Lipid Environment of Gastric Potassium Ion-Stimulated Adenosine Triphosphatase

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The K⁺-stimulated ATPase associated with the purified gastric microsomal fraction can be completely inactivated by treatment with 15% (v/v) ethanol for 60s at 37°C, but not at 25°C. Sequential exposure of the microsomal fraction to 15% ethanol at 25°C and 37°C caused release of 2.5% and 2.9% of the total membrane phospholipids respectively. Restoration of the enzyme activity was achieved by sonication with phosphatidylcholine in the presence of Mg²⁺, K⁺ and ATP, which were essential for the reconstitution. Our data suggest that the phospholipids extracted by 15% ethanol at 37°C are derived primarily from the immediate lipid environment of the enzyme, and ATP, together with the metal ions, helps the partially delipidated enzyme to retain the appropriate configuration for the subsequent reconstitution.

Most membrane-bound enzymes fail to maintain their activities when isolated from the lipid environment. Re-activation of the delipidated enzymes occurs when supplemented with various lipids, specially phospholipids (Sandermann, 1978; Fourcans & Jain, 1974; Dahl & Hokin, 1974). Thus a variety of different phospholipids have been demonstrated to be effective in the reconstitution of (Na⁺ + K⁺)-stimulated ATPase (Korenbrod, 1977; Palatini *et al.*, 1977; Kimelberg, 1976; Le Maire *et al.*, 1976; Knowles & Racker, 1975; Warren *et al.*, 1975). It is noteworthy that for Ca²⁺-stimulated ATPase, a shell of 30 phospholipids, intimately associated with the purified enzyme and essential for its activity, has been demonstrated (Warren *et al.*, 1975; Warren & Metcalfe, 1977). It was suggested (Warren *et al.*, 1975; Warren & Metcalfe, 1977) that the enzyme imposes a lateral segregation of phospholipids in its microenvironment within the membrane to ensure the maintenance of functional configuration.

In all cases mentioned above, the general approach has been to isolate and purify the membrane-bound enzymes in a lipid-free state by using detergents and then to perform the reconstitution in detergent medium, followed by removal of the detergents. We used an alternate approach to study the effects of lipid environment of the activity of membrane-bound enzyme without any detergent treatment. Our approach involves a brief and mild treatment of the microsomal preparation with 15% (v/v) ethanol for 60s at various temperatures and then relating the remaining enzyme activity to the small amount of lipid extracted from the membrane. Brief ethanol treatment at 37°C caused the release of only 2.9% of the total microsomal phospholipids, with complete inactivation of the K⁺-stimulated ATPase activity.

Treatment of the microsomal preparation with ethanol at 0°C and 25°C did not inactivate the K⁺-stimulated ATPase, although 2.5% of the total membrane lipid was extracted at the latter temperature. The enzyme activity of the ethanol-treated membranes at 37°C could be restored by sonication with phospholipids under appropriate conditions and in presence of ATP, K⁺ and Mg²⁺. Our data suggest that lipids intimately associated with the microsomal K⁺-stimulated ATPase can be preferentially removed by brief treatment with 15% ethanol at 37°C, the temperature for optimal performance of the enzyme. Furthermore, ATP, K⁺ and Mg²⁺ play a critical role during restoration of the enzyme with phospholipids, presumably by ensuring that the enzyme maintains the appropriate configuration during lipid insertion.

Part of this work was reported at the annual meeting of the Federation of American Societies for Experimental Biology (Sen & Ray, 1979).

Materials and Methods

Purified microsomal membranes from rabbit gastric mucosa were prepared by the method of Ray (1978). Briefly, the fundic mucosa of rabbit was desquamated and scraped (Forte *et al.*, 1972) to collect the oxyntic-cell-enriched fractions. The mucosal cells were homogenized in 0.25M-sucrose containing 0.2mM-EDTA and 0.2mM-1,4-piperazine-diethanesulphonic acid buffer (pH 6.8) by using a loose-fitting pestle in a Dounce homogenizer. The homogenate was centrifuged at 8000g for 5min. The process was repeated three times. All supernatants were pooled together and layered over 40ml of 37% (w/v) sucrose in 84ml screw-cap tubes and centrifuged at 100000g for 5h in a type 35 Beckman angle

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Table 1. *Lipid composition of rabbit gastric microsomal fraction and supernatants after treatment with 15% ethanol for 60s at 25°C and 37°C*

For the direct ethanol treatment, rabbit gastric microsomal fraction (2mg) was treated with 15% ethanol at 25°C or 37°C. After 60s, the microsomal fractions were diluted with 10 vol. of ice-cold 100 mM-Tris/HCl buffer (pH 7.4) containing 0.5 mM-dithiothreitol and centrifuged at 150000g for 60min. For the sequential treatment the pellet from 25°C was suspended in Tris buffer as above and treated again with 15% ethanol at 37°C. After 60s the membranes were diluted and centrifuged as above. The supernatants were separately extracted three times with chloroform/methanol (1:2, v/v), evaporated, dissolved in a known volume of chloroform and assayed for phospholipids and cholesterol as described in the Materials and Methods section.

Treatment	Phospholipids ($\mu\text{g}/\text{mg}$ of membrane protein)	Cholesterol ($\mu\text{g}/\text{mg}$ of membrane protein)	Cholesterol/ phospholipid molar ratio
Gastric microsomal fraction	117.0 $\pm$ 2.0	69.0 $\pm$ 2.1	1.6
Ethanol, 37°C (direct)	6.1 $\pm$ 0.06	18.7	7.7
Ethanol, 25°C (direct)	2.9 $\pm$ 0.06	7.7	7.2
Pellet (25°C), ethanol, 37°C (sequential)	3.3 $\pm$ 0.12	11.2	9.2

rotor. The microsomal-membrane band appeared at the interface of the clear soluble supernatant and 37% sucrose. The membrane bands were collected, diluted with homogenizing medium and centrifuged at 100000g for 90min. The pellet was resuspended in homogenizing medium at an appropriate protein concentration and used for the study. Proteins were assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

The ATPase was assayed as previously described (Ray, 1978). The incubation mixture contained, in a total volume of 1 ml, 50 μmol of Tris/HCl (pH 7.4), 0.05 μmol of EDTA, 1 μmol of MgCl_2 , 2 μmol of Tris/ATP, 10 μg of membrane protein, in the presence or absence of 2 μmol of KCl. After 15 min incubation at 37°C, the reactions were stopped by adding 1 ml of 12% (w/v) trichloroacetic acid. The P_i was assayed by the procedure of Sanui (1974). The K^+ -stimulated ATPase activity was calculated by subtracting the basal rate (with Mg^{2+} as the only cation) from the rate of hydrolysis of ATP in presence of both Mg^{2+} and K^+ .

Microsomal membranes [1–2 mg in 4 ml of 0.1 M-Tris/HCl (pH 7.4)/0.5 mM-dithiothreitol/1 mM- Mg^{2+}] were treated with 15% ethanol at 0°C, 25°C and 37°C for 60s, diluted 10-fold with ice-cold 0.1 M-Tris/HCl buffer (pH 7.4) and centrifuged at 150000g for 60min. The pellet was assayed for K^+ -stimulated ATPase activity and the supernatant was assayed for lipids. The supernatants were vigorously extracted four times with equal volumes of chloroform/methanol (1:2, v/v). After separation of the two phases by brief centrifugation, the chloroform layer was collected, dried under N_2 and dissolved in a desired volume of chloroform for assay of lipids.

The lipids were extracted from the purified microsomal membrane by the methods of Bligh & Dyer (1959). A nitrogen atmosphere was maintained during extraction to prevent aerial oxidation. Lipid phosphorus was assayed by the Bartlett (1959)

procedure and cholesterol by using $\text{FeCl}_3/\text{H}_2\text{SO}_4$ (King & Wotton, 1956).

Phospholipids (50 $\mu\text{g}/\text{ml}$; egg or gastric microsomal phosphatidylcholine) were dispersed in 0.1 M-Tris/HCl buffer (pH 7.4) containing 0.5 mM-EDTA by sonication for 10min at 37°C. Restoration of K^+ -stimulated ATPase activity of the ethanol-treated membranes was done by mixing equal volumes of phospholipid suspension and the membranes, suspended in 0.1 M-Tris/HCl (pH 7.4)/1 mM- MgCl_2 /2 mM-KCl/2 mM-ATP. The mixture was sonicated vigorously (Ultrasonic, model W-225 R, 20 kHz at 6 A) at 37°C for 10min and centrifuged at 150000g for 60min. The membrane pellet was resuspended in 0.1 M-Tris/HCl buffer (pH 7.4) and assayed for K^+ -stimulated ATPase activity.

The variability of samples is expressed as mean $\pm$ S.E.M. of three separate experiments.

Results and Discussion

The sum total of the phospholipids and cholesterol released from the gastric microsomal fraction by sequential treatments with 15% ethanol at 25°C and 37°C for 60s equals the amounts of the corresponding lipids extracted by direct treatment with ethanol at 37°C (Table 1). The cholesterol/phospholipid molar ratios in the ethanol extracts are 7.2 and 9.2 at 25°C and 37°C (sequential treatment) respectively and are much higher than that of the total microsomal fraction (Table 1). Such substantial differences in the cholesterol/phospholipid ratios might suggest that those lipids are extracted largely from different regions of the membrane surface at different temperatures. This view is also suggested by our data (Table 2) on the differential effects of 15% ethanol on the activity of membrane-bound K^+ -stimulated ATPase at various temperatures. Thus the data in Table 2 showing the inactivation of microsomal ATPase at 37°C, but not at 25°C,

Table 2. *Microsomal K⁺-stimulated ATPase activity and release of membrane lipids after sequential treatment with 15% ethanol for 60s at various temperatures*

Gastric microsomal fraction (2mg) was sequentially treated with 15% ethanol for 60s at 0°C, 25°C and 37°C as follows. The microsomal fraction was exposed to 15% ethanol at 0°C. After 60s the membranes were diluted 10-fold with ice-cold 100mM-Tris/HCl (pH 7.4) containing 0.5mM-dithiothreitol and centrifuged at 150000g for 60min. The pellet was resuspended in the same Tris/dithiothreitol buffer, treated with ethanol at 25°C for 60s, diluted 10-fold with ice-cold Tris/dithiothreitol buffer and centrifuged as above. The pellet was again treated with ethanol at 37°C and processed as above. The membranes after each treatment were tested for K⁺-stimulated ATPase activity and the supernatants were extracted with chloroform/methanol (1:2, v/v) as described in Table 1 and the Materials and Methods section for lipid analysis. The K⁺-stimulated ATPase activity represents the rate of hydrolysis of ATP in the presence of Mg²⁺ and K⁺ minus the basal rate (Mg²⁺ alone). The basal activity was 10.2 μmol/h per mg of protein.

Temperature (°C)	K ⁺ -stimulated ATPase		Phospholipids released (% of total)
	(μmol/h per mg)	(% of activity at 0°C)	
0	18.5	100	0
25	18.1	98	2.5
37	0	0	2.9

suggest that the lipids solubilized by 15% ethanol at 37°C are derived primarily from the immediate vicinity of the K⁺-stimulated ATPase within the membrane, unlike those lipids solubilized at 25°C. However, the effect of 15% ethanol on gastric microsomal ATPase at 37°C may also in part be due to an indirect effect arising from the extraction of lipid from the membrane as a whole.

An Arrhenius plot of the data on the kinetics of the K⁺-stimulated ATPase activity of the untreated membranes at various temperatures show two discontinuities, one at 15°C and the other at 35°C (P. C. Sen & T. K. Ray, unpublished work). The activities of the enzyme at 25°C and 15°C are about 40% and 7% of the optimal activity (at 37°C) respectively. Studies with several membrane-bound enzymes (Raison *et al.*, 1971; Grisham & Barnett, 1973; Kimelberg & Papahadjopoulos, 1974) suggest that the membrane lipids must be appropriately fluid for the enzymes to function. In view of the

preceding information it is likely that the lipid microenvironment of the membrane-bound K⁺-stimulated ATPase would be in an appropriate fluid state at 37°C to allow the enzyme to undergo necessary conformational changes during efficient operation, but to be in a more crystalline state at 25°C or below. Hence it appears that the preferential extraction of some membrane lipids at 25°C and 37°C by 15% ethanol (Table 2) is possibly due to changes in fluidity of those lipids at the respective temperatures.

Microsomal K⁺-stimulated ATPase activity, abolished by 15% ethanol at 37°C (Table 2), can be restored by the gastric microsomal phosphatidylcholine (Table 3). Egg phosphatidylcholine is equally effective in the restoration of K⁺-stimulated ATPase activity (Table 3). Cholesterol is completely ineffective in the reconstitution process. Cholesterol is known to interfere with the activity of membrane-bound Ca²⁺-stimulated ATPase (Warren *et al.*,

Table 3. *Reconstitution of ethanol (37°C)-inactivated microsomal K⁺-stimulated ATPase activity by phosphatidylcholine*

Microsomal membranes (approx. 200 μg) were treated with ethanol at 37°C for 60s as described in Table 2. The ethanol-treated membranes were then resuspended in a medium containing 1 mM-Mg²⁺, 2 mM-ATP and 2 mM-K⁺ and sonicated with an equal volume of phosphatidylcholine (50 μg/ml) dispersed in Tris/EDTA medium as described in the Materials and Methods section. The reconstitution was done at 37°C. After 10 min sonication, the membranes were harvested by centrifugation at 150000g for 60 min and tested for K⁺-stimulated ATPase activity. Details are given in the Materials and Methods section. The control (untreated) and the ethanol-treated (37°C) membranes were also sonicated in parallel in a medium of similar composition to that used for the reconstitution experiments without the phospholipids.

Treatment	K ⁺ -stimulated ATPase activity (μmol/h per mg)
Untreated microsomal fraction	18.2 ± 0.6
Ethanol-treated (37°C)	0
Ethanol-treated + cholesterol	0
Ethanol-treated + microsomal phosphatidylcholine	16.3 ± 0.5
Ethanol-treated + egg phosphatidylcholine	16.0 ± 0.6

Table 4. *Effects of Mg^{2+} , K^+ and ATP on the reconstitution of gastric K^+ -stimulated ATPase activity*

The activity of K^+ -stimulated ATPase in control membranes (without ethanol treatment) was taken as 100. When present, the concentrations of Mg^{2+} , K^+ and ATP were 2mM each. After sonication of the membranes (200 μ g) with phosphatidylcholine (50 μ g) for 10 min at 37°C in the presence of various agents, the membranes were collected by centrifugation and tested for K^+ -stimulated ATPase activity. Details are given in Table 3 and the Materials and Methods section.

Agents tested	K^+ -stimulated ATPase activity (% of control)
None	0
Mg^{2+}	0
K^+	0
ATP	12
Mg^{2+} +ATP	15
K^+ +ATP	20
K^+ + Mg^{2+} +ATP	90 $\pm$ 3

1975), where replacement of the neighbouring phospholipids by cholesterol inhibits the enzyme activity. It would be interesting to know if cholesterol has similar inhibitory effects on the gastric K^+ -stimulated ATPase system.

Our data show that an appropriate lipid environment is essential for the maintenance of K^+ -stimulated ATPase activity. In the absence of a proper lipid milieu, as in ethanol-treated membranes at 37°C, the state of the enzyme molecule becomes such that it would neither support any hydrolysis of ATP nor allow reconstitution with phospholipids in the absence of Mg^{2+} , K^+ and ATP (Table 4). The enzyme activity can be restored almost completely by phospholipids in the presence of Mg^{2+} , K^+ and ATP. As shown in Table 4, Mg^{2+} , K^+ and ATP alone or a combination of any two are without much effect. Thus it appears that Mg^{2+} , K^+ and ATP somehow help the ethanol-treated inactive enzyme to maintain the proper conformational state during the reconstitution so that the phospholipid molecules may occupy the right places in the microenvironment of the enzyme molecule.

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