THE INTERACTION OF THE D(-)β-HYDROXYBUTYRIC APOENZYME WITH LECITHIN

Peter Jurtshuk, Jr.*, Ichiro Sekuzu* and D. E. Green

Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin

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In a companion communication (Sekuzu et al., 1961) we have described the isolation of the mitochondrial β-hydroxybutyric apodehydrogenase in soluble form with a specific activity 135 times higher than that of mitochondria. This is the first dehydrogenase system to be described which shows an absolute requirement for a particular phospholipid, viz., lecithin, and this requirement is additional to that for DPN -- the specific electron acceptor. The present communication deals with some of the many facets of the interaction of enzyme protein with lecithin.

Regardless of the source of isolation (mitochondria, egg, soybean), lecithin fractions were uniformly active. But there were considerable variations between lecithins from different sources and even between different samples of lecithin from the same source with respect to the concentration levels required for achieving maximal reactivation of the enzyme. We have recognized at least three factors which determine the potency of a given lecithin fraction: (1) the degree of unsaturation of the fatty acid residues; (2) the physical (micellular) state of the added lecithin in the assay medium; and (3) the extent and nature of phospholipid impurities.

Synthetic lecithins kindly supplied to us by Professor D. Hanahan of the University of Washington (Hanahan and Brockerhoff, 1960) were invaluable for demonstrating the correlation between the potency of a lecithin preparation and the degree of unsaturation. The saturated synthetic lecithin -- α'β-dipalmitoyl lecithin (added in ethanolic solution) showed only slight activity at low concentration levels (< 10% of the maximum) and at higher concentration levels

*Postdoctoral trainee, Institute for Enzyme Research, Univ. of Wisconsin, Madison

there was no increase in activity. However, the unsaturated lecithin -- α'-palmitoyl, β-oleoyl lecithin was fully active but the concentration required for saturation of the enzyme was some ten times higher than that of the more unsaturated lecithins isolated from natural source, such as those of mitochondria and egg (cf. Fig. 1).

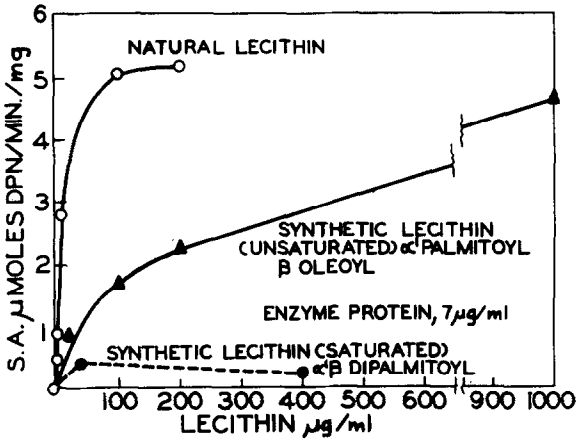

Fig. 1: Comparison of the activities of natural, polyunsaturated lecithin and synthetic lecithins (monounsaturated and saturated) in the β-hydroxybutyric dehydrogenase system.

For details of the assay system, cf. legend of Fig. 1 in the companion paper by Sekuzu et al. in this issue. A mitochondrial phospholipid mixture containing 40% by weight of lecithin was used in lieu of natural lecithin. The two are roughly equivalent in activity. Note that the mitochondrial phospholipid preparation is not a mixture of different phospholipid micelles, but rather a micelle preparation containing multiple phospholipids.

Lecithins were added to the assay medium either in the form of ethanolic solutions or in the form of aqueous dispersions of varying degrees of fineness. When an ethanolic solution of lecithin is introduced rapidly into an aqueous solution, a fine dispersion of lecithin is achieved, but in the comparison of different lecithins introduced by this procedure, there is always the uncertainty whether the observed extent of reactivation is the maximum achievable for the particular lecithin sample.

Fleischer and Klouwen (1961) have pioneered in the technique of preparing soluble phospholipid micelles in aqueous media. Such micelle solutions which are water-clear are ideal for the study of the potency of lecithin and other phospholipid preparations. Fleischer and Klouwen (1961) have observed

that hydrogenated phospholipids lose the capacity to form soluble micelles and the low potency of the synthetic lecithin with saturated fatty acid residues could be related to this incapacity to form soluble micelles.

The highest activity per unit weight of lecithin (ca. 35 μmoles DPN reduced/min/mg lecithin) is achieved when lecithin of natural origin, i.e., with a relatively high degree of unsaturation, is added in the form of a water-soluble micelle solution or in ethanolic solution. Micelle solutions of mitochondrial phospholipid (a mixture of phospholipids containing 40% by weight of lecithin) are equivalent in potency to solutions of purified lecithin. The apoenzyme is maximally reactivated when the lecithin:protein ratio (mg/mg) is about 2 (cf. Fig. 2). The combination takes place rapidly even at 0° and is independent of the absolute concentration of the two reactants. When a lecithin or phospholipid preparation is contaminated with lysolecithin or as yet unidentified phospholipids, a quite different picture is obtained. Maximal reactivation of the apoenzyme is achieved only at relatively high lecithin:protein ratios (> 30:1) or relatively high protein:lecithin ratios (> 60:1). When these low potency preparations of lecithin are purified by paper chromatography or column techniques, the purified lecithin thus obtained now shows activity at greatly reduced lecithin:protein ratios (ca. 2:1) (cf. Fig. 2).

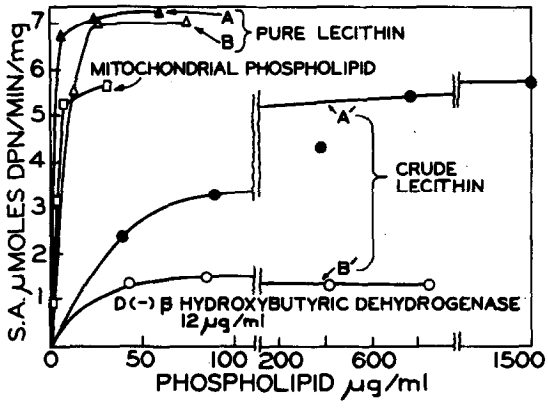

Fig. 2: Activity-concentration relationship for lecithins of varying degree of purity. See Sekuzu et al. (1961) for details of assay procedure.

A is the chromatographically pure sample of lecithin prepared from sample A'; B stands in the same relationship to B'. Cell samples were used in the form of ethanolic solutions.

The purified lecithin preparations were kindly supplied to us by Drs. S. Fleischer and G. Brierley.

Although phospholipids other than lecithin show no activation effect over wide concentration ranges, these do inhibit the combination of enzyme protein with lecithin to a greater or lesser degree. Table I lists the concentrations of the various phospholipids required for 50% inhibition of the rate of oxidation of β-hydroxybutyrate at various concentration levels of lecithin. It is to be noted that the acidic phospholipids (inositol phosphatide, cardiolipin and phosphatidic acid) are highly inhibitory, whereas the amphoteric phospholipids (phosphatidyl ethanolamine and sphingomyelin) are either non-inhibitory or inhibitory only in relatively high concentrations. The inhibition by the acidic phospholipids cannot be reversed by increasing the concentration of lecithin in the assay medium.

TABLE I

Inhibition by phospholipids of the interaction of β-hydroxybutyric apoenzyme with lecithin.

Phospholipid	Concentration of Phospholipid required for 50% inhibition μg/ml	Concentration of lecithin μg/ml
Cardiolipin*	8	27
	11	270
	12	1,100
Phosphatidic acid*	10	27
	7	270
	6	1,100
Phosphatidyl inositol*	4	22
	7	220
	7	870
Phosphatidyl ethanolamine*	--	27
	437	270
	565	1,100
Sphingomyelin**	--	22
	460	220
	535	870

* Added in the form of soluble micelle solution.
** Added in ethanolic solution.

The experimental observations reported above and in the previous communication have demonstrated (1) the chemical specificity of lecithin for activation;

(2) the absolute requirement for lecithin; and (3) the dependence of maximal enzymatic activity on the concentration of lecithin. The conclusion is clear that lecithin is an essential component of the β-hydroxybutyric dehydrogenase system. The ultracentrifuge data are compatible with a molecular weight of the apoenzyme in the range of 5×10^4 - 20×10^4. Assuming a 2:1 stoichiometry (2 mg lecithin/mg protein), then each molecule of protein would combine with 100-400 molecules of lecithin. This high molar ratio argues against a simple coenzyme function for lecithin. We incline to the interpretation that the active enzyme is a lecithinoprotein and that lecithin contributes to the tertiary structure of the enzyme.

With the accumulation of evidence from our laboratory that each of the four complexes of the electron transport chain contains lipid and that lipid is essential for all the enzymatic activities of these four basic complexes, it becomes increasingly clear that the interactions between the β-hydroxybutyric apodehydrogenase and lecithin should point the way to an understanding of the precise role of lipid in mitochondrial oxidations (Green and Lester, 1959; Fleischer, Klouwen and Brierley, 1961).

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