FAD-DEPENDENT MALATE DEHYDROGENASE FROM MYCOBACTERIUM SP. STRAIN TAKEO*: A POSSIBLE ROLE OF PHOSPHOLIPID

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SUMMARY: Using a homogeneously purified FAD-dependent malate dehydrogenase, a phospholipid-requiring enzyme from the particulate fraction of Mycobacterium sp. strain Takeo, specific and near stoichiometric binding of FAD to the apoenzyme-cardiolipin complex, but not to the free apoenzyme, was demonstrated by sucrose density gradient centrifugation in the presence of FAD. The result strongly indicates that the FAD-binding site of the apoenzyme is formed by the protein-phospholipid interaction. This is also supported by the fact that the protective effect of FAD on the stability and on pCMB inhibition of the apoenzyme was observed only in the presence of cardiolipin.

In previous papers (1,2), we reported that partially purified malate dehydrogenase requires both FAD and phospholipid for its enzymatic activity. Many similar enzymes have been reported in various bacteria (3-8). One such enzyme was purified from Mycobacterium phlei to 85% homogeneity and its properties were studied by Brodie and his colleagues (9,10).

Our previous work was performed with a partially purified apoenzyme, so that the purification of the enzyme was reexamined to study its physicochemical properties and molecular interaction between the enzyme and phospholipid. Recently we succeeded in preparing an electrophoretically homogeneous apoenzyme, and its

* The name Mycobacterium avium strain Takeo has been used in previous papers. Refer to the text for details.
Abbreviations: pCMB, p-chloromercuribenzoate: DCIP, 2,6-dichlorophenol indophenol.

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purification method and some properties were reported (11). The present paper describes the results in experiments on binding of FAD to the enzyme aimed at clarifying the role of phospholipid.

MATERIALS AND METHODS

Chemicals Chemicals were purchased from commercial sources as described previously (11). Cardiolipin (beef heart) was kindly donated by Sumitomo Chemical Co..

Enzyme assay Assay method was essentially the same as in the previous paper (11), the reduction rate of DCIP at 600 nm with vitamin K_3 as an intermediatory electron acceptor being measured spectrophotometrically using a Hitachi 323 spectrophotometer. One unit of the enzyme is the amount which catalyses the reduction of one μmole of DCIP per min, and specific activity is expressed as units per mg of protein.

Protein Protein concentration was determined by the method of Lowry et al. (12), with bovine serum albumin as standard.

FAD and FMN Concentration of each standard flavin solution was determined spectrophotometrically at 453 nm using a millimolar extinction coefficient of 11.7×10^3 (13). Flavin concentration of fractionated solutions after sucrose density gradient centrifugation was determined fluorometrically according to the method of Burch (14) after FAD had been hydrolyzed into FMN in 10% trichloroacetic acid.

Cardiolipin liposome Cardiolipin liposome was prepared by the method of Saha et al. (15).

Bacterium and bacterial growth Kusunose et al. recently reported that Mycobacterium avium strain Takeo was most closely related to Mycobacterium smegmatis but not to Mycobacterium avium among mycobacteria and norcadia tested in an immunological study of superoxide dismutase (16). Therefore the name Mycobacterium sp. strain Takeo was tentatively used until its classification is established. Bacterial growth was the same as in the previous paper (1).

FAD-dependent malate dehydrogenase FAD-dependent malate dehydrogenase was purified from the particulate fraction of Mycobacterium sp. strain Takeo according to the previous purification method (11). The purified apoenzyme was homogeneous as judged by SDS-polyacrylamide gel electrophoresis.

Sucrose density gradient centrifugation Sucrose density gradient centrifugation was performed by the method of Martin and Ames (17). A sample in 0.1 ml of 0.1 M potassium phosphate buffer, pH 6.8, containing 0.5 μg of disodium FAD per ml and 1 mM EDTA disodium salt, was layered on top of 4.7 ml of 5-20% (W/V) sucrose linear gradient in the above medium. Centrifugation was performed at 3-5° using a Hitachi 55P-2 centrifuge with a Hitachi RPS-40 rotor.

RESULTS

Binding of FAD The apoenzyme was incubated with cardiolipin liposome for 30 min at 0° and then the incubated mixture was subjected to the sucrose density gradient centrifugation. In a

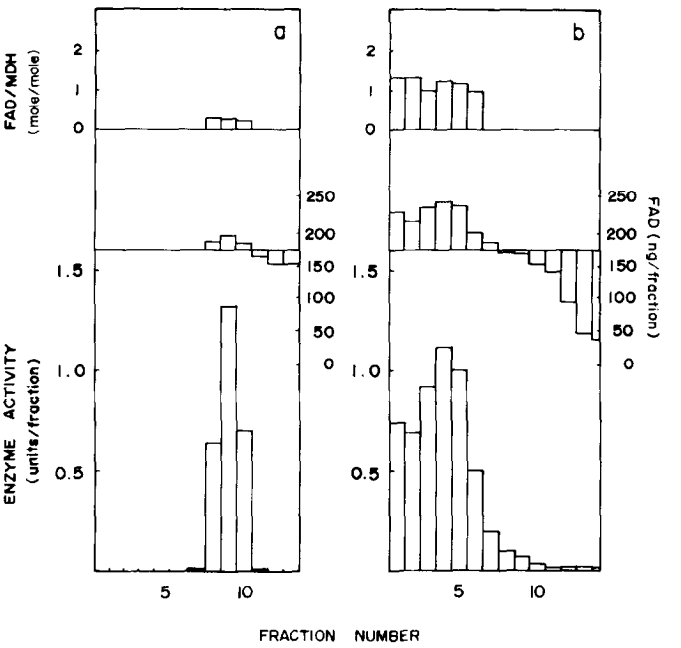

Fig. 1 Binding of FAD to the free apoenzyme and apoenzyme-cardiolipin complex. Apoenzyme (30 μg in 5 μl) in Fig. 1a, and apoenzyme (30 μg) plus cardiolipin (10 μg) in Fig. 1b were incubated for 30 min at 0° in 0.1 ml of the medium described in "MATERIALS AND METHODS". Centrifugation was performed for 22 hr at 39,000 rpm in Fig. 1a and 14 hr at 28,000 rpm in Fig. 1b. As a slight increase of fluorescence at the bottom and decrease at the top were observed when the centrifugation was performed in the absence of the apoenzyme and cardiolipin, the values of FAD (middle parts) were corrected by substracting the values of these control experiments.

sedimentation profile of the centrifugation, all the enzyme activity was found in the fast-moving fraction, which was quite different in position from the slow-moving fraction of the free apoenzyme. Furthermore, the fast-moving fraction showed enzymatic activity in the absence of cardiolipin while cardiolipin liposome alone remained at the top fractions of the gradient after the centrifugation. These results indicate the formation of the apoenzyme-cardiolipin complex. Details of complex formation between apoenzyme and cardiolipin will be reported in near future.

The experiments of FAD binding to the free apoenzyme and the

apoenzyme-cardiolipin complex were studied under the same condition except that prolonged centrifugation was performed for the free apoenzyme, and the results are shown in Fig. 1. FAD was able to bind to the apoenzyme-cardiolipin complex to a considerable extent, but in the case of the free apoenzyme, the binding was to a much lesser extent (Fig. 1, middle parts). Assuming that (a) the molecular weight of the enzyme is 53,000 daltons (11), and (b) only enzyme which shows its activity after the centrifugation is involved in binding with FAD, the amounts of FAD bound to one mole of the enzyme were calculated to be only 0.21-0.30 mole in the free apoenzyme, but in the apoenzyme-cardiolipin complex they were 0.80-1.30 moles (Fig. 1, upper parts). Amounts of the enzyme in each fraction were calculated from their observed enzymatic activity and specific activity of 269 and 280 for apoenzyme and apoenzyme-cardiolipin complex, respectively (11).

No significant binding of FMN to the apoenzyme or the apoenzyme-cardiolipin complex was observed under the same conditions except that 0.3 μg of monosodium-FMN per ml were contained in the medium instead of 0.5 μg of FAD disodium salt. Fluorescence due to enzyme protein was negligible in the fluorometrical measurement.

Protective effect of FAD on stability and pCMB inhibition

The protective effects of FAD on the enzyme are summarized in Table I. The stability of the apoenzyme-cardiolipin complex upon standing for 28 hr at 0° was greatly increased in the presence of FAD in contrast to only a slight increase in the case of the free apoenzyme. Approximately half of the enzymatic activity of the free apoenzyme was lost irrespective of the absence or presence of FAD. The activity of the complex was reduced to about one-fourth of the original activity in the absence of FAD while 94%

Table I

Effect of FAD on stability and pCMB inhibition of the free apoenzyme and apoenzyme-cardiolipin complex

Treatment	Enzyme	Activity Remaining (%)	
		no FAD	plus FAD
Experiment 1 (standing)	Apoenzyme	44	50
	Apoenzyme plus cardiolipin	23	94
Experiment 2 (pCMB inhibition)	Apoenzyme	3	25
	Apoenzyme plus cardiolipin	1	83

Apoenzyme (0.22 μg) was incubated in 0.1 ml of 0.1 M potassium phosphate buffer, pH 6.8, containing cofactors as indicated in the table, where the amounts were 10 μg of FAD and 10 μg of cardiolipin liposome. The activity remaining is expressed as percent of the respective enzyme activities at zero time in Experiment 1 and after 1 hr in the absence of pCMB in Experiment 2. In Experiment 2, 2 μM pCMB was added to the incubation mixture after the addition of cofactors.

of the activity remained in the complex in the presence of FAD. This result suggests binding of FAD to the apoenzyme only in the presence of cardiolipin; and consequently, the apoenzyme is stabilized by forming the holoenzyme as similar to the case of the enzyme in the membrane, which is stable on prolonged standing at 0° (unpublished observation).

Sulfhydryl reagent, pCMB, was inhibitory for both the free apoenzyme and the complex in the absence of FAD. When FAD had been added prior to the addition of the reagent, the protective effect of FAD was quite obvious in the complex, but not to such an extent in the free apoenzyme. This result again indicates that FAD can bind to the apoenzyme-cardiolipin complex.

DISCUSSION

A number of enzymes requiring phospholipids for their enzymatic activity have appeared in the literature and roles of phospholipids are suggested. Little is known, however, about the precise role of phospholipid in enzyme function by reason of difficulty in preparing a lipid-depleted homogeneous enzyme. In a previous paper (2), which showed that this enzyme required phospholipid for its activity even in an assay system using a water soluble phenzine methosulfate as an electron acceptor, the role of phospholipid was assumed to be such that the interaction of phospholipid with the apoenzyme results in the formation of an "active center" and inevitably involves a structural change of the enzyme molecule. This seems to be experimentally proven by the results of the present study: near stoichiometric binding of FAD to the apoenzyme-cardiolipin complex and a protective effect of FAD on the stability of the apoenzyme at standing, and on pCMB inhibition in the presence of cardiolipin. The phospholipid requirement for the enzymatic activity can be explained by the present study, but any other role(s) of phospholipid must still be considered in the light of its location in the membrane and of the fat soluble electron acceptor, vitamin K_2 derivative of the enzyme (18).

Several methods have been used for the measurement of coenzyme-enzyme binding, but these were not applicable to this enzyme due to relative instability and limited availability of the enzyme. Instead, we employed a method, namely, sucrose density gradient centrifugation in the presence of FAD, based on the principle of gel chromatography (19) to measure the FAD-binding to the apoenzyme. This method would be a useful tool in coenzyme binding experiments, if the binding affinity of FAD to

the apoenzyme and to the apoenzyme-cardiolipin complex is not affected during the sedimentation as it passes through sucrose density gradient.

In the absence of cardiolipin, 0.21-0.30 moles of FAD were found to be bound to one mole of the free apoenzyme. The purified apoenzyme preparation used in this study contained about 0.2 mole of phospholipid as cardiolipin per mole of the enzyme; however, its enzymatic activity was only 3% of the maximal enzyme activity in the absence of cardiolipin (11). It is not clear whether or not the unremovable phospholipid influences the result, nor whether or not the value represents the binding affinity of FAD to the apoenzyme under the experimental conditions.

One of about three half cystines in the amino acid composition of the enzyme was titratable by pCMB (11) which was inhibitory for the enzyme activity (unpublished observation). The result of the pCMB inhibition indicates that one cystein residue might be involved in the binding of FAD to the apoenzyme, or it might be located at or near the binding site of FAD of the enzyme.

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