fig. 1, panel C). These findings indicate that non-latent microsomal transhydrogenase activity is not due to the activity of an adsorbed cytoplasmic enzyme.

The topology of microsomal glutathione-insulin transhydrogenase was investigated by determining enzyme activity in microsomes following treatment with increasing concentrations of Triton X-100 (a non-ionic detergent), deoxycholate (an ionic detergent) and phospholipase [18,19]. As shown in Fig. 2, exposure of intact microsomes to any of the three agents results in a concentration-dependent increase in the amount of measurable transhydrogenase activity with maximum activation occurring at 0.06% (v/v) Triton X-100, 0.05% (w/v) deoxycholate and 0.3 μg phospholipase A_2 per ml. A near quantitative recovery of microsomal transhydrogenase in the high-speed supernatant (Fig. 2) following treatment with each agent indicates that the unmasking of microsomal transhydrogenase

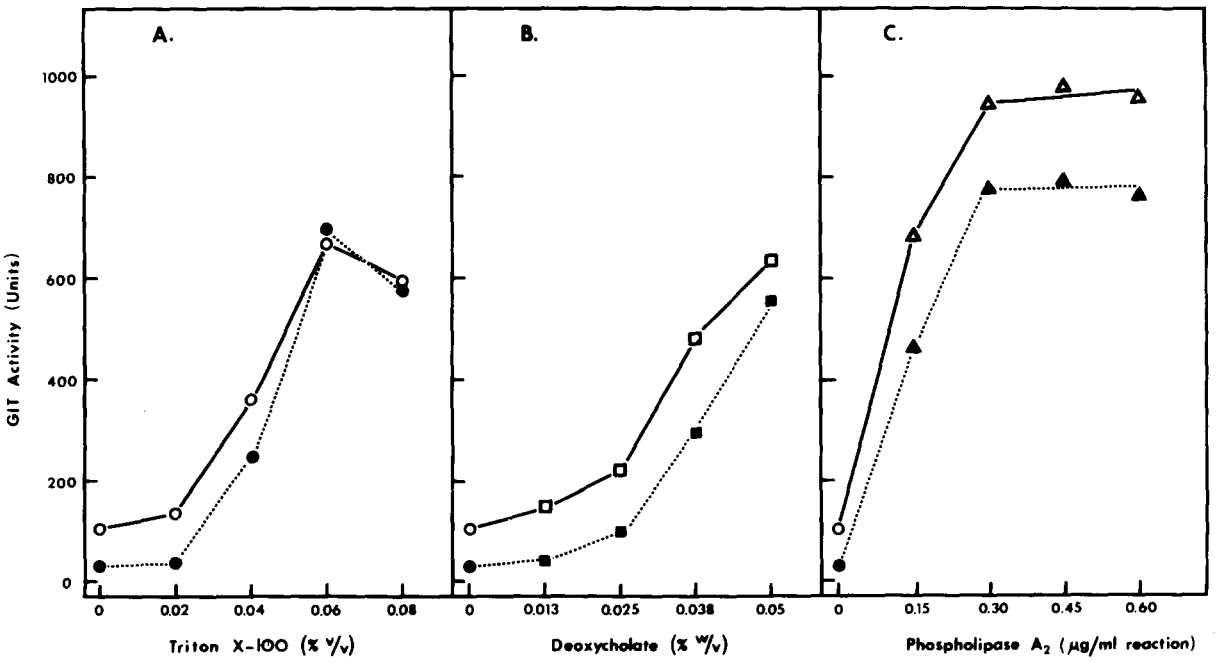

Fig. 2. Glutathione-insulin transhydrogenase activity of microsomes treated with varying concentrations of Triton X-100, deoxycholate or phospholipase A_2. Microsomes (approx. 3.5 mg protein/ml) were treated with the indicated concentrations of Triton X-100 (panel A), deoxycholate (panel B) or phospholipase (panel C) and subjected to high-speed centrifugation. Glutathione-insulin transhydrogenase activity was measured using the treated microsomes before centrifugation (open symbols) and in the high-speed supernatants following centrifugation (closed symbols). Units of the transhydrogenase activity indicated on the ordinate are per mg protein in the original microsomal fraction. See text for other experimental details. GIT, glutathione-insulin transhydrogenase.

activity by detergents and phospholipase is accompanied by a parallel release of enzyme into the surrounding medium. Although exposure of the microsomes to the three agents results in a nearly 20-fold increase in soluble transhydrogenase activity (Fig. 2), the protein in the high-speed supernatant increased by 2-fold only, resulting in a 10-fold net increase in the specific activity of glutathione-insulin transhydrogenase of the high-speed supernatant (Fig. 3). These findings suggest that Triton, deoxycholate and phospholipase cause the release of certain selective microsomal proteins including glutathione-insulin transhydrogenase. Thus, microsomal transhydrogenase appears to be among the group of loosely bound microsomal proteins which are released from the vesicle by exposure to non-disruptive conditions [22,26].

The topology of insulin transhydrogenase in microsomes was further probed by determining the susceptibility of the enzyme to proteolytic inactivation by trypsin (Table I) and thermolysin

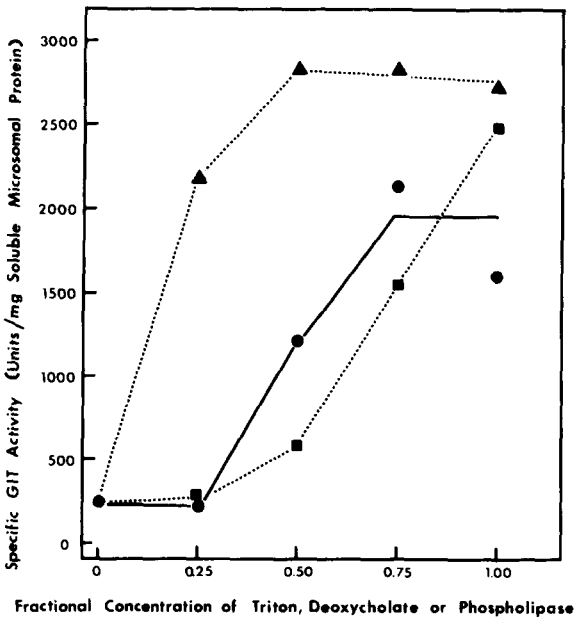

Fig. 3. The specific activity of solubilized microsomal glutathione-insulin transhydrogenase (GIT) following treatment of microsomes with various agents. The high-speed supernatant transhydrogenase activity data shown in Fig. 2 are replotted as units of transhydrogenase activity/mg of the supernatant protein. The fractional concentration of 1.00 on the abscissa represents 0.08% for Triton X-100 (●), 0.05% for deoxycholate (■) and 0.6 μg/ml for phospholipase (▲).

(Table II) using microsomes having different degrees of permeability. In Table I, the set of experiments designated I shows the specific activity of transhydrogenase in untreated microsomes (i.e., non-latent transhydrogenase) and indicates vesicle integrity under basal conditions. It has been previously shown [27–29] that, in the absence of detergent treatment, microsomal vesicles remain impermeable to large molecules and the action of trypsin is limited to the vesicle surface. As can be seen from Table I, in non-detergent-treated closed vesicles, trypsin reduces non-latent transhydrogenase activity by 25% (approx. 20 units/mg) (Expt. II) and the subsequent exposure of these vesicles to either deoxycholate (Expt. IIIA) or Triton X-100 (Expt. IIIB) shows the presence of full transhydrogenase activity (cf. Fig. 2) except for the same small reduction (approx. 20 units/mg) in transhydrogenase activity. When microsomal vesicles are treated with 0.03% deoxycholate, they become semipermeable to high molecular weight components [22] but still retain 80% of their available transhydrogenase activity in the latent state (cf. Fig. 2, panel B). Therefore, microsomes in 0.03% deoxycholate were exposed to trypsin; after 30 min trypsin inhibitor was added and the microsomes were treated with 0.05% (final concentration) deoxycholate to unmask completely the transhydrogenase activity. Results of these experiments (Expt. IV) indicate that in semipermeable vesicles trypsin inactivates nearly 60% of the total available transhydrogenase (Expt. IV). In similar experiments with microsomes in which the permeability barrier is completely disrupted by treatment with 0.05% deoxycholate (Expt. VA) or 0.08% Triton X-100 (Expt. VB), trypsin inactivates 67 and 56% of the total transhydrogenase activity, respectively. Hence, trypsin treatment produces the same amount of inactivation of the transhydrogenase in semipermeable vesicles (where transhydrogenase is still an integral part of the membrane) as in completely permeable microsomal vesicles (where transhydrogenase is in the soluble state).

Additional experiments to define the microsomal permeability requirements for proteolytic inactivation were done using thermolysin, a proteinase with a broad substrate specificity (Table II). Thermolysin treatment of intact microsomal

TABLE I

SUSCEPTIBILITY TO TRYPSIN OF GLUTATHIONE-INSULIN TRANSHYDROGENASE ACTIVITY CONTAINED IN MICROSOMAL VESICLES HAVING DIFFERENT DEGREES OF PERMEABILITY

Microsomes (approx. 3.5 mg protein/ml) with impermeable (Expts. II and III), semi-permeable (Expt. IV), or fully-permeable (Expt. V) membranes were pretreated by incubation at 25°C in the absence or presence of trypsin (50 μg/mg microsomal protein). After 30 min, trypsin activity was terminated by the addition of trypsin inhibitor (160 μg/ml). I, unincubated microsome preparations, which exhibit non-latent transhydrogenase activity, were carried through in all experiments as an additional control. II, intact microsomes (i.e., containing impermeable membranes) were pretreated with or without trypsin, trypsin inhibitor was added and the microsomes were immediately assayed for transhydrogenase activity. III, intact microsomes were pretreated with and without trypsin, trypsin inhibitor was added and incubations were continued for another 30 min in 0.05% deoxycholate (IIIA) or 0.08% Triton X-100 (IIIB) to unmask all transhydrogenase activity. IV, microsomes were pretreated with and without trypsin in submaximal concentrations of deoxycholate (0.03%), trypsin inhibitor was added and incubations continued for another 30 min in 0.05% deoxycholate to unmask total available transhydrogenase activity. V, microsomes were pretreated with and without trypsin in maximal concentrations of deoxycholate (0.05%) (VA) or Triton X-100) (0.08%) (VB) for 30 min, trypsin inhibitor was added and transhydrogenase activity was determined. Glutathione-insulin transhydrogenase activity data shown are mean ± S.E. of the number of determinations indicated in parentheses; *P* values are for comparison between trypsin-treated and buffer-treated reactions in the same set of experiments. Differences in transhydrogenase activity in different experiments represent different preparations of microsomes used. See text for other details. DOC, deoxycholate; ns, not significant.

Experiment	Treatment of microsomes		Glutathione-insulin transhydrogenase (units/mg protein)		*P*-value
	First incubation	Second incubation	Buffer-treated	Trypsin-treated	
I	—	—	101 ± 7(12)	—	
II	± Trypsin	—	87 ± 2 (5)	63 ± 2(5)	< 0.001
IIIA	± Trypsin	0.05% DOC	509 ± 10 (4)	488 ± 6(4)	ns
IIIB	± Trypsin	0.08% Triton	565 ± 7 (4)	556 ± 7(4)	ns
IV	0.03% DOC ± trypsin	0.05% DOC	773 ± 14 (4)	336 ± 23(4)	< 0.001
VA	0.05: DOC ± trypsin	—	490 ± 28 (9)	160 ± 11(9)	< 0.001
VB	0.08% Triton ± trypsin	—	513 ± 17 (9)	226 ± 10(9)	< 0.001

TABLE II

SUSCEPTIBILITY TO THERMOLYSIN OF GLUTATHIONE-INSULIN TRANSHYDROGENASE ACTIVITY CONTAINED IN MICROSOMAL VESICLES HAVING DIFFERENT DEGREES OF PERMEABILITY

I, intact microsomes (i.e., containing impermeable membranes) were pretreated at 25°C with thermolysin in the absence and presence of 15 mM EDTA, which inactivates thermolysin. After 30 min, both sets were assayed for transhydrogenase activity. II, intact microsomes were incubated for 30 min with or without thermolysin, 15 mM EDTA was added to terminate the activity of thermolysin and transhydrogenase activity was unmasked by another 30 min incubation in 0.08% Triton X-100. III, microsomes with fully permeable membranes were prepared by incubating microsomes for 30 min in the presence of 0.5 μg phospholipase/ml with or without thermolysin, 15 mM EDTA was added and transhydrogenase activity was determined. The concentration of microsomal protein was approx. 3.5 mg/ml and that of thermolysin (where included) was 50 μg/mg microsomal protein. All reactions contained 5 mM Ca^{2+}. Glutathione-insulin transhydrogenase activity data shown are mean ± S.E. of the number of determinations indicated in parentheses. *P*-values are for comparison within the same set of experiments. See text for other details. PLP A_2, phospholipase A_2.

Experiment	Treatment of microsomes		Glutathione-insulin transhydrogenase (units/mg protein)		*P* value
	First incubation	Second incubation	Control	Thermolysin-treated	
I	Thermolysin ± EDTA	—	72 ± 4(5)	61 ± 5(5)	0.05
II	± Thermolysin	Triton	514 ± 5(4)	471 ± 8(8)	< 0.01
III	PLP A_2 ± thermolysin	—	654 ± 15(5)	494 ± 17(5)	< 0.001

vesicles caused a 15% (11 units/mg) loss of non-latent transhydrogenase activity (Expt. I) and subsequent unmasking of all transhydrogenase showed only an 8% (43 units/mg) loss of total transhydrogenase activity (Expt. II). However, when microsomal vesicles are rendered completely permeable by phospholipase (0.5 μg/ml) and subjected to thermolysin treatment, thermolysin produced a 25% (160 units/mg) inactivation of total microsomal transhydrogenase activity (Expt. III). Thus, both trypsin and thermolysin caused greater inactivation of transhydrogenase in permeable vesicles than in intact vesicles.

Phospholipase may increase microsomal glutathione-insulin transhydrogenase activity through two mechanisms, by disruption of the protein-phospholipid interactions in the microsomal membranes and/or through a detergent-like effect of the products of the phospholipase reaction (free fatty acids and lysophosphatides) on the microsome surface which can be inhibited by albumin [22,28]. The data given in Fig. 4 indicate that albumin partially inhibits the dose-dependent activation of microsomal transhydrogenase, the degree of inhibition being inversely proportional to the concentration of phospholipase present. Hence, albumin (50 mg/ml) inhibition of phospholipase-induced transhydrogenase activation varied from 80 to 50% when microsomes were incubated with submaximal concentrations of phospholipase (0.1–0.5 μg/ml respectively) and from 50 to 33% when saturating phospholipase concentrations (0.5–1.0 μg/ml respectively) were used. Whether fatty-acid-depleted albumin would have been more effective was not investigated.

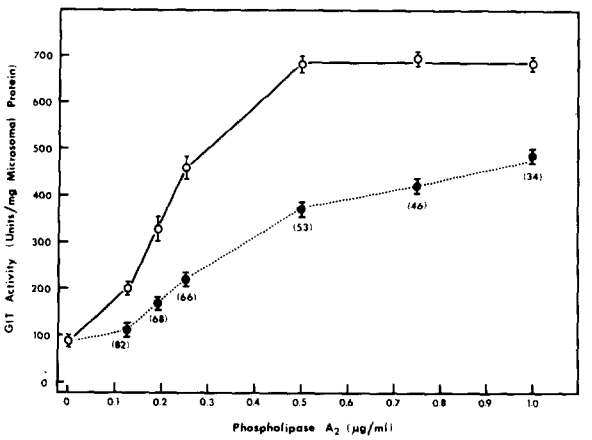

Fig. 4. Inhibition by bovine serum albumin of phospholipase A_2-induced microsomal glutathione-insulin transhydrogenase activation. Microsomes (approx. 3.5 mg protein/ml) were incubated at 4°C with varying concentrations of phospholipase A_2 in the absence (○) or presence (●) of 50 mg bovine serum albumin/ml reaction. After 20 min, transhydrogenase activity was determined. The data shown are mean ± S.E. of 2–4 determinations. Values in parentheses are the percent of inhibition by albumin of the net increase in transhydrogenase activity at each level of phospholipase. See text for other details. GIT, glutathione-insulin transhydrogenase.

Discussion

In the present study, microsomes were isolated under conditions where the relative proportion of non-latent glutathione-insulin transhydrogenase activity is not altered following an extensive wash procedure (Fig. 1). This indicates that the small fraction (approx. 14%) of measurable transhydrogenase activity found in untreated microsomes is a membrane-associated enzyme and not a contaminant. These isolated microsomes were used to explore the intravesicle orientation of transhydrogenase and the nature of its latency.

During prolonged storage of isolated microsomes, a time- and temperature-dependent increase occurs in the fraction of total transhydrogenase found in the non-latent (i.e., active) state (Fig. 1). The percentage of non-latent transhydrogenase increases during storage at −15°C or at a higher temperature but remains stable during storage at −65°C or at a lower temperature. These results confirm and extend previous work from this laboratory which indicated a time-dependent increase in microsomal transhydrogenase activity at −15°C [19]. The time- and temperature-dependent increase in active transhydrogenase is slowed by the inclusion of 5 mM EDTA. Although the role of EDTA in stabilizing latent transhydrogenase is not known, it is suggestive of a role for cations such as Mg^{2+} or Ca^{2+} in either membrane destabilization or as direct modifiers of transhydrogenase latency.

Previous reports have indicated that the unmasking of microsomal transhydrogenase by detergents and phospholipase is accompanied by enzyme solubilization [18,19] and solubilization by Triton X-100 has been used as a major step in

microsomal transhydrogenase purification [16]. However, since the ratio of effectors to microsomal protein in these studies was greater than is required for non-disruptive membrane perturbation [26,28,30], previous reports do not define a functional relationship between latent microsomal transhydrogenase activation and its release from the membrane. In the present study we have used effector (detergents and phospholipase) to microsomal protein ratios which allow the release of vesicle contents without producing vesicle disassembly [26,28,30] to explore a possible relationship between latent transhydrogenase activation and enzyme solubilization. The results of these experiments (Figs. 2 and 3) indicate that a close association exists between the unmasking of latent transhydrogenase activity and its subsequent translocation to the soluble fraction. Moreover, the nearly 10-fold increase in the specific activity of the transhydrogenase of the soluble fraction further indicates that transhydrogenase translocation is partially selective for this enzyme and does not result from general vesicle deterioration.

Combinations of detergents, phospholipase and proteolytic enzymes have been used to explore the topological location of specific microsomal components [22,28,31]. In the present study the topological location of microsomal transhydrogenase was investigated using proteolytic enzymes having a very narrow (trypsin) and a very broad (thermolysin) peptide bond specificity. In the absence of detergents where proteolysis is restricted to the surface of the microsomal vesicle [27–29], only non-latent transhydrogenase is susceptible to trypsin and then only 25% of the activity is inactivated (Table I, Expts. II and III). At 0.03% deoxycholate, high molecular weight components are permitted into microsomal vesicles [22] which still retain a major portion of the transhydrogenase in their membranes (Fig. 2, panel B); in these vesicles, 60% of the total transhydrogenase activity is inactivated by trypsin (Table I, Expt. IV). No additional inactivation by trypsin of total transhydrogenase occurs in vesicles in which the permeability barrier is completely disrupted and transhydrogenase completely solubilized (Table I, Expt. V). Although not as effective as trypsin in inactivating microsomal transhydrogenase, the action of thermolysin upon isolated microsomes was qualitatively similar to that of trypsin (Table II). While only limited proteolytic inactivation of non-latent transhydrogenase occurred when intact microsomes were treated with thermolysin, transhydrogenase inactivation was potentiated when phospholipase was used to increase the vesicle permeability. These results indicate that the latent portion of transhydrogenase is inaccessible to large molecules in intact impermeable microsomes and therefore is localized on the cisternal surface of the vesicle.

When free fatty acids and lysophosphatides, the products of phospholipid hydrolysis by phospholipase action, are bound by albumin, the detergent effect of phospholipase is nullified and the action of this enzyme is then restricted to phospholipid hydrolysis at the vesicle surface [22,28]. The ability of albumin to inhibit latent transhydrogenase activation by phospholipase (Fig. 4) is, therefore, germane to two aspects of the transhydrogenase-microsomal vesicle relationship; intravesicle transhydrogenase location and vesicle-enzyme interaction. That albumin significantly inhibits the unmasking of transhydrogenase activity by low phospholipase concentrations supports the concept that latent transhydrogenase is located on the cisternal surface of the microsome and requires some detergent-like action for complete unmasking of the activity. Conversely, that even a limited unmasking of glutathione-insulin transhydrogenase occurs in the presence of albumin argues that phospholipid hydrolysis is also necessary for microsomal transhydrogenase activation. This supports the hypothesis that latent transhydrogenase is bound to the microsomal membrane through phospholipid-protein interactions [19]; the most likely phospholipids being phosphatidylinositol and/or sphingomyelin, which appear to be localized in the inner half of the vesicle bilayer [22].

In summary, rat liver glutathione-insulin transhydrogenase has been shown to be an integral component of the endoplasmic reticulum. In microsomal vesicles, the enzyme has an asymmetrical distribution with more than 80% of the enzyme located on the cisternal surface, which corresponds to the luminal surface of the endoplasmic reticulum. The physiological significance of this orientation of transhydrogenase in the endoplasmic re-

ticulum remains to be elucidated. Although the exact nature of the interaction between the transhydrogenase and the microsomal membrane is not known, it appears to be weakly bound to the membrane phospholipids [19]. The unmasking of latent transhydrogenase activity occurs by selective translocation of the enzyme to the soluble (cytoplasmic) fraction and not through increases in substrate availability (permeability). In both orientation and mechanism of activation, transhydrogenase resembles the nucleoside diphosphotases [22].

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