Vol. 133, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1985 Pages 1-7

FAD-DEPENDENT MALATE DEHYDROGENASE FROM *MYCOBACTERIUM SMEGMATIS*: ACTIVATION OF THE LIPID-DEPLETED ENZYME BY INCORPORATION INTO CARDIOLIPIN LIPOSOME

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Received September 19, 1985

SUMMARY: The lipid-depleted, enzymatically inactive malate dehydrogenase isolated from *Mycobacterium smegmatis* membrane was found to be incorporated spontaneously into cardiolipin liposome, but not into phosphatidylcholine liposome, as was revealed by electron spin resonance spectra with the use of 5-doxylstearic acid as a spin probe in the phospholipid liposomes. In addition, sucrose density gradient centrifugation in 0.5 M KCl showed hydrophobic interaction of the enzyme with cardiolipin liposome and further proved that the enzyme thus interacted hydrophobically with cardiolipin liposome became enzymatically active. From the results obtained above, it was concluded that the lipid-depleted, enzymatically inactive malate dehydrogenase isolated from *M. smegmatis* membrane was found to be activated by incorporating into the hydrophobic region of cardiolipin liposome.

Phospholipid-requiring enzymes are one of the useful tools for studying lipid-protein interactions in biomembranes [1]. *Mycobacterium smegmatis* malate dehydrogenase is a membrane-bound flavoprotein and became require FAD and cardiolipin, which is the major phospholipid species of the bacterium, for enzyme activity when solubilized from the membrane by the treatment with acetone [2,3]. Later, the enzyme was solubilized with *n*-butanol instead of acetone and was purified to homogeneity in the lipid-depleted, enzymatically inactive form [4]. With this enzyme preparation, we have already reported that phospholipid is required for binding of FAD to the enzyme [5]. And furthermore, we have found that anionic phospholipids including cardiolipin but not zwitter-

Abbreviation: ESR, electron spin resonance.

0006-291X/85 $1.50

ionic phospholipids including phosphatidylcholine could activate the lipid-depleted enzyme [6]. In this communication, we will show, by the use of spin-labeling and sucrose density gradient centrifugation techniques, that the lipid-depleted, inactive malate dehydrogenase isolated from *M. smegmatis* membrane could be incorporated into cardiolipin liposome only by standing at room temperature, while incorporation of the enzyme into phospatidylcholine liposome was not observed, and the enzyme thus incorporated into cardiolipin liposome became enzymatically active.

MATERIALS AND METHODS

Malate Dehydrogenase: *M. smegmatis* malate dehydrogenase was purified in a lipid-depleted, enzymatically inactive form as described in [4] from the bacterium grown on the glycerol-bouillon-pepton medium as described [2]. The purified enzyme preparation was homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [7].

Enzyme Assay: Malate dehydrogenase activity was measured at 20°C as in a previous paper [4], as the reduction rate of 2,6-dichlorophenolindophenol at 600 nm with cardiolipin (activator), FAD (coenzyme), vitamine K_3 (intermediate electron acceptor) and L-malate (substrate) using a Hitachi 124 spectrophotometer. One enzyme unit is defined as the amount which catalyze the reduction of one μmol of 2,6-dichlorophenolindophenol per minute and specific activity is expressed as units per mg of protein.

Phospholipid Liposome: Phospholipid liposome was prepared as follows: each phospholipid (1 mg) or phospholipid (1 mg) plus 5-doxylstearic acid (11 mg) dried under a stream of dry nitrogen and under a reduced pressure was suspended in 10 mM Tris-HCl / 1 mM EDTA, pH 7.4 (1 ml). The resultant turbid suspension was dispersed in an ice bath with sonication under nitrogen atmosphere using a microprobe at a setting of 2-3 (Branson Sonifier model 350) for 5-15 min until no further decrease in turbidity of the sample was observed. Then the dispersed sample was centrifuged for 200,000 x *g* for 60 min and the clear supernatant obtained was used for each experiment.

Electron Spin Resonance Spectra: Electron spin resonance spectra were recorded on a JEOL ME-3X spectrophotometer operating near 9.3 GHz. The spectrometer settings were: microwave power 6 mW, modulation amplitude 6G, scan range 200 G and time constant 0.03 sec. All spectra were recorded at room temperature (∿23°C). Order parameter was calculated from a equation $S = (T_{\parallel} - T_{\perp}) / (T_{zz} - T_{xx})$, where T_{zz} (32.9 gauss) or T_{xx} (5.9 gauss) is the electron-nuclear hyperfine tensor directed to the molecular *z* axis or the molecular *x* axis for the nitroxide, respectively, and $T_{\parallel}$ and $T_{\perp}$ are experimental values of the nitroxide radical

hyperfine constants which can be derived from the obtained spectra [8].

Sucrose Density Gradient Centrifugation: The method used in this study was almost the same as in [9]. A sample (0.1 ml) was layered on top of a 4.7 ml of sucrose density linear gradient (5-20% w/v) in 0.1 M potassium phosphate, pH 7.0 containing 0.5 M KCl and 1 μmol FAD. Centrifugation was performed at 3-5°C for 14 hours at 28,000 rpm using a Hitachi 55P-2 ultracentrifuge with a Hitachi RPS-40 rotor.

Assay Methods: Protein concentration was estimated by the method of Lowry *et al.* [10] with the use of bovine serum albumin as the standard. Phospholipid-phosphorus was determined by the method of Bartlett [11].

Chemicals: The chemicals used were purchased as follows: highly purified egg yolk phosphatidylcholine and horse heart cytochrome *c* from Sigma; 5-doxylstearic acid, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl was from Syva Research Chemicals, Palo Alto, California. Other chemicals were obtained from commercial sources. Beef heart cardiolipin was kindly donated from Sumitomo Chemical Company, Osaka, Japan.

RESULTS AND DISCUSSION

Spin-labeling —— Interactions of the lipid-depleted malate dehydrogenase with phospholipid liposomes were investigated by electron spin resonance technique with the use of a stearic acid spin label, 5-doxylstearic acid. The malate dehydrogenase was incubated at 20°C for 60 min with each phospholipid liposome containing 5-doxylstearate and then the ESR spectra were recorded. The spectra obtained with cardiolipin liposome are shown in Fig. 1. The spin-labeled fatty acid in cardiolipin liposome alone was rather mobile evident from an existence of the spectral components characteristic to the fluid lipid bilayers (Fig. 1, soild line spectrum). Addition of the lipid-depleted enzyme to cardiolipin liposome caused a broadening of an interval between the two outermost peaks of the spectrum (Fig. 1, dashed line spectrum). This may suggest an appearance of an outer hyperfine extrema: immobilized components [8], suggesting that the malate dehydrogenase may be incorporated into cardiolipin liposome by standing at 20°C for 60 min. On the other hand, in the case of phosphati-

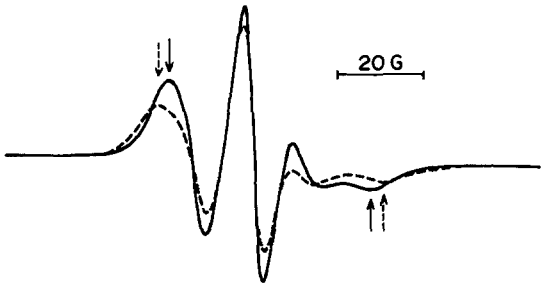

Fig. 1 The effect of the lipid-depleted malate dehydrogenase on the ESR spectrum of cardiolipin liposome spin-labeled with 5-doxylstearic acid. (——); Co-dispersion of cardiolipin (140 μg) and 5-doxylstearic acid (1.5 μg) in 10 μl of 0.1 M potassium phosphate, pH 7.0 containing 1 μM FAD prepared as described under "MATERIALS AND METHODS", (----); similar preparation with added the malate dehydrogenase, *i.e.* the enzyme (60 μg) was incubated at 20°C for 60 min with the co-dispersion of cardiolipin – 5-doxylstearic acid (as described above) in 10 μl of 0.1 M potassium phosphate, pH 7.0 containing 1 μM FAD. A horizontal bar indicates the magnitude of magnetic field in gauss and vertical arrows of solid and dashed lines indicate the outermost lines of each spectrum.

dylcholine liposome used, such a distinct spectral change was not observed when the enzyme was incubated with the liposome (data not shown). To show the extent of immobilization of the spin-label, we calculated the order parameter(s) from each spectrum obtained [8]. From the data shown in Table I, it was clearly

Table I. Order parameters (S) of phospholipid liposomes spin-labeled with 5-doxylstearic acid under various conditions

	Sample	Order parameter(s)
(1)	(a) cardiolipin	0.52
	(b) cardiolipin + enzyme	0.62
	(c) (cardiolipin + enzyme) / 0.5 M KCl*	0.64
	(d) cardiolipin + cytochrome *c***	0.53
(2)	(a) phosphatidylcholine	0.53
	(b) phosphatidylcholine + enzyme	0.53

ESR spectra were obtained under the same condition as in Fig. 1 unless stated. The order parameter was calculated from each ESR spectrum with a equation $S = (T_{\parallel} - T_{\perp}) / (T_{zz} - T_{xx})$ [8].

* 2 M KCl solution was added to the preincubated mixture of the enzyme and cardiolipin liposome (as prepared in (1)-(b)) at the final concentration of 0.5 M.

** Thirty micrograms of horse heart cytochrome *c* (instead of the enzyme) was added to cardiolipin liposome as in (1)-(b).

demonstrated that the addition of the malate dehydrogenase caused an increase of the order parameter of cardiolipin liposome, whereas the order parameter of phosphatidylcholine liposome was not changed. In order to make sure that the increase of the order parameter of 5-doxylstearate in cardiolipin liposome by the enzyme addition could be attributed to its incorporation into the hydrophobic region of the liposome but not its electrostatic binding to the liposome, horse heart cytochrome *c* was used as an example which had been revealed to associate electrostatically with cardiolipin liposome [12], that is, cardiolipin liposome incubated with cytochrome *c* was analyzed by ESR spectra. The calculated order parameter shown in Table I clearly indicated that the addition of horse heart cytochrome *c* to cardiolipin liposome did not affect significantly on the ESR spectrum. Besides, addition of 0.5 M KCl to the performed cardiolipin-malate dehydrogenase complex caused no change of the ESR spectrum, suggesting that the malate dehydrogenase was not dissociated from cardiolipin liposome by washing with 0.5 M KCl. From the results obtained above, the enzyme was incorporated into the hydrophobic region of cardiolipin liposome only by standing at 20°C for 60 min without "cholate dialysis procedure" [13] or "sonic oscillation method" [14].

Sucrose Density Gradient Centrifugation —— An another binding experiment was performed by sucrose density gradient centrifugation method. The malate dehydrogenase was incubated with cardiolipin liposome or phosphatidylcholine liposome for 60 min at 20°C, and then the incubated sample was centrifuged in the sucrose gradient containing 0.5 M KCl. The fractionated samples after centrifugation were analyzed for enzyme activity both in the presence and absence of added cardiolipin. The results obtained are shown in Fig. 2. The malate dehydrogenase alone sedimented slowly and the enzyme sedimented exhibited very low

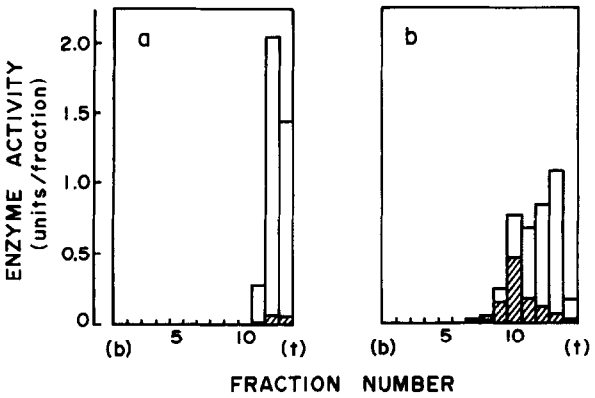

Fig. 2 Sucrose denstiy gradient centrifugation of the lipid-depleted malate dehydrogenase alone and complexed with cardiolipin liposome in 0.5 M KCl. a, the enzyme (30 μg) alone; b, the enzyme (30 μg) was incubated with 5.6 μg of cardiolipin liposome in 0.1 M potassium phosphate, pH 7.0 containing 1 μM FAD at 20°C for 60 min. After centrifugation, the sample was fractionated and analyzed for enzyme activity in the presence (▭) and absence (▨) of the added beef heart cardiolipin. Symbols (t) and (b) represent the top and the bottom of the sucrose gradient, respectively.

enzyme activity (Fig. 2a). The malate dehydrogenase incubated with phosphatidylcholine liposome also sedimented slowly and exhibited little enzyme activity without extraneous addition of cardiolipin (data not shown). On the other hand, as shown in Fig. 2b, when the malate dehydrogenase incubated with cardiolipin liposome was centrifuged in the same sucrose gradient, about half of the malate dehydrogenase was sedimented rapidly, while the remainder sedimented slowly. The rapidly-sedimenting fractions (around fraction number 10) exhibited high enzyme activity without extraneous addition of cardiolipin (shaded areas), while the slowly-sedimenting fractions (around fraction number 13) showed low enzyme activity under the same assay conditions. Therefore, the slowly-sedimenting fractions seemed to contain the lipid-depleted enzyme which had not be incorporated into cardiolipin liposome. In contrast, presence of the enzymatically active, rapidly-sedimenting band in 0.5 M KCl strongly suggested that the malate dehydrogenase must be inserted into the hydrophobic region of cardiolipin liposome.

In conclusion, the above spin-labeling and sucrose density gradient centrifugation experiments revealed that the lipid-depleted, enzymatically inactive malate dehydrogenase isolated from *M. smegmatis* membrane became active when incorporated into cardiolipin liposome. Further studies are now in progress to characterize these enzyme-phospholipid interactions.

ACKNOWLEDGMENTS

The authors wish to express their sincere gratitude to Mr. Y. Kageyama for his technical assistance and to Mrs. M. Taketsugu for her preparing the manuscript. They also thank for generous gift of beef heart cardiolipin to Sumitomo Chemical Company, Osaka, Japan. This research was supported in part by a grant from Matsunaga Science Foundation.

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