THE ASSOCIATION OF CARDIOLIPIN WITH DETERGENT-SOLUBILIZED CYTOCHROME *c* OXIDASE

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Cardiolipin seems to enjoy a specific association with cytochrome *c* oxidase. This phospholipid copurifies with beef heart enzyme (1, 2) and could be removed quantitatively only by denaturing the enzyme (1). Recently Robinson (3) developed a procedure for using high ionic strength for reversibly removing 1 or 2 mol of the remaining 3 mol of cardiolipin from the Triton X-100 solubilized enzyme. Restoration of activity was specific for cardiolipin.

RESULTS AND DISCUSSION

Our laboratory has recently found that the spin-labeled cardiolipin analogue 1-(3-*sn*-phosphatidyl)-3-(1-acyl-2-[16-doxylstearoyl]glycero[3]phospho)-*sn*-glycerol described earlier (4) can associate relatively strongly with detergent solubilized cytochrome oxidase containing 8 mol of lipid phosphorous (4 mol cardiolipin) per mole of enzyme (Fig. 1). Even in the absence of added phospholipid the spin labeled fatty acid analogues were not detectably associated; spin-labeled phosphatidylcholine associated very weakly. In Fig. 1, unlabeled cardiolipin (2 mol lipid phosphorous per mol) was added as competing lipid. Spin-labeled phosphatidic acid was bound nearly as strongly as was spin-labeled cardiolipin; spin-labeled phosphatidylglycerol was bound considerably less strongly. These conclusions follow from the interpretation of the relative slopes of the lines shown in Fig. 1 and the values of the intercepts with the abscissa (5). All three analogues bind with the same stoichiometry, 6 mol lipid phosphorous per mol enzyme, which implies binding sites from three mol cardiolipin per mol enzyme. These three binding sites may be identified with those shown by Robinson (3) to be required for activity. Our assumptions are that all the lipid phosphorous was cardiolipin and that all three sites were accessible to the spin label. Equivalent results could be obtained if only one of the sites immobilized the spin label. Further, the spectra obtained were composite, containing, in addition to the spectrum of the detergent solubilized spin-labeled lipid analogue, both a highly immobilized component and a less highly immobilized component as previously observed for spin-labeled cardiolipin (6). The analysis presented in Fig. 1 was carried out by treating both immobilized components as associated.

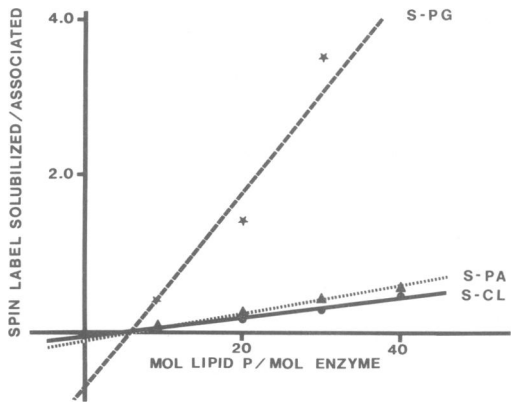

FIGURE 1 Graphic estimation of the number of binding sites and relative partition coefficients for phospholipids to cytochrome oxidase. The ratios of moles of spin-label solubilized to total spin-label immobilized using spin-labeled cardiolipin (SCL), spin-labeled phosphatidic acid (SPA) and spin-labeled phosphatidylglycerol (SPG) are shown vs. the total cardiolipin (added plus intrinsic) given as mol lipid phosphorous/mol enzyme. The sample contained 1 mol spin-labeled phospholipid/mol of cytochrome oxidase at a concentration of 15 mg protein/ml in 10 mM Tris-HCl (pH 7.4) and 2% sodium cholate.

The effects of high ionic strength and high pH in increasing the solubilization and release of spin-labeled cardiolipin from the lipid depleted cytochrome oxidase clearly implicate charge effects between the protein and the polar head group in the binding of cardiolipin to this membrane enzyme. Cholate was more effective than Tween 20, which was in turn more effective than Tween 80 in releasing spin-labeled cardiolipin from cytochrome oxidase. Cytochrome oxidase has been shown to have particularly good activity in Tween 80. Cholate has seemed best for purification.

The detergent-solubilized preparations of cytochrome oxidase exhibit considerable rotational mobility as judged from the saturation transfer electron spin resonance spectroscopy using a protein-localized maleimide spin label[1] (7). This rotational mobility clearly distinguishes these delipidated and detergent-solubilized preparations from the relatively immobile membranous cytochrome oxidase preparations employed earlier (6) as judged from our saturation transfer measurements. The results obtained using spin-labeled cardiolipin with these two different preparations of cytochrome oxidase, which also differ markedly in lipid content, were also distinct.

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[1]Gwak, S. H., and G. L. Powell. Unpublished observations.

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resonance spectrometer (Varian Associates Inc., Palo Alto, CA) at Furman University, Greenville, South Carolina.

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A SOLUBILIZED AND ACTIVE ADENYLATE CYCLASE LIPIDATED WITH NATIVE ANNULAR LIPIDS

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The critical role of phospholipids in the modulation of adenylate cyclase in plasma membranes has often been speculated upon, but unequivocal experimentation has been lacking. The modulating role of the membrane phospholipid milieu upon the enzyme has been demonstrated in intact membranes and cells; however, a requirement for specific phospholipids has been difficult to investigate due to the ease of disruption of lipid-protein associations in detergent solution and the lability of the soluble enzyme during purification (1).

In this communication we present a solubilization of rat liver plasma membranes which liberates a population of membrane proteins surrounded by an annulus of the phospholipids that were in residence at the lipid-protein interface at the moment of solubilization. Unlike K^+-cholate-solubilized adenylate cyclase, which retains an annular phospholipid milieu yet is highly inactive and shows no ligand sensitivity (2), our solubilization procedure yields an enzymatically active and ligand-sensitive adenylate cyclase.

METHODS

Purified rat liver plasma membranes were isolated as described earlier (3). Adenylate cyclase was assayed as described previously (4). Membrane lipid-protein-detergent complexes were isolated by sucrose density gradient centrifugation at 360,000 *g* (5). Phospholipid-phosphorous was determined as a malachite green complex with phosphomolybdate (6) after predigestion of sucrose with nitric acid.

RESULTS AND DISCUSSION

The solubilization of rat liver plasma membrane adenylate cyclase by detergents was examined to determine the extent of lipid association with the liberated soluble membrane proteins. The protein-detergent and protein-lipid-detergent interactions of the soluble membrane components were evaluated. Lubrol-PX, a non-ionic detergent (an ethylene oxide-fatty alcohol adduct [C_{12}–E_9]), is widely used to solubilize membrane-bound adenylate cyclase from rat liver plasma membranes. Purified plasma membranes were solubilized with 100 mM, 50 mM, 10 mM and 1 mM Lubrol-PX at detergent:protein ratios of 12.5, 6.25, 1.25 and 0.125, respectively. We have found that all concentrations of Lubrol-PX used to solubilize adenylate cyclase releases > 89% of the phospholipid present in the soluble membrane fraction as free micellar lipid. Under these conditions, a delipidated enzyme was found in all cases except with 10 mM Lubrol-PX, which yielded a soluble enzyme partially (minimally) associated with lipid. The presence of this lipid-enzyme complex was demonstrated by extended centrifugation at high gravitational fields of the detergent-soluble membrane preparation across a sucrose boundary. The soluble membrane protein was found to bind 4 nmol of phospholipid per 100 μg of soluble protein. These boundary lipids may be associated with high affinity lipid binding sites of the membrane proteins, since they do not represent a complete annular shell of boundary lipids and are the most resistant to detergent substitution.

Since the isolated protein-detergent complex which lacks phospholipids is still active, we suggest that Lubrol-PX provides a supportive milieu for the enzyme, mimicking native phospholipids. The enzyme that is minimally associated with lipid is more stable than the delipidated form and possesses higher enzyme activity and sensitivity to fluoride and guanylyl 5′ imidodiphosphate (GMP-PNP). Maximal fluoride:basal ratios and GMP-

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