ROLE OF LECITHIN IN D-β-HYDROXYBUTYRATE DEHYDROGENASE FUNCTION

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SUMMARY

Binding of NADH to D-β-hydroxybutyrate dehydrogenase, a lecithin-requiring enzyme from beef heart mitochondria, has been studied using a homogeneous enzyme which is soluble and, most important, free of lipid. The enzyme complexed with lecithin or with a phospholipid mixture containing lecithin binds NADH with a dissociation constant of 6-16 μM, while the apoenzyme or phospholipid alone or complexes formed with non-reactivating phospholipids bind no NADH. The results show that the binding of NADH to the dehydrogenase is dependent upon the formation of an enzyme-lecithin complex. This is the first demonstration of a role of lipid in a particular step of the reaction mechanism of a specific lipid-requiring enzyme.

It is well established that phospholipids are required for the functions of many membrane-bound enzymes (1-3). However, little evidence exists as to the precise roles of lipids in determining the functions of these proteins.

D-β-Hydroxybutyrate dehydrogenase, first released from the mitochondrial membrane by cholate treatment, has a specific requirement for lecithin for enzymic activity (4). The enzyme can also be released by phospholipase A treatment (5); the partially purified enzyme (6), when reactivated by total mitochondrial phospholipids (MPL), exhibits kinetic constants similar to those of the original membrane-bound enzyme (the reaction seems to use an ordered BiBi mechanism-Cleland nomenclature-with NAD the first substrate bound and NADH the last to be released). The phospholipase A-solubilized enzyme has recently been purified to apparent homogeneity without adding detergents (7). The enzyme has also been puri-

Abbreviations used: total mitochondrial phospholipids, MPL; phosphatidylethanolamine, PE; cardiolipin, DPG; dioleoyl-lecithin, PC 18:1; dilauroyl-lecithin, PC 12:0; N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid, HEPES.

fied (8) using the method of Jurtshuk et al. (9). In contrast to their preparation, our enzyme is soluble, completely inactive, and most important, free of lipid. These properties make our enzyme preparation ideal for studying protein-lipid interactions leading to the restoration of enzymic activity. Natural and synthetic lecithins activate the enzyme; maximal activation is with MPL (100 micromoles NAD reduced/minute/mg protein) (7,10).

We now present a study of the molecular interactions between the purified enzyme, its cofactor NADH, and several phospholipids. We show conclusively that the rebinding of lecithin to the apoenzyme restores the coenzyme-binding capacity of the dehydrogenase.

MATERIALS AND METHODS

The enzyme was released from mitochondria using phospholipase A and subsequently purified by ammonium sulfate precipitation and column chromatography (7). Enzymic activity was measured at 37°C by following the reduction of NAD at 340 nm (6). However, the assay was buffered with 10 mM potassium phosphate buffer, pH 7.3.

MPL, containing about 40% lecithin, was prepared from heavy beef heart mitochondria (11) and microdispersed in aqueous solution (12). Phosphatidylethanolamine (PE) and cardiolipin (DPG) were separated from MPL, and a mixture of PE with DPG (4 to 1) was prepared. Dioleoyl-lecithin (PC 18:1) and dilauroyl-lecithin (PC 12:0) were prepared as described by Cubero Robles and Van den Berg (13). A mixture of PC 12:0 and PE (1 to 0.8) was prepared. All phospholipids, except MPL, were microdispersed by sonication (10). All phospholipid microdispersions were centrifuged at 65,000 xg for 30 minutes before use.

[^{3}H]-NAD (adenine-2,8-labelled, from New England Nuclear) was diluted to 3.7 mC/mmole with unlabelled NAD and purified by DEAE chromatography (14). The [^{3}H]-NAD was reduced using yeast alcohol dehydrogenase (15), and [^{3}H]-NADH was separated from [^{3}H]-NAD by DEAE chromatography.

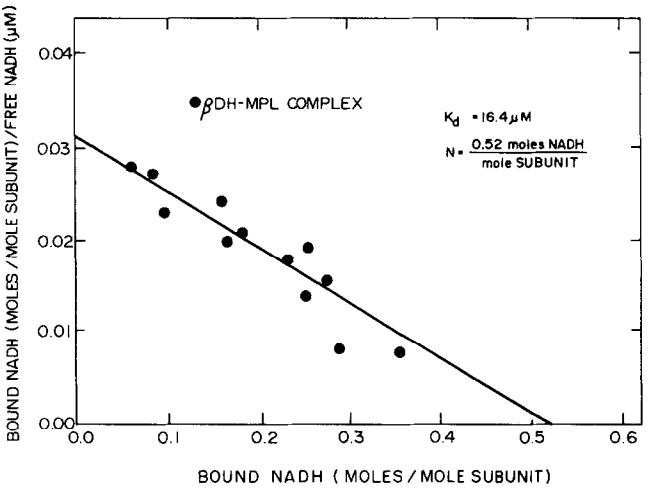

Figure 1. Binding of NADH by D-β-hydroxybutyrate dehydrogenase in the presence of MPL. Equilibrium dialysis was carried out at 4°C with 10 μg enzyme (0.32 nmoles, using a molecular weight of 31,500 - ref. 7) and 85 nmoles of MPL in 25 μl. The final buffer contained 120 mM LiBr, 10 mM phosphate pH 7.3, and 2.5 mM dithiothreitol. [^{3}H]NADH and enzyme samples (25 μl each) were added on opposites sides of the dialysis membrane. Samples (10 μl) were removed after equilibrium had been reached (after 9 and 12 hours). The enzyme-MPL complex and the apoenzyme retained 100% and 85% enzymic activity, respectively, during the dialysis.

Equilibrium dialysis was carried out using microcells with dialysis chambers that hold 30 μl each (16). Cellulose dialysis tubing, boiled for 5 minutes in 5% Na_2CO_3-50 mM EDTA, was used as dialysis membrane. Fluoresence studies were carried out in a 0.3 ml quartz cuvette using an Aminco Bowman spectrophotofluorometer. All slit widths were 0.5 mm.

RESULTS AND DISCUSSION

The binding of [^{3}H]-NADH to the apoenzyme (the enzyme alone and free of lipid) and to a complex formed between the apoenzyme and MPL was studied using equilibrium dialysis. No [^{3}H]-NADH was bound by the apoenzyme or by MPL alone. [^{3}H]-NADH does, however, bind to the dehydrogenase after a complex has been formed between the apoenzyme and MPL. Analysis of the binding data, in the form of a Scatchard plot, indicates that 1 mole of the enzyme subunit, when reactivated by MPL, binds 0.5 moles of NADH with a dissociation constant of 16.4 μM (Fig. 1). The binding of lecithin to

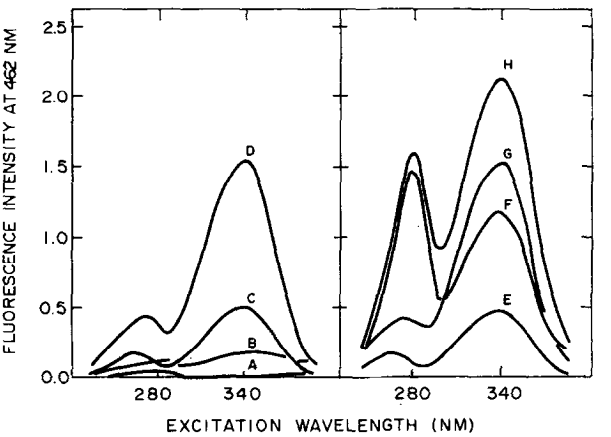

Figure 2. Excitation spectra of NADH fluorescence in the presence of the apoenzyme or the enzyme-MPL complex. Measurement of the fluorescence at 462 nm was carried out at 23°C using 34 µg protein (1.1 nmoles) and 420 nmoles MPL in an initial volume of 0.2 ml containing 125 mM LiBr, 1 mM HEPES pH 7.0 and 4 mM dithiothreitol. The enzyme and MPL were preincubated together at 23°C for 7 minutes before dilution to 0.2 ml. Aliquots of 1.25 mM NADH were added. The excitation spectra shown are from: A) the apoenzyme alone; B) the enzyme-MPL complex; C) 30 µM NADH alone; D) 115 µM NADH alone; E) the apoenzyme with 30 µM NADH; F) the enzyme-MPL complex with 30 µM NADH; G) the apoenzyme with 115 µM NADH; H) the enzyme-MPL complex with 115 µM NADH.

the apoenzyme, thus, restores the dehydrogenase's ability to bind the co-enzyme.

Fluorescence studies were carried out to confirm the results of the equilibrium dialysis as well as to measure the binding of NADH to several enzyme-phospholipid complexes. The amount of enzyme-bound NADH was determined by: a) measuring the energy transfer from tryptophan to NADH (by exciting the enzyme at 280 nm and recording the NADH fluorescence emission at 462 nm); and b) by directly exciting NADH at 340 nm (bound NADH seems to have a relative fluorescence between 5 and 10 times that of free NADH in the case of this dehydrogenase) (Fig. 2). The intensity of NADH fluorescence was greatest at 462 nm, and no change in the NADH emission spectrum was seen when the apoenzyme or phospholipid alone or an enzyme-lipid complex was added. NADH by itself has excitation peaks at 260 nm and 340 nm, corresponding to the absorption maxima of adenine and the dihydropyridine ring. The addition of the apoenzyme or lipid alone does not change the exci-

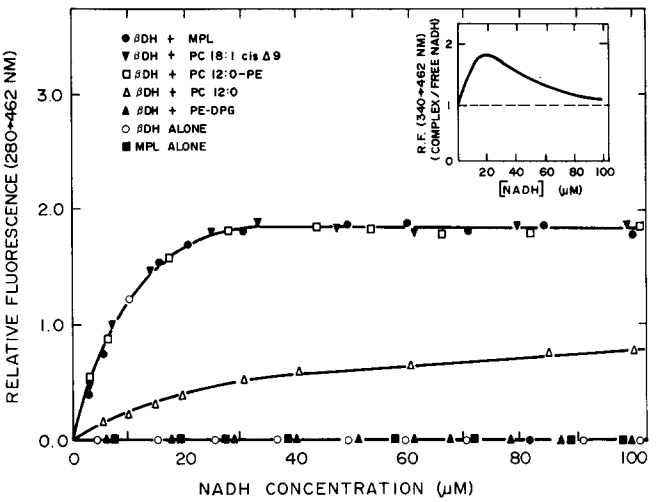

Figure 3. Effect of phospholipids on the binding of NADH to D-β-hydroxybutyrate dehydrogenase. Fluorescence measurements were carried out as described in Fig. 2. When testing PE-DPG, the initial salt concentration was only 20 mM LiBr. The amounts of lipid used were: MPL (420 nmoles); PC 18:1 (390 nmoles); PC 12:0 (250 nmoles); PC 12:0-PE (440 nmoles, 1 to 0.8); and PE-DPG (260 nmoles, 4 to 1). NADH fluorescence, measured by excitation at 280 nm (energy transfer from the enzyme),was corrected for dilution due to the addition of NADH. The fluorescence data shown were corrected by subtracting the fluorescence observed with NADH alone from the fluorescence observed with the samples. The fluorescence data of the inset, obtained by direct excitation of NADH at 340 nm, are plotted as the ratio (NADH fluorescence in the presence of the enzyme-phospholipid complex)/(fluorescence of NADH alone).

tation spectrum of NADH. Addition of an enzyme-MPL complex, however, results in a new excitation peak at 280 nm. Further, the NADH fluorescence, directly excited at 340 nm, is increased. Both observations strongly suggest that NADH binds to the enzyme-MPL complex.

The binding of NADH to several phospholipid-enzyme complexes was tested (Fig. 3). When NADH is added to the complex formed between the apoenzyme and lecithin, the intensity of the emission peak at 462 nm due to bound NADH increases as more NADH is bound until saturation of the coenzyme-binding sites is reached (using either energy transfer to, or direct excitation of, NADH). An apparent dissociation constant of 6-7 μM was estimated from the point of half-maximal fluorescence. The complexes formed between the apoenzyme and MPL, PC 18:1 or the mixture PC 12:0-PE (1 to 0.8) bind NADH better

than the enzyme-PC 12:0 complex. In agreement with this data, we find that the apoenzyme binds to MPL, PC 18:1 or the mixture PC 12:0-PE better than to PC 12:0 (10). The fluorescence due to bound NADH can be completely abolished by titrating the enzyme-lecithin (or MPL)-NADH complex with NAD (not shown). When the apoenzyme or MPL alone or complexes between the apoenzyme and PE-DPG (4 to 1) or PE (not shown) are titrated with NADH (up to 250 μM NADH), no increase in fluorescence relative to that of NADH by itself was observed. NADH can bind to the dehydrogenase only after the apoenzyme binds lecithins. Studies are now in progress to define more precisely the coenzyme-binding parameters.

We have shown in this paper the direct effect of phospholipids on a particular step of the reaction mechanism of a specific lipid-requiring enzyme. Both the overall activity of the dehydrogenase and the binding of NADH (and presumably of NAD also) to the enzyme are dependent upon the apoenzyme's binding to lecithin. The NADH (NAD) binding step may be the one which confers the specific requirement for lecithin to the enzyme.

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