REQUIREMENT OF FLAVIN ADENINE DINUCLEOTIDE AND PHOSPHOLIPID FOR THE ACTIVITY OF MALATE DEHYDROGENASE FROM MYCOBACTERIUM AVIUM

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In a previous communication, Kimura and Tobari (1963) described the occurence of a flavoprotein in Mycobacterium avium concerned with the dehydrogenation of malate to oxaloacetate. This is in contrast to the NAD-linked malate dehydrogenase, well documented in animal tissues and other microorganisms. Later, Asano and Brodie (1963) reported that the activity of malate-vitamine K reductase from M.phlei requires the addition of FAD. Optimal rate of activity was achieved only when vitamine K_1 was suspended in phospholipid. The involvement of phospholipid has also been reported for the β-hydroxybutyrate dehydrogenase isolated from beef heart mitochondria (Jurtshuk, Sekuzu and Green, 1961).

In the present paper the requirement for FAD and phospholipid in order to observe the maximal activity of the soluble malate dehydrogenase purified from the particulate fraction of M.avium is described.

MATERIALS and METHODS

An acetone powder of the particulate fraction from M.avium was prepared as previously reported (Kimura and Tobari, 1963). Apomalate dehydrogenase was extracted from the acetone powder by 0.05 M phosphate buffer (pH 8.9). The extract was acidified to pH 4.8 with 2 N acetic acid and then the apo-enzyme was precipitated by ammonium sulfate between 30 and 60 % saturation. The dialyzed solution was placed on a DEAE-cellulose column which was previously treated with 0.005 M phosphate buffer (pH 5.5). The column was washed with the same buffer and then the apo-enzyme was eluted with 0.1 M phosphate buffer (pH 7.0). At this stage, the specific activity was 1.30 μmoles per min. per mg. protein at 26°C. Rechromatography of

the active fraction on the same column results in another 4 fold purification.

All enzyme assays were performed spectrophotometrically by measurement of reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 mμ in the presence of either phenazine methosulfate, vitamine K_1 or menadione as an intermediate electron carrier.

Bacterial lipids were partially purified from M.avium cells. It was shown by thin layer chromatography that the bacterial lipid preparation contained cardiolipin as the major component, phosphatydyl inositololigomannoside and neutral lipid as minor components and no phosphatydyl ethanolamine. Other experimental procedures are as previously reported (Kimura and Tobari, 1963).

RESULTS and DISCUSSIONS

As shown in Table I, the phospholipid preparation isolated from M.avium cells was able to restore the activity of malate dehydrogenase in the presence of FAD. If FAD and phospholipid were ommitted, less than 20% of the full activity was observed. After the extraction of the apo-enzyme, the additions of FAD and phospholipid were required for the activity.

Table I

Requirement of FAD and Bacterial Phospholipid for the Activity of Malate Dehydrogenase

Reaction Mixture	Malate Oxidized
Complete System	23.2 mμmoles/min.
without Bacterial Lipid	1.3
without FAD	4.2
without Menadione	2.4
without Apo-enzyme	0.0
without Malate	0.0

The complete system contains: 150 μmoles of K-phosphate buffer (pH 7.0), 60 μmoles of L-malate, 3.5 mμmoles of FAD, 400 mμmoles of menadione, 80 mμmoles of DCPIP, 180 μg of bacterial lipid, 18μg of apo-enzyme (fraction after column separation) in 3.0 ml. of total volume. Temperature at 24°C.

Bacterial lipids were extracted from the residue after the extraction of apo-enzyme by stirring for 10 hours at room temperature with methanol-ether (2:1). The organic layer was removed and dried in vacuo. The dried residue was extracted three times with acetone. The acetone insoluble fraction was reextracted with methanol. The methanol extractable phospholipid was used for the enzyme assay.

Since vitamine K's are not freely soluble in water, phospholipid may act to solubilize vitamine K's, resulting in homogeneous solution of the reaction mixture. However, as indicated in Table II, the restoration of the activity by phospholipid was observed in the system containing phenazine, in which system all components are readily soluble in water. Further, beef cardiolipin can completely replace the bacterial phospholipid, whereas two other emulsifying agents, Tween-80 and desoxycholate are inactive for the restoration. Similar results were obtained in the system containing menadione as an acceptor. In the latter system, various cardiolipin analogues were tested. The results show that bis-(dipalmytoyl DL-glycerophosphoryl-1,3-propanediol was able to give the same maximal activity as obtained with cardiolipin, although about 6 times the amount of this material as cardiolipin was required. Also, bis-(dipalmytoyl glyceryl)-phosphate was able to give about 50% of the full activity. In contrast to phenazine and menadione, the use of vitamine K_1 as the

Table II

Specificity of Phospholipid as Factor Required for the Activity of Malate Dehydrogenase When Assayed by Various Electron Acceptors

Phospholipid Added	Electron Acceptor		
	Phenazine	Menadione	Vitamine K_1
None	0.0	0.0	0.0
Cardiolipin	11.4	33.0	31.7
Bacterial Lipid	12.0	30.3	26.5
Lecithin	3.8	5.2	28.7
Tween-80	0.0	0.0	25.8
Desoxycholate	0.0	0.0	8.3

All values are expressed as the enzymic activity (mμmoles/min.). The reaction mixture are similar to Table I, except phospholipid and electron acceptor. Phospholipids and detergents were added at the amount of 10 μg. 205 μg of apo-enzyme (ammonium sulfate fraction) was used. Phenazine methosulfate, menadione and vitamine K_1 were added 1.6 μmoles, 400 mμmoles and 200 mμmoles respectively. Temperature at 25°C.

Egg yolk lecithin was purified essentially as described by Pangborn (1951). Highly purified beef cardiolipin containing 4.2% of P and less than 0.05% of N is a gift from Sumitomo Chemical Company.

electron acceptor permits activation of enzymic activity by a much broader spectrum of surface active compounds. In this system, egg lecithin and Tween-80 can restore full activity and desoxycholate gives partial restoration of activity. It is perhaps pertinent to point out here that the K_m for vitamine K_1 is much lower than that for menadione or phenazine, and that vitamine K_1 and menadione give higher V_{max} than phenazine. Whether the behaviour of vitamine K_1 is related to the less stringent requirement of phospholipid is not clear (Table III).

Table III

Michaelis Constants and V_{max} for Essential Components

Component	K_m	V_{max}
FAD	1.6×10^{-7} M	33.0 mμmoles/min.
Cardiolipin	1.5×10^{-7}	33.0
Vitamine K_1	3.3×10^{-6}	31.7
Menadione	3.0×10^{-5}	33.0
Phenazine	2.5×10^{-4}	13.7

The most striking feature of this phospholipid reactivation is the minute amounts of phospholipid required. For maximal activation, the ratio of cardiolipin to protein is 1:40 by weight. If one assumes a molecular weight for the enzyme (as monomer) to be 40,000, then each mole of the enzyme would contain one mole of cardiolipin. This suggests that the role of the phospholipid can not be such an unspecific one as the emulsification of vitamine K_1, but indicates the formation of a specific phospholipid enzyme complex with 1:1 molar ratio. This hypothesis is also supported by the observation that K_m for for cardiolipin is identical to that for FAD and smaller than the K_m for electron acceptors (Table III). The requirement for cardiolipin

in a completely soluble system, with phenazine as electron acceptor, is also in agreement with this hypothesis. Another interesting feature is the ability of a synthetic phospholipid without any unsaturated fatty acid, to restore the enzymic activity. This eliminates the possibility of the participation of the unsaturated fatty acids in cardiolipin during the oxidation-reduction reaction. Rather it seems more reasonable to suggest that the phospholipid reacts stoichiometrically with the enzyme and results in the formation of an "active center". Whether this involves changes in the tertiary structure of the enzyme molecule must await further work.

Previously, all attempts to solubilize the dehydrogenase gave essentially negative results. This phenomenon may be readily explained by the fact that the particulate preparation contains phospholipid which is necessary for the activity, while the soluble preparation has no phospholipid and therefore inactive even though the apo-enzyme may by present.

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